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Differential geographic patterns of mitochondrial DNA variation in two sympatric species of Japanese wood mice, *Apodemus speciosus* and *A. argenteus*

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We examined the gene sequences of mitochondrial cytochrome *b* (cyt *b*) in two Japanese wood mouse species, *Apodemus speciosus* (n = 89) and *A. argenteus* (n = 46), which are distributed on the four main islands of Japan (Hokkaido, Honshu, Shikoku, and Kyushu) and on the small islands surrounding them. *Apodemus speciosus*, the larger of the two species, showed substantial genetic variation, with a maximum of 3% sequence divergence, and remarkable phylogenetic subdivision with two major clades. One clade represents haplotypes from a central region, including Honshu, Shikoku, Kyushu, and their adjacent islands; the other clade includes haplotypes from Hokkaido and the peripheral islands, forming four subclades: a) Hokkaido, b) Sado Island, c) Satsunan Islands, and d) the Izu Islands. Sequence divergence among the four subclades was 1.0 to 1.5%, implying that *A. speciosus* colonized these geographic regions 0.2 to 0.3 million years ago, assuming a substitution rate of 2.4% per million years. The population on the Izu Islands has preserved haplotypes that are distinct from those in any other region, providing good evidence for the natural colonization of the volcanic islands of the Izu Islands. The cyt *b* sequence variation had no relation to the karyotypic dimorphism for the eastern (2n = 48) and western (2n = 46) geographic groups, between which a strict border exists at central Honshu. On the other hand, *Apodemus argenteus*, the smaller of the two species, showed a similar level of sequence divergence (maximum of 3%) but no substantial geographic differentiation: populations in Hokkaido, Sado, and Yakushima shared similar haplotypes with each of the central populations, suggesting that genetic exchanges occurred among the localities in the last 0.15 million years. The apparent genetic structure of the mitochondrial DNA found in the *A. speciosus* population might be caused solely by long-term existence in insular regions, presumably due to ecological superiority relative to *A. argenteus*.

Key words: *Apodemus argenteus, A. speciosus,* geographic variation, mitochondrial DNA, wood mouse

INTRODUCTION

The four main islands of Japan (Hokkaido, Honshu, Shikoku, and Kyushu) form an arc along the eastern edge of the Eurasian continent and harbor about one hundred species of mammals, most of which are native. This is especially true of smaller mammals, such as rodents and moles. The island chain extends a great distance from north to south, and thus has great potential usefulness for phylogeographic studies from which to infer the impact, of the Quaternary glacial age on genetic diversity

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Table 1. List of samples and nucleotide diversity (π) with and without (in parenthesis) islet populations.

	Collection locality	n	DNA Code (HS)
I. *Apodemus speciosus*			
Hokkaido (2n=48)			
1	Rishiri I.	2	252, 253
2	Kunashiri I.	1	1103*
3	Bibai	1	97
4	Otofuke	1	2783#
5	Naganuma	3	237, 238, 240#**
6	Onuma	1	359
7	Okushiri I.	3	210, 211, 214
		π = 0.3% (0.28%)	
Northern Honshu (2n=48)			
8	Aomori, Aomori Pref.	2	389, 390
9	Karumai, Iwate Pref.	1	375
10	Mt. Moriyoshi, Akita Pref.	1	301
11	Tono, Iwate Pref.	1	1492
12	Sado I.	2	101#, 104#
13	Tainai, Niigata Pref.	1	65
14	Mt. Urabandai, Fukushima Pref.	1	52
15	Namie, Fukushima Pref.	1	77
16	Koriyama, Fukushima Pref.	1	127
17	Oze, Gunma Pref.	1	303
18	Mt. Haruna, Gunma Pref.	1	162
19	Tsumagoi, Gunma Pref.	1	247
20	Kimitsu, Chiba Pref.	1	192
21	Esumi-gun, Chiba Pref.	1	653
22	Katsuura, Chiba Pref.	1	378
23	Kamaridani, Kanagawa Pref.	1	22
24	Izuoshima I.	2	181#, 186
25	Niijima I.	2	3259#, 3260#
26	Shikinejima I.	1	96
27	Miyakejima I.	4	49#, 50, 51, 302
28	Mt. Ashitaka, Shizuoka Pref.	3	194-196
29	Subashiri, Shizuoka Pref.	1	418
30	Mishima, Shizuoka Pref.	1	200
31	Mt. Amagi, Shizuoka Pref.	1	189
32	Sakuma A, Shizuoka Pref.	1	1436
		π = 1.5% (0.7%)	
Southern Honshu (2n=46)			
33	Sakuma B, Shizuoka Pref.	1	1435
34	Mt. Chausu, Nagano Pref.	2	1441, 1442
35	Kanazawa, Ishikawa Pref.	1	145
36	Asiu, Kyoto Pref.	1	282
37	Rokko, Hyogo Pref.	1	203
38	Ise, Mie Pref.	1	93
39	Kawayu, Wakayama Pref.	1	342
40	Nachi, Wakayama Pref.	2	95, 349
41	Minachi, Wakayama Pref.	1	279
42	Dogo, Oki Is.	1	172
43	Beppu, Dozen, Oki Is.	2	173, 175
44	Hiwa, Hiroshima Pref.	1	73
		π = 0.8% (0.8%)	
Shikoku (2n=46)			
45	Shodoshima I.	2	136,140
46	Omishima I.	1	299
47	Mt. Tsurugi, Tokushima Pref.	1	310
48	Saga, Kochi Pref.	1	53
		π = 0.9% (1.0%)	
Kyushu (2n=46)			
49	Tsushima I.	2	68#, 70
50	Fukuejima I.	1	3010#
51	Mt. Shiragami, Kumamoto Pref.	1	143
52	Ebino, Miyazaki Pref.	1	308
53	Yamanokuchi, Kagoshima Pref.	2	3002#, 3003#
54	Uchiura, Kagoshima Pref.	1	406
55	Koshikijima Is.	1	277#
56	Tanegashima I.	3	107, 109, 117
57	Yakushima I.	1	28
58	Kuchinoerabujima I.	3	1176, 3017#, 3018#
59	Kuchinoshima I.	3	1177-1179
60	Nakanoshima I.	4	14, 306, 1180, 1181
		π = 2.1% (1.6%)	
II. *Apodemus argenteus*			
Hokkaido			
1	Sapporo	1	2778#
2	Naganuma	3	222, 226, 361#**
		π = 0.0%	
Northern Honshu			
3	Aomori, Aomori Pref.	1	392
4	Hachinohe, Aomori Pref.	1	294
5	Sado I.	2	102, 105#
6	Koriyama, Fukushima Pref.	1	284
7	Oze, Gunma Pref.	1	295
8	Nikko, Tochigi Pref.	2	82, 83
9	Mt. Haruna, Gunma Pref.	2	153, 158
10	Chichibu, Saitama Pref.	1	88
11	Aokigahara, Yamanashi Pref.	1	285
12	Subashiri, Shizuoka Pref.	1	424
13	Ito, Shizuoka Pref.	1	1217
14	Mt. Amagi, Shizuoka Pref.	3	187#, 188, 191
15	Sakuma A, Shizuoka Pref.	1	1433#
		p = 1.1% (1.2%)	
Southern Honshu			
16	Mt. Chausu, Aichi Pref.	1	1438
17	Ashiu, Kyoto Pref.	1	290#
18	Minachi, Wakayama Pref.	2	35, 37
19	Mt. Nachi, Wakayama Pref.	1	350
20	Dogo, Oki Is.	3	177#, 178, 179
21	Beppu, Dozen, Oki Is.	1	180#
22	Kakinoki, Shimane Pref.	1	142
23	Akiyoshidai, Yamaguchi Pref.	1	3028
		π = 1.5% (1.1%)	
Shikoku			
24	Shodoshima I.	1	135#
25	Shioe, Kagawa Pref.	2	1147#, 1216#
		π = 2.5% (3.0%)	
Kyushu			
26	Soeda, Fukuoka Pref.	1	360
27	Omura, Nagasaki Pref.	1	3026#
28	Mt. Shiragami, Kumamoto Pref.	1	292#
29	Ebino, Miyazaki Pref.	2	43, 45
30	Miyazaki, Miyazaki Pref.	2	1807, 1811
31	Fukuejima I.	1	3027#
32	Yakushima I.	2	29, 31#
		π = 1.8% (1.9%)	

