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Phylogenetic Position and Geographic Differentiation of the Japanese Dormouse, *Glirulus japonicus*, Revealed by Variations among rDNA, mtDNA and the *Sry* Gene

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ABSTRACT—The Japanese dormouse, *Glirulus japonicus*, is the only extant lineage that represents this genus and it has been classified as a single species distributed on the three main islands of Japan, namely Honshu, Shikoku and Kyushu. However, individuals collected from Fukui, Wakayama and Kochi Prefectures (southwestern part of Japan) yielded distinctly different profiles of restriction fragments of the nuclear ribosomal DNA (rDNA) spacer from those collected from Yamanashi and Nagano Prefectures (central Japan). The estimated sequence divergence between the two groups was 2.8% on average, which corresponds to a putative divergence some two million years ago. Representing mitochondrial DNA (mtDNA) sequences, 402 bases of cytochrome *b* gene were determined by direct sequencing and the estimated extent of the sequence divergence between the two groups was 6.5-7%. Differences between the two geographic groups were also substantial in the sequences of about 300 base-fragments from the Y-linked, sex-determining locus, *Sry*. To assess the phylogenetic relationships between the Japanese dormouse and members of the family Myoxidae, we compared sequences of mitochondrial 12S rRNA gene of Japanese dormice with those of the forest dormouse (*Dryomys nitedula*) and the common dormouse (*Muscardinus avellanarius*), two continental genera thought to be closely related to the genus *Glirulus*. The results showed that the sequences from Japanese dormice were distinct from any sequences of the two continental species and the extent of the differences were somewhat similar to that between the rat (*Rattus norvegicus*) and the hamster (*Mesocricetus auratus*).

INTRODUCTION

Japan has many species of mammals because it is located near the three developmental zones of animals, Central Asia, the Oriental Region and Siberia and there were several periods of attachment during the past several million years. Japanese mammals are also complex since Japan is long in a south to north direction and there are many geographic borders, especially for small mammals. To understand the organization of Japanese mammals, we have performed molecular phylogenetic studies (Suzuki *et al.*, 1990, 1994a,c; Hosoda *et al.*, 1993; Wakana *et al.*, 1996) . Here we investigated the origin and geographic differentiation of the Japanese dormouse (*Glirulus japonicus*).

The genus *Glirulus* represents fossil and extant species of dormice. The oldest fossil relative was discovered in the geological layers of the early Miocene, Pliocene and early Pleistocene in Europe (Holden, 1993). The single extant species within this genus is the Japanese dormouse, which currently lives on the three main islands of Japan: Honshu, Shikoku, and Kyushu (Fig. 1). Studies of this rare species have raised two major phylogenetic issues.

First, morphological and behavioral characteristics vary geographically (S. Minato, unpublished data). For example, the fur of individuals in central areas of Japan is grayish-brown, while that of individuals in southeastern area of Japan is dark brown (S. Minato, unpublished data). There are also differences in some ecological behavior, such as the mating season (S. Minato, unpublished data). Hence it is necessary to estimate the genetic differentiation between the two morphological forms of the Japanese dormouse.

Second, its phylogenetic position among the members of family Myoxidae remains to be determined (Holden, 1993;

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Fig. 1. Geographic distribution of the Japanese dormouse. Numbers refer to the collection sites of the dormice listed in Table 1.

Wahlet *et al.*, 1993). At present, there are three independent notions (Holden, 1993; Wahlet *et al.*, 1993) as follows. *Glirulus* and related fossil genera belong to a unique subfamily Glirulinae, independent of the seven other well-defined subfamilies of Myoxidae; they belong to the subfamily Dromyinae which is represented by the genus *Dryomys*, or they belong to the subfamily Myoxinae which is represented by the genus *Muscardinus*.

We examined these issues using four phylogenetic markers: restriction site length polymorphism (RFLP) of the nuclear gene for ribosomal RNA (rDNAs; Hosoda *et al.*, 1993; Suzuki *et al.*, 1990, 1994a,b); sequence differences in the Y-linked, sex-determining locus, *Sry* (Gubbay *et al.*, 1990); and sequence differences in the cytochrome *b* and 12S rRNA genes (Kocher *et al.*, 1989) within the mitochondrial DNA (mtDNA). Using the first three markers, we examined intraspecies differences among four populations of the Japanese dormouse. We analyzed the RFLP of rDNA and variations in the 12S rRNA sequences, to determine genetic relationships among the Japanese, the forest (*Dryomys nitedula*, subfamily Dromyinae), and the common dormouse (*Muscardinus avellanarius*, subfamily Myoxinae). We found that there are at least two phylogenetically and geographically distinct populations of the Japanese dormouse. We also found that this species diverged from *Dryomys nitedula* and *Muscardinus avellanarius* at about the same time, perhaps during the early Miocene period.

