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Intra- and Interspecific Genetic Complexities of Two Eothenomys Species in Honshu, Japan

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ABSTRACT—Differences in the nuclear ribosomal DNA (rDNA), mitochondrial DNA (mtDNA), cytochrome b (Cytb), and Y chromosomal Sry genes were used to assess intra- and interspecific relationships in two Japanese red-backed voles, Eothenomys andersoni and E. smithii, focusing on areas where the two species might come into contact. In the Kii Peninsula, southwestern Honshu, which contains an allopatric population of E. andersoni isolated from its main range, the rDNA-RFLP data provide robust evidence of past mutual interspecific gene introgression, while the Cytb and Sry sequences were specific to this population. In central Honshu, where E. andersoni and E. smithii inhabit higher and lower altitudes, respectively, with a narrow sympatric zone, the rDNA-RFLP and Sry variation was specific for each species, while introgression of the mtDNA from E. smithii to E. andersoni was seen. These complex patterns in the gene markers are consistent with our previous notions derived from sex chromosome variation. Our previous and present data strongly suggest that the evolution of these vole species, which are morphologically and cytogenetically distinct, involves complex genetic interactions and the resultant combinations of genes are sometimes peculiar, mainly due to the Cytb haplotypes. However, phylogenetic analysis using a combination of maternal, paternal, and biparental markers has proven useful for understanding the evolutionary history given the complex phylogenetic background.

Key words: Eothenomys, Honshu, ribosomal DNA gene, cytochrome b, Sry

INTRODUCTION

The Japanese Islands, which cover a large distance from north to south and have many alpine areas, harbor many subjects for biogeographical studies. Small mammals in Japan, especially those from Honshu, Shikoku, and Kyushu, tend to have sibling species or local populations where speciation is going on to various extents. Morphological, cytogenetic, and molecular genetic studies of red-backed voles, which are typical small mammals, have revealed unexpected patterns of evolution that are complex and conflicting (e.g., Iwasa, 1998; Suzuki et al., 1999; Iwasa et al., 1999; Iwasa and Suzuki, 1999; Iwasa and Suzuki, 2002a).

The Japanese Eothenomys voles (Rodentia, Arvicolinae) E. andersoni and E. smithii inhabit all the Japanese islands except Hokkaido (Abe et al., 1994). Eothenomys andersoni is distributed in the eastern half of Honshu, with an allopatric relic population in the Kii Peninsula of Honshu (Iwasa and Suzuki, 2002a; Fig. 1). E. smithii is distributed in Kyushu, Shikoku, and Honshu except for the northern part (Abe et al., 1994; Fig. 1), and is horizontally parapatric with E. andersoni in the Kii Peninsula and vertically parapatric in central Honshu. Both species include several local populations that have been characterized morphologically (Imaizumi, 1979). Researchers have examined the genetic features of these species using karyotypes, nuclear rDNA-RFLP, and the sequences of mitochondrial and nuclear genes (Tsuchiya, 1981; Kitahara and Harada, 1996; Iwasa et al., 1999; Suzuki et al., 1999; Iwasa and Tsuchiya, 2000; Iwasa and Suzuki, 2002a, b). However, the genetic patterns seen within and between species are very complex and the intra- and interspecific relationships are poorly understood, especially in the areas where two species are in contact historically and contemporarily, as in the Kii Peninsula and central Honshu.

Suzuki et al. (1999) showed that E. andersoni and E. smithii differ in four restriction sites along the spacer regions of the 18S and 28S genes (rDNA) based on a restriction fragment length polymorphism (RFLP) analysis, but the Kii Peninsula population of E. andersoni in Honshu (Fig. 1) car-
ries both restriction patterns. Therefore, Suzuki et al. (1999) hypothesized that the Kii Peninsula population of *E. andersoni* (cited as a valid species, *E. imaizumii*) originated via ancient hybridization between the ancestors of *E. andersoni* and *E. smithii*. The rDNA-RFLP provided reliable phylogenetic information because rDNA exists as a multi-gene family that consists of several hundred copies in the mammalian genome (Babu and Verma, 1985). In agreement with Suzuki et al. (1999), Iwasa et al. (1999) also suggested that the Kii Peninsula population of *E. andersoni* (cited as *E. imaizumii*) was derived from a cross-species event between the ancestors of the two species according to an analysis of meiotic chromosome behavior in their specific sex chromosomes based on observed synaptonemal complex.

