Phylogenetic implications of variations in rDNA and mtDNA in red-backed voles collected in Hokkaido, Japan, and in Korea

Shigeharu WAKANA¹, Mitsuru SAKAIZUMI², Kimiyuki TSUCHIYA³, Mitsuhiro ASAKAWA⁴, Sang Hoon HAN⁵, Keisuke NAKATA⁶ and Hitoshi SUZUKI^{7*}

¹Department of Genetics, Central Institute for Experimental Animals, Nogawa, Miyamae-ku, Kawasaki 213, ²Department of Environmental Science, Faculty of Science, Niigata University, Niigata 950-21, ³Experimental Animal Center, Miyazaki Medical College, Kiyotake, Miyazaki 889-16, ⁴Department of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069, ⁵Institute of Applied Zoology, Faculty of Agriculture, Hokkaido University, Sapporo 060, ⁶Hokkaido Forest Experiment Station, Bibai, 079-01, ⁷Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University, Kita 10, West 5, Kita-ku, Sapporo 060, Japan

(*To whom correspondence should be addressed)

Fax. 011-706-2225, E-mail. htsuzuki @ eesbio. hopudai. ac. jp

Abstract. Restriction fragment length polymorphisms (RFLPs) in nuclear ribosomal DNA (rDNA) spacers and mitochondrial DNA (mtDNA) were examined in red-backed voles collected in Hokkaido (Japan), and Korea. These voles have been classified into six species on the basis of morphological characteristics, such as dental morphology. The RFLPs of the rDNA allowed us to classify the voles into three distinct groups: rCrt (*Clethrionomys rutilus*), rCrf (*C. rufocanus*, *C. sikotanensis* and *Eothenomys regulus*) and rCrx (*C. rex* and *C. montanus*). The estimated sequence divergence between rCrt and rCrf and that between rCrf and rCrx were 4.8% and 2.3%, respectively. In the rCrf group, no major differences in mtDNA were observed among the populations from the mainland of Hokkaido, Rishiri Island, and Daikoku Islet. Similarly, in the rCrx group, mtDNA haplotypes from the mainland of Hokkaido and Rishiri I. were closely related each other, indicating that there have been genetic exchanges between the populations after speciation, or those haplotypes are derived from recent common origin. The Korean red-backed vole, which is sometimes referred to *E. regulus*, had rDNA identical to that of the rCrf group from Hokkaido. By contrast, the mtDNA haplotype of the Korean vole was substantially different from that of *C. rufocanus* in Hokkaido (8% sequence divergence). These results imply that the Korean red-backed vole and *C. rufocanus* in Hokkaido are very closely related and that ancestrally diverged mtDNA haplotypes have been maintained in the different geographic regions.

Key words. *Clethrionomys*, mitochondrial DNA, restriction fragment length polymorphism, ribosomal DNA, the red-backed vole.

Red-backed voles, which are small rodents that live in the fields and mountains of the Palaearctic Region, are extremely complicated in terms of taxonomy. Red-backed voles have been traditionally classified into two genera, *Clethrionomys* and *Eothenomys*, on the basis of differences in morphological characteristics, such as the presence or absence of rooting of the molars (Hinton 1926). However, it is uncertain whether or not such criteria are phylogenetically appropriate. In terms of morphological criteria, the red-backed voles living in Hokkaido, Japan, are classified as *Clethrionomys* because of the presence of rooting of the molars. On the mainland of Hokkaido, three different forms of vole are known: the Northern red-backed vole (*C. rutilus* or *C. r. mikado*); the gray red-backed vole (*C. rufocanus* or *C. r. bedfordiae*); and a recently identified form, represented by *C. rex* and *C. montanus* (Imaizumi 1971, 1972). Morphologically, *C. rufocanus* and *C. rex* are similar and some taxonomists question the classification of *C. rex* as a distinct species (Aimi 1980, Corbet 1978, Musser and Carleton 1993). Another taxonomic issue is the classification of voles on the peripheral islands of Hokkaido, and on Rishiri Island in particular, where the existence of two different forms of the red-backed vole, namely, *C. sikotanensis* and *C. rex*, have been reported (Imaizumi 1971). *C. sikotanensis* has, however, been considered as synonymous with *C. rufocanus* by Abe (1984) and Kaneko and Sato (1993). According to Imaizumi (1972), *C. rex*-like voles on the mainland should be designated *C. montanus*, a species different from *C. rex* on Rishiri I. However, Abe (1984) considered that *C. rex* and *C. montanus* are synonymous. It is of interest, moreover, that voles living on Daikoku Islet, adjacent to the southeastern coast of Hokkaido, are usually classified as *C. rufocanus* but display morphological characteristics of both *C. rufocanus* and *C. rex* (Abe 1984). Therefore, the unequivocal taxonomic classification of the voles on Daikoku I. has not yet been made (Abe 1984).