MATERIALS AND METHODS

DNA preparation

Permission from the Environmental Agency and the Agency for Cultural Affairs of Japan was given to S. Minato to examine specimens of the Japanese dormouse. A total of seven individual Japanese dormice, one forest dormouse and one common dormouse (Table 1, Fig. 1) were phylogenetically compared. Skin samples were cultured by a standard method without sacrificing the animals, then DNA was isolated as described by Maniatis *et al.* (1982). Nuclear DNA was also prepared from the liver tissue of a Syrian hamster, which was a gift from Dr. A. Matsuda (Institute of Medical Science, University of Tokyo).

Construction of restriction maps of the nuclear rDNA spacers

Southern blots were performed as described by Suzuki *et al.* (1994b). To construct restriction maps for the various types of repeating unit of rDNA (repetypes), genomic DNA was digested with the restriction enzymes, *Aat*I, *Bam*HI, *Bcl*I, *Bgl*II, *Dra*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, and *Xba*I and resolved by electrophoresis. The fragments were transferred to nylon filters and sequentially hybridized with the ^{32}P-labeled rDNA probes, 18SB, 28S, and INT (see Fig. 2). We constructed restriction maps for the coding and spacer regions of genes for rRNA based on hybridization profiles after single digestions (Suzuki *et al.*, 1994b).

Direct sequencing

"Semi-nested" polymerase chain reactions (PCRs) and direct sequencing were performed according to instructions supplied with the automated sequencer (ABI, Division of Applied Biosystems, Perkin Elmer). DNA was amplified in 20-μl reaction mixtures containing 10 mM Tris (pH 8.3), 50 mM KCl, 0.01% gelatin, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.05 μM each primer (1 pmol of each primer per reaction), 0.5 units of Amplitaq DNA polymerase (Perkin Elmer) and 0.1-0.5 μg of template total genomic DNA using an automated thermal cycler (model PJ2000; Perkin Elmer). The thermal cycling parameters were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 60°C for 1 min. A 0.5-μl aliquot of each reaction mixture after PCR, was used as the template for the second PCR in a 20-μl reaction mixture with the same reagents and under the same conditions as the first PCR, apart from the primer pairs and the concentration of $MgCl_2$ (1.25 mM). To construct the primer pair for the second PCR, the sequence of the 18-meric dye-labeled primer, M13RP1 (R) or -21M13 (U), from ABI was attached to the 5' end of each of the gene-specific primers. Both 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).

Sequencing the mitochondrial gene for cytochrome b

A 1.2-kb fragment of the gene for cytochrome *b* was amplified as described above for the first PCR using the primers [universal primers of Kocher *et al.* (1989)] L14724 and H15915 (Fig. 2b). L and H refer to light and heavy strands. Numbers refer to the position of the 3' base of the primer in the complete sequence of human mtDNA (Anderson *et al.*, 1981). For the second PCR, the 3' part of the forward primer, R-L14724 (5'-CAGGAAACAGCTATGACCGATATGAAAAA-CCATCGTTG-3'), was a shortened version of Kocher's universal primer (Kocher *et al.*, 1989) and the reverse primer, U-H15155 (5'-TGTAAAACGACGGCCAGTTGCCCCTCAAAAGGATATTTG-3'), was a sequence that should anneal to all mammalian mtDNAs around nucleotide positions 15155-15176 in view of the known sequences of mammalian genes for cytochrome *b* (Irwin *et al.*, 1991).

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Sequencing of the mitochondrial gene for 12S rRNA

A 0.9-kb fragment of the 12S rRNA gene was amplified by PCR (Fig. 2c) with the primer pair L613 and H1478 (Kocher *et al.*, 1989). Then first half of the fragment was amplified with the primer pair R-L613 (5'-CAGGAAACAGCTATGACCACACAAAGCATGGCACTG-AA-3') and U-H1066 (5'-TGTAAAACGACGGCCAGTGGGGTATCTA-ATCCCAGTTTG-3'). The second half was amplified with the primer pair R-L1091 (5'-CAGGAAACAGCTATGACCACTGGGATTAGATAC-CCCACTAT-3') and U-H1478 (5'-TGTAAAACGACGGCCAGTCAGA-GGGTGACGGGCGGTGTG-3'). To sequence the region around nucleotide position 1091, a fragment of 0.9 kb was amplified with the primer pair R-L613 and U-H1478, and the product was purified by agarose gel electrophoresis using a Geneclean Kit (BIO 101 Inc.). The amplified fragments were subsequently sequenced described above.

