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# Geographic Patterns of Cytochrome *b* and *Sry* Gene Lineages in the Gray Red-Backed Vole *Clethrionomys rufocanus* from Far East Asia Including Sakhalin and Hokkaido

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**ABSTRACT**—The gray red-backed vole, *Clethrionomys rufocanus*, from Far East Asia including Sakhalin and Hokkaido is known to harbor intraspecific morphological and cytogenetic variations. Here we analyzed geographic variation in the mitochondrial cytochrome *b* (*Cytb*) gene and Y chromosome specific *Sry* gene by a PCR direct sequencing technique. Determination of sequences in the *Cytb* gene (1140 bp) in 29 individuals provided 28 haplotypes and construction of a neighbor joining tree clearly indicated that they were grouped into four major lineages, which predominated in Primorskyi territory, Kamchatska-Magadan, Sakhalin, and Hokkaido-Kuril, with 0.02–0.04 sequence divergence (Kimura's distance, all substitutions, all codon positions). The sequences for the *Sry* region (336–366 bp) included a variable repetitious region of microsatellites such as $TG(TC)_2$. In total five sequences were recognized which can be grouped into two forms, continental and insular (Sakhalin and Hokkaido) based on the presence or absence of a segment of $TG(TC)_3TG(TC)_4$. It was revealed that, therefore, the population of Sakhalin possessed their own type of mitochondrial DNA but the same *Sry* gene as Hokkaido. A similar trend can be seen in the continental populations. Our results suggested that the population of *C. rufocanus* in each of the geographic domains accumulated own genetic elements in part but genetic exchanges between neighboring populations occurred during the course of evolution. It is noteworthy that the insular domains, Sakhalin and Hokkaido, have played an important role in raising the amount of genetic diversity in small rodent species.

## INTRODUCTION

The gray red-backed vole, *Clethrionomys rufocanus*, which can be characterized by the specific enamel pattern of its upper third molar (Kaneko *et al*., 1998), occurs in northeastern Eurasia from Scandinavia in the west through Siberia and Primorskyi territory to Sakhalin and Hokkaido (and peripheral islands including the southern Kuril Islands) in the east (Corbet, 1978; Corbet and Hill, 1991; Kaneko *et al*., 1998). The species shows morphological variations among geographic regions. For example, the tail length is quite short in Magadan and Kamchatska and rather long in Hokkaido (Kaneko *et al*., 1998; Iwasa, unpublished data), and individuals from Hokkaido are darker in body color than those from Russia (Imaizumi, 1960; Kaneko *et al*., 1998). Then taxonomists tend to treat these geographic populations as subspecies: populations of Russia have been separated to several subspecies (Gromov and Polyakov, 1992; Kartavtseva *et al*., 1998) while those of Hokkaido have been regarded as *C. rufocanus bedfordiae* (Imaizumi, 1960; Gromov and Polyakov, 1992; Kaneko *et al*., 1998). Furthermore, populations of the small peripheral islands of Hokkaido are known to have some specific morphological characteristics (Imaizumi, 1960; Miyao, 1968). The population of Rishiri Island, for example, tends to have a larger body size than that of Hokkaido (Miyao, 1968).

Intraspecific karyological variation has been also investigated in *C. rufocanus*, and it has been revealed that the karyo-

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type is essentially the same (Vorontsov *et al.*, 1980; Sokolov *et al.*, 1990; Kartavtseva *et al.*, 1998). However, the size of the Y chromosome is known to differ between populations of the continent and Sakhalin-Hokkaido (Vorontsov *et al.*, 1980; Tsuchiya, 1981; Kashiwabara and Onoyama, 1988; Kartavtseva *et al.*, 1998). To expand our knowledge on the morphological and chromosomal variations underlying the evolution of the *Clethrionomys* species, several genetic approaches have been taken. Recently, molecular analyses have been attempted using microsatellites, a nuclear ribosomal RNA gene and mitochondrial DNA markers to clarify the genetic background of this species (Ishibashi *et al.*, 1995, 1997a, b; Suzuki *et al.*, 1999; Wakana *et al.*, 1996). In addition, several studies on the phylogenetic relationships among related species of red-backed voles in East Asia, including *C. rutilus* and *C. rex*, have been made. *Clethrionomys rufocanus* is shown to have a close affinity with *C. rex* that inhabits Hokkaido and its neighboring islands (Imaizumi, 1971, 1972; Kaneko, 1994; Kaneko *et al.*, 1998) rather than with *C. rutilus* that is distributed in a wide area of the northern hemisphere, based on karyological (Gamperl, 1982; Kashiwabara and Onoyama, 1988; Obara *et al.*, 1995; Iwasa, 1998) and molecular phylogenetic aspects (Suzuki *et al.*, 1999; Wakana *et al.*, 1996).

To obtain a clear phylogenetic view of geographic populations, it is necessary to understand the genetic constitution as a whole, since multiple genetic exchanges among populations as well as random lineage sorting would have occurred during the course of populational differentiation (Harrison, 1989; Avise, 1991). To this end, the study of these geographic populations with multiple phylogenetic markers would be highly effective. However, to date, only variation in the mitochondrial DNA (mtDNA) in a small number of specimens from Primorskyi territory and Hokkaido has been reported (Suzuki *et al.*, 1999; Wakana *et al.*, 1996).

Here we conducted a more intensive and extensive study on the intraspecific variation of *C. rufocanus* collected from several geographic areas of Far East Asia, Primorskyi territory, Kamchatska, Sakhalin, Hokkaido, and Kuril Islands, with two molecular markers, one each for maternal and paternal lineages. Using a PCR direct sequencing technique, we determined sequences for the mitochondrial cytochrome *b* (*Cytb*) gene and the sex-determination gene, *Sry*. The *Sry* gene is omnipresent on the Y chromosome in mammalian species and the chromosomal site is the region un-paired with the X chromosome in mouse and human, implying that the gene is a useful marker for tracing paternal lineages without degradation by meiotic recombination events, as is mtDNA for maternal lineages (Harrison, 1989).