The Korean red-backed vole provides another intriguing question with respect to classification. It has been included in the genus *Clethrionomys* and classified as a species, *C. regulus*, that is endemic to Korea (Corbet 1978). In a detailed morphological study of voles from Russia and Korea, Kaneko (1990) showed that all of the examined specimens from Korea had rootless teeth, in contrast to the individuals from Russia and have subsequently been classified as *Eothenomys regulus* (Corbet and Hill 1991). The true geographical demarcation line between *C. rufocanus* and *E. regulus* lies on the western and southern boundary of the Kaima Plateau, North Korea (Kaneko 1990).

Morphological studies have not provided sufficient information about the classification of red-backed voles and a single morphological characteristic, such as rooting of molars, is insufficient for classification of a given species. Karyological studies have been made on some members of the complicated genera of *Clethrionomys* and *Eothenomys*. However, the karyotypes appear to be very similar and no informative variations have been reported (Kashiwabara and Onoyama 1988, Tsuchiya 1981, Yoshida *et al.* 1989).

Both rDNA and mtDNA provide powerful diagnostic markers for the identification of populations because 1) both mtDNA and rDNA exist as

multiple copies in the mammalian genome and, thus, are easily analyzed, and 2) many RFLPs are specific to each population. Combined analyses, exploiting both cytoplasmic and nuclear markers, should provide much more reliable information on the timing of divergence and the topology of the phylogenetic tree among populations of given animal species.

In the present study, we compared variations in rDNA and mtDNA among six morphologically different forms of red-backed voles collected on the mainland of Hokkaido, on Rishiri I., on Daikoku I. and in Korea. Here we demonstrate that the six taxa can be classified as three distinct species.

MATERIALS AND METHODS

1. Voles

A total of 36 voles (Table 1), collected from eight localities (Fig. 1), was used for the analysis of rDNA and mtDNA.

2. Blot analysis

Nuclear DNA was prepared from the liver of each vole as described by Maniatis *et al.* (1982). Southern blot analysis was carried out as described by Suzuki *et al.* (1994b). DNA was digested with eight restriction enzymes for the analysis of mtDNA (*Aat*I, *Apa*I, *Bam*HI, *Dra*I, *Eco*RI, *Hind*III, *Pst*I and *Xba*I) and with ten restriction enzymes for the analysis of rDNA (*Aat*I, *Bam*HI, *Bgl*II,

Table 1. List of samples used and specific types of morphology, rDNA and mtDNA.

Serial no. and locality	Name of species, as typed with morphological characteristics	rDNA reptype	mtDNA haplotypes (no. of individuals with common haplotypes)
Hokkaido, Japan			
1. Bekkai	<i>C. rutilus</i>	rCrt	mCrt1 (1)
2. Teshio	<i>C. rufocanus</i>	rCrt	mCr1 (4)
	<i>C. montanus</i>	rCrX	mCrX1 (4)
3. Tobetsu	<i>C. rufocanus</i>	rCr1	mCr1 (2), mCr2 (5)
			mCr3 (1)
4. Naganuma	<i>C. rufocanus</i>	rCr1	mCr1 (1), mCr2 (2)
			mCr4 (3)
5. Ohtaki	<i>C. rufocanus</i>	rCr1	mCr3 (1)
6. Rishiri I.	<i>C. sikotanensis</i>	rCr1	mCr5 (1)
	<i>C. rex</i>	rCrX	mCrX2 (1)
7. Daikoku I.	<i>C. rufocanus</i>	rCr1	mCr1 (8)
Korea			
8. Mt. Chiri	<i>E. regulus</i>	rCr1	mEr1 (2)

Fig 1. Localities at which red-backed voles were sampled. Numbers assigned to localities are the same as in Table 1.