* Chelomina et. al. (1998), **Serizawa et al. (2000)

Taxa subjected to phylogenetic analysis with 1140 bp sequence data.

and speciation (Hewitt, 1996). In fact, molecular genetic studies have revealed a substantial degree of intraspecies genetic variation in dormice (Suzuki et al., 1997), voles (Suzuki et al., 1999; Iwasa and Suzuki, 2002), and moles (Tsuchiya et al., 2000) and have provided interesting portraits of not only the evolutionary history of animals but also of episodes in the geographic history of the islands. For example, Japanese red-backed voles (genus *Eothenomys*), with eastern (*E. andersonii*) and western (*E. smithii*) species, show apparent population genetic structures and historical genetic exchanges among local populations and between the species, implying that expansion and separation occurred repeatedly among the islands in association with the climatic changes during the Quaternary ice age (Iwasa and Suzuki, 2002). Similarly, it has been suggested that the complicated geographic variation of mitochondrial DNA (mtDNA) in two Japanese moles with distributions mainly in the eastern (*Mogera imaizumii*) and western (*M. wogura*) regions was influenced by climatic changes during the Quaternary ice age (Tsuchiya et al., 2000). Comparable analysis with many species of Japanese mammals is expected to provide a more comprehensive view of the dynamics of climatic change and its effects on population genetic structures. The most common of small mammals in Japan, the wood mouse genus *Apodemus*, however, has not yet been studied using the common tools of molecular genetics.

Members of the genus *Apodemus* inhabit broad-leaf forests in the temperate zone of the Palearctic region (Corbet, 1978; Corbet and Hill, 1992; Musser and Carleton, 1993), and depend on forest resources such as acorns, insects, and other small invertebrates (Sekijima and Sone, 1994; Shimada, 2001). *Apodemus* species have limited species-specific distributions, with two or more species often cohabiting in a forest (Corbet, 1978; Corbet and Hill, 1992; Musser and Carleton, 1993). Japan today hosts three different species of *Apodemus*, two of which are endemic, the large Japanese wood mouse and lesser Japanese wood mouse. The third species, the Korean wood mouse *A. peninsulae*, has a Japanese branch in Hokkaido, and based on mitochondrial sequence variation it has been suggested that the Hokkaido population became established in the latter part of the Quaternary (Serizawa et al., 2002). The large Japanese wood mouse, *Apodemus speciosus,* occurs throughout the four main islands and their peripheral islands, including Sado Island, the Izu Islands (Izu-oshima, Niijima, Shikinejima, and Miyakejima, from north to south in this order), and the Satsunan Islands (Tanegashima, Yakushima, and Kuchinoerabujima, *etc.*). The small one, *A. argenteus*,