## MATERIALS AND METHODS

Analyzed specimens of *C. rufocanus* (34 individuals) were captured from own localities as shown in Table 1 and Fig. 1. The species identification was performed according to Kaneko (1994) and Kaneko *et al.* (1998), mainly based on the enamel pattern of molar dentition. All specimens are preserved at the authors' laboratories.

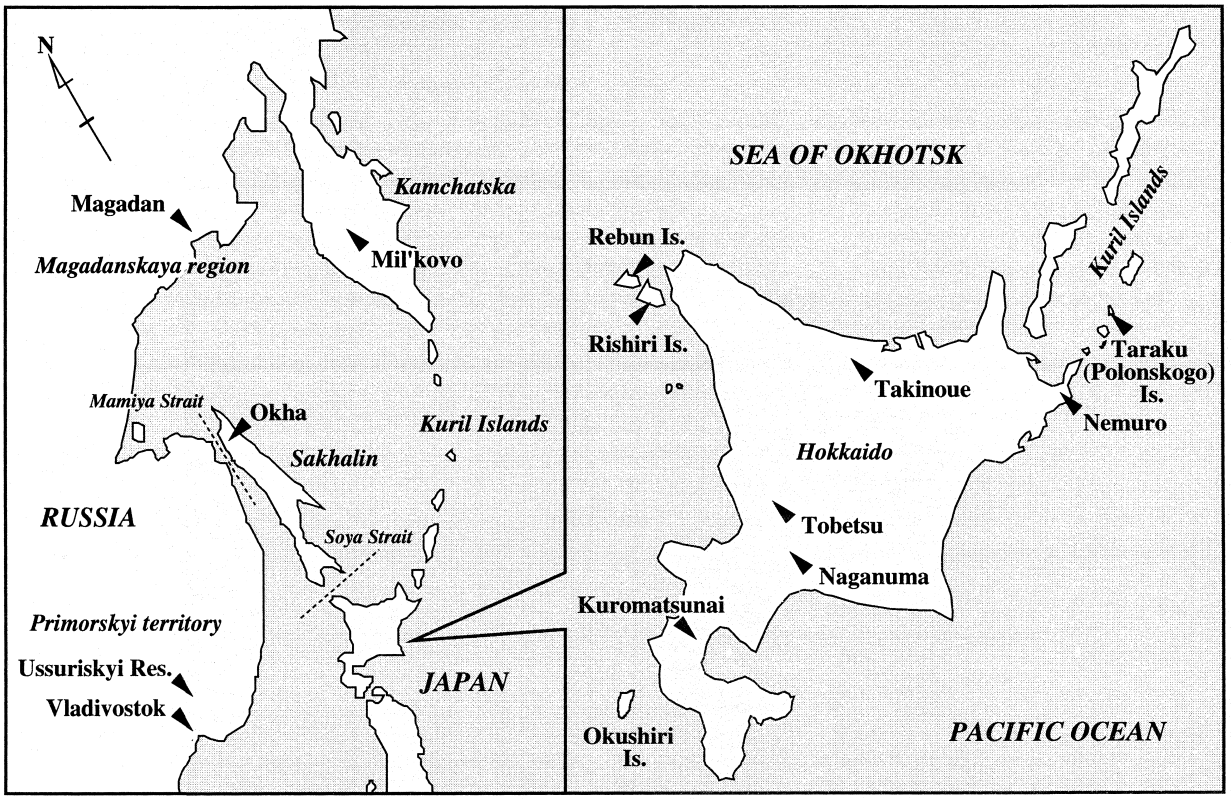


**Fig. 1.** Localities (arrowheads) from which individuals of *Clethrionomys rufocanus* were collected.

**Table 1.** Collecting localities, specimen numbers, and mitochondrial DNA (mtDNA) and Sry haplotypes of *Clethrionomys rufocanus* examined in this study.

| Collecting locality | Specimen No. | mtDNA haplotype | *Sry* haplotype |
|---|---|---|---|
| Takinoue, Hokkaido, Japan | HEG117-98 | TK-HK1 | – |
| | HEG121-98 | TK-HK2 | A |
| | HEG118-98 | TK-HK3 | – |
| Nemuro, Hokkaido, Japan | HEG141-98 | NM-HK1 | A |
| | HEG149-98 | NM-HK2 | A |
| | HEG140-98 | – | A |
| Tobetsu, Hokkaido, Japan | HEG1-97 | TB-HK1 | – |
| Naganuma, Hokkaido, Japan | HS231 | NG-HK1 | – |
| | HS234 | – | A |
| Kuromatsunai, Hokkaido, Japan | KN98107 | KM-HK1 | – |
| | KN98108 | KM-HK2 | – |
| Rishiri Island, Hokkaido, Japan | HS227 | RS-HK1 | – |
| | HS228 | – | B |
| Rebun Island, Hokkaido, Japan | HEGRB-2 | RB-HK1 | – |
| Taraku Island, Kuril Islands, Russia | IN28-98 | TK-KR1* | – |
| | IN38-98 | TK-KR1* | – |
| Okha, Sakhalin, Russia | IK61-97 | OH-SH1 | – |
| | IK64-97 | OH-SH2 | A |
| | IK65-97 | OH-SH3 | A |
| | HS639 | OH-SH4 | – |
| Ussuriskyi Reservation, Primorskyi territory, Russia | IK122-96 | US-RS1 | – |
| | IK115-96 | US-RS2 | – |
| | IK116-96 | US-RS3 | – |
| | IK120-96 | US-RS4 | – |
| | IK123-96 | US-RS5 | – |
| | IK124-96 | US-RS6 | E |
| Vladivostok, Primorskyi territory, Russia | HS943 | VS-RS1 | – |
| | HS944 | VS-RS2 | – |
| Mil'kovo, Kamchatska region, Russia | IK101-97 | MK-KC1 | D |
| | IK103-97 | MK-KC2 | – |
| | IK100-97 | MK-KC3 | D |
| | IK128-97 | – | C |
| | IK110-97 | – | D |
| Magadan, Magadanskaya region, Russia | DK29-97 | MG-RS1 | D |

* IN28-98 and IN38-98 showed identical sequences in mtDNA, therefore, they were assigned the same haplotype as TK-KR1.

