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Differentiation of Restriction Sites in Ribosomal DNA in the Genus *Apodemus*

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Southern blot analysis of ribosomal DNA (rDNA) from seven species of Apodemus *was carried out in order to examine the genetic relationships between the species. Analysis of heterogeneity in rDNA spacers in* A. sylvaticus, A. flavicollis, A. semotus, A. agrarius, A. argenteus, A. speciosus, *and* A. peninsulae, *using 13 different restriction enzymes and cloned mouse rDNA probes, revealed that the families of rDNA in these species can be characterized by restriction maps which show the major constituents of rDNA repeating units (repetypes). Based on differences in the arrangement of restriction sites, sequence divergence among the different major repetypes was estimated. Among the seven species of* Apodemus *examined, the major repetypes of* A. flavicollis *and* A. sylvaticus *were the most closely related, having only 1.0% sequence divergence. These repetypes and those of the remaining five species differ substantially from one another, with 4.3–8.5% divergence.*

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INTRODUCTION

Wood mice (*Apodemus,* Muridae), of which there are 13 known species, are the most common small rodents living in fields and mountains around the Old North region (Corbet, 1978). Unlike house mice or rats, whose migration depends largely on human actions, dispersal of wood mice is thought to be rather independent of such influences. Thus, analysis of genetic differences between isolated populations may provide useful data for evolutionary biology and zoogeography. Although *Apodemus* species have been compared in terms of morphology, karyology, and biochemical genetics (Imaizumi, 1962, 1964; Kral, 1970; Tsuchiya, 1974; Bekasova *et al.,* 1980; Bonhomme *et al.,* 1984), there is no consensus as to their phylogenetic relationships. In the last decade, qualitative genetic analysis of related species has been performed at the DNA level based on restriction enzyme fragment length polymorphism (RFLP), for cytoplasmic mitochondrial DNA (mtDNA) (Yonekawa *et al.,* 1981; Ferris *et al.,* 1983) and nuclear genomic rDNA (Arnheim *et al.,* 1980; Wilson *et al.,* 1984; Hillis and Davis, 1986, 1988; Hillis, 1987; Suzuki *et al.,* 1986, 1987).

The rRNA locus exists as a multigene family which consists of several hundred copies in the mammalian genome. The copies are usually separated into tandemly repeated clusters at several different sites on chromosomes. Each rDNA repeating unit is composed of three rRNA genes, namely, those for 28, 5.8, and 18 S RNA, which are separated from each other by spacers. The spacers are known to evolve rapidly (Arnheim, 1983), and particularly rapid evolution is observed in nontranscribed spacers (NTS), where RFLPs are generated predominantly by base changes at restriction sites and the deletion, insertion, or duplication of DNA segments.

We constructed restriction maps of an approximately 40-kb portion of the repeating units of *Apodemus* rDNA, encompassing the rRNA coding region, the internal spacer regions, and the flanking spacer regions, using four different probes. In this report, we compare the major repetypes of seven species of *Apodemus* and discuss the genetic relationships among the seven species.

MATERIALS AND METHODS

Wood Mice

A total of 18 individual mice from seven species of *Apodemus* was used (Table I). Wood mice were trapped and kept at the Experimental Animal Center of Miyazaki Medical College prior to experiments.

Table I. List of Samples Used

Apodemus species	Locality	No. of mice examined
A. sylvaticus	1. Bonn (West Germany)	2
A. flavicollis	2. Lübeck (West Germany)	1
A. semotus	3. Mt. Lisan (Taiwan)	4
A. agrarius	4. Lübeck (West Germany)	1
	5. Jindo Island (Korea)	4
A. argenteus	6. Miyazaki (Japan)	1
A. speciosus	7. Fukushima (Japan)	3
A. peninsulae	8. Piacol (Korea)	1
	9. Hokkaido (Japan)	1

