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# Evolution of Restriction Sites of Ribosomal DNA in Natural Populations of the Field Mouse, *Apodemus speciosus*

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**Abstract.** An analysis by restriction endonuclease digestion of ribosomal DNA (rDNA) was carried out in natural populations of *Apodemus speciosus*, a field mouse that is endemic to Japan. Two restriction sites, the *Eco*RI (E3) and *Dra*I (D4) sites, in the nontranscribed spacer region downstream from the gene for 28S RNA showed polymorphism within and between individuals in the populations from the Japanese main islands. By contrast, populations from the small adjoining islands which are thought to have separated from the main islands 1–2 × $10^4$ years ago showed relatively low levels of polymorphism within and between individuals, i.e., one of the polymorphic bands in the case of each enzyme was predominant in these populations, irrespective of the variants. These results indicate that the rate of fixation of site variations depends on population size and that the direction of fixation is random. Furthermore, each polymorphic restriction site seems to be fixed independently.

**Key words:** Concerted evolution — Island population — Restriction-fragment-length polymorphism — Ribosomal DNA — *Apodemus speciosus*

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## Introduction

The rRNA locus comprises a multigene family that 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 genes for rRNA—namely, genes for 28S, 5.8S, and 18S RNA—which are separated from one another by spacers. The units are known to evolve rapidly (Arnheim 1983), especially in the nontranscribed spacer regions, and concertedly (see Ohta 1980 or Dover 1982 for review) within the genome by homogenization mechanisms and within Mendelian populations by mating. Although a theoretical analysis has been well developed in the case of concerted evolution (e.g., Smith 1976; Ohta 1980; Dover 1982), only a few studies of this issue in natural populations have been reported. Hence, there is disagreement with respect to the mechanism and process of fixation in natural populations. For example, Nevo and Beiles (1988) suggested that natural selection affects fixation and Dover (1982) claimed directional fixation mechanism was operative, while Ohta (1980) suggested that the fixation can be explained only as a result of random drift.

Field mice (*Apodemus*, Muridae), of which 13 species are known, are the most common small ro-

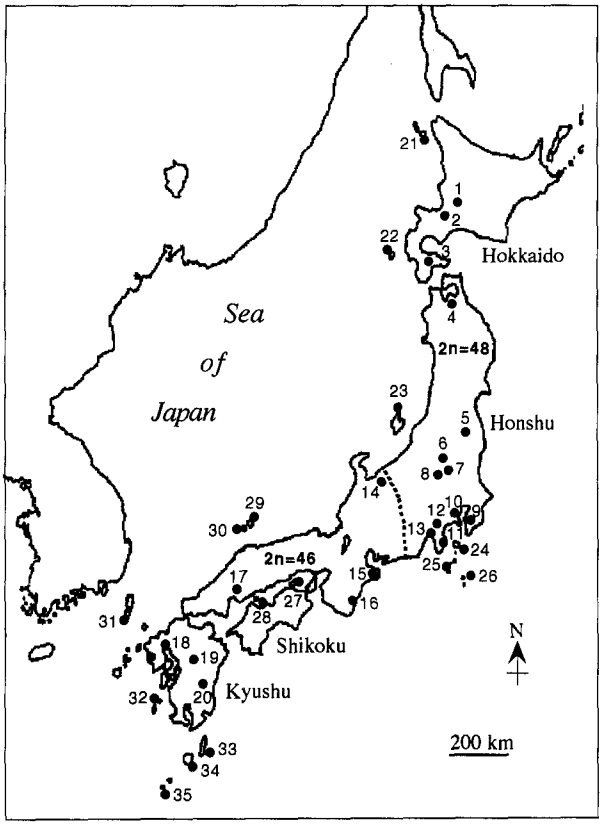


**Fig. 1.** Locations at which populations of *Apodemus speciosus* were sampled on the main islands (Nos. 1–20) and adjoining small islands (Nos. 21–35). The locality numbers are explained in Tables 1 and 2. The *dotted line* represents the Toyama-Hamamatsu line that divides this species into two distinct types with respect to karyotype: $2n = 46$ (western) and $2n = 48$ (eastern).

