A Biogeographic View of *Apodemus* in Asia and Europe Inferred From Nuclear and Mitochondrial Gene Sequences

Hitoshi Suzuki · Maria Grazia Filippucci · Galina N. Chelomina · Jun J. Sato · Keiko Serizawa · Eviatar Nevo

Abstract Sequences of the mitochondrial cyt *b* gene and nuclear IRBP, *RAG1*, *I7*, and vWF genes were used to assess the evolutionary history of major lineages of *Apodemus*, in particular to better understand dispersal between Asia and Europe. Our data show eight extant lineages of Late Tertiary origin: *Apodemus agrarius*, *A. semotus*, *A. peninsulae*, *A. speciosus*, *A. argenteus*, *A. gurkha*, *A. mystacinus*, and *A. sylvaticus*. Monophyly of two European lineages (*A. mystacinus* and *A. sylvaticus*) and four Asian lineages (*A. agrarius*, *A. semotus*, *A. peninsulae*, and *A. speciosus*) was confirmed with high bootstrap support. Together with literature data, the available molecular data depict three crucial evolutionary events: (1) initial wide dispersal and subsequent radiation around 6 million years ago, (2) region-specific radiations in Europe and southern China around 2 million years ago, and (3) westward dispersal of *A. agrarius* to Europe in the Late Quaternary.

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Introduction

Based on the recent molecular phylogenetic analyses of small mammals, the last 10–20 million years were very important for establishing the current distributions of extant species (e.g., Michaux et al. 2002, 2003, 2004; Serizawa et al. 2000; Suzuki et al. 2003, 2004). Molecular studies of small mammals, which use specific ecological habitats, provide important clues for understanding how extant flora and fauna were established. In addition, they are also useful for understanding how environmental changes in the Late Tertiary and Quaternary affected the movements of lineages across areas and even across continents. Factors that affected speciation events must be considered from a biogeographic perspective. Among the various groups of small mammals, the wood mouse genus *Apodemus* is the most common rodent in temperate Eurasia and may be one of the best subjects for biogeographic study.

Species in the genus *Apodemus* are the most common rodents in the temperate zone of the Palaearctic region (Corbet 1978; Corbet and Hill 1992; Orlov et al. 1996), and more than 20 *Apodemus* species are now recognized, half from Asia and half from Europe (Musser and Carleton 2005). *Apodemus* species occur in a variety of habitats, including woodlands, forests, and grasslands, depending on resources such as acorns, insects, and other small invertebrates. Two or more species often coinhabit, showing resource partitioning among syntopic species, which is thought to be associated with the biogeographic history of this genus and its speciation processes. The framework for an *Apodemus* molecular phylogeny has been provided by analyses of the mitochondrial cytochrome *b* (cyt *b*) gene and the nuclear interphotoreceptor retinoid binding protein (IRBP) gene (Michaux et al. 2002; Serizawa et al. 2000; Suzuki et al. 2003), indicating four distinct lineages among the taxa examined to date: the *Sylvaemus*, the *Apodemus*, the *A. argenteus*, and the *A. gurkha* groups. These lineages are somewhat comparable to the subgeneric grouping system of Musser et al. (1996); however, *Apodemus mystacinus*, which is sometimes classified as the distinct subgenus *Karstomys*, was not included in the molecular analyses covering an entire set of the distinct lineages. Meanwhile, through ongoing morphological and molecular analyses, the taxonomic situation has improved somewhat, but there are still some difficult cases dealing with closely related species (see Musser and Carleton 2005).

In particular, the systematics of western Palaearctic species, included in the subgenus *Sylvaemus*, are complicated because of the description of several new species in southern Europe and the Middle East [*A. alpicola*, *A. uralensis*, *A. fulvipectus, A. hermonensis* (*whiterbyi* in Musser and Carleton 2005), *A. arianus, A. hyrcanicus, A. ponticus, A. rusiges*, and *A. wardi* (*pallipes* in Musser and Carleton 2005)]. The phylogenetic relationships among these taxa are still not solidly established, and the distribution of the two most widespread species, *A. sylvaticus* and *A. flavicollis*, in southeastern Europe and the Middle East is still unclear. Several subspecies of *A. sylvaticus* appear to be geographical forms of *A. uralensis* (Mezhzherin and Mikhailenko 1991). Vorontsov et al. (1992) proposed that four species inhabit the Caucasus and Transcaucasus based on morphological, chromosomal, and allozyme data: *A. ponticus* (previously considered a subspecies