There are still many taxonomic and phylogenetic questions concerning *E. andersoni* (Kaneko et al., 1992; Kimura et al., 1994, 1999; Suzuki et al., 1999; Iwasa et al., 1999; Iwasa, 2000; Iwasa and Tsuchiya, 2000; Iwasa and Suzuki, 2002a, b). In particular, it is thought that *E. andersoni* occurs in alpine areas in central Honshu (Imaizumi, 1960; Kaneko et al., 1992; Abe et al., 1994). However, a small population of an *andersoni*-like vole, which has not been identified, was discovered at quite low altitudes (≤1,000 m) near Nagano in central Honshu, in 1972 (cited as a *Eothenomys* sp. in Miyao et al., 1979a, b). The voles from Nagano are smaller than *E. andersoni* from alpine regions (Miyao et al., 1979a, b; Iwasa, 2000). In addition, the *E. andersoni* from Nagano carried a *smithii*-like X chromosome and an *andersoni*-like Y chromosome (Iwasa, 1998; Iwasa et al., 1999; Iwasa and Tsuchiya, 2000). The Y chromosomal features of the Nagano individuals were identical to those of *E. andersoni* from alpine regions in central Honshu (Iwasa and Tsuchiya, 2000). Based on karyological and morphological data, the individuals from Nagano (alt. 380–800 m) are tentatively regarded as *E. andersoni* (Iwasa, 2000; Iwasa and Tsuchiya, 2000). A few local populations of *E. andersoni* show genetic and morphologic differentiation; however, phylogenetic and taxonomic problems of such small relic populations of *E. andersoni* at lower altitudes still remain, as pointed out by some vole researchers (Kaneko, 1981, 1998; Kaneko et al., 1992; Kimura et al., 1994, 1999; Iwasa, 2000).

In this study, we analyzed three gene regions using Southern blotting for rDNA-RFLP and sequencing for the maternally inherited mitochondrial cytochrome *b* gene and paternally inherited Y chromosomal sex-determining gene. We reconsidered the evolution of *E. andersoni*, including Nagano individuals, and *E. smithii* in Honshu, which was hypothesized to be a hybridization event based on our previous studies (Iwasa et al., 1999; Suzuki et al., 1999; Iwasa and Tsuchiya, 2000; Iwasa and Suzuki, 2002a, b).

Fig. 1. Distributions of *Eothenomys andersoni* (a) and *E. smithii* (b). Solid areas indicate each horizontal distribution. Collection localities (c) of *Eothenomys* voles examined in this study. All locality numbers are identical to those in Table 1.
MATERIALS AND METHODS

Vole samples

Forty-five Eothenomys samples from Honshu were used for the phylogenetic analyses in this study (Table 1 and Fig. 1; Iwasa, 2000; Iwasa and Tsuchiya, 2000; Iwasa and Suzuki, 2002a). Species identification was based on morphological characteristics (Kaneko et al., 1992; Iwasa, 2000). Total DNA was prepared from liver tissue by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. Fourteen specimens of both species were subject to rDNA-RFLP analysis. Mitochondrial cytochrome b (Cytb) gene sequences were determined for 12 specimens and the male

Table 1. Japanese Eothenomys specimens from Honshu examined in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Collection locality</th>
<th>Specimen No.</th>
<th>Sex</th>
<th>rDNA##</th>
<th>Cytb##</th>
<th>Sry##</th>
</tr>
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<td>E. andersoni</td>
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<td>I*</td>
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<td>Oze, Fukushima Pref. (5)</td>
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<td>Northeastern Honshu</td>
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<td>Kii Peninsula</td>
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<td>mEsm-31</td>
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<td>VI*</td>
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<td>VI</td>
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<td>Ashiu, Kyoto Pref. (19)</td>
<td>HS258</td>
<td>m</td>
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<td>mEsm-35*</td>
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</table>