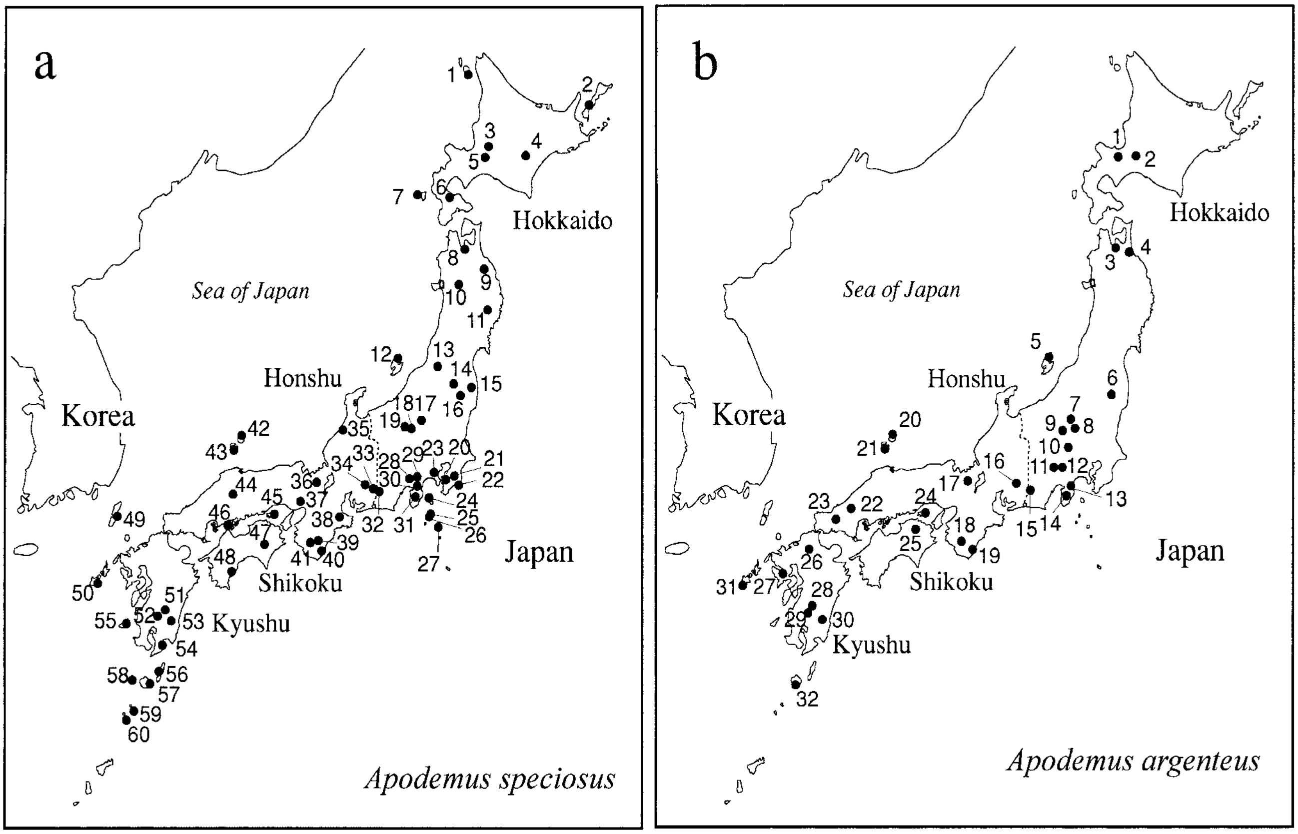
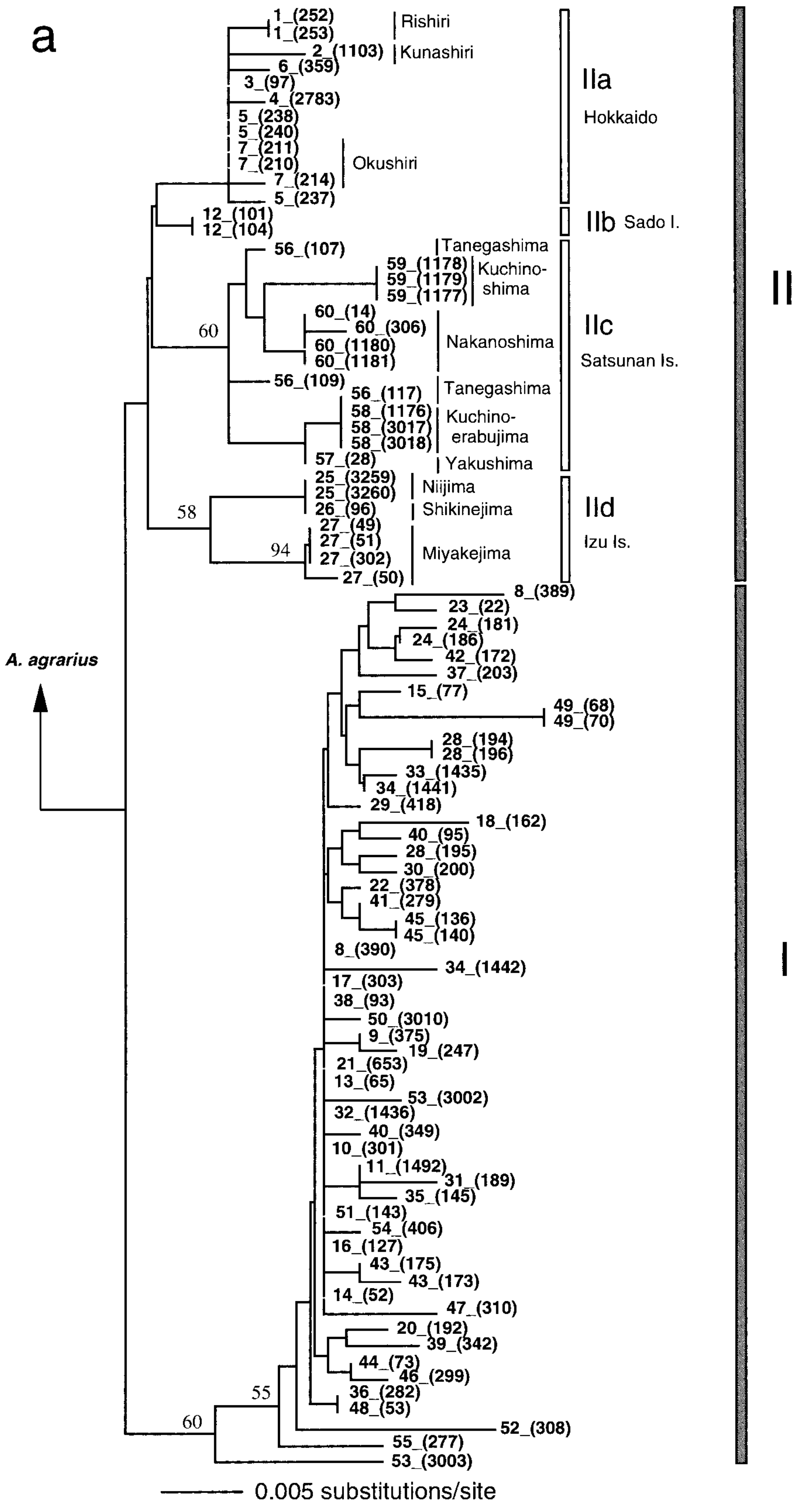


Fig. 1. Locations at which populations of *Apodemus speciosus* (a) and *A. argenteus* were sampled on the main islands and adjacent small islands. The locality numbers are explained in Table 1. The dotted line represents the Toyama-Hamamatsu line that divides *A. speciosus* into two distinct types with respect to karyotype: 2n = 48 (eastern) and 2n = 46 (western).