of *A. flavicollis*), *A. hyrcanicus, A. uralensis*, and *A. fulvipectus.* In western Anatolia, allozyme and morphological data indicate the presence of four *Sylvaemus* species (Filippucci et al. 1996): *A. sylvaticus* (rare and restricted to a small area near the coast of the Black Sea, previously considered a widely distributed species in Asia Minor), *A. flavicollis* (widely distributed, previously considered limited to eastern Anatolia and the Caucasus), *A. hermonensis* (widely distributed, previously known only from the type locality, Mount Hermon in Israel; Filippucci et al. 1989), *A. uralensis* (confined to the humid mountainous areas of northern Asia Minor). These results were confirmed by Macholán et al. (2001), extending the study to eastern Turkey, Armenia, and Iran, where populations of *A. flavicollis, A. hermonensis*, and *A.* cf *hyrcanicus* were found. The systematics of wood mice from Iran are still incomplete, and data from eastern Iran and central Asia are fragmentary. Musser and Carleton (2005) suggested that *A. arianus* inhabited Iran and *A. wardi* was distributed in Nepal, Kashmir, Pakistan, Afghanistan, and northwestern Iran. According to these authors, *A. fulvipectus* (in the north) and *A. ponticus* (in the northwest) probably also occur in Iran.

Filippucci et al. (2002) analyzed the genetic variation of 28–38 gene loci by protein electrophoresis in ten species of the genus *Apodemus* from Europe, the Middle East, and North Africa, and found high genetic distance in *A. mystacinus* between *epimelas* from Europe and *mystacinus* from Israel and Turkey, suggesting that the two taxa could represent distinct species and supporting Storch's (1977) hypothesis of their ancient separation dating to the Pleistocene. The separation of the two taxa has been supported by molecular phylogenetic studies (Bellinvia 2004; Michaux et al. 2005). *Apodemus mystacinus* and *A. epimelas* appeared as sister taxa to other species of the *Sylvaemus* group (Bellinvia 2004; Michaux et al. 2002), in agreement with Musser et al. (1996), although the validity of the subgeneric name *Karstomys*, used by several authors (Rietschel and Storch 1974; Storch 1975) for the two former taxa, is not generally accepted.

Molecular analyses of the East Asian subgeneric group *Apodemus* (Serizawa et al. 2000; Suzuki et al. 2003) have indicated four distinct lineages represented by *Apodemus agrarius*, *A. draco*, *A. peninsulae*, and *A. speciosus*, in which the former two lineages include at least two (*agrarius* and *chevrieri*) and three species (*draco*, *latronum*, and *semotus*), respectively, forming species groups *agrarius* and *draco*, respectively. Liu et al. (2004) examined cyt *b* gene sequences, focusing on Chinese taxa, and showed that the Yunnan taxon *A. ilex*, which is often treated as a synonym of *A. draco* (Musser and Carleton 1993; Suzuki et al. 2003), possessed a taxon-specific mitochondrial lineage, as is seen in *A. draco* from other mainland areas and *A. semotus* from Taiwan.

Apodemus agrarius has a wide range across several geographic domains. A branch of *A. agrarius* is found in Europe, including Turkey. To determine the evolutionary episodes of the European population, a comparative study with Asian populations is necessary. Through biochemical analysis, Filippucci (1992) showed intraspecific variation in this species, as observed in other European *Apodemus* species. Although Nei's genetic distance between two subspecies of *A. agrarius* (*istrianus* from northeastern Italy and *agrarius* from Yugoslavia, according to Krystufek 1985) is not very high ($D = 0.027$), populations from the two geographic

areas displayed polymorphism at different loci (Italian population: *Me-1, 6-Pgdh, Got-1*, and *Ada*; Yugoslavian population: *Idh-2, Got-1, Pgm-1*, and *Pgm-2*), and *6-Pgdh* was partially different between the two taxa. Currently, however, no data are available on the genetic relationships between Asian and European populations.

In this study, we conducted phylogenetic analyses with a larger set of both Asian and European species. We examined 17 species of *Apodemus* using five phylogenetic markers: the mitochondrial cyt *b* (1,140 bp) gene and the nuclear single-copy genes *IRBP* (1,152 bp), recombination activating gene 1 (*RAG1*, 1,002 bp), and von Willebrand factor (*vWF*, 1,192 bp). Exon regions of the nuclear genes *IRBP, RAG1*, and *vWF* are used to assess phylogenetic relationships across mammalian orders (Huchon et al. 1999, 2002; Springer et al. 1997; Stanhope et al. 1992, 1996). These nuclear genes are also useful for defining relationships among lower-level taxa (e.g., Chinen et al. 2005; Jansa and Voss 2000; Serizawa et al. 2000; Suzuki et al. 2000, 2003, 2004). We also examined the *I7* gene (an olfactory receptor, 792 bp), a member of the olfactory receptor gene family, which is the largest gene family in the mammalian genome, with more than 1,000 members. This study is a good opportunity to assess the utility of olfactory receptor genes for phylogenetic inference. We also addressed the issue of genetic relatedness of Asian and European *A. agrarius* populations using both mitochondrial and nuclear gene variation.