**Table 2.** PCR nested primers for the mitochondrial *Cytb* gene and the *Sry* gene.

| Primer | Primer sequence |
|---|---|
| N-L14724 | 5'-CAGGAAACAGCTATGACCGATATGAAAAACCATCGTTG-3' |
| N-L15135 | 5'-CAGGAAACAGCTATGACCGCTATAATAGCAACAGCATTCATAGG-3' |
| N-L15561 | 5'-CAGGAAACAGCTATGACCCCACATATTAAACCAGAATG-3' |
| N-H15155 | 5'-TGTAAAACGACGGCCAGTTGCCCCTCAAAAGGATATTTG-3' |
| N-H15919 | 5'-TGTAAAACGACGGCCAGTGTCATCTCCGGTTTACAAGA-3' |
| N-H15599 | 5'-TGTAAAACGACGGCCAGTGAGTCCTCCTAGTTTGTTGGG-3' |
| R-SRY306 | 5'-CAGGAAACAGCTATGACCACTTGGATATTTTGTTACAG-3' |
| U-HMG597* | 5'-TGTAAACGACGGCCAGTCACACGATAAATGCGTTCATGGG-3' |

* from Suzuki *et al.* (1997)

**Direct sequencing of the mitochondrial cytochrome *b* gene**

The total DNA was extracted from liver tissues by the conventional phenol-chloroform method. Amplifications of a fragment of the mitochondrial *Cytb* gene were performed with universal primers (L14724 and H15915; Irwin *et al.*, 1991) at 1st PCR, and followed by a specific 2nd PCR amplification with nested (N-) primers that were designed for vole specimens (N-L14724, N-L15135, N-L15561, N-H15155, N-H15599 and N-H15919; Table 2). The reactions were carried out for 35 cycles each consisting of 30 sec at 96°C for denaturation, 30 sec at 50°C for annealing and 30 sec at 60°C for extension. These reaction mixtures (20 **m**l) contained 50 **m**M dNTPs, 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$ (1.25 mM at 2nd PCR), 50 nM primers and 0.5 units Ampli Taq polymerase (Perkin Elmer). Both DNA strands of the product of the second PCR were directly sequenced by an automated method using the Dye Primer Cycle Sequencing Kit (ABI) and an automated sequencer (model 373A, ABI).

**Direct sequencing of the Y chromosomal *Sry* gene**

Amplifications of a fragment of the *Sry* gene at 1st PCR were performed with primers designed from human and murine sequences (SRY286 and HMG777, Fig. 3; Sinclair *et al.*, 1990; Suzuki *et al.*, 1997), and followed by a 2nd PCR with nested primers of R-SRY306 and U-HMG597 (Table 2; Suzuki *et al.*, 1997) to obtain an approximately 350 bp fragment of the flanking region of the HMG box. The reaction of the 1st PCR was identical to that for the *Cytb* gene. The reaction for the 2nd PCR was carried out for 35 cycles each consisting of 30 sec at 96°C for denaturation, 30 sec at 62°C for annealing and 30 sec at 72°C for extension. The products of the 2nd PCR were directly sequenced as for the *Cytb* gene.

**Construction of a phylogenetic tree**

We constructed a phylogenetic tree by the neighbor-joining (NJ) method (Saitou and Nei, 1987) and performed bootstrap analysis (1000 replications), with genetic distances computed by Kimura's two parameter method (Kimura, 1980), using CLUSTAL W program ver. 1.6 (Thompson *et al.*, 1994) for *Cytb* sequences. We sequenced the same gene region for *C. rutilus* and *C. rex* from Hokkaido and used them as an outgroup.

The DNA sequence data of *C. rufocanus*, *C. rutilus* and *C. rex*, which were determined in this study will be placed in the GenBank, EMBL and DDBJ nucleotide sequence databases with the following accession numbers, AB031553-AB031582 (*Cytb*) and AB031858-AB031862 (*Sry*).

## RESULTS

Sequence determination of the *Cytb* gene (1140 bp) in 29 individuals of *C. rufocanus* provided 28 haplotypes (Table 1). And pairwise sequence divergences among the haplotypes were calculated to be 0.001–0.040 by Kimura's two parameter method (Kimura, 1980) considering all substitutions at all codon positions (Table 3). In the comparisons of these sequences within *C. rufocanus*, saturation bias would not be expected since transition to transversion ratios were high throughout the analysis with a minimum value of 10.94. A neighbor joining tree was constructed with the sequence divergences, and indicated the presence of four prominent clusters that were essentially specific to each geographic domain, Primorskyi territory, Sakhalin, Kamchatska, and Hokkaido (Fig. 2). Each cluster was supported by high bootstrap values (>87%, Fig. 2). There was substantial sequence divergence between the four prominent lineages with a Kimura's distance of 0.02–0.04 (Table 3). However, the branching patterns of these four lineages were unclear: the lengths between the nodes were short and the bootstrap values that supported the branching were low (<68%, Fig. 2).