Blot Analysis

Nuclear DNAs were prepared from the livers of wood mice as described by Maniatis *et al.* (1982). The DNAs were digested with restriction enzymes and subjected to electrophoresis on 0.6–0.7% agarose gels at 2 V/cm for 13–15 hr in 40 mM Tris–acetate buffer (*p*H 7.8) that contained 2 mM EDTA. Double-stranded fragments of DNA were transferred to nylon filters, baked at 80°C for 2 hr, and hybridized with a probe as described by Southern (1975). Labeling of probes was carried out using (α-^{32}P)-dCTP and random primers. The specific radioactivity of probes was 4–8 $\times$ 10^8 cpm/μg. Prior to hybridization, the filters were incubated at 66°C for 2–4 hr in 6$\times$ SSC (1$\times$ SSC = 0.15 M NaCl–0.015 M Na citrate at *p*H 7.0) which contained 0.01% (w/v) sodium dodecyl sulfate and Denhardt's solution with denatured salmon sperm DNA (100 μg/ml). Hybridization was performed at 66°C for 20 hr, and filters were washed twice with 0.1$\times$ SSC for 30 min at room temperature and then wrapped in Saran wrap (Dow Chemicals) to prevent drying. Autoradiography was performed on Fuji-RX film for 12–24 hr at room temperature with an intensifying screen. Then filters were boiled in distilled water at 95°C for 5–10 min to remove the probe DNA. After air-drying the filters were reused for hybridization with another probe.

Construction of Restriction Maps

To construct the restriction maps for the various types of rDNA repeating unit (repetypes), the genomic DNAs from seven species of *Apodemus, A. sylvaticus, A. flavicollis, A. semotus, A. agrarius, A. argenteus, A. speciosus,* and *A. peninsulae,* were subjected to single and double digestion with 13 restriction enzymes, namely, *Aat*I, *Apa*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, and *Xba*I. The digested DNAs on nylon filters were

hybridized sequentially with four ^{32}P-labeled rDNA probes, "18SA," "18SB," "28S," and "INT." These probes were prepared from clones of mouse rDNA according to Kominami *et al.* (1981, 1982). The specific probes are indicated in Fig. 2.

Since the sites of cleavage by *Eco*RI sites on the genes for 18 and 28 S rRNA, designated E1 and E2 in Fig. 2, were judged to be conserved in *Apodemus* rDNA, as they are in most mammalian species, hybridization data after double digestion with *Eco*RI plus another enzyme were usually quite useful for the construction of restriction maps.

Construction of Phylogenetic Trees

To estimate the sequence divergence among seven major repetypes represented in Fig. 2, we compared the arrangement of restriction sites between pairs of rDNA repetypes and counted common and different sites. Employing a method developed by Gotoh *et al.* (1979) in which backward mutations and parallel mutations are taken into account (Jukes and Cantor, 1969), we produced a matrix of sequence divergences among all possible combinations of repetypes (Table II). Then we constructed phylogenetic trees using the unweighted pair-group (UPG) method (Sokal and Sneath, 1963) and the neighbor-joining (NJ) method (Saitou and Nei, 1987). Since insertions and deletions of small DNA fragments frequently occur in rDNA spacers, we selected informative restriction sites near the two probes (see Fig. 2) and conducted a phylogenetic analysis using the maximum parsimony (MP) method (Fitch, 1971) employing a computer program developed by Saitou and Imanishi (1989).

RESULTS

Restriction Maps of Major rDNA Repetypes from Seven Species of *Apodemus*

From the patterns of hybridization after single and double digestions, we first constructed restriction maps for the coding and internal spacer regions of genes for rRNA. Most restriction sites in the coding region were conserved, indicating little significant differentiation within each species and also among the seven species examined. Exceptionally, digestion with *Sac*I and *Apa*I of the gene for 18 S rRNA showed some difference between species, but the genes were almost monomorphic within each genome and within each species. Since E1/E2 fragments from the seven species were digested by *Apa*I into several small segments, we could not localize the *Apa*I sites within E1/E2. For all samples investigated, *Dra*I and *Kpn*I sites in the internal spacers were also

Table II. Sequence Divergence Among the Major rDNA Repetypes of Seven Species of *Apodemus* (Upper Right), Based on the Number of Common and Different Restriction Sites (Lower Left)