a
1_(252)
1_(253)
Rishiri
2_(1103)
Kunashiri
6_(359)
3_(97)
4_(2783)
5_(238)
5_(240)
7_(211)
7_(210)
7_(214)
Okushiri
5_(237)
IIa
Hokkaido
12_(101)
12_(104)
IIb
Sado I.
56_(107)
Tanegashima
59_(1178)
59_(1179)
59_(1177)
Kuchino-shima
60_(14)
60_(306)
60_(1180)
60_(1181)
Nakanoshima
60
56_(109)
56_(117)
Tanegashima
58_(1176)
58_(3017)
58_(3018)
Kuchino-erabujima
57_(28)
Yakushima
IIc
Satsunan Is.
25_(3259)
25_(3260)
Niijima
26_(96)
Shikinejima
58
27_(49)
27_(51)
27_(302)
27_(50)
94
Miyakejima
IId
Izu Is.
II
8_(389)
23_(22)
24_(181)
24_(186)
42_(172)
37_(203)
A. agrarius
15_(77)
49_(68)
49_(70)
28_(194)
28_(196)
33_(1435)
34_(1441)
29_(418)
18_(162)
40_(95)
28_(195)
30_(200)
22_(378)
41_(279)
45_(136)
45_(140)
8_(390)
34_(1442)
17_(303)
38_(93)
50_(3010)
9_(375)
19_(247)
21_(653)
13_(65)
53_(3002)
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40_(349)
10_(301)
11_(1492)
31_(189)
35_(145)
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52_(308)
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0.005 substitutions/site

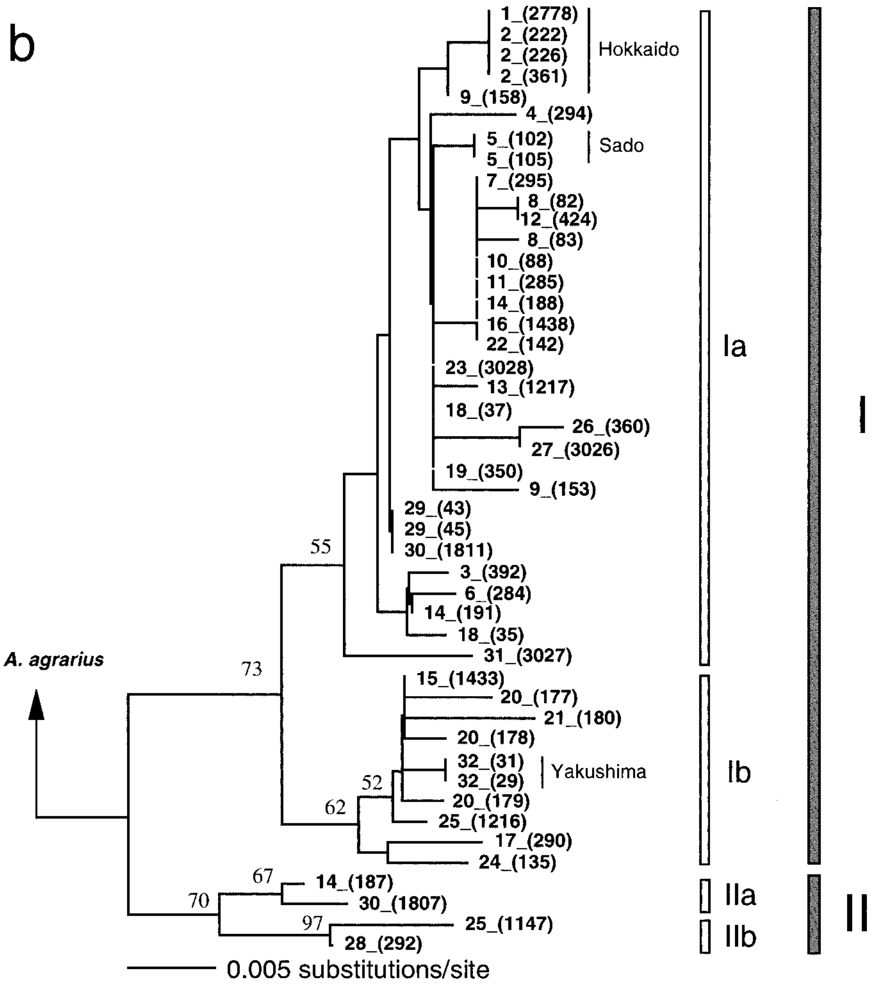

Fig. 2. Phylogenetic relationships among 77 sequences of the mitochondrial cytochrome *b* gene (402 bp) from *Apodemus speciosus* (a) and among 44 sequences from *A. argenteus* (b). The tree was constructed using Kimura's (1980) distances and the neighbor-joining (NJ) algorithm considering all codon positions. Numerical values associated with nodes are those supported by both the NJ analyses with high bootstrap values (1000 replicates, > 50%). The taxon names correspond to those given in Table 1 (locality number followed by individual number).

which shows sympatric and often even syntopic distribution with *A. speciosus*, has a distribution range similar to that of *A. speciosus*, including some remote islands such as Sado Island and Yakushima Island. These two species have been shown to have notably long evolutionary histories independent from those of the continental species, with divergence times of five or six million years, based on molecular phylogenetic studies (Serizawa et al., 2000; Suzuki et al., 2003). Because the presence or absence of connections among the remote and the main islands and the geological timing of such connections have been debated, these two mouse species have the potential to provide useful clues for the elucidation of the geological history of the Japanese islands, including the formation and disappearance of land bridges among the insular domains.

Apodemus speciosus has long attracted research interest because of the substantial morphological variation seen in specimens from the main and the peripheral islands. The population on Hokkaido is sometimes treated as a valid species, and populations on remote islands such as Miyakejima and Tsushima are regarded as different subspecies from the mainland species (Imaizumi, 1962, 1964). The species is also known to have dimorphic variation in karyotype; populations with 2n = 48 and 2n = 46 are located in the eastern and western parts of the mainland, respectively, with a strict border in central Honshu (Tsuchiya et al., 1973). Heterozygotic specimens of *A. speciosus* with 2n = 47 have been trapped only in this border zone, which is about 5 to 20 km wide (Harada et al., 1984). This dimorphism can also be seen on isolated peripheral islands where patterns are the same as those on the adjacent mainland areas (Tsuchiya et al., 1973). Genetic variation has been examined in *A. speciosus* using allozymes (Saitoh et al., 1989) and restriction fragment-length polymorphism (RFLP) of the spacer regions of the 18S and 28S ribosomal RNA genes (rDNA; Suzuki et al., 1994), revealing that there is no substantial divergence between the two chromosomal groups.