# Each locality number in parenthesis is identical to that in Fig. 1.
## rDNA, repetype based on RFLP for nuclear ribosomal RNA genes; Cytb, haplotype number of mitochondrial cytochrome b gene; Sry, haplotype based on Y chromosomal Sry sequences; –, no data.
*Data from Iwasa and Suzuki (2002a).
sex-determining region of the Y chromosome (Sry) was sequenced in nine. These sequences were compared with other sequences from the databases (Iwasa and Suzuki, 2002a).

**Southern blotting for rDNA-RFLP**

Total DNA from 14 specimens of both species was digested using nine restriction enzymes: AatI (abbreviation=A), BamHI (B), BglII (G), DraI (D), EcoRI (E), PvuII (V), SacI (S), and XbaI (X) (Fig. 2). The digested DNA was immobilized on nylon membranes and allowed to hybridize with digoxigenin-labeled (Roche DIG DNA Labeling and Detection Kit) rDNA probes (0.8 µg/ml), namely, 28S, 18SB, and INT (Fig. 2), derived from the BALB/c mouse (Suzuki et al., 1994a, b; Iwasa et al., 2001). The probes were detected on the membrane using material supplied with the kit.

To identify blotting band patterns, restriction maps for various rDNA repeating units (repetypes) were constructed using restriction sites in the 18S, 5.8S, and 28S coding regions that are known to be conserved in mammals (Suzuki et al., 1994a, b; Iwasa et al., 2001). The restriction site maps were constructed from RFLP blotting patterns for the 5'-upstream external spacer region of the 18S gene (18S region), the 3'-downstream external spacer region of the 28S gene (28S region), and the internal spacer regions (ITS1 and 2) (Fig. 2).

**Direct sequencing of the mitochondrial Cytb gene**

A fragment of the mitochondrial Cytb gene was amplified with universal primers (L14724 and H15915; Irwin et al., 1991) in a primary PCR, followed by specific secondary PCR amplification with nested primers that were designed for voles (L14724, L15135, L15561, H15155, H15599, and H15919; Iwasa et al., 2000). The PCR reactions were carried out according to Iwasa et al. (2000). Both DNA strands of the product of the second PCR were sequenced directly using a Dye Primer Cycle Sequencing Kit (ABI) and an automated sequencer (model 373A, ABI).

**Phylogenetic analyses**

We constructed median-joining (MJ) network trees (Bandelt, 1994; Bandelt et al., 1995, 1999) using the rDNA-RFLP data (0–1 matrix for all restriction sites), using the program Network 2.0 and setting the explicit parameter ε = 0 (Röhö, 1997) based on the maximum parsimony heuristic calculations (Farris, 1970).

A maximum likelihood (ML; Felsenstein, 1981) tree was constructed using the quartet-puzzling method (10,000 puzzling steps) with the program Tree-Puzzle ver. 5.0 (Strimmer and von Haeseler, 1996) from the Cytb data. The ML analysis was performed using the HKY algorithm (Hasegawa et al., 1985) with unequal base frequencies and a discrete approximation to the gamma-distribution. The transition/transversion ratio, fraction of invariable sites, and shape parameter were estimated from the model. The mixed model of the heterogeneity rate (one invariable rate + eight gamma-distribution rates) was executed with the program (Strimmer and von Haeseler, 1996). To assess confidence, we used the quartet-puzzling scores (Strimmer and von Haeseler, 1996) for the ML tree. In addition, to confirm the relationships among all the Cytb haplotypes, a minimum-spanning tree was constructed according to the number of substitutions using ARLEQUIN ver. 2.000 (Schneider et al., 2000).