The members of each of the clusters showed a strict geographic preference. Exceptionally, a haplotype (VS-RS1) from Vladivostok, Primorskyi territory was incorporated into the cluster Kamchatska, while the other seven haplotypes from Primorskyi territory formed a unique cluster (Fig. 2, Table 1). Further subdivision within the clusters is possible but the extent of differentiation was small (Fig. 2). For example, the Hokkaido cluster was likely to be further subdivided into three clades (monophyletic subclusters) that reflected the geographic source of the haplotypes: Hokkaido (NM-HKs, KM-HKs,TB-HK1, NG-HK1, and TK-HKs), Rishiri-Rebun Islands

**Table 3.** Pairwise distances of mitochondrial cytochrome *b* gene sequences (1140 bp) using the Kimura two parameter method (Kimura,

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *C. rex* | | | | | | | | | | | | | | | | | | |
| 2 | RS-HK1 | .067 | | | | | | | | | | | | | | | | | |
| 3 | RB-HK1 | .066 | .006 | | | | | | | | | | | | | | | | |
| 4 | NM-HK1 | .067 | .008 | .011 | | | | | | | | | | | | | | | |
| 5 | NM-HK2 | .068 | .009 | .012 | .003 | | | | | | | | | | | | | | |
| 6 | KM-HK1 | .068 | .010 | .012 | .005 | .008 | | | | | | | | | | | | | |
| 7 | KM-HK2 | .068 | .010 | .012 | .005 | .006 | .005 | | | | | | | | | | | | |
| 8 | NG-HK1 | .066 | .006 | .009 | .005 | .006 | .007 | .007 | | | | | | | | | | | |
| 9 | TB-HK1 | .067 | .009 | .012 | .004 | .007 | .004 | .004 | .006 | | | | | | | | | | |
| 10 | TK-HK1 | .066 | .010 | .012 | .005 | .008 | .005 | .005 | .007 | .004 | | | | | | | | | |
| 11 | TK-HK2 | .065 | .010 | .012 | .002 | .004 | .007 | .007 | .007 | .006 | .007 | | | | | | | | |
| 12 | TK-HK3 | .070 | .008 | .011 | .005 | .006 | .007 | .007 | .005 | .006 | .007 | .007 | | | | | | | |
| 13 | TK-KR1 | .068 | .008 | .011 | .009 | .008 | .011 | .009 | .007 | .010 | .011 | .011 | .009 | | | | | | |
| 14 | US-RS1 | .069 | .030 | .030 | .027 | .028 | .027 | .027 | .027 | .028 | .029 | .027 | .027 | .029 | | | | | |
| 15 | US-RS2 | .070 | .031 | .031 | .030 | .031 | .030 | .030 | .028 | .031 | .032 | .030 | .030 | .030 | .004 | | | | |
| 16 | US-RS3 | .071 | .032 | .032 | .031 | .032 | .031 | .031 | .029 | .032 | .033 | .031 | .031 | .031 | .007 | .004 | | | |
| 17 | US-RS4 | .068 | .029 | .031 | .030 | .031 | .030 | .032 | .028 | .031 | .030 | .030 | .030 | .028 | .010 | .011 | .013 | | |
| 18 | US-RS5 | .070 | .031 | .031 | .030 | .031 | .030 | .030 | .028 | .031 | .032 | .030 | .030 | .030 | .003 | .005 | .008 | .011 | |
| 19 | US-RS6 | .069 | .030 | .030 | .029 | .030 | .029 | .029 | .027 | .030 | .031 | .029 | .029 | .029 | .004 | .004 | .007 | .010 | .004 |
| 20 | VS-RS1 | .066 | .033 | .035 | .034 | .035 | .034 | .034 | .034 | .035 | .036 | .034 | .036 | .034 | .026 | .025 | .026 | .024 | .027 |
| 21 | VS-RS2 | .069 | .028 | .028 | .027 | .028 | .027 | .027 | .025 | .028 | .029 | .027 | .027 | .027 | .004 | .004 | .005 | .010 | .004 |
| 22 | OH-SH1 | .069 | .035 | .035 | .038 | .039 | .038 | .040 | .036 | .039 | .040 | .038 | .038 | .034 | .020 | .022 | .023 | .024 | .021 |
| 23 | OH-SH2 | .067 | .035 | .034 | .036 | .037 | .034 | .038 | .034 | .037 | .038 | .036 | .036 | .033 | .018 | .021 | .023 | .022 | .019 |
| 24 | OH-SH3 | .068 | .036 | .035 | .037 | .038 | .037 | .039 | .035 | .038 | .039 | .037 | .037 | .034 | .021 | .023 | .024 | .023 | .022 |
| 25 | OH-SH4 | .068 | .036 | .035 | .037 | .038 | .035 | .037 | .035 | .036 | .037 | .037 | .037 | .034 | .019 | .022 | .024 | .023 | .020 |
| 26 | MK-KC1 | .064 | .028 | .031 | .028 | .029 | .028 | .028 | .028 | .029 | .030 | .028 | .030 | .028 | .019 | .018 | .019 | .020 | .020 |
| 27 | MK-KC2 | .063 | .028 | .030 | .027 | .028 | .027 | .027 | .027 | .028 | .029 | .027 | .029 | .027 | .018 | .017 | .018 | .019 | .019 |
| 28 | MK-KC3 | .064 | .029 | .031 | .028 | .029 | .028 | .028 | .028 | .029 | .030 | .028 | .030 | .028 | .019 | .018 | .019 | .020 | .020 |
| 29 | MG-RS1 | .067 | .035 | .037 | .034 | .035 | .034 | .034 | .034 | .035 | .036 | .034 | .034 | .033 | .027 | .024 | .025 | .028 | .028 |