Here we examined mitochondrial sequence variation (cytochrome *b* gene, 1140 bp) in the two Japanese wood mice to understand their population structures. A comparison of the patterns of geographic differentiation provides useful insights into not only the consequences of geological events, such as the formation and disappearance of land bridges to the remote islands, but also into other unknown factors in the genetic patterns of animal populations.

MATERIALS AND METHODS

Animals. Between 1980 and 2003, 89 specimens of *A. speciosus* and 46 specimens of *A. argenteus* were collected from throughout their distribution range (Table 1, Fig. 1).

Sequencing and Phylogenetic Analysis. A 1.2-kb fragment of the gene for cyt *b* was first amplified by polymerase chain reaction (PCR) with the universal primers L14724 and H15915 (Irwin et al., 1991). For the second PCR, nested PCRs were performed according to methods previously described (Suzuki et al., 1997, 2000). Single segments were amplified from the first PCR products, using the nested primer set R-L14724/U-H15155 (Suzuki et al., 1997) to produce a 402-bp sequence, and R-L14724/U-H15402 and R-L15244/U-H15915 (Yasuda et al., submitted) to produce an 1140-bp sequence. Both strands of the 402-bp product of the second PCR, and of the 1140-bp product for selected individuals, were then sequenced directly by an automated method with a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an automated sequencer (Model 3100; Applied Biosystems). The nucleotide sequences have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession numbers AB164483-AB164614.

Trees were constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987) and the maximum parsimony (MP) method, using PAUP* 4.0b10 (Swofford, 2003). For the NJ analysis we used Kimura's two-parameter distances (*d*; Kimura, 1980). For the MP analyses, 10 heuristic searches were conducted with the tree bisection-reconnection (TBR) option, in which the input order of the taxa is randomized. The robustness of the resulting phylogenies was assessed by bootstrap analysis with 1000 resamplings. Nucleotide diversities (π; Nei, 1987) were calculated using ARLEQUIN software (Schneider et al., 2000). Genetic distances between clusters of sequences were computed with PHYLTEST (Kumar, 1996)

RESULTS

Sequencing of the cyt *b* gene of 89 *A. speciosus* and 46 *A. argenteus* specimens generated 58 and 31 haplotypes, respectively. Of the 402 base pairs sequenced, 70 sites were variable and 30 sites were informative for parsimony analysis in *A. speciosus*, whereas there were 38 variable sites and 12 informative sites in *A. argenteus*.

Phylogenetic analysis (Fig. 2a) revealed that the 58 haplotypes from *A. speciosus* fell into two major clades with clear geographic affinity: Clade I comprised haplotypes from the three main islands of Honshu, Shikoku, and Kyushu and their closely associated islands, such as Oki Islands, Izu-oshima, and Tsushima Islands; and clade II encompassed haplotypes from Hokkaido and the remote islands. Bootstrap analysis weakly or moder-

Table 2. Fst values based on genetic distance

a. *A. speciosus*	n	1	2	3	4
1. Hokkaido, Sado, Izu Is.#, Satsunan Is.	35				
2. Eastern Honshu (ex. Sado, Izu Is.#)	25	0.57937*			
3. Western Honshu	15	0.54587*	0.01291		
4. Shikoku	5	0.51135*	0.07798	0.02121	
5. Kyushu (ex. Satsunan Is.)	9	0.45635*	0.07729*	0.05610*	0.02245

b. *A. argenteus*	n	1	2	3	4
1. Hokkaido	4	–			
2. Eastern Honshu	18	0.24994*	–		
3. Western Honshu	11	0.34884*	0.12499*	–	
4. Shikoku	3	0.54717*	0.45103*	0.1797	–
5. Kyushu	10	0.25305*	0.11963*	0.0386	0.1075

* Significance level, P <0.05

Izu-oshima I. was included in the group of Eastern Honshu

ately (50–60%) supported the grouping. The maximum extent of sequence divergence was 3%. The second clade could be divided into four geographically separated subclades with weak or moderate bootstrap support: subclades from Hokkaido (IIa; the main island, Kunashiri, Okushiri, and Rishiri), Sado Island (IIb), the Izu Islands (IId; Miyakejima, Niijima, and Shikinejima), and the Satsunan Islands (IIc; Kuchinoshima, Kuchinoerabujima, Nakanoshima, Tanegashima, and Yakushima).

The 31 haplotypes from *A. argenteus* fell into two clades, I and II (Fig. 2b), with genetic diversity of up to 3%. Clade I could be further divided into two subclades,