Sequencing of flanking portion of the Sry *gene*

The *Sry* gene is located on the Y chromosome and is the most likely sex-determining gene in mammalian genomes (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990). The *Sry* gene has a conserved DNA-binding motif, known as the HMG box, while its flanking regions have rapidly evolved and are male-specific phylogenetic markers for related species and subspecies (Lundrigan and Tucker, 1994; Nagamine *et al.*, 1994). The HMG box provided one side of the primer-anchoring regions for amplification by PCR. To choose other primer-anchoring sites, we compared the sequences of the human and murine *Sry* genes (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990) by analyzing a homology plot and found an appropriate region about 300 bp upstream from the 3' end of the HMG box. PCR was performed first with the primer pair SRY286 (5'-GTTGGGCGGGGTTGAGGGGGTGTTGAGGGCGGAG-3') and HMG777 (5'-GGCCGATACTTATAGTTTCGGGTATTT-3'). Fragments were then amplified with R-SRY286 (5'-CAGGAAACAGCTATGACCTGAGGGGGTGTTGAGGGCGGAG-3') and U-HMG597 (5'-TGTAAAACGACGGCCAGTCACACGATAAATG-CGTTCATGGG-3') as primers. The number of each primer refers to the position of the 3' base of the primer in the sequence of the human *Sry* gene (Sinclair *et al.*, 1990).

Construction of phylogenetic trees

To estimate sequence divergence among major rDNA repetypes, we compared the arrangement of restriction sites between pairs of rDNA repetypes. We counted common and different sites for a total of about 27 sites, namely 24 sites from both sides of the external spacer region and a few from the internal spacer regions, as described criteria (Suzuki *et al.*, 1994b). We used the method developed by Gotoh *et al.* (1979), in which backward mutations and parallel mutations are taken into account (Jukes and Cantor, 1969), to produce a matrix of sequence divergence among all possible combinations of repetypes. Sequences of the gene for 12S rRNA were aligned manually, with introduction of gaps to maximize homology. Excluding gap regions, we selected 804 bp with which to consider sequence divergence. We produced a matrix of sequence divergence for all possible combinations of sequences of genes for cytochrome *b* (402 bp) and 12S rRNA (830 bp) using a computer program (DNADIST), with a "Kimura's 2-parameter" option, in PHYLIP 3.5 developed by Felsenstein (1993). We constructed phylogenetic trees by the neighbor-joining (NJ) method (Saitou and Nei, 1987) and performed a bootstrap analysis (500 replications) to check the topology for robustness using software (SEQBOOT and NEIGHBOR) in PHYLIP 3.5.

RESULTS

Intraspecies variation in the Japanese dormouse

We analyzed restriction length polymorphism (RFLP) in the spacers of the genes for rRNA, which provides genetic relationships between closely related species or populations (Suzuki *et al.*, 1990, 1994a; Wakana *et al.*, 1996). DNA from a total of nine individuals (Table 1), including seven Japanese dormice from four localities (Fig. 1) and two individuals of continental species, the common dormouse and the forest dormouse, was Southern hybridized after digestion with 12 restriction enzymes. Five restriction maps of rDNA (Fig. 2) constructed from the hybridization profiles, represented the major repetypes of these individuals. We compared the repetypes and estimated the sequence divergence (Table 1) according to the criteria described in Materials and Methods. We then constructed a phylogenetic tree by the NJ method

Table 1. List of samples and sequence divergence among the major repetypes of rDNA

Species Collection site	n*	Repetype	Sequence divergence (%)** Nagano	Wakayama	Kochi	Forest	Common
Glirulus japonicus (Japanese dormouse)							
1. Nagano Pref., Japan	2	Nagano	–	2.9	3.3	15.7	11.1
2. Yamanashi Pref., Japan	2	Nagano					
3. Fukui Pref., Japan	1	Wakayama	19.5/7.5	–	0.6	15.1	16.8
4. Wakayama Pref., Japan	2	Wakayama					
5. Kochi Pref., Japan	1	Kochi	19.5/8.5	26/2	–	15.7	16.8
Dryomys nitedula (forest dormouse)							
6. Russia	1	Forest	6.5/19.5	7/20	7/21	–	8.9
Muscardinus avellanarius (common dormouse)							
7. Russia	1	Common	9.5/17.5	6.5/21.5	6.5/21.5	10.5/14.5	–

*Number of samples examined.