DraI, *EcoRI*, *HindIII*, *PstI*, *PvuII*, *SacI*, and *XbaI*). The digested DNAs were immobilized on nylon filters and then allowed to hybridize sequentially with ^{32}P -labeled probes of rDNA, namely, 28S, 18SB, and INT (Suzuki *et al.* 1994b). A mtDNA probe used was the whole mtDNA genome that was prepared from the liver of a hamster, as described by Wakana *et al.* (1986). After hybridization, filters were washed twice with $2 \times \text{SSC}$ (0.3 M NaCl-0.03 M Na citrate solution with pH 7.0) containing 0.1% (w/v) sodium dodecyl sulfate at room temperature. Autoradiographs were obtained either by exposing hybridized membranes to X-ray film or with an image analyzer (Bio Image Analyzer, BAS2000, Fuji Film, Japan).

3. Construction of restriction maps of the rDNA

From the patterns of hybridization after single digestions, restriction maps were constructed for the coding and internal spacer regions of rDNA because most restriction sites in the coding region as well as a *Dra*I site in the internal spacer were conserved. By reference to restriction-site maps of the coding and the internal spacer regions, the location of the restriction sites on the external spacer region, which flanked the genes for 18S and 28S rRNA, was estimated by single digestion and hybridization with the 18SB and 28S probes. Since the probes were localized to the distal end of the coding regions, only the restriction sites nearest to the distal end of the genes for 18S or 28S rRNA could be mapped. Although length polymorphisms within the genome were observed in certain regions of the external spacer in most samples investigated, only the most prominent bands were taken into account for construction of the physical maps.

4. Construction of phylogenetic trees

To estimate the sequence divergence among the three major rDNA repetypes we compared the arrangement of restriction sites between pairs of repetypes and counted the number at common and different sites. The sequence divergence among haplotypes of mtDNA was estimated from the number of common and different restriction fragments observed. Employing a method developed by Gotoh *et al.* (1979), in which backward mutations and parallel mutations are taken into account, we produced a matrix of sequence divergences among all possible combinations of rDNA repetypes (Table 2) and mtDNA haplotypes (Table 3). Then we constructed phylogenetic trees using the unweighted pair-group (UPGMA) method (Sokal and Michener 1958) and the neighbor-joining (NJ) method (Saitou and Nei 1987).

Table 2. Sequence divergence among the rDNA repetypes (upper right), based on the number of common and different restriction sites (lower left).

		Sequence divergence (%)		
		rCrt	rCrX	rCrF
rCrt		—	6.4	4.8
rCrX	28S*	6/4**		
	18S	5.5/4.5	—	2.3
	INT	1/3		
	total	12.5/11.5		
rCrF	28S	6/4	8/2	
	18S	6.5/3.5	8/2	—
	INT	2.5/2.5	2.5/1.5	
	total	15/10	18.5/5.5	

* The external (28S, 18S) spacer region and the internal spacer and coding regions (INT).

**Number of sites (common/different).

Table 3. Sequence divergence among the mtDNA haplotype (upper right), based on the number of common and different restriction fragments (lower left).

Haplo- type	Sequence divergence (%)								
	mCrt1	mCrX1	mCrX2	mCrF1	mCrF2	mCrF3	mCrF4	mCrF5	mErg1
mCrt1	—	11.7	11.8	8.3	8.3	10.3	8.4	8.4	11.3
mCrX1	5/49*	—	0.3	7.5	6.8	7.5	8.6	6.8	7.1
mCrX2	5/50	26/3	—	7.8	6.1	7.8	8.8	7.6	8.2
mCrF1	8/43	8/37	8/39	—	0.3	0.7	0.3	0.7	7.1
mCrF2	8/43	9/36	11/37	25/3	—	1.1	0.3	0.7	8.3
mCrF3	6/47	8/37	8/39	24/6	25/3	—	0.7	0.7	7.1
mCrF4	8/44	7/40	7/42	26/3	24/3	24/6	—	0.7	7.3
mCrF5	8/44	9/36	8/38	24/6	22/6	22/6	24/6	—	7.9
mErg1	5/46	8/34	7/37	8/34	7/38	8/34	8/35	7/35	—

*Number of fragments (common/different).