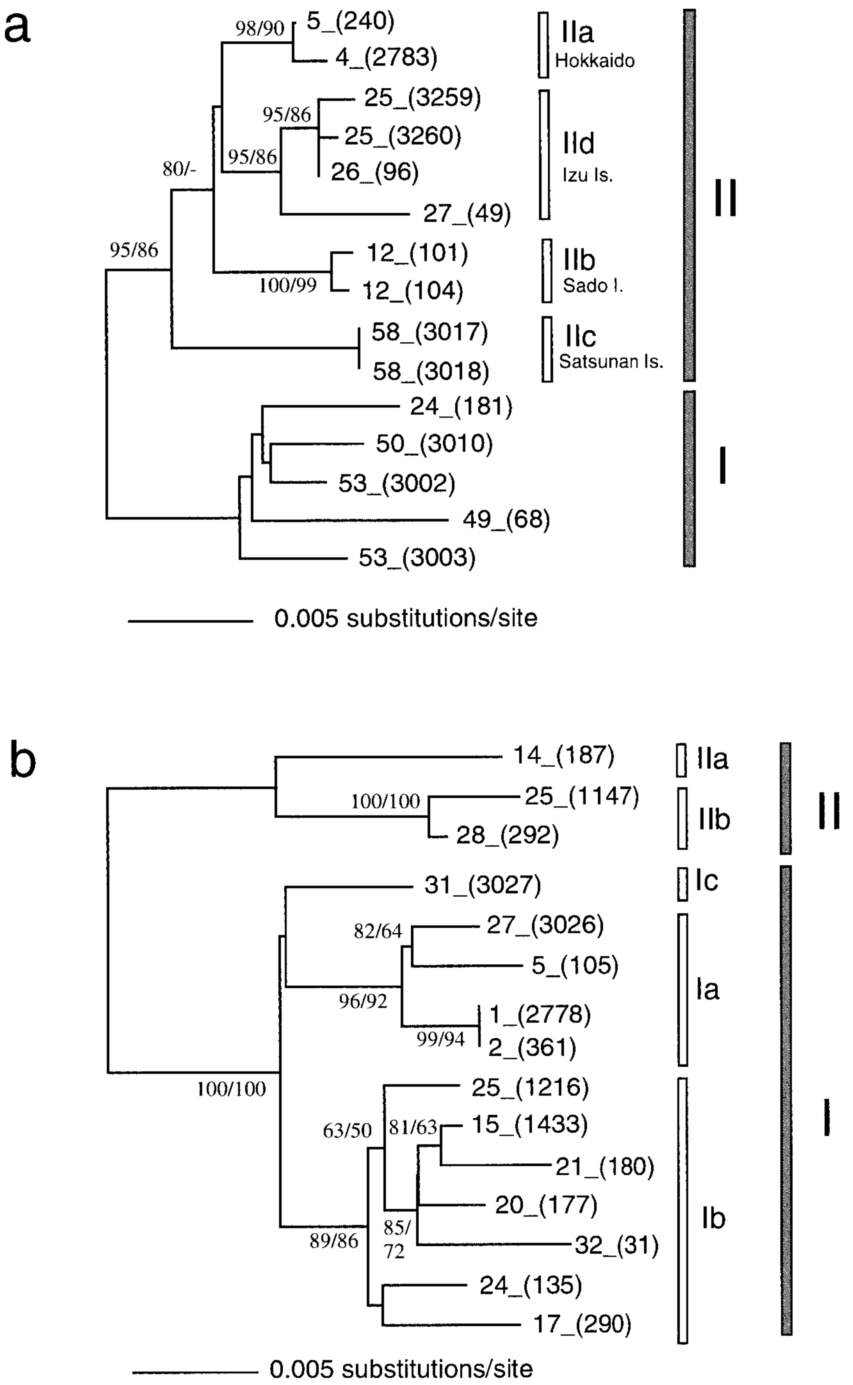

Fig. 3. Phylogenetic relationships among 15 sequences of the mitochondrial cytochrome *b* gene (1140 bp) from *Apodemus speciosus* (a) and among 15 sequences from *A. argenteus* (b). The tree was constructed using Kimuras (1980) distances and the neighbor-joining (NJ) algorithm considering all codon positions. Nodes that are supported by both the NJ and maximum parsimony methods with high bootstrap values (1000 replicates, > 50%) are marked (NJ/MP). The taxon names correspond to those given in Table 1 (locality number followed by individual number).

Ia and Ib. The larger subclade, Ia, encompasses the whole distribution range from Hokkaido to Kyushu, while members of subclade Ib were confined to the western part of Japan. Clade II consists of four haplotypes from the eastern part of Honshu (n = 1, Amagi, location 14 in Fig. 1b), Shikoku (n = 1), and Kyushu (n = 2). Thus, in sharp contrast to *A. speciosus*, which occurred as clade II on islands such as Sado, Hokkaido, and Yakushima, and as clade I on the three mainlands, *A. argenteus* specimens from those islands had mtDNA genotypes similar to those of mainland populations.

The minimum spanning network (data not shown; Schneider et al., 2000) demonstrated a substantial difference between *A. speciosus* clades I and II, with eight mutational steps, and between clades I and II of *A. argenteus*, with nine mutational steps. Clade I of *A. speciosus*, from all of Japan except Hokkaido and the peripheral islands, and subclade Ia of *A. argenteus*, from the entire Japanese archipelago, had a star-shaped topology, which has been interpreted as being the result of a recent expansion from a small number of individuals (Avice, 2000). The distribution range of *A. argenteus* subclade Ib is limited to the western region, where the *A. speciosus* 2n = 46 karyotype prevails, as mentioned above, while *A. speciosus* clade I occurs on both sides of the genetic border defined by the 2n = 48 and 2n = 46 karyotypes.

Population analysis was carried out with the cyt *b* sequences in both species. The genetic variation within regions, based on nucleotide diversity (π), tended to be high at low latitudes and low at high latitudes (Table 1). Here we divided Honshu into two geographic regions, north (or east) and south (or west) of the karyotype border of *A. speciosus* (Fig. 1a). The values of π for *A. speciosus* were 0.3, 1.5, 0.8, 0.9, and 2.1% in Hokkaido, eastern Honshu, western Honshu, Shikoku, and Kyushu, respectively. The values for *A. argenteus* were 0.0, 1.1, 1.5, 2.5, and 1.8%, respectively, for the same regions. To assess regional differentiation, pairwise Fst values were obtained based on distance methods (Table 2). We grouped *A. speciosus* haplotypes into five groups, taking into account the clustering patterns of haplotypes (Fig. 2a) and the karyotype groups (Tsuchiya et al., 1973): remote islands, eastern Honshu, western Honshu, Shikoku, and Kyushu. No substantial difference was seen between the eastern and western haplotype groups of Honshu, in spite of the border between the karyotypes (Table 2). In contrast, a geographic subdivision was seen in *A. argenteus* with respect to the *A. speciosus* karyotypic border in central Honshu: the Hokkaido and eastern Honshu groups could be differentiated from the other groups.

To assess the divergence times for the haplotype subdivisions, we sequenced the longer nested PCR product (1140 bp) for representative individuals (Table 1). The resulting phylogenetic trees (Fig. 3) were essentially consistent with those derived from the 402-bp sequences. Exceptionally, the sequence of *A. argenteus* from Fukuejima I., Kyushu (HS3027, Locality number 31 in Fig. 1b) nested in clade Ia formally in the 402-bp analysis appeared to be independent from Ia and Ib in the 1140-bp analysis (and was therefore designated as distinct clade Ic). Using average genetic distances and an estimated evolutionary rate of 0.024/million years/one way for cyt *b* (Suzuki et al., 2003), rough estimates of the divergence times of the representative sequences for each monophyletic group were obtained (Table 3). The divergence of clades I and II of *A. speciosus* was calculated to have occurred 0.5 million years ago (mya), while the divergence of *A. argenteus* clades I and II was estimated to have occurred about 0.6 mya. Phylogenetic analysis suggested that in *A. speciosus,* the Satsunan Islands subclade IIc diverged from the members of clade II early (Fig. 3a); the lineage split of the Satsunan Islands (IIc) was

Table 3. Degree of intra- and inter-clade sequence divergences with Kimura two-parameter distance (cyt *b*, 1140 bp)