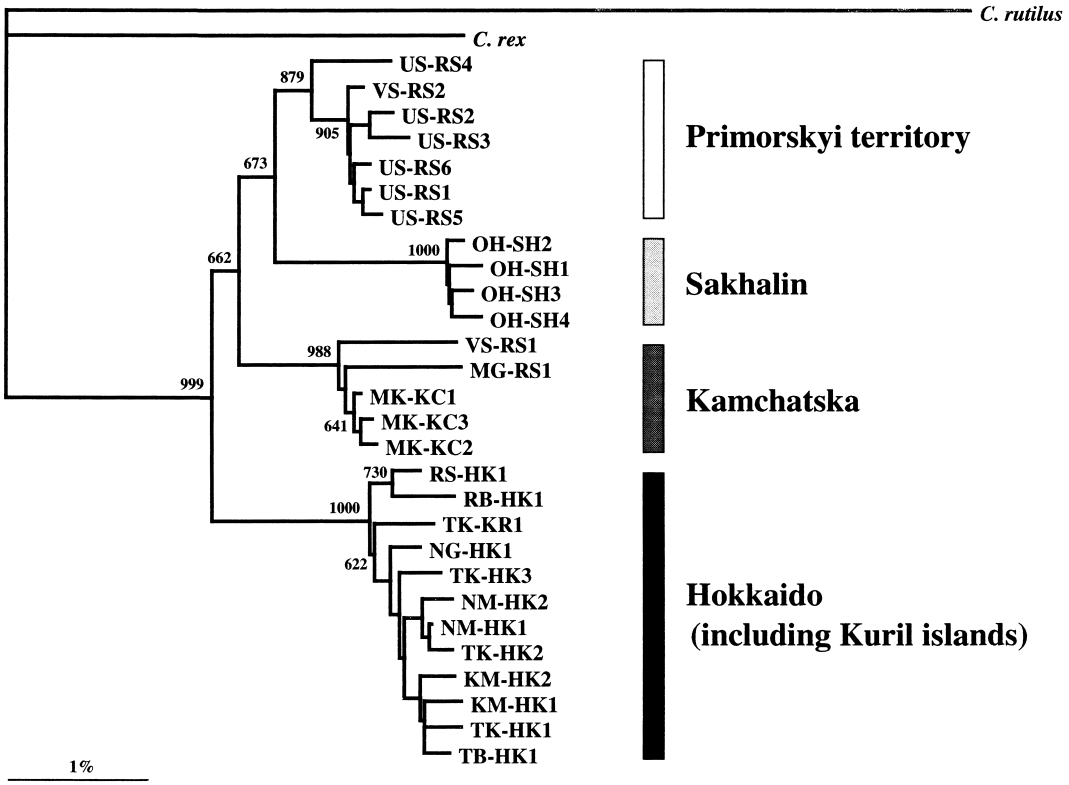


**Fig. 2.** A phylogenetic analysis with the complete sequence of the mitochondrial cytochrome *b* gene (1140 bp) in *Clethrionomys rufocanus* from Far East Asia. A neighbor-joining tree was constructed with pairwise sequence divergences that were calculated by the Kimura's two parameter method (Kimura, 1980) considering all substitutions at all codon positions. Bootstrap values related to the nodes are indicated (1000 replicates). Sources of the haplotypes are listed in Table 1.

1980). Abbreviated haplotype names are shown in Table 1.

| 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 |
|---|---|---|---|---|---|---|---|---|---|
| .026 | | | | | | | | | |
| .004 | .024 | | | | | | | | |
| .020 | .034 | .022 | | | | | | | |
| .022 | .034 | .020 | .004 | | | | | | |
| .022 | .035 | .022 | .004 | .004 | | | | | |
| .021 | .035 | .021 | .004 | .003 | .004 | | | | |
| .019 | .011 | .017 | .026 | .026 | .027 | .027 | | | |
| .018 | .001 | .016 | .025 | .025 | .026 | .026 | .001 | | |
| .019 | .012 | .017 | .026 | .026 | .027 | .027 | .002 | .003 | |
| .027 | .017 | .025 | .035 | .035 | .035 | .035 | .010 | .009 | .012 |

(RS-HK1 and RB-HK1), and Taraku (=Polonskogo) Island, southern Kuril Islands (TK-KR1). The sequence divergences between the three clades were approximately 0.01 of the Kimura's distance.

We determined the sequences of the *Sry* region (336–366 bp) in 14 males of *C. rufocanus* (Table 1). The region showed a variable state within species, which was largely due to the presence of several repeats of microsatellite-like elements such as $TG(TC)_2$ (Fig. 3). From the status of the polymorphic region, we recognized five types of *Sry* gene in our samples (types A–E). In addition, types D and E differed from the others in one base substitution (position 50, marked with an asterisk; Fig. 3). These *Sry* gene types showed a certain geographic distribution, although the tendency was not consistent with that for the mtDNA (Table 1). Types A and B were observed in individuals from Sakhalin-Hokkaido and Rishiri Island, respectively, while the other three types were obtained from those of the continent. Types A and B differed slightly with respect to the repeat number of $TG(TC)_2$ and both contained a segment $TG(TC)_3TG(TC)_4$, associated with the microsatellite region, whereas the continental types, C, D, and E, lacked the segment (Fig. 3). Then, taking into account that the constitution of the repetitious number of the $TG(TC)_2$ motif is labile but insertion or deletion of $TG(TC)_3TG(TC)_4$ is not reversible, we grouped the *Sry* gene types into two, continental (C, D, and E) and insular (A and B) forms, respectively.