dents living in fields and mountains around the Old-North region (Corbet 1978). One species of *Apodemus*—namely, *A. speciosus*— which is endemic to Japan, is widely distributed over the four main islands (Hokkaido, Honshu, Shikoku, and Kyushu) as well as the small adjoining islands (Imaizumi 1962, 1964). Tsuchiya et al. (1973) revealed that *A. speciosus* has two distinct karyotypes: $2n = 46$ (western) and $2n = 48$ (eastern). The boundary between the two karyotypes, designated the Toyama-Hamamatsu line, divides Japan at approximately the center of the largest island (Fig. 1). Unlike house mice or rats, field mice are thought to be dispersed rather independently of human actions. Thus, analysis of genetic differentiation between geographically isolated populations of *A. speciosus* may provide useful data on the process of evolution of particular genes.

We previously constructed restriction maps of a portion of about 40 kb of repeating units of rDNA from *A. speciosus* that encompass the rRNA-coding region, both sides of the flanking spacer regions, and the internal spacer regions (Suzuki et al. 1990). We also demonstrated that *Eco*RI and *Dra*I fragments that contain the 3′ end of the gene for 28S rRNA are polymorphic within and between individuals (Suzuki et al. 1990). In this report we describe the geographical distribution of the polymorphic sites, *Eco*RI and *Dra*I, as identified in individuals collected from 35 different localities. We discuss the process of concerted evolution of rDNA with emphasis on homogenization within an individual and within a population.

## Materials and Methods

*Field Mice.* A total of 129 individuals of *A. speciosus* trapped in 35 different localities was used (Fig. 1, Tables 1 and 2).

*Blot Analysis.* Nuclear DNA was prepared from the livers of the field mice as described by Maniatis et al. (1982). DNAs were digested with restriction enzymes and subjected to electrophoresis on 0.6–0.7% agarose gels at 2 V/cm for 13–15 h in 40 mM Tris-acetate buffer (pH 7.8) that contained 2 mM EDTA. Then the fragments of double-stranded DNA were transferred to nylon filters (Amersham), heated at 80°C for 2 h, and hybridized with a probe. Labeling of the probe for 28S rDNA (Kominami et al. 1981; Fig 2) was carried out using [$\alpha$-$^{32}$P]-dCTP and a random primer DNA labeling kit (Amersham). The specific radioactivity of the probe was 4–8 × $10^8$ cpm/μg. Prior to hybridization, the filter was incubated at 66°C for 2–4 h in 6× SSC (1× SSC = 0.15 M NaCl–0.015 M sodium citrate solution at pH 7.0) that contained 0.01% (w/v) sodium dodecyl sulfate and Denhardt's solution plus denatured salmon-sperm DNA (100 μg/ml). Hybridization was performed at 66°C for 20 h, and the filter was washed twice with 0.1× SSC for 30 min at room temperature. Autoradiography was performed on Fuji-RX film (Fuji Film Co., Japan) for 12–24 h at room temperature with an intensifying screen.

## Results

The *Eco*RI (E3) and *Dra*I (D4) sites that lie about 1 kb downstream from the 3′ end of the gene for 28S rRNA are highly polymorphic within and between individuals (Suzuki et al. 1990). By Southern blot analysis with the probe for 28S rDNA (Fig. 2), we found distinct patterns of bands generated by *Eco*RI and *Dra*I among the populations investigated. (See, for example, Fig. 3.) Since no polymorphisms were found for restriction sites within the coding region, such as D2 and E2 (Suzuki et al. 1990), the polymorphisms can be attributed to the presence or absence of the nearest restriction sites, E3 and D4. The rDNA repeating unit with E3 generated the 1.3-kb *Eco*RI fragment and was designated E3(+), while that lacking the E3 site generated a larger fragment, such as the 5.0-kb and 6.2-kb fragments, and was designated E3(−). D4(+) and D4(−) repeating units generated the 4.1-kb and 4.7-kb fragments, respectively (Fig. 2). To estimate the fre-