**Sequence divergence (upper right) and total number of sites (common/different) for the spacer upstream from the gene for 18S rRNA, the internal spacer region, and the spacer region downstream from the gene for 28S rRNA (lower left).

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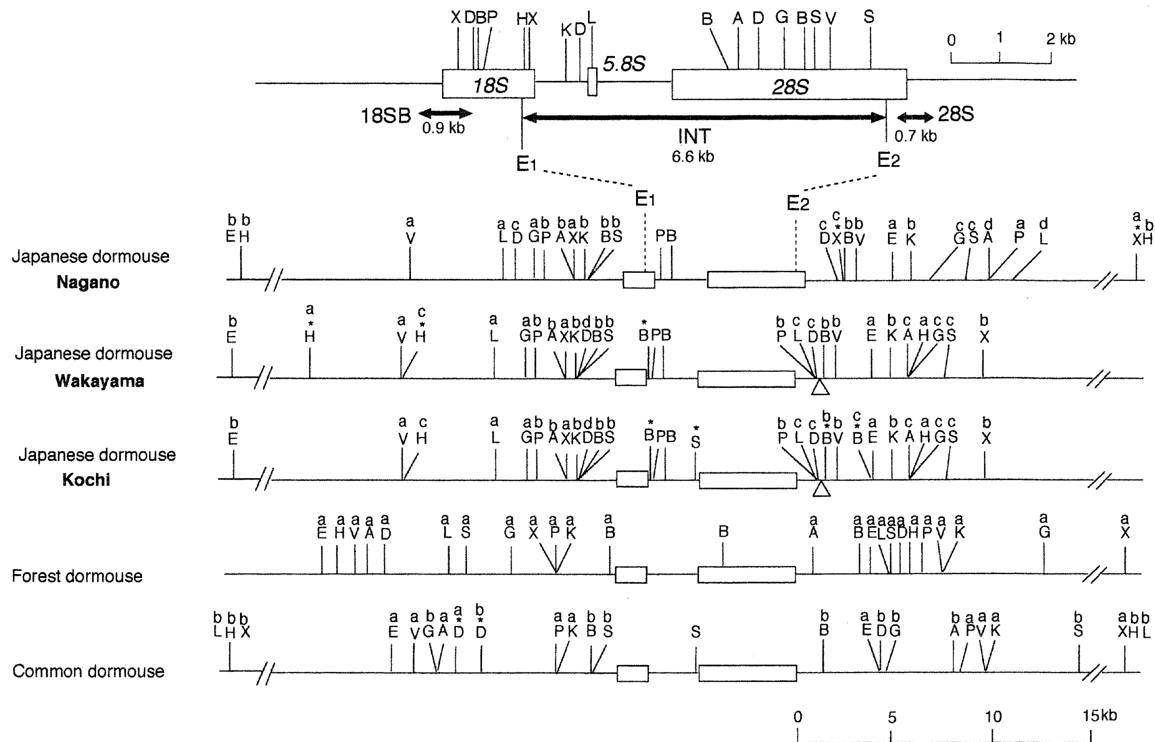

Fig. 2. Restriction maps of the major rDNA repetypes of the Japanese dormice from Nagano, Wakayama, and Kochi Prefectures, and of continental forest and common dormice. With respect to the restriction sites on the flanking spacers, only those nearest the distal end of the genes for 18S or 28S rRNA are shown. The top diagram shows the conserved restriction sites in the coding and internal spacer regions of the genes for 18S and 28S RNA (see text), which are not represented in the lower maps. Positions of probes are also shown by arrows. Asterisks and brackets indicate polymorphic sites that show substantial heterogeneity within genomes and among localities, respectively. Small characters represent types of sites identified after comparing restriction maps. A, *Aat*I; B, *Bam*HI; D, *Dra*I; E, *Eco*RI; G, *Bgl*II, H, *Hind*III; K, *Kpn*I; L, *Bcl*I; P, *Pst*I; S, *Sac*I; V, *Pvu*II; X, *Xba*I.