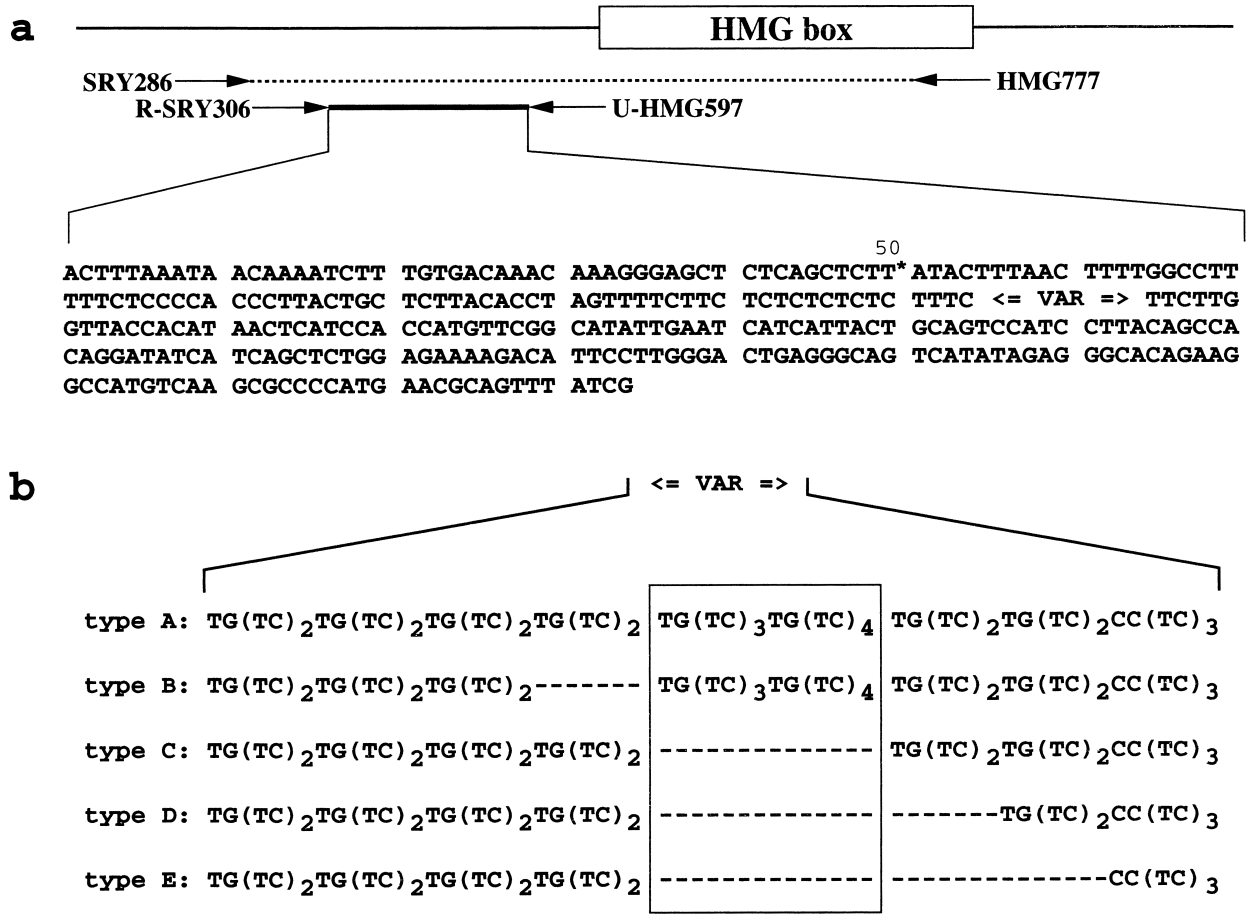


**Fig. 3.** Sequence polymorphism of the *Sry* gene in *Clethrionomys rufocanus* from Far East Asia. The *Sry* gene region analyzed (thick line) and primers used for the PCR amplifications are indicated (a). A flanking region (336–366 bp) upstream from the 5' end of the HMG box was sequenced. Five types (A–E) are recognized from a base substitution and a variable region that contains microsatellite-like elements (b). A representative sequence of the region and sequences for a polymorphic region are shown. The asterisk indicates the polymorphic site at which types A, B and E exhibit "T" and types C and D exhibit "G". The variable region marked by "<=VAR=>" includes polymorphic repeats of $TG(TC)_n$.

## DISCUSSION

With markers for the *Cytb* and *Sry* genes we revealed that there was substantial intraspecific variation in the populations of *C. rufocanus* from Far East Asia and that each of the genes had its own geographic distribution (Figs. 2 and 3). The results obtained here, hence, would be useful not only for the evaluation of the phylogenetic relatedness among the geographic populations of *C. rufocanus* but also for learning how molecular markers with different modes of inheritance have behaved during the course of evolution. In addition, our data provide a clue as to the roles of several zoogeographic domains in Far East Asia, especially Hokkaido, in the mode of genetic diversity in small rodents.

### Genetic relationships of the geographic populations from Far East Asia

The mtDNA data provides us a rationale for the subdivision of *C. rufocanus* and approximate discrimination of geographic boarders (Fig. 2). Accordingly, we can denote four geographic domains for *C. rufocanus* in the Far East; Primorskyi territory, Sakhalin, Kamchatska and Hokkaido. This notion is somewhat consistent with the morphological classification where the populations of Primorskyi territory, Kamchatska, and Sakhalin are regarded as different subspecies (e.g., Gromov and Polyakov, 1992).

The mtDNA tree showed that the divergences between the lineages for the four geographic domains were distinct as indicated by their long branch lengths (0.03–0.04 for pairwise sequence divergence, Table 3 and Fig. 2), implying that considerable time has passed since their ancestors colonized these geographic areas. The divergence time of the four branches is likely to be a few million years, taking account of the traditional assumption of the divergence rate in mitochondrial DNA (0.01–0.02 per million years per two lineages; Brown *et al.*, 1979). However it is uncertain whether such an estimation is accurate since the evolutionary rate would vary in each organism (Zardoya and Meyer, 1996) and, probably, in each lineage of the clusters of the voles.

In contrast to the mtDNA variation, the *Sry* gene data indicate a populational demarcation of *C. rufocanus* in Far East Asia into essentially two groups, continental and insular,

based on the constitution of repetitious elements (Fig. 3). The different modes of geographic differentiation between both genes examined would be attributable to differences in their inheritance. In the case of red-backed voles, it has been reported that male individuals travel further than females (Ishibashi *et al*., 1997b) during their lifetime. Thus it is reasonable that mtDNA tends not to be replaced with neighboring types irrespective of the presence of gene flow which mainly occurs through male individuals. Such a phenomenon is sometimes recognized as a "founder events" with respect to mtDNA (Harrison, 1989). Even though we can not specify the reason for the contradiction at this moment, our results suggest that ancestral populations communicated genetically within the continental populations and between the Sakhalin and Hokkaido populations. This notion is consistent with the geographic variation of Y chromosomes in *C. rufocanus* in Far East Asia where acrocentric Y is observed in Sakhalin and Hokkaido and metacentric Y observed at several continental localities (Vorontsov *et al*., 1980; Kartavtseva *et al*., 1998). It is also consistent with the morphological view that the populations of Sakhalin and Hokkaido are the same subspecies (Gromov and Polyakov, 1992; Kartavtseva *et al*., 1998). The data of mtDNA from Vladivostok that showed affinity with those of Kamchatska also support the idea of interpopulational gene flow as an evolutionary trend between neighboring geographic populations (Fig. 2).