Species	Sequence divergence (%)						
	syl.	*fla.*	*sem.*	*agra.*	*arg.*	*spe.*	*pen.*
1. *sylvaticus*	—	1.0	4.7	6.1	7.2	8.5	8.5
2. *flavicollis*							
a[a]	11/1	—	5.1	6.8	7.2	8.5	7.2
b	4/0						
c	10/2						
Total	25/3						
3. *semotus*							
a	8/4	7/6	—	4.3	6.8	8.1	7.2
b	3/1	3/1					
c	6/6	7/5					
Total	17/11	17/12					
4. *agrarius*							
a	7/5	6/6	8/5	—	8.1	6.8	6.8
b	3/2	3/2	3/1				
c	6/7	6/7	8/5				
Total	16/14	15/15	19/11				
5. *argenteus*							
a	6/6	6/6	6/6	4/9	—	6.8	6.8
b	2/4	2/4	2/3	3/1			
c	7/6	7/6	7/6	6/6			
Total	15/16	15/16	15/15	13/16			
6. *speciosus*							
a	5/8	4/9	4/9	5/8	5/8	—	8.1
b	2/3	2/3	2/2	2/2	3/1		
c	6/6	7/5	7/5	8/5	7/6		
Total	13/17	13/17	13/16	15/15	15/15		
7. *peninsulae*							
a	5/8	5/8	6/7	5/7	7/6	4/9	—
b	2/2	2/2	2/1	2/1	2/2	2/1	
c	6/7	7/5	6/7	7/6	6/7	7/6	
Total	13/17	14/15	14/15	14/14	15/15	13/16	

[a]Number of sites (common/different) for the spacer upstream from the gene for 18 S rRNA (a), the internal spacer region (b), and the NTS region downstream from the gene for 28 S rRNA (c).

observed in the corresponding regions of samples from house mice and rats (data not shown).

By reference to the maps of the coding and the internal spacer regions, the location of the restriction sites on the external spacer regions, which flanked the genes for 18 and 28 S rRNA, was estimated by hybridization, after single digestion, with the 18 SB and 28 S probes, both of which are

localized to the distal end of the coding regions. The arrangement of restriction sites was confirmed by hybridization after double digestions (see Fig. 1).

The restriction maps of the major rDNA repetypes are presented schematically in Fig. 2. These maps display only the nearest restriction sites on the external spacers which flank the genes for 18 or 28 S RNA.

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. Some restriction sites also showed heterogeneity within a genome, within a population, and between populations of the same species. Then we arranged the restriction sites that provided evidence for the most predominant repetypes in each species. Significant variations with respect to the presence or absence of certain restriction sites were observed in the case of some restriction sites among individuals from the same locality. Such sites are marked on the restriction maps in Fig. 2 with asterisks.

In the present study, we analyzed two populations [Jindo (Korea) and Lübeck (West Germany)] of *A. agrarius*. We found that a variation in restriction sites on rDNA was present in the *Aat*I site in the internal spacer. The *Aat*I site was observed in 72% of rDNA units for Jindo samples on average and in 22% in the case of the Lübeck sample. In the case of *A. peninsulae*, no significant difference in the arrangement of major restriction sites was observed between the two individuals collected from Piacol (Korea) and Hokkaido (Japan), which have been taxonomically classified as belonging to different subspecies of *A. peninsulae*, namely, *A. p. peninsulae* and *A. p. giliacus* (Kobayashi and Hayata, 1971).

DISCUSSION

In the present study, we investigated the phylogenetic relationships among seven species of *Apodemus* based on the differentiation of rDNA sequences. It was clear that multiple mutations, including mutations in size and at restriction sites, had accumulated in rDNA, especially in its spacer regions. However, most of the mutations appeared to be have been fixed to yield specific repetypes within populations or species during the course of differentiation. Changes in such a manner, designated as evidence of concerted evolution, have been well documented in families of repetitive DNA, especially in the rDNA family (see Dover, 1982, for review). In this study we were easily able to characterize the rDNA family of each species with single restriction maps of the major repetypes and we used these maps as an index of genetic diversity among the seven species examined. To estimate the degree of the sequence divergence between repetypes, comparisons of site differences are very useful (Gotoh *et al.*, 1979). Accordingly, we neglected differences in size, and only