Clades compared	Genetic distance	mya
A. speciosus		
Clades I vs II	2.44 ±0.36	0.51
IIa/IIb/IId vs IIc	1.72 ±0.34	0.36
IIa/IId vs IIb	1.26 ±0.28	0.26
IIa vs IId	1.06 ±0.27	0.22
Miyake vs Niijima/Shikine	0.88 ± 0.27	0.18
Niijima/Shikine	0.013 ± 0.08	0.03
A. argeneteus		
Clades I vs II	3.07 ±0.44	0.64
Ia/Ic vs Ib	1.69 ±0.31	0.35
IIa vs IIb	1.74 ±0.38	0.36

mya: divergence time (million years ago) based on evolutionary rate of 2.4%/mya/lineage (Suzuki et al., 2003).

estimated to have occurred 0.3–0.4 mya, based on the genetic distances of 1.72 ± 0.34 (*d* x 100; mean, standard error). The time of separation of the Sado (IIb), Hokkaido (IIa), and Miyakejima/Niijima/Shikinejima (IId) populations was estimated to be 0.2–0.3 mya (Table 3). On the other hand, in *A. argenteus,* isolation of the Sado and Satsunan Islands' (represented by Kuchinoerabujima) populations from the mainland populations was estimated to have occurred less than 0.15 million years ago, based on the genetic distances of 0.6−0.8 (*d* x 100). Our present data for *A. speciosus* and *A. argenteus* thus provide robust evidence that migrations have occurred among the islands, including Sado Island and the Satsunan Islands, at least twice during the last half million years.

DISCUSSION

Differential patterns of geographic variation. The haplotypes found in *A. speciosus* were grouped into two major clades (Figs. 2, 3). Clade I represents haplotypes from the Honshu, Shikoku, and Kyushu mainlands and their closely associated islands, while clade II comprises haplotypes from four peripheral island groups: Sado, Hokkaido, the Izu Islands, and the Satsunan Islands. The geographic distribution of the mtDNA variation in *A. speciosus* did not show any association with that of chromosomal dimorphism. In contrast, the sequences of *A. argenteus* from Sado, Hokkaido, and Yakushima were closely related to those from the Honshu, Shikoku, and Kyushu mainlands. The two clades identified in the *A. argenteus* samples, which are comparable to those of *A. speciosus* in terms of genetic distances, are likely to be commingled throughout the Japanese islands, though the members of clade II were recovered from western Japan. Thus, our data indicate that unknown factor(s) acted differentially to generate the mtDNA population structure in each of these species.

The geographic structure of mtDNA variation in *A. speciosus*, which indicated a division between central (Honshu, Shikoku, and Kyushu) and peripheral (Hokkaido, Sado, Izu Is., and Satsunan Is.) populations, is puzzling, because genetic subdivision of organisms in Japan is usually observed between eastern and western populations. One explanation for the *A. speciosus* structure is that this species might have developed some degree of reproductive separation between the mainlands and the remote insular populations, allowing the remote insular populations to sustain their mtDNA types irrespective of secondary contact between the peripheral and central islands by reconstruction of land bridges. Imaizumi (1962, 1964) found substantial differences in the morphological characteristics of large Japanese wood mouse specimens from Hokkaido and Miyakejima, as compared to those from other regions. The populations belonging to *A. speciosus* clade II have, in fact, sometimes been regarded as different species or subspecies. The hypothesis that assumes reproductive isolation, however, seems inconsistent with the fact that the peripheral islands have a karyotype identical to that of the nearby mainlands, suggesting the existence of gene flow when land bridges were present.

Alternatively, and more plausibly, the differential patterns of mtDNA variations between *A. speciosus* and *A. argenteus* can be attributed to a differential ability to survive on the small islands during long periods of separation from major populations. The presence of island-specific haplotypes of *A. speciosus* simply indicates that the islands maintained populations for a considerable geological time, while *A. argenteus* was probably susceptible to extinction during isolation for long periods of evolutionary time. The observed differences in the geographical distribution of genetic variation might reflect differences in the survival ability of the two species: the survival of *A. speciosus* is superior to that of *A. argenteus* on small islands (Kaneko, 1992). Although both species share resources and coinhabit the same forests (Sekijima and Sone, 1994; Shimada, 2001), islands of less than 150 km^2 tend to sustain only one species, probably because of limited resources subject to interspecies competition; on the majority of these islands, the surviving species is *A. speciosus* (Kaneko, 1992). This notion leads us to conclude that differences in survival ability among closely related species are possibly involved in producing the population genetic structure. Thus, it is possible to say that *A. speciosus* maintained its insular populations for such long evolutionary periods and preserved anciently diverged mtDNA regardless of secondary contact between the insular and mainland domains because of the particular properties of maternally inherited genetic materials. Persistence of such anciently diverged mtDNA haplotypes is also seen in other small mammals of the Japanese islands (voles, Suzuki et al., 1999; Iwasa and Suzuki, 2002; dormice, Suzuki et al., 1997), probably owing to female philopatry.

Population genetic structure. The mtDNA data presented here allow us to assess the level of genetic diversity in the two wood mouse species, which appears to vary from north to south among the Japanese islands. Excluding the peripheral islands and Shikoku, where sample sizes were very small, comparisons of nucleotide diversity (π) among the regions of Hokkaido, eastern Honshu, western Honshu, and Kyushu clearly showed that the values were substantially lower in Hokkaido and higher in Kyushu (Table 1). These results can be attributed to variations in population size (abundance of resources), topographic diversity, and ice age impact. Though further work will be needed to clarify which factors shaped the cline of genetic variability, one interesting point is that *A. argenteus*, which lives at higher altitudes and thus is thought to be more tolerant to cold, also shows the same apparent tendency toward lower diversity at higher latitudes (Table 2). In *A. argenteus*, the nucleotide diversity is nearly 0 in Hokkaido (Table 1) and the genetic distances between haplotypes from Hokkaido and Honshu is at least 0.008, suggesting a recent migration of mtDNA from Honshu to Hokkaido. Therefore, it is possible to hypothesize that *A. argenteus* was more sensitive to the impact of the ice age. The difference in survival ability may be owing to interspecies competition in some local habitats where the resources became insufficient to maintain both species. This is somewhat consistent with our prediction that *A. speciosus* was better adapted for insular survival than was *A. argenteus*, and that this resulted in the extinction of island-specific mtDNA in *A. argenteus*.