(Fig. 3a). In agreement with the morphological and behavioral findings, we found considerable intraspecies differences among the Japanese dormouse. As shown in Fig. 2 and Table 1, individuals from Nagano and Yamanashi Prefectures, had the same repetype, Nagano. Individuals from Fukui and Wakayama Prefectures, also had the same repetype, Wakayama. The Wakayama repetype differed slightly from that of the individual from Kochi Prefecture, with an estimated SD of 0.6% (Table 1). However, the Nagano repetype was quite distinct from the those of southwestern Japan, namely, Fukui, Wakayama and Kochi. The average SD between them was 2.8% (7-7.5 sites were different among 28-29 sites examined), which is relatively high for populations that belong to the same species: the SD value was higher than between two subspecies of mouse, *Mus musculus domesticus* and *M. m. musculus* (1.5% SD, four sites differed among 24 examined; Suzuki *et al.*, unpublished data), or between two related species of field mice, *Apodemus flavicollis* and *A. sylvaticus* (1% SD; Suzuki *et al.*, 1990). Assuming that the rate of evolution of the spacer of rDNA is 1-2% per million years (Myr; Suzuki *et al.*, 1994b), the estimated time of the split between the Nagano-Yamanashi and Wakayama-Fukui-Kochi groups of the Japanese dormouse was 1.4-2.8 million years ago (Mya). The Wakayama and Kochi repetypes differed slightly, with estimated an SD of 0.6% (Table 1).

An apparent intraspecies divergence in sequences of mitochondrial genes for cytochrome *b* was also observed in populations of Japanese dormice. An examination of 402 bp fragments of the cytochrome *b* gene from ten dormice from six localities (Table 1) revealed six sequences (haplotypes) that were specific to each locality. We constructed a phylogenetic tree by the NJ method based on these sequences (Fig. 3b). The sequences from individuals from the central Japan, namely, Nagano and Yamanashi Prefectures were similar to each other but distinct from those from southwestern Japan, namely, Fukui, Wakayama and Kochi Prefectures (Fig. 3b). The SD was over 7%, which corresponds to divergence 2.8 Mya if the rate of evolution of the gene for cytochrome *b* is 2.5% per Myr (Irwin *et al.*, 1991; Masuda and Yoshida, 1994). In contrast to the results obtained for rDNA, the mtDNA haplotype from Wakayama was distinct from those from Fukui and Kochi with SD of 9.2 and 9.1%, respectively. The inconsistency between the results for rDNA and mtDNA might reflect the specific nature of mtDNA inheritance. The mtDNA phylogeny reflects only the movement of female individuals. Genetic interchanges among various geographic populations might have occurred during the ice age (1-2 Mya), when Honshu and Shikoku (Fig. 1) were attached by land bridges

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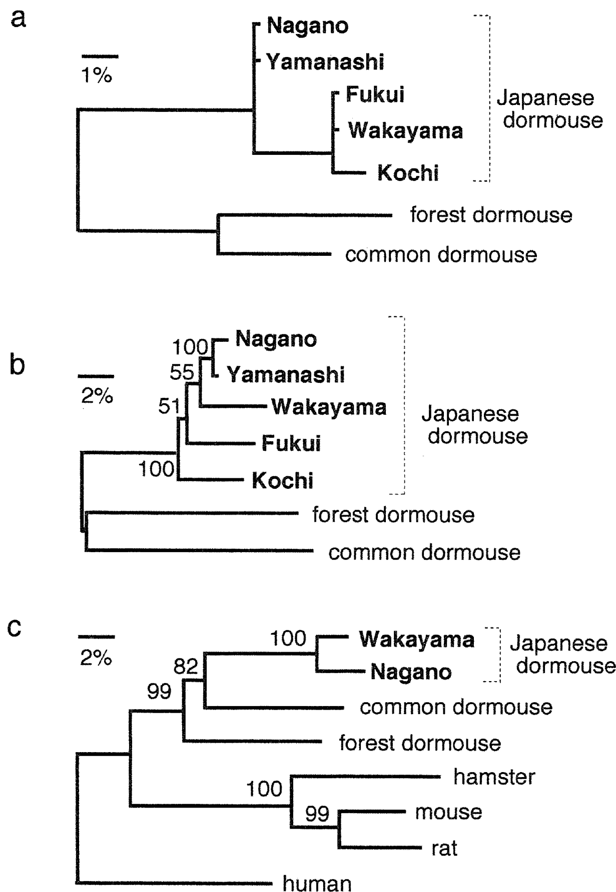