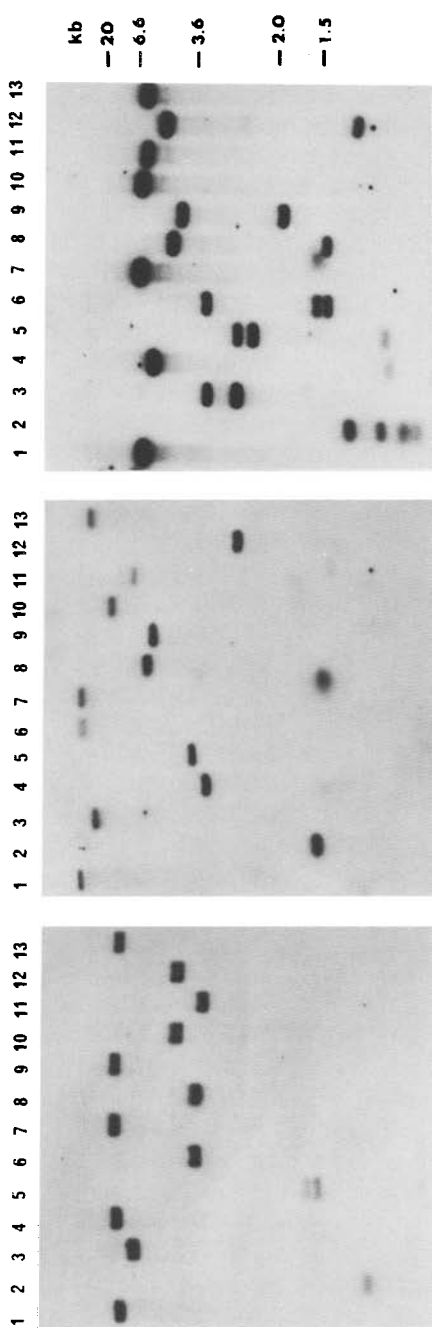

Fig. 1. Southern blot patterns of *Apodemus peninsulae peninsulae* DNA cleaved with *Eco*RI (1) and *Eco*RI plus *Apa*I (2), *Aat*I (3), *Kpn*I (4), *Dra*I (5), *Bam*HI (6), *Hin*dIII (7), *Sac*I (8), *Bgl*II (9), *Pst*I (10), *Xba*I (11), *Pvu*II (12), or *Eco*RV (13), using a 0.7-kb "28 S" probe (a), a 0.9-kb "18 SB" probe (b), and a 6.6-kb "INT" probe (c).