It will be of interest to examine the mechanisms that govern the generation and maintenance of genetic diversity in central Honshu that demarcates the eastern and western groups, as in the case of the 2n = 48 and 2n = 46 karyomorphs of *A. speciosus* (Fig. 1). Our present data revealed that the border has no apparent effect on the genetic subdivision for either wood mouse species, based on the mtDNA sequences examined to date. The mtDNA patterns (Figs. 2, 3) imply that genes have been transmitted across the border, as was predicted by the allozyme and rDNA-RFLP analyses of *A. speciosus* (Saitoh et al., 1989; Suzuki et al., 1994). These results imply that the karyotype difference exhibiting the Robertsonian rearrangement does not cause a substantial reproductive isolation. Hybrid individuals with 2n = 47 have been collected from the field in the border zone (*e.g.*, Tsuchiya, 1975; Saitoh et al., 1989) and can be obtained in the laboratory (Tsuchiya, 1975). The fertility of F1 hybrids has also been demonstrated by experimental work (Tsuchiya, 1975). These considerations lead us to conclude that the karyotypic border zone permits gene flow, at least for some genes that are thought to be evolving neutrally.

Another interesting point to consider is why the chromosomal dimorphism has been sustained in a specific geographic region, in spite of apparent gene flow. *Apodemus speciosus* is thought to have come to the Japanese islands as long as 5 to 7 million years ago (Serizawa et al., 2000; Suzuki et al., 2003) and the species-specific karyotypes differ from those of the continental species *A. agrarius* and *A. peninsulae* (Yoshida et al., 1975; Saitoh and Obara, 1986; Orlov et al., 1996; Matsubara et al., unpublished), so the chromosome arrangement seen in *A. speciosus* probably originated somewhere in western Japan and expanded, forming an ever-lengthening border in the manner of the statipatric speciation model (White, 1968) and ceasing at the Toyama-Hamamatsu line. The formation of such geographically defined genetic borders has been attributed to heterozygote disadvantage, *i.e.*, the reproductive inferiority of F1 hybrids, as is predicted for Rh blood polymorphisms (Nei et al., 1981). Heterozygote disadvantage has been used to explain the maintenance of a structured karyotypic border between Israeli and Turkish mole rats despite gene flow (*e.g.*, Suzuki et al., 1987, 1996). Saitoh and Obara (1988), in fact, have shown that F1 karyotype hybrids of *A. speciosus* have meiotic cell numbers of up to 25% below normal levels.

Utility of mtDNA data for the assessment of geological history. The present data provide valuable evidence for the presence of land bridges in the Japanese islands during the last half-million years. The distinctness of mtDNA sequences in the insular populations of *A. speciosus* indicates the long-term persistence of these populations before human colonization. The two species of *Apodemus* are thought to have inhabited the Japanese islands a long time ago, such as the late Tertiary (Serizawa et al., 2000), which implies that migrations from the continent do not need to be taken into consideration for generation of genetic diversity. Unlike rodents with conspecific populations on the Asian continent, such as other rodents that inhabit Hokkaido (Iwasa et al., 2000; Serizawa et al., 2002), the genetic variation seen in the endemic species would be the result of long evolutionary processes that involved only the Japanese Islands. The distinctness of the island haplotypes can thus be associated with the geological events of the formation and disappearance of land bridges between the islands and the neighboring mainlands. A minimum value for the genetic distances between the insular and mainland haplotypes could be used to estimate the timing of the formation or disappearance of land bridges. The accuracy of estimates based on the available datasets is limited because closer haplotypes might exist undiscovered at other sites or might be extinct. Therefore the estimates derived from the minimum genetic distances reflect the ‘maximum time’ for the occurrence of a given geological event.

The timing of the formation and disappearance of the connection between Hokkaido and Honshu, namely the land bridge across the Tsugaru Strait, is a source of major debate in biogeographic studies. Our data clearly indicate that land bridges were established at least twice, approximately 0.2–0.4 mya and less than 0.15 mya, based on the genetic variation seen in *A. speciosus* and *A. argenteus*, respectively. Similarly, a connection between Sado Island and Honshu was apparently established twice, 0.2–0.3 mya and less than 0.15 mya. The genetic distinctness of insular *A. speciosus* populations is somewhat comparable to that seen in the case of moles (Tsuchiya et al., 2000), although the evolutionary rate of the cyt *b* gene may differ between wood mice and moles. The existence of land bridge connections between the Izu Islands and Honshu has long been debated because the islands are thought to have emerged by volcanic activity. Our data clearly indicate that the Izu Islands were connected with Honshu at least once, at 0.2–0.3 mya (Table 3), providing good evidence for the natural colonization of the Izu Islands. Interestingly, the islands of the Izu group adjoined one another in more recent times; a connection existed between Miyakejima, Niijima, and Shikinejima approximately 0.2 mya, and between Niijima and Shikinejima during the last glacial period. These findings are rather unexpected because the Izu Islands are now separated by deep sea (over 200 m even at the shallowest point between Shikinejima and Miyakejima), as well as from mainland Honshu. A similar situation is seen for Okushiri Island, which is separated from mainland Hokkaido by a deep strait (over 400 m deep), but the mtDNA haplotypes from the island are quite similar to those from Hokkaido, with an estimated divergence time of less than 0.1 mya (Fig. 2a). For the Satsunan Islands, including Kuchinoerabujima and Yakushima, land bridges were apparently established at least twice, 0.3–0.4 mya and less than 0.15 mya, based on the genetic variation seen in *A. specious* and *A. argenteus*, respectively. This is also consistent with findings for other mammals with insular populations exhibiting minimal genetic distinctness from the mainland populations. Data collected for moles from Tanegashima and Yakushima show a genetic distance of < 0.01 (Tsuchiya et al., 2000), and Japanese weasels from Yakushima have a genetic distance of 0.005 (Hosoda et al., 2000), relative to the nearest mainland populations.

In conclusion, phylogeographic studies of the Japanese wood mouse can potentially provide robust evidence for the geologic events of the Japanese islands during the Quaternary. These studies are also useful in assessing the impact of environmental changes on the level of genetic diversity and the framework of genetic structures. In addition, these studies shed light on the evolutionary consequences of genetic structures, such as the interspecies ecological competition underlying the persistence of local populations. The two species of wood mice that inhabit Japan, with similar geographic ranges, can be viewed as valuable bio-resources for phylogeographic research to better understand the impact on genetic diversity of historical geological events, topographic structure, and the ecological relationships between congeneric species.

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