The close relationships between Sakhalin and Hokkaido can be seen in other small mammals. For example, the soricine shrew species, *Sorex gracillimus*, with a similar habitat to *C. rufocanus*, shows close genetic relationships between Hokkaido and Sakhalin populations in *Cytb* gene sequence data (Ohdachi *et al*., 1997). In the *Cytb* gene variation of the East-Asiatic wood mice, *Apodemus peninsulae*, which is distributed across Far East Asia including Sakhalin and Hokkaido, a similar tendency can be seen (Serizawa *et al*., unpublished data). Therefore, it can be considered that the fauna of small mammals in Sakhalin and Hokkaido tends to have similar genetic constitutions that differ from those of the continent. Gene flow across land bridges on the Soya Strait (45 m in depth in present; Oshima, 1990) during the glacial periods would explain such genetic similarity.

**Evolutionary aspects of the Hokkaido population**

*Clethrionomys rufocanus* populations inhabiting small peripheral islands of Hokkaido, such as Rishiri Island, are known to have specific morphological characteristics (Miyao, 1968). These insular populations are sometimes classified as distinct species or subspecies (Imaizumi, 1960; Japanese Environmental Agency, 1993). The present data, however, indicated that the populations of Hokkaido and its offshore islands including the Taraku Islands, in the southern Kuril Islands, share similar phylogenetic elements (Figs. 2 and 3). Wakana *et al*. (1996) reported a close affinity between the Hokkaido population and island ones (Rishiri and Daikoku Islands) as determined from mtDNA analysis. These close relationships are easily explained by geological factors such as the narrow straits between the islands (Oshima, 1990). It is noteworthy that on Okushiri Island, near the southwestern part of Hokkaido (Fig. 1) which is separated from Hokkaido by deep strait (>400 m; Oshima, 1990), there is no extant population of *C. rufocanus*.

From our data, and cytogenetic and morphological data in the literature (Vorontsov *et al*., 1980; Gromov and Polyakov, 1992, Kaneko *et al*., 1998), the *C. rufocanus* populations of Hokkaido including neighboring small islands can be considered to be undergoing further differentiation from Russian continental populations through a certain kind of geographic isolation. We consider that such processes may have been involved in the establishment of *C. rex* which is now confined to Hokkaido and neighboring islands (Kaneko *et al*., 1998) and is phylogenetically very close to *C. rufocanus* among red-backed vole species (Wakana *et al*., 1996; Kaneko *et al*., 1998; Suzuki *et al*., 1999). It is possible that the common ancestor of *C. rex* and *C. rufocanus* was once distributed across Far East Asia including Hokkaido like *C. rufocanus* presently is and a branched lineage that had been retained in Hokkaido is now recognized as *C. rex*. This notion can be supported by several lines of evidence. *Clethrionomys rex* is known to bear primitive morphological characteristics compared to other species of *Clethrionomys* (Imaizumi, 1972; Gromov and Polyakov, 1992). This species now exhibits a fragmented geographic distribution in Hokkaido, probably due to the invasion of *C. rufocanus* with ecological superiority (Nakata, 1995). In addition, *C. sikotanensis*, which is presumed to be a synonym of *C. rex*, is retained in the southern Kuril islands, peripheral part of Hokkaido (Tokuda, 1935; Gromov and Polyakov, 1992) from morphological features (Iwasa, unpublished data), suggesting oldness of the lineage of *C. rex* in the Hokkaido area.

Therefore, Hokkaido can be characterized zoogeographically as a phylogenetically important area that raises the level of species and genetic diversity in small mammals. Finally it is worth noting that the two species of red-backed voles, *C. rex* and *C. rufocanus*, in Hokkaido could represent a high level of genic endemism among the mammalian fauna of Hokkaido, in contrast to the low levels of genetic endemism in the East-Asiatic wood mice *A. peninsulae* (Serizawa *et al*., unpublished data), red fox *Vulpes vulpes* (Tsuda *et al*., 1997), and sable *Martes zibellina* (Hosoda *et al*., 1999).

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## REFERENCES

Avise JC (1991) Ten unorthodox perspectives on evolution prompted by comparative population genetic findings on mitochondrial DNA. Ann Rev Genet 25: 45–69