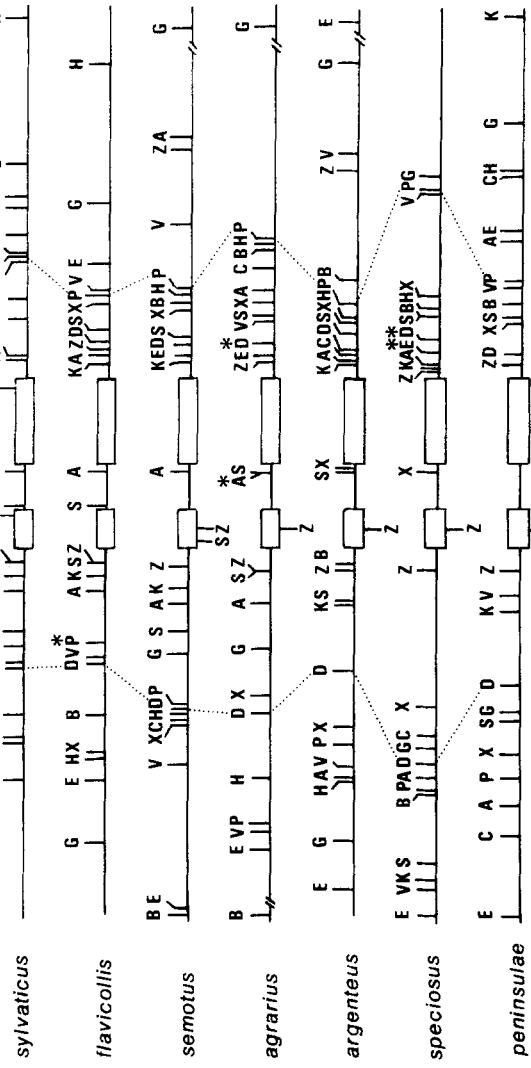

Fig. 2. Restriction maps of the major rDNA repetypes of *Apodemus sylvaticus* (1), *A. flavicollis* (2), *A. semotus* (3), *A. agrarius* (Jindo) (4), *A. argenteus* (5), *A. speciosus* (6), and *A. peninsulae* (7). The short and long open boxes indicate the genes that encode 18 and 28 S rRNA, respectively. With respect to the restriction sites on the flanking spacers, only those nearest to the distal end of the genes for 18 or 28 S rRNA are shown. The top diagram shows the conserved restriction sites in the coding and the internal spacer regions of the genes for 18 and 28 S RNA (see text), which are not represented in the lower maps. Positions of probes are also shown with arrows. Asterisks indicate polymorphic sites among individuals of a given species. The restriction sites between two dotted lines are used for the analysis of the maximus parsimony methods. A = *Aat*I; B = *Bam*HI; C = *Eco*RV; D = *Dra*I; E = *Eco*RI; G = *Bgl*II, H = *Hin*dIII; K = *Kpn*I; S = *Sac*I; P = *Pst*I; V = *Pvu*II; X = *Xba*I; Z = *Apa*I.

differences in the arrangement of restriction sites along the restriction maps of the rDNA spacers were taken into account for estimations of the degree of divergence among the repetypes. It should be noted that the values used to quantitate the divergence of sequences (Table II) are somewhat speculative because site rearrangements are generated not only by point mutations but also by segment mutations which include deletion, insertion, duplication and inversion.

From the estimates of the amount of sequence divergence, we constructed two hypothetical phylogenetic trees for these repetypes, by the UPG and NJ methods (Figs. 3a and b). We also constructed a phylogentic tree by the MP method. Restriction sites were used as characters and informative sites were selected from the restriction maps as listed in Table III. We obtained two equally parsimonious tree topologies which were similar to those by the UPG and NJ methods (Fig. 3). Based on these analysis, we now propose a new set of criteria for the classification of our seven species of *Apodemus*. The average sequence divergence of six repetypes (Nos. 2–7 in Table II) was 7.0%, while that between the human and the Japanese macaque (*Macaca fuscata*) and between *Mus musuculus domesticus* and *M. m. molossinus* are 7.3 and 1.5%, respectively, when analyzed under the same conditions as those used here for *Apodemus* (Suzuki *et al.*, unpublished data). The divergence of human from macaque and of *M. m. domesticus* from *M. m. molossinus* is considered to have occurred $2–3 \times 10^7$ (Hasegawa *et al.*, 1988) and $1–2 \times 10^6$ years ago (Yonekawa *et al.*, 1981), respectively. Extrapolating from these data, we can assume that divergence of the various species of *Apodemus* occurred approximately 10^7 years ago. This value agrees with Bonhomme's (1984) suggestion that *A. agrarius* is distantly related to *A. sylvaticus* (1984).

Ribosomal DNA spacer difference indicates that *A. sylvaticus* and *A. flavicollis* are the most closely related species among the seven species investigated. A sequence divergence between the major rDNA repetypes of two species of 1% is similar to that between the two mouse subspecies described above and to that between *Rattus rattus* and *R. norvegicus* (Suzuki *et al.*, unpublished data). The close relationship between *A. sylvaticus* and *A. flavicollis* is also supported by analyses of karyotypes (Kral, 1970) isozymes (Gebczynski *et al.*, 1986), satellite DNA (Brown and Dover, 1979) and mtDNA (Wakana *et al.*, manuscript in preparation). Although Gebczynski *et al.* (1986) estimated the divergence of *A. flavicollis* from *A. sylvaticus* to have occurred $15–30 \times 10^4$ years ago, based on their biochemical analysis, our results indicate that the divergence occurred $67–133 \times 10^4$ years ago. In conclusion, our results suggest the following process of species differentiation. The ancestors of the seven species first differentiated into five groups, *sylvaticus/flavicollis, semotus/agrarius, argenteus, speciosus,* and *peninsulae.* Then, *semotus* and *agrarius* were separated. About 1 million years ago *sylvaticus*