The Cytb and Sry sequences of the specimens analyzed in
Iwasa and Suzuki (2002a) were included in this study (GenBank/EMBL/DDBJ accession numbers: AB037281-AB037316 (Cytb) and AB037317-AB037323 (Sry)). Our new Cytb data (mEan-12, 13, 14, 30, 31 and 34) were added to the DNA databases under accession numbers AB104503-AB104508.

RESULTS

Genetic cline from rDNA repertypes

Restriction maps were constructed from the rDNA-RFLP patterns for the 14 Eothenomys specimens examined. In total, nine repetitive genotypes (repertypes) were detected, ignoring the size variation due to the insertion/deletion of small DNA fragments (a–i in Fig. 2). They were divided into seven groups: northeastern Honshu, central Honshu, Nagano, and the Kii Peninsula for E. andersoni, and central Honshu, southwestern Honshu, and the Kii Peninsula for E. smithii (Table 1 and Fig. 2).

The repertypes for E. andersoni and E. smithii consis-

![Fig. 3. Median-joining network tree based on the restriction site matrix by current rDNA-RFLP data. NE Honshu, northeastern Honshu; CT Honshu, central Honshu; SW Honshu, southwestern Honshu. Abbreviation of the nine repertypes (a–i) marked with open circles are the same those in Fig. 2. Small solid circles indicate hypothetical junction points. Branch lengths indicate distances based on the 0 1 distance matrix.](https://bioone.org/journals/Zoological-Science)

![Fig. 4. Maximum likelihood tree based on the Cytb gene sequence data using quartet-puzzling method. Numbers near nodes of the tree are support values (>50%, 10,000 steps) to assign the confidences of nodes. All haplotype numbers are listed in Table 1.](https://bioone.org/journals/Zoological-Science)
tentatively differed by three restriction sites: site S in the 18S region, and sites D and V in the 28S region (Fig. 2). In addition, a comparison of the repetypes of *E. andersoni* from northeastern Honshu and *E. smithii* from central and southwestern Honshu identified differences at ten sites (sites A, D, G, S, V, and X in the 28S region; Fig. 2). *E. smithii* at the base of the Kii Peninsula possessed some locality-specific restriction patterns and, unexpectedly, shared the same variation with the adjacent population of *E. andersoni* from the Kii Peninsula at D, G, and X in the 18S region and A and E in the 28S region (Fig. 2). *E. andersoni* from northeastern Honshu and *E. smithii* from central and southwestern Honshu identified differences at ten sites (sites A, D, G, S, V, and X in the 28S region; Fig. 2). *E. andersoni* from Nagano had similar patterns, sharing several variable sites (sites A, E, S, and V in the 28S region; Fig. 2) with *E. andersoni* from central Honshu and the Kii Peninsula. By contrast, in *E. smithii*, only the individual from the Kii Peninsula repertype had a higher level of variability than those from central and southwestern Honshu (Fig. 2).

The repetypes from the Kii Peninsula of both species showed a close relationship in the MJ tree that was constructed from the rDNA-RFLP data (Fig. 3). Similarly, the repetypes from northeastern and central Honshu of *E. andersoni* were closely related. The repetypes of *E. andersoni* from Nagano showed some affinity with the central Honshu *E. andersoni* (Fig. 3).

### Intra- and interspecific relationships of Cytb sequences

Based on our previous work (Iwasa and Suzuki, 2002a) and the present Cytb data (Table 1), we constructed an ML tree (\(\ln L=3370.01\), unresolved quartets=10.5\%, transition/transversion ratio=10.26, pyrimidine transition/purine transition ratio=2.09, gamma distribution parameter alpha=0.15\pm0.02 SE) and a minimum spanning network tree (Figs. 4 and 5). Both trees included a single specimen of *E. andersoni* (HEG167-98; Cytb haplotype No. mEan-25) in the *E. smithii* group (Cytb haplotype mEsm series, Figs. 4 and 5). In addition, *E. andersoni* was subdivided into the following distinct local groups: the Kii Peninsula (two sub-groups), central Honshu, Nagano, and northeastern Honshu groups.