Fig. 3. Neighbor-joining trees showing the phylogenetic position and geographic variation of the Japanese dormouse inferred from (**a**) the nuclear rDNA-RFLP, (**b**) mitochondrial cytochrome *b* gene sequences, and (**c**) mitochondrial 12S ribosomal RNA gene sequences. In trees **b** and **c**, bootstrap scores expressed in percentages out of 500 replicates are given above each branch. The human sequence (Anderson *et al.*, 1981) was used to root tree **c**. Japanese dormice were collected from Nagano, Yamanashi, Fukui, Wakayama, and Kochi Prefectures.

on several occasions. Such discontinuous geographic communication might have influenced the close relationship between the rDNA repetypes from Wakayama and Kochi. Since mtDNA is maternally inherited and the choice of certain haplotypes after populational fusion and subsequent isolation must be determined in a stochastic manner, ancestrally diverged haplotypes might have been preserved in some localities.

While the geographic distribution of mtDNA haplotypes depends on the movement of female individuals, intraspecific differentiation of the *Sry*-associated region must reflect the movement of male individuals. To examine the extent of the geographic differentiation in the Y chromosome-specific region in the Japanese dormouse, we sequenced approximately 300 bp of the flanking region of the HMG box in three male individuals, one from Yamanashi, one from Wakayama, and one from Kochi Prefecture (Fig. 4). The extent of sequence differences was not very high but one base change and one gap differed between the sequences from Yamanashi and Wakayama, while those from Wakayama and Kochi differed by only a single base change. In a 515-bp region from *Sry* in *M. musculus* (Lundrigan and Tucker, 1994), the intraspecific sequence difference ranges from 0.39% (2/515, between European *M. m. musculus* and *M. m. domesticus*) to 0.78% (4/512, between Asian *M. m. musculus* and *M. m. domesticus*). Our data indicate that phylogenetic differences between the two groups of Japanese dormice from central and southwestern Japan, are also evident in the *Sry*-associated region. In addition, our data indicate that the Sry variation will be useful in analyzing populational differentiation.

Interspecies relationships of the Japanese dormouse

To estimate the extent of interspecies differences between Japanese and continental common and forest dormice, we examined sequences of the mitochondrial DNA as well as the RFLP of the nuclear rDNA. The estimated SD among the genes for cytochrome *b* from the three species was over 20%. The extent of substitution is likely to be saturated and thus, these data may not reflect the true interspecies phylogenetic relationship. The estimated SD among the three species on the basis of the RFLP of nuclear rDNA was over 13% (Table 1, Fig. 3a). Although the data suggest that these species diverged at quite an ancient time, for example 10 Mya, the reliability of the estimation might be not very high because the extent of changes in restriction sites tended to be saturated. Sequence analysis of the mitochondrial gene for 12S rRNA should provide useful information over a longer evolutionary period (Mindel *et al.*, 1991). We sequenced about 830 bp of the gene for 12S rRNA from two individuals of the Japanese dormouse from Wakayama and Nagano, one common and one forest dormouse. To compare the extent of divergence with those of other relatively well-examined rodents, we also sequenced this region from the Syrian hamster (*Mesocricetus auratus*) and obtained the analogous human, mouse and rat sequences from a database (Anderson *et al.*, 1981; Bibb *et al.*, 1981; Kobayashi *et al.*, 1981). As shown in Fig. 3c, the extent of divergence between the Japanese and forest dormice was similar to that between the Japanese and common dormice, suggesting that these three species diverged at about the same time. The extent was similar to that of divergence between the rat and the hamster.

DISCUSSION

Japanese dormice have been classified into a single taxon without any subdivision into subspecies or local races. However, according to variations in the rDNA, the mtDNA and the *Sry* gene, Japanese dormice can be subdivided into at least two groups, which we tentatively refer to as Nagano-Yamanashi and Wakayama-Fukui-Kochi populations. From the extent of divergence it can be assumed that the split occurred some two Mya. The extent of the divergence is similar to that between species, such as field mice (Suzuki *et al.*, 1990) and voles (Wakana *et al.*, 1996). This subdivision of

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```
           1        10        20        30        40        50        60        70
Yamanashi  AATGAATCGTTACAAAGGTTAAGGTGGTA---CCATTTTGAATCATACAATAAGTTTCCGAACTCTTACA
Wakayama   .............................AAG......................................
Kochi      ...T.........................AAG......................................