**Table 1.** Frequencies of the E3 and D4 sites in the populations from the main islands

| No. | Locality | $N^a$ | E3[b] Relative freq. (%) | E3[b] $I$ | D4[b] Relative freq. (%) | D4[b] $I$ |
|---|---|---|---|---|---|---|
| 1 | Bibai | 1 | 36.0 | 0.54 | 54.8 | 0.50 |
| 2 | Sapporo | 10 | 8.9 ± 8.4 | 0.85 ± 0.12 | 19.0 ± 19.3 | 0.77 ± 0.21 |
| 3 | Oonuma | 1 | 7.7 | 0.86 | 14.0 | 0.76 |
| 4 | Aomori | 3 | 24.5 ± 4.2 | 0.63 ± 0.04 | 18.7 ± 7.9 | 0.71 ± 0.11 |
| 5 | Fukushima | 3 | 71.5 ± 10.1 | 0.61 ± 0.10 | 1.3 ± 1.1 | 0.97 ± 0.02 |
| 6 | Oze | 1 | 28.6 | 0.59 | 6.5 | 0.88 |
| 7 | Haruna | 8 | 23.6 ± 17.4 | 0.70 ± 0.12 | 9.7 ± 5.9 | 0.83 ± 0.09 |
| 8 | Asama | 3 | 50.8 ± 16.2 | 0.55 ± 0.04 | 12.2 ± 8.3 | 0.80 ± 0.13 |
| 9 | Chiba | 1 | 17.6 | 0.71 | 9.6 | 0.83 |
| 10 | Yokohama | 6 | 77.7 ± 21.3 | 0.74 ± 0.17 | 25.4 ± 10.2 | 0.64 ± 0.09 |
| 11 | Yugashima | 1 | 87.9 | 0.79 | 1.0 | 0.98 |
| 12 | Mishima | 3 | 41.7 ± 11.4 | 0.54 ± 0.05 | 2.8 ± 0.9 | 0.95 ± 0.02 |
| 13 | Numadu | 6 | 35.5 ± 8.1 | 0.56 ± 0.05 | 8.3 ± 8.3 | 0.86 ± 0.13 |
| 14 | Kanazawa | 6 | 26.0 ± 20.1 | 0.70 ± 0.13 | 15.9 ± 7.9 | 0.75 ± 0.12 |
| 15 | Ise | 1 | 2.3 | 0.96 | 24.6 | 0.63 |
| 16 | Nachi | 6 | 24.5 ± 28.9 | 0.80 ± 0.13 | 70.4 ± 22.6 | 0.69 ± 0.12 |
| 17 | Hiroshima | 3 | 60.3 ± 6.3 | 0.53 ± 0.03 | 16.8 ± 11.8 | 0.75 ± 0.14 |
| 18 | Saga | 2 | 70.1 ± 6.3 | 0.59 ± 0.05 | 22.0 ± 3.3 | 0.66 ± 0.04 |
| 19 | Shiraga | 2 | 27.5 ± 11.6 | 0.63 ± 0.10 | 14.9 ± 4.9 | 0.75 ± 0.07 |
| 20 | Miyazaki | 1 | 5.0 | 0.91 | 41.1 | 0.41 |
| | Total | Total | | | | |
| | 20 | 68 | 38.2 ± 24.5[c] | 0.66 ± 0.13[c] | 19.3 ± 16.5[c] | 0.76 ± 0.13[c] |

[a] *N:* No. of individuals examined
[b] Mean ± SD between individuals, *I:* Restriction-site identity
[c] Average (mean ± SD between localities)