RESULTS

We analyzed rDNA from 36 individual voles, namely, 34 from Hokkaido and two from Korea. The voles represented all six possible different morphological forms, as listed in Table 1. The analysis revealed that there were three major rDNA repetypes (rCrt, rCrF and rCrX) and nine different mtDNA haplotypes (mCrt1, mCrX1, mCrX2, mCrF1, mCrF2, mCrF3, mCrF4, mCrF5 and mErg1) among the voles examined. The restriction maps of the three repetypes of rDNA are presented schematically in Fig. 2. Although most of the mutations appeared to have been fixed within populations, some mutations were not fixed within a genome. The restriction sites that showed such intragenomic polymorphisms are marked on restriction maps with asterisks. In this study, we counted substantial heterogeneous variation at a particular site as one half of a common site and one half of a different site. Minor bands on the blotting patterns were ignored.

On the basis of the extent of sequence divergence (Tables 2 and 3), we constructed phylogenetic trees for the mtDNA haplotypes and rDNA repetypes by the UPGMA method (Fig. 3). We also constructed phylogenetic trees by the NJ method and obtained trees with topology essentially identical to those presented in Fig. 3. The trees revealed new criteria for classification of the voles. The rDNA and mtDNA trees indicated that *C. rutilus* (rCrt/mCrt1) diverged first from the others. Two lineages of *C. rufocanus* (rCrF/mCrF1-5) and *C. rex* (rCrX/mCrX1, 2) then diverged. Interpopulational differentiation has been low in both cases.

The mtDNA haplotype of the Korean vole (mErg1) was substantially different (8% sequence divergence calculated from Table 3) from those of *C. rufocanus* from Hokkaido (mCrF1, mCrF2, mCrF3, mCrF4 and mCrF5), even though individuals from Korea and Hokkaido had the same rDNA repetype rCrF.

Fig. 2. Restriction maps of the major rDNA repetypes of *Clethrionomys rutilus* (rCrt), *C. rufocanus* (rCrF), and *C. rex* (rCrX). With respect to the restriction sites on the flanking spacers, only those nearest to the distal end of the genes for 18S or 28S rRNA are shown. The top diagram shows the conserved restriction sites in the coding and the internal spacer regions of the genes for 18S and 28S rRNA (see text), which are not represented in the lower maps. Positions of probes are also shown by arrows. Small characters represent types of restriction sites identified after a comparison of restriction maps. Asterisks indicate polymorphic sites within the genome of a given species. A, *Aat*I; B, *Bam*HI; D, *Dra*I; E, *Eco*RI; G, *Bgl*II; H, *Hind*III; P, *Pst*I; S, *Sac*I; V, *Pvu*II; X, *Xba*I.

Fig. 3. Phylogenetic trees for the mtDNA haplotypes and major rDNA repetypes constructed by the UPGMA methods. Abbreviations are the same as in Table 1. Divergence time were estimated by assuming the rate of evolution to be 2-4% per Myr for mtDNA RFLPs and 1-2% per Myr for rDNA RFLPs.

DISCUSSION

In the present study, we obtained a new perspective on the phylogeny of red-backed voles in Hokkaido and Korea. Our data indicate that three true species of red-backed voles exist in Hokkaido, namely, *C. rex*, *C. rufocanus* and *C. rutilus*. The two morphological forms of the red-backed voles reported by Imaizumi (1971, 1972), namely, *C. rex* from Rishiri I. and "*C. montanus*" from the Hidaka Mountains, Hokkaido, can be regarded as synonymous species, as described by Abe (1984). Similarly, we found that "*C. sikotanensis*" from Rishiri I. (Imaizumi 1971) and voles from Daikoku I. were very closely related to *C. rufocanus* from the mainland of Hokkaido on the basis of rDNA and mtDNA sequences. Thus "*C. sikotanensis*" from Rishiri I. and the voles from Daikoku I. can all be regarded as *C. rufocanus*.