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**Fig. 5.** Minimum spanning network tree based on the Cytb gene sequence data. All haplotype numbers are listed in Table 1. Number of open small circles along all branches mean number of substitutions between haplotypes connected. Branch lengths showing large number of substitutions (>20) are more reduced than actual genetic distances and number of substitutions are indicated by italicized numbers.
Intra- and interspecific relationships of the Sry sequences

Five distinct Sry haplotypes (I–V) were recognized after considering the sequence and size variation from previous (Iwasa and Suzuki, 2002a) and present analyses (Table 1). No variation within E. smithii was seen; a unique haplotype (VI) was recovered from all six males (Table 1; Iwasa and Suzuki, 2002a). The Sry sequences of both species contained a TG(TC)n repeat and a variable region in the central part, resulting in size variation ranging from 328 to 356 bp, identical to previous data in Iwasa and Suzuki (2002a). Two sequences with a single nucleotide substitution were observed in individuals from the western part of the central Honshu population of E. andersoni, although they are treated as one haplotype (III) here (Table 1).

In E. andersoni, the six variants of the Sry sequence showed specific geographic affinity (Table 1). Specimen HEG167-98 carrying the smithii-type Cytb sequence (mEan-14) had a typical andersoni-type Sry haplotype (III; Table 1) as well as karyological and morphological characteristics (Iwasa, 2000; Iwasa and Tsuchiya, 2000). In addition, the other specimens from Nagano had the same Sry haplotype (III; Table 1) as specimen HEG167-98. Considering Y chromosome criteria for Eothenomys voles (e.g., Iwasa, 1998; Iwasa and Tsuchiya, 2000), specimens with a small entirely heterochromatic Y chromosome, including specimen HEG167-98, carried the andersoni-type Sry haplotype and those bearing a medium-sized partially heterochromatic Y chromosome carried the smithii-type Sry, irrespective of the Cytb haplotype.

**DISCUSSION**

Our previous studies revealed the complexity of the genomes of two Japanese Eothenomys species (Iwasa et al., 1999; Suzuki et al., 1999; Iwasa and Tsuchiya, 2000; Iwasa and Suzuki, 2002a, b). In particular, E. andersoni from Nagano and the Kii Peninsula exhibits locality-specific and unique features in its sex chromosomes and combinations of Cytb and Sry sequences (Iwasa and Tsuchiya, 2000; Iwasa and Suzuki, 2002a). In this study, we examined the nuclear rDNA variation in Eothenomys samples, including new specimens, and found concrete evidence for interspecific genetic introgression between the sibling species E. andersoni and E. smithii during the course of evolution. Our findings suggest a specific mechanism for the generation and persistence of genetic diversity in small terrestrial organisms from Honshu, where there is topographic complexity over a broad range of latitude and a history of dramatic Quaternary climate changes.

Two phylogenetic markers, the mitochondrial Cytb gene and Y-chromosome Sry gene, which are both free from recombination, showed relatively marked genetic variation. By contrast, the nuclear rDNA-RFLP data showed less genetic variation, although these data provide reliable information for assessing phylogenetic status because of the multiple genetic elements within a genome (ca. 500 copies), and the specific concerted evolution that drives sequence similarity within a genome and within the same reproductive population (Coen et al., 1982). Distinct species differences were seen in E. andersoni and E. smithii at three restriction sites in the rDNA-RFLP array (Fig. 2). Using these mitochondrial and nuclear DNA markers, we reassessed the genetic make-up of local vole populations from Honshu, focusing on the samples of E. andersoni from the Kii Peninsula and Nagano.