**Table 2.** Frequencies of the E3 and D4 sites in small-island populations

| No. | Locality ($km^2$) | | $N^a$ | E3[b] Relative freq. (%) | E3[b] $I$ | D4[b] Relative freq. (%) | D4[b] $I$ |
|---|---|---|---|---|---|---|---|
| 21 | Rishiri I. | (183) | 6 | 30.0 ± 21.0 | 0.67 ± 0.12 | 9.7 ± 5.2 | 0.83 ± 0.09 |
| 22 | Okushiri I. | (143) | 11 | 1.2 ± 0.6 | 0.98 ± 0.01 | 63.7 ± 21.3 | 0.63 ± 0.14 |
| 23 | Sado I. | (857) | 3 | 13.3 ± 18.5 | 0.84 ± 0.19 | 94.7 ± 7.0 | 0.91 ± 0.12 |
| 24 | Oshima I. | (91) | 6 | 56.3 ± 10.7 | 0.53 ± 0.03 | 2.9 ± 1.3 | 0.94 ± 0.02 |
| 25 | Shikine I. | (4) | 1 | 0.6 | 0.99 | 0.5 | 0.99 |
| 26 | Miyake I. | (55) | 3 | 0.9 ± 0.1 | 0.98 ± 0.00 | 0.5 ± 0.0 | 0.99 ± 0.00 |
| 27 | Shodoshima I. | (152) | 6 | 24.5 ± 22.5 | 0.73 ± 0.15 | 13.8 ± 9.9 | 0.78 ± 0.14 |
| 28 | Omishima I. | (64) | 1 | 99.5 | 0.99 | 0.5 | 0.99 |
| 29 | Dogo I. | (243) | 1 | 10.6 | 0.81 | 28.6 | 0.59 |
| 30 | Nishinoshima I. | (57) | 4 | 1.1 ± 0.9 | 0.98 ± 0.02 | 7.5 ± 4.1 | 0.86 ± 0.07 |
| 31 | Tsushima Is. | (698) | 6 | 97.6 ± 1.3 | 0.95 ± 0.02 | 4.3 ± 3.3 | 0.92 ± 0.06 |
| 32 | Kamikoshiki I. | (44) | 1 | 33.5 | 0.55 | 1.0 | 0.98 |
| 33 | Tanegashima I. | (446) | 6 | 0.5 ± 0.0 | 0.99 ± 0.00 | 3.1 ± 2.6 | 0.94 ± 0.05 |
| 34 | Yakushima I. | (503) | 1 | 0.5 | 0.99 | 46.2 | 0.50 |
| 35 | Nakanoshima I. | (28) | 5 | 1.0 ± 0.0 | 0.98 ± 0.00 | 5.7 ± 9.9 | 0.91 ± 0.15 |
| | Total | | Total | | | | |
| | 15 | | 61 | 24. ± 34.1[c] | 0.85 ± 0.18[c] | 20.4 ± 28.3[c] | 0.85 ± 0.16[c] |

[a] *N:* No. of individuals examined
[b] Mean ± SD between individuals, *I:* Restriction-site identity
[c] Average (mean ± SD between localities)

quencies of E3(+) and D4(+) repeating units semiquantitatively, we carried out densitometric analysis and measured the amounts of the 1.3-kb *Eco*RI and 4.1-kb *Dra*I fragments relative to the total amount of fragments generated by each restriction enzyme in each individual. Average frequencies of E4(+) and D4(+) in each population are shown in Tables 1 and 2.

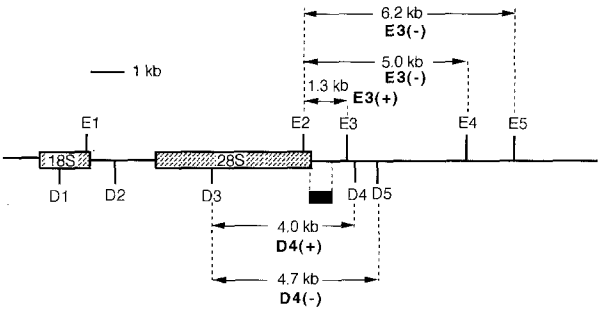


**Fig. 2.** A restriction map of ribosomal RNA repeating units from *Apodemus speciosus* constructed by the method of Suzuki et al. (1990). A 0.7-kb probe for mouse 28S rDNA is indicated by a *black box*. The expected fragments generated by *Eco*RI from various rDNA repeating units are represented by *arrows* marked 6.2-kb E3(−), 5.0-kb E3(−), and 1.3-kb E3(+); those generated by *Dra*I are represented by *arrows* marked 4.0-kb D4(+) and 4.7-kb D4(−). *D* and *E* represent the sites recognized by restriction endonucleases *Dra*I and *Eco*RI, respectively.