The Korean red-backed vole collected on Mt. Chiri, which was a member of a species that is usually known as *E. regulus* (Corbet and Hill 1991), had the same rDNA RFLP profile as individuals collected in Hokkaido with respect to the 24 restriction sites that were investigated. Since the rDNA RFLP exhibits similar patterns within the same reproductive population and distinct patterns when different reproductive populations are compared (Arnheim *et al.* 1980, Coen *et al.* 1982, Suzuki *et al.* 1986, 1987, 1990, 1994a, 1994b), these data indicate that the Korean red-backed vole is closely related phylogenetically to *C. rufocanus*. The absence of rooting of the molars in the Korean vole (Kaneko 1990, 1992) is a characteristic that may have developed within a short period of evolutionary time in the Korean population. Although the Korean red-backed vole has been classified as *Eothenomys* on the basis of its dental characteristics (Hinton 1926), it is closely related to *C. rufocanus*. Hinton's criterion, whereby voles are classified into different genera on the basis of rooting of molars, may include such exception or itself be inappropriate.

The mtDNA haplotype of the Korean vole was substantially different from those of *C. rufocanus* from Hokkaido, Japan (Fig. 3). Since the major rDNA repetype was identical for the Korean vole and the populations in Hokkaido, the existence of a distinct mtDNA haplotype might be due to the difference in the mode of inheritance between rDNA and mtDNA. Such phenomena are frequently observed in many organisms, including mammalian species (e.g.; Ferris *et al.* 1983, Tegelström 1987, Yonekawa *et al.* 1988). For example, in the case of Japanese house mice, the major genetic elements are those of *Mus musculus musculus*, which were introduced from the Asian continent, and the minor genetic elements are those of *M. m. castaneus*, which is thought to have existed on the Japanese islands in ancient times. In Hokkaido and the northern part of Honshu, *castaneus*-type mtDNA exists dominantly as a relict component (Yonekawa *et al.* 1988). In the case of the Korean red-backed vole described in this study, the difference in the rDNA and mtDNA phylogenies may have been due to the maintenance of ancestrally diverged mtDNA molecules in the different geographic area, Korean Peninsula and Hokkaido. The

timing of the divergence of mtDNAs of the Korean vole and *C. rufocanus* from Hokkaido (approximately 8% sequence divergence, Table 3) is estimated to be 2–4 million years ago (Mya), assuming that the rate of evolution of mtDNA to be 2–4% per 1 million years (Myr) (Wilson *et al.* 1985). The timing of the divergence corresponds to that of the divergence of *C. rufocanus* and *C. rex* (Fig. 3).

The sequence divergence between the rDNA repetypes of *C. rufocanus* and *C. rex*, rCrF and rCrX, was 2.3% (Table 2) and the timing of divergence between the two repetypes was estimated to be 1.2–2.4 Mya on the assumption that the rate of evolution of the spacers of rDNA has been 1–2% per 1 Myr (Hosoda *et al.* 1993, Suzuki *et al.* 1994b). The timing of the divergence of mtDNAs of *C. rufocanus* and *C. rex* (8% sequence divergence, Table 3) is estimated to be 2–4 Mya, as mentioned above. Thus, it is suggested that dispersal and species differentiation occurred in the ancestral populations of *rufocanus/rex* about 2 Mya, at the beginning of the glacial age. The sequence divergence between the repetypes of rCrF/rCrX and rCrT was about 6% (Table 2 and Fig. 3) and the timing of divergence between the two group was estimated to be 3–6 Mya. Analysis of mtDNA also support the estimation of time of the *rufocanus/rex-rutilus* split (Fig. 3).

We now propose the following process of species differentiation. (1) The early expansion of red-backed voles in the Palaearctic Region occurred 2.5–5 Mya (see Fig. 3). At that time, *C. rutilus* was diverged from the lineage of *rufocanus/rex*. (2) About 1–2 Mya, the lineage of *rufocanus/rex* differentiated into two species. If *C. rex* is endemic to Hokkaido, the ancestral population that migrated to Hokkaido at that time was the founder population of *C. rex*. (3) In the following glacial period, *C. rufocanus* moved to Hokkaido from the Asian continent. Finally, 0.01–0.02 Mya, when Hokkaido and the continent were last joined with a land bridge (Japan Association for Quarternary Research 1987), populations of *C. rufocanus* communicated genetically among the mainland of Hokkaido and peripheral islands, and the continent. Similarly, populations of *C. rex* on the mainland of Hokkaido and Rishiri I. also communicated genetically at that time.

The evolutionary history of the Korean red-backed vole, *E. regulus*, is currently unknown, but is likely to be well correlated to those of *C. rufocanus*. Molecular identification with various markers will clarify the extent of genetic interchanges between the Korean vole and *C. rufocanus*.

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