Brown WM, George M Jr, Wilson AC (1979) Rapid evolution of ani-

mal mitochondrial DNA. Proc Natl Acad Sci USA 6: 1967–1971
Corbet GB (1978) The Mammals of the Palaearctic Region: A Taxonomic Review. Cornell Univ Press, Ithaca
Corbet GB, Hill JE (1991) A World List of Mammalian Species. 3rd ed, Oxford Univ Press, Oxford
Gamperl R (1982) Chromosomal evolution in the genus *Clethrionomys*. Genetica 57: 193–197
Gromov IM, Polyakov IY (1992) Voles (Microtinae), Fauna of the USSR, Mammals vol III, No 8. E J Brill Pub Com, Netherlands
Harrison RG (1989) Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. Trends Ecol Evol 4: 6–11
Hosoda T, Suzuki H, Iwasa MA, Hayashida M, Watanabe S, Tatara M, Tsuchiya K (1999) Genetic relationships within and between the Japanese marten *Martes melampus* and the sable *M. zibellina*, based on variation of mitochondrial DNA and nuclear ribosomal DNA. Mammal Study 24: 25–33
Imaizumi Y (1960) Colored Illustrations of the Mammals of Japan. Hoikusha, Osaka (in Japanese)
Imaizumi Y (1972) Land mammals of the Hidaka mountains, Hokkaido, Japan, with special reference to the origin of an endemic species of the genus *Clethrionomys*. Bull Nat Sci Mus, 5: 131–149 (in Japanese with English summary)
Irwin DM, Kocher TD, Wilson AC (1991) Evolution of the cytochrome *b* gene of mammals. J Mol Evol 32: 128–144
Ishibashi Y, Saitoh T, Abe S, Yoshida MC (1995) Polymorphic microsatellite DNA markers in the grey red-backed vole *Clethrionomys rufocanus bedfordiae*. Mol Ecol 4: 127–128
Ishibashi Y, Saitoh T, Abe S, Yoshida MC (1997a) Cross-species amplification of microsatellite DNA in Old World microtine rodents with PCR primers for the gray-sided vole, *Clethrionomys rufocanus*. Mammal Study 22: 5–10
Ishibashi Y, Saitoh T, Abe S, Yoshida MC (1997b) Sex-related spatial kin structure in a spring population of grey-sided voles *Clethrionomys rufocanus* as revealed by mitochondrial and microsatellite DNA analyses. Mol Ecol 6: 63–71
Iwasa, MA (1998) Chromosomal and molecular variations in red-backed voles. Honyurui Kagaku [Mammalian Science] 38: 145–158 (in Japanese with English abstract)
Japanese Environmental Agency (1993) A list of Japanese Wild Animals: Vertebrates. Japan Wildlife Research Center, Tokyo (in Japanese)
Kaneko Y (1994) Rodentia. In "A Pictorial Guide to the Mammals of Japan" Ed by H Abe, Tokai Univ Press, Tokyo, pp 90–109 and 168–183 (in Japanese)
Kaneko Y, Nakata K, Saitoh T, Stenseth NC, Bjørnstad ON (1998) The biology of the vole *Clethrionomys rufocanus*: a review. Res Popul Ecol 40: 21–37
Kartavtseva IV, Pavlenko MV, Kostenko VA, Chernyavskii FB (1998) Chromosomal variation and abnormal karyotypes in the red-backed mouse *Clethrionomys rufocanus* (Rodentia, Microtinae). Russian J Genet 34: 928–935
Kashiwabara S, Onoyama K (1988) Karyotypes and G-banding patterns of the red-backed voles, *Clethrionomys montanus* and *C. rufocanus bedfordiae* (Rodentia, Microtinae). J Mamm Soc Japan 13: 33–41
Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111–120
Miyao T (1968) The gray red-backed vole, *Clethrionomys rufocanus bedfordiae*, from Rishiri Island. Hoppou-Ringyou 20: 169–172 (in Japanese)
Nakata K (1995) Microhabitat selection in two sympatric species of voles *Clethrionomys rex* and *Clethrionomys rufocanus bedfordiae*. J Mamm Soc Japan 20: 135–142
Obara Y, Kusakabe H, Miyakoshi K, Kawada S (1995) Revised karyotypes of the Japanese northern red-backed vole, *Clethrionomys rutilus mikado*. J Mamm Soc Japan 20: 125–133
Ohdachi S, Masuda R, Abe H, Adachi J, Dokuchaev NE, Haukisalmi V, Yoshida MC (1997) Phylogeny of Eurasian soricine shrews (Insectivora, mammalia) inferred from the mitochondrial cytochrome *b* gene sequences. Zool Sci 14: 527–532
Oshima K (1990) The History of straits around the Japanese islands in the late—Quaternary. Quat Res 29: 193–208 (in Japanese with English abstract)
Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–452
Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R, Goodfellow PN (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346: 240–244
Sokolov VY, Aniskin VM, Serbenyuk MA (1990) Comparative cytogenetics of 6 species of the genus *Clethrionomys* (Rodentia, Microtinae). Zool Zhur 69: 145–151 (in Russian with English summary)
Suzuki H, Minato S, Sakurai S, Tsuchiya K, Fokin IM (1997) Phylogenetic position and geographic differentiation of the Japanese dormouse, *Glirulus japonicus*, revealed by variations among rDNA, mtDNA and the Sry gene. Zool Sci 14: 167–173
Suzuki H, Iwasa M, Harada M, Wakana S, Sakaizumi M, Han SH, Kitahara E, Kimura Y, Kartavtseva I, Tsuchiya K (1999) Molecular phylogeny of red-backed voles in Far East Asia based on variation in ribosomal and mitochondrial DNA. J Mamm 80: 512–521
Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nuc Acids Res 22: 4673–4680
Tokuda M (1935) *Neoaschizomys*, a new genus of Microtinae from Sikotan, a south Kurile island. Mem Coll Sci Kyoto Imp Univ, Ser B X-3: 241–250
Tsuchiya K (1981) On the chromosome variations in Japanese cricetid and murid rodents. Honyurui Kagaku [Mammalian Science] 21: 51–58 (in Japanese with English summary)
Tsuda K, Kikkawa Y, Yonekawa H, Tanabe Y (1997) Extensive interbreeding occurred among multiple matriarchal ancestors during the domestication of dogs: Evidence from inter-and intraspecies polymorphisms in the D-loop region of mitochondrial DNA between dogs and wolves. Genes Genet Syst 72: 229–238
Vorontsov NN, Lyapunova EA, Borissov YM, Dovgal VE (1980) Variability of sex chromosomes in mammals. Genetica 52/53: 361–372
Wakana S, Sakaizumi M, Tsuchiya K, Asakawa M, Han SH, Nakata K, Suzuki H (1996) Phylogenetic implications of variations in rDNA and mtDNA in red-backed voles collected in Hokkaido, Japan, and in Korea. Mammal Study 21: 15–25
Zardoya R, Meyer A (1996) Phylogenetic performance of mitochondrial protein-coding genes in resolving relationships among vertebrates. Mol Biol Evol 13: 933–942

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