To compare the levels of fixation of the site variations within a population, an estimate of restriction-site diversity was calculated, by analogy with the estimate of gene identity discussed by Nei (1987, p. 178):

$$\text{Restriction-site identity } (I) = [x_i^2 + (1 - x_i)^2]$$

where $x_i$ is the relative frequency of E3(+) or D4(+) in an individual. The averages of the restriction-site identities within populations are summarized in Tables 1 and 2. Populations on the large main islands gave small values of $I$ relative to those of the populations on small peripheral islands. Average values of the restriction-site identity of E3(+) and D4(+) in 15 populations from small islands are 0.85 ± 0.18 and 0.85 ± 0.16, respectively, while those in 20 populations from the main islands are 0.66 ± 0.13 and 0.76 ± 0.13, respectively. Smaller islands, such as Shikine I. and Miyake I., gave the highest levels (close to 1.0) of restriction-site identity among the island populations. However, complete elimination of the E3 site or the D4 site from the genome was not observed in any of the individuals investigated: prolonged exposure resulted in detection of a faint signal from 1.3-kb *Eco*RI and 4.1-kb *Dra*I bands.

## Discussion

The Japanese field mouse, *Apodemus speciosus,* is represented by many island populations that are thought to have been evolving independently for 1–2 × $10^4$ years. To examine the evolutionary process that affects the restriction sites in the repeats of the genes for ribosomal RNA, we focused on the polymorphic restriction sites, E3 and D4, in the spacer region and examined the level of fixation in these populations.

A possible factor that modulates the level of polymorphism is the chromosomal location of the rDNA clusters, since homogenization events involving rDNA within a genome are thought to occur among members of the rDNA clusters on nonhomologous chromosomes (Arnheim et al. 1980; Arnheim 1983; Ohta 1980). However, the clusters of rDNA of this species are found to be on a single pair of chromosomes, as judged from silver staining (Saitoh and Obara 1986) and in situ hybridization (Suzuki et al., unpublished data). Thus, in the case of homogenization events that relate to rDNA of *A. speciosus,* the situation is less complicated and we can ignore any participation of "communication" between nonhomologous chromosomes.

In this study, we found that levels of polymorphism within populations are lower in populations on the small islands than those in populations on the main islands. The finding that population size has a large effect on variability of the rDNA indicates that intrapopulational homogenization by random mating contributes significantly to the acceleration of fixation of a given type of cluster that is subjected to the intragenomic homogenization process. With respect to the mechanisms of intragenomic homogenization, there are several informative studies, such as those on human rDNA by Seperack et al. (1988) and the histone genes of *Drosophila* by Matsuo and Yamazaki (1989). These authors examined genetic variation within an individual and between individuals, and they indicated that the rate of spreading on a chromosome, by mechanisms such as, for example, sister chromatid exchanges, is high relative to rates of change due to other evolutionary forces.

In our previous study, we showed that E3 and D4 are present in the genome of other species of *Apodemus,* such as *A. semotus* and *A. agrarius,* which are thought to have diverged from *A. speciosus* five million years ago (Suzuki et al. 1990). Therefore, both E3(+) and D4(+) must be the ancestral forms. E3(−) and D4(−) repeating units seem to have spread during the course of evolution of *A. speciosus* and are now predominant both in small-island and main-island populations. Thus, it seems that E3(+) and D4(+) are now in the process of being lost from the gene pool of *A. speciosus.*

The direction of fixation at the E3 and D4 sites differs between populations—in particular, among those on small islands. For example, E3(+)/D4(−) is dominant on Tsushima Is., E3(−)/D4(+) on Sado I., and E3(−)/D4(−) on Miyake I. and Tanegashima I. From these results, we can assume that such types of repeating unit were generated by occasional recombinations within the two restriction sites and that intrachromosomal homogenization