          71        80        90       100       110       120       130       140
Yamanashi  AATTTTGGATTGTGTTCCTTTGTTTTTTGTTTTGTTTTTAACGGTGCAATCTTAGGCTTCTGCTATGTTC
Wakayama   ...................C..................................................
Kochi      ...................C..................................................

         141       150       160       170       180       190       200       210
Yamanashi  AGAGAATTGGGCGGCGGTAATTACAGTCTAACCCCGCAGCAACGGGACGACCTTACCTTTCGGAAAAACT
Wakayama   ......................................................................
Kochi      ......................................................................

         211       220       230       240       250       260       270       280
Yamanashi  CTTCTTTCCCTTGGACCGACAACCCTAGCTTAAATTATCAGCGTGATATGGGAGGAAGCAGTAAAGAGGG
Wakayama   ......................................................................
Kochi      ......................................................................

         281       290       300
Yamanashi  AGTCCAGGATCGGGTAAAACGA 3'
Wakayama   ......................
Kochi      ......................
```

Fig. 4. Sequences of the region of the Y-linked, sex-determining locus, *Sry*, of Japanese dormice from Yamanashi, Wakayama, and Kochi Prefectures.

Japanese dormice populations corroborates the differences in morphological and behavioral features of the two groups (see Introduction).

The mechanism or process which is responsible for such subdivision in the populations of Japanese dormice is unclear. However, Honshu Island harbors well-differentiated, but related geographic populations or species of small mammals. The borders that divide many genetically differentiated populations or species are considered to be located around central Honshu, such as those of field mice and moles. For instance, the border that separates karyotypic differences in the Japanese field mouse, *Apodemus speciosus*, is located in central Honshu (Tsuchiya *et al.*, 1973). The Kii Peninsula, which includes Wakayama prefecture, also includes several borders that demarcate regions of genetic differentiation in moles and voles. *Eothenomys imaizumii*, the so-called Wakayama red-backed vole, occurs only on the southern part of the Kii Peninsula (Jameson, 1961). In future studies, it is necessary to compare the location of borders and extent of differentiation on the Japanese islands with several DNA markers for each of these small terrestrial species of mammals, including dormice, to understand their evolutionary history and the mechanisms of the genetic subdivision of species.

The Japanese dormouse has a phylogenetic lineage distinct from those of forest and common dormice (Fig. 3). The extent of the divergence of genes among the three species of dormouse examined was similar to that between the rat and the hamster (Fig. 3c). Comparisons of various DNA sequences indicate that the murid-hamster split is about 1.6 times older than the rat-mouse split, which is thought to have occurred between 20 and 29 Mya (O'hUigin and Li, 1992). If these estimates are valid, the murid-hamster split must have occurred between 32 and 46 Mya (O'hUigin and Li, 1992) and, thus, the splitting of the three species of dormouse is assumed to have occurred in the early Oligocene (37 Mya; Savage and Long, 1986). Alternatively, if the date of the rat-mouse split is considered to be 12 Mya, as suggested by available rodent fossils (see O'hUigin and Li, 1992), the calculated date of the split of the three species of dormouse occurred 19 Mya (1.6 × 12 Mya). Therefore, at present there are two possibilities. The three species of dormouse examined here separated either during the early Oligocene or the early Miocene (21 Mya; Savage and Long, 1986). According to the description of Holden (1993), most extant genera are surviving members of distinct evolutionary lineages which were already clearly differentiated by the early to medial Miocene. The generation of the Japanese islands is considered to have started 20 Mya, when the land core separated from the Asian continent (Otofuji *et al.*, 1985). Thus, the differentiation of the Japanese dormouse might have been associated with the generation of the Japanese islands. To clarify these unresolved issues, a careful examination of fossils and genes is required.

Our results provide useful clues for reconsidering of the taxonomic status of the Japanese dormouse. From a

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phylogenetic perspective, the sequences of mitochondrial genes for 12S rRNA suggest that the Japanese dormouse belongs to its own subfamily, Glirulinae. In addition, it is also evident that Japanese dormice can be subdivided into at least two genetically different groups according to variations in various DNA markers. Taxonomic reconstruction of this species, in conjunction with morphological studies, should be performed in the future.

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