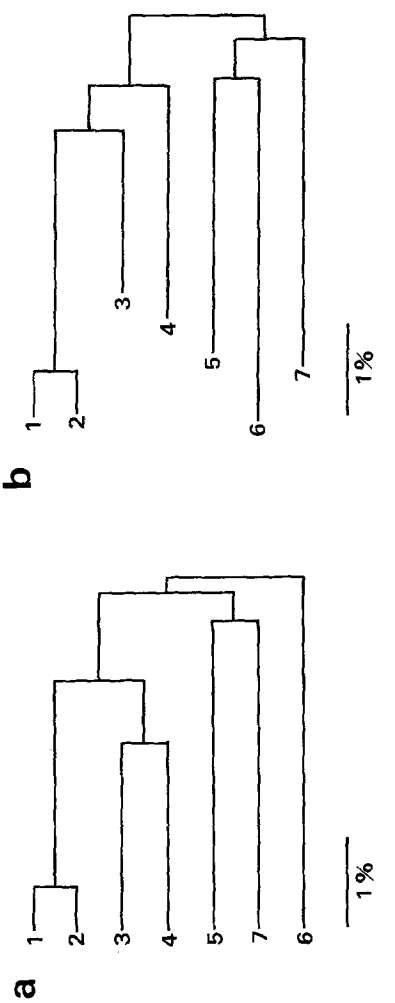

Fig. 3. Hypothetical phylogenetic trees for the major rDNA repetypes from the seven species of *Apodemus* by the UPG (a), NJ (b), and MP (c) methods. 1 = *sylvaticus*; 2 = *flavicollis*; 3 = *semotus*; 4 = *agrarius*; 5 = *argenteus*; 6 = *speciosus*; 7 = *peninsulae*.

Table III. Presence (+) or Absence (−) of Hypothetical Common Restriction Sites Within the Major rDNA Repetypes of Seven Species of *Apodemus*

	Restriction site[a]																									
Species	D	V	P	X	G	A	K	S	Z	Z	S	A	S	X	Z	K	A	Z	E	D	S	X	B	H	V	P
1. *syl.*	+	+	+	−	+	+	+	+	+	−	+	+	−	−	−	−	+	+	−	+	+	+	−	−	−	+
2. *fla.*	+	+	+	−	−	+	+	+	+	−	+	+	−	−	−	+	+	+	−	+	+	+	−	−	−	+
3. *sem.*	+	−	+	−	+	+	+	−	+	+	−	+	−	−	−	+	−	−	+	+	+	+	+	+	−	+
4. *agr.*	+	−	−	+	+	+	−	+	+	+	−	+	+	−	+	−	−	−	+	+	+	+	+	+	−	+
5. *arg.*	+	−	−	−	−	−	+	+	+	+	−	−	+	+	−	+	+	−	−	+	+	+	−	+	−	+
6. *spe.*	+	−	−	+	−	−	−	−	+	+	−	−	−	+	+	+	+	−	+	+	+	−	+	+	+	+
7. *pen.*	+	−	−	−	−	−	+	−	+	+	−	−	−	−	+	−	−	−	−	+	+	−	+	−	+	+
Informative site		0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0			0	0	0	0	

[a]Restriction sites shared by more than one repetype between *Dra*I sites upstream of the 18 S rRNA gene and the *Pst* I sites downstream of the 28 S rRNA gene were analyzed (see Fig. 2). The hypothetical common sites were selected to maximize the number of pluses in this table. Abbreviations of the restriction sites and their arrangement are as in the legend to Fig. 2.

and *flavicollis* were differentiated from their common ancestor. Our results do not provide any genetic basis for the classification into two "ecotypes," i.e., a division into field types (*agrarius, flavicollis, peninsulae,* and *speciosus*) and wood types (*sylvaticus, semotus,* and *argenteus*).

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