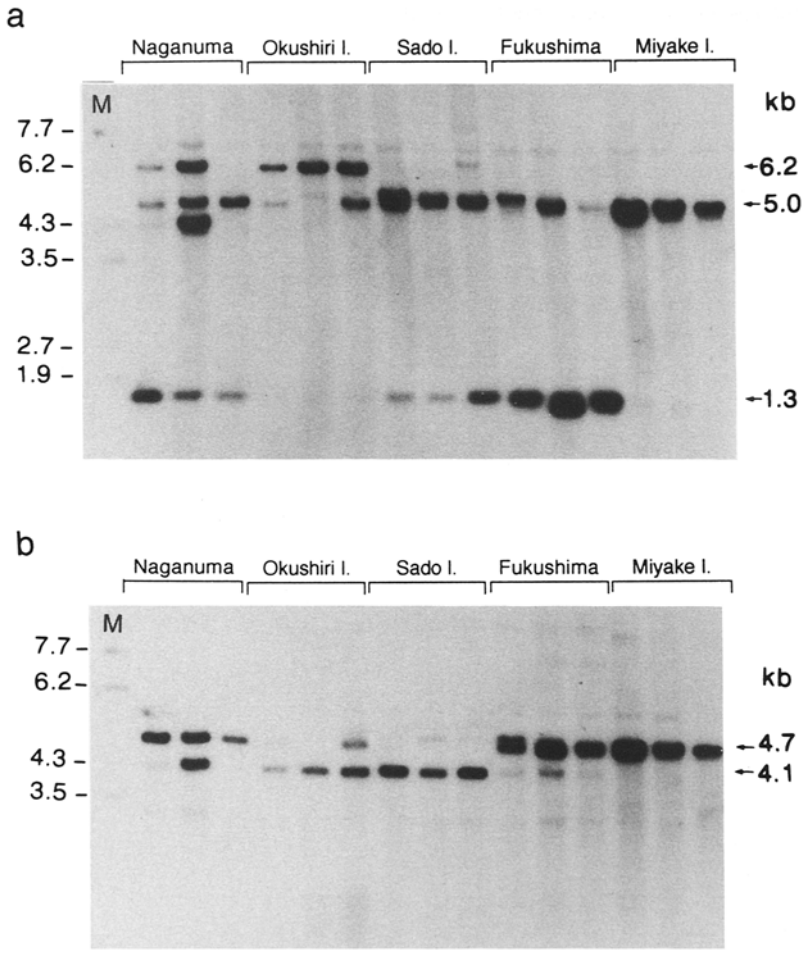


**Fig. 3.** Examples of restriction fragment patterns obtained with *Eco*RI **a** and *Dra*I **b**, and probing with the fragment of 28S rDNA. Three individuals from each location are represented. Lambda DNA digested with *Eco*T14I was used for generation of size markers (lane M).

mechanisms fixed the types of repeating unit. It is likely that the restriction sites have been evolving independently, even if two restriction sites lie within 1 kb of one another (Fig. 2).

Another issue of interest is whether new variants of rDNA repeating units have some advantage over old ones in the homogenization process (Ohta 1980; Dover 1982; Nevo and Beiles 1988). Some theories predict that newly developing variants have an advantage in the homogenization process (Dover 1982) or that certain environmental factors, such as humidity or temperature, affect the direction of fixation of variants (Nevo and Beiles 1988). Since the direction of fixation of restriction sites differs between islands, it seems that new variants, such as E3(−) and D4(−), have little advantage as compared to the original type. In our opinion, the choice of the direction of fixation with respect to each restriction-site sequence during the homogenization process seems to be random, as predicted by Ohta (1980).

In conclusion, our data indicate that the rate of fixation of variants of restriction sites within rDNA spacer regions depends on the size of the population and that the direction of the fixation is random. Furthermore, we have shown that each restriction site within the same rDNA array evolved independently, even though restriction sites on homologous regions of rDNA arrays have evolved concertedly.

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