The Kii Peninsula population of E. andersoni, which is separated from the main population in Honshu (Abe et al., 1994; Mammalogical Society of Japan, 1997) and is sometimes treated as a valid species (E. imaizumii, e.g., Jameson, 1961), has intriguing genetic characteristics. The Cytb and Sry gene variation showed historic gene flow from the neighboring population of E. andersoni in central Honshu (Iwasa and Suzuki, 2002a), resulting in two haplotypes of gene markers: one specific to the Kii Peninsula and one similar to the neighboring populations. Conversely, the rDNA-RFLP suggests interspecific hybridization (Figs. 2 and 3; Suzuki et al., 1999). Therefore, the Kii Peninsula population of E. andersoni likely has a hybrid origin, incorporating genetic elements from its sibling species, E. smithii (Fig. 3). This is consistent with our previous hypothesis based on a cytogenetic study, which showed that E. andersoni from the Kii Peninsula possessed a mixed type of sex chromosome variation that was thought to have originated from both species (Iwasa et al., 1999; Iwasa and Tsuchiya, 2000). Although morphological and cytogenetical markers distinguish E. smithii from E. andersoni (Miyao, 1967; Iwasa, 2000; Iwasa and Tsuchiya, 2000), one E. smithii (HEG96Ess-1) from the base of the Kii Peninsula shared several restriction sites with E. andersoni from the Kii Peninsula in the rDNA-RFLP analysis (Kii Peninsula E. smithii; Figs. 2 and 3), implying that introgression of the rDNA elements occurred in both directions during the course of evolution in the Kii Peninsula and the adjacent area. Therefore, the data from the Kii Peninsula support the hypothesis (Iwasa and Suzuki, 2002a, b) that the evolution of Japanese vole species involves frequent introgression, both between conspecific local populations and between closely related species.

The seven E. andersoni from Nagano, in central Honshu, exhibited another complex genetic pattern. The Sry genotypes were the same as in E. andersoni from the Kii peninsula and alpine areas in central Honshu (Table 1). In contrast, the Cytb haplotype was an admixture of E. andersoni and E. smithii (Figs. 4 and 5), providing robust evidence for interspecific genetic exchange, although no E. smithii individuals have been trapped near Nagano (Morozumi and Miyao, 1974; Morozumi, 1977; Miyao et al., 1979a, b; Morozumi and Morozumi, 1988; Iwasa, 2000; Iwasa and Tsuchiya, 2000). The rDNA-RFLP shows no sign of genetic
introgression and is the *E. andersoni* type, based on diagnostic variation in the three restriction sites mentioned above, although there are some changes (Figs. 2 and 3). The sequences of the *Cytb* gene from Nagano are also distinct, with approximately 1% genetic distance from the type for each species (Fig. 4), showing separation of the Nagano population from other conspecific and non-conspecific populations. Therefore, interspecific genome introgression occurred at some time in the past and the polymorphism in the mitochondrial DNA has been maintained (founder events; Mayr, 1963; Harrison, 1989). This postulate explaining the presence of historical interspecific introgression in the Nagano sample is in agreement with our previous studies of chromosomes (Iwasa and Tsuchiya, 2000) and X-linked gene sequences (Iwasa and Suzuki, b).

Our previous (Iwasa et al., 1999; Iwasa and Tsuchiya, 2000; Iwasa and Suzuki, 2002a, b) and current data indicate that the evolutionary course of Japanese *Eothenomys* is puzzling and highly complex, countering initial expectations (Suzuki et al., 1999). These complex patterns (Figs. 3, 4 and 5) are a consequence of complex evolutionary processes, such as past interspecific genome introgression, judging from intra- and interspecific gene divergence (Iwasa and Suzuki, 2002a). During the last glacial age, these vole species would not only have been split further into local populations with topographic changes arising from climatic change, but would have undergone genome introgression between the local populations (Iwasa and Suzuki, 2002a). Similar examples are seen in other Japanese small mammals, including shrews (Iwasa et al., 2001; Ohdachi et al., 1997, 2001, 2003; Motokawa et al., 2000), shrew-moles (Harada et al., 2001), moles (Tsuchiya et al., 2000; Kawada et al., 2001), and voles (Iwasa and Suzuki, 2002a, b; Iwasa et al., 2000, 2002).

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