Materials and Methods

Samples and Sequence Data

In addition to the samples used in the previous studies (Chelomina and Suzuki 2006; Chelomina et al. 1998; Han et al. 1996; Sato and Suzuki 2004; Serizawa et al. 2000; Suzuki et al. 2003), the cyt *b*, *IRBP, RAG1, I7*, and *vWF* sequences of *A. agrarius* (Treviso, Venetia, Italy), *A. hermonensis* (Mount Hermon, Israel), and *A. mystacinus* (Masada Forests, Golan Heights, Israel) were determined (Table 1). We used the species name *A. draco* for individuals from Yunnan, China (sample codes HS 505, 1628; Suzuki et al. 2003) in this study, but it is notable that the taxon is sometimes classified as *A. ilex* and differs from *A. draco* from other parts of southern China (see Liu et al. 2004). *RAG1* sequences were also determined for 13 ingroup taxa and an outgroup taxon, the mole rat *Spalax ehrenbergi* (Kerem Ben Zirma, Israel; sample code KBZ500). Sequences of other ingroup and outgroup taxa (*Micromys minutus, Mus musculus, Rattus norvegicus*, and *Mesocricetus auratus*) were obtained from the previous studies (Sato and Suzuki 2004; Serizawa et al. 2000; Suzuki et al. 2003, 2004).

Sequencing

Polymerase chain reactions (PCR) and direct sequencing of the cyt *b* (1,140 bp), *IRBP* (1,152 bp), and *RAG1* (1,002 bp) genes were performed according to the previously described methods (Sato et al. 2004; Serizawa et al. 2000; Suzuki et al.

Table 1 *Apodemus* species analyzed in the interspecies phylogenetic analysis

Species	Main range	Sample code (source)	Data source[a]
A. agrarius	East Asia, Europe	HS1253 (Primorye, Russia)	2; 2; 1; 1; 1
		HAN43 (Yang san, South Korea)	1; –; 1; 1; –
		HAN37 (Gansung, South Korea)	1; –; –; –; –
		HS328 (Shanghai, China)	3; 3; –; –; –
		HS1733 (Guizhou, China)	3; –; 1; –; –
		HS86 (Taiwan)	3; 3; –; 1; –
		2F (Italy)	1; –; –; 1; –
A. chevrieri	Yunnan (China)	HS503 (Yunnan)	3; 3; 1; –; 1
		HS504 (Yunnan)	3; 3; –; –; –
		HS510 (Yunnan)	3; –; 1; –; –
		HS1729 (Yunnan)	3; –; 1; 1; –
A. draco (*A. ilex*)[b]	Southern China	HS505 (Yunnan)	3; –; 1; 1; 1
		HS1628 (Yunnan)	3, 3; –; 1; –
		HS2365 (Yunnan)	3, 3; –; –; –
A. latronum	Yunnan (China)	HS2515 (Yunnan)	3; 3; 1; 1; –
		HS2516 (Yunnan)	3; 3; 1; 1; 1
		HS2366 (Yunnan)	3; –; 1; –; 1
A. peninsulae	Northern East Asia	HS329 (Hokkaido, Japan)	2; 2; 1; 1; –
		HS1390 (Primorye, Russia)	–; –; –; –; 1
A. semotus	Taiwan	HS2 (Taiwan)	6; 2; 4; 1; 1
A. speciosus	Japan	HS240 (Hokkaido, Japan)	2; 2; 1; 1; 1
		HS391 (Aomori, Japan)	–; –; –; –; 1
A. argenteus	Japan	HS361 (Hokkaido, Japan)	2; 2; 1; 1; 1
A. gurkha	Nepal	HS1317 (Nepal)	2; 2; 1; 1; 1
A. mystacinus	Europe	MAS2 (Israel)	1; 1; 1; 1; 1
A. alpicola	Europe	HS596 (Austria)	2; 2; 1; 1; 1
A. flavicollis	Europe	HS591 (Switzerland)	2; 2; 1; 1; 1
		HS1105 (Ukraine)	5; 5; 1; 1; –
A. hermonensis	Middle East	Her102 (Israel)	1; 1; 1; –; –
		Her103 (Israel)	1; 1; 1; 1; –
		HS1102 (Armenia)	5; 5; 1; 1; 1
A. ponticus	Caucasus; Transcaucasus	HS1101 (Georgia)	5; 5; 1; 1; 1
A. sylvaticus	Europe	HS1290 (Holland)	6; 2; 4; 1; 1
		HS1104 (Ukraine)	5; 5; 1; 1; –
A. uralensis	Northern Asia Minor	HS1744 (Kazakhstan)	3; 3; 1; 1; 1
		HS1106 (Altai)	5; 5; 1; 1; –
		HS1107 (Moldova)	5; 5; 1; 1; –
A. wardi	Nepal	HS1307 (Nepal)	3; 3; 1; 1; 1

[a] Reference codes are given for genes cyt *b*, *IRBP*, *RAG1*, *I7*, *vWF*, respectively. Reference code: 1, this study; 2, Serizawa et al. (2000); 3, Suzuki et al. (2003); 4, Sato and Suzuki (2004); 5, Chelomina and Suzuki (2006); 6, Suzuki et al. (2000)

[b] Individuals from Yunnan are considered to be *A. ilex*, according to Liu et al. (2004)

2000, 2003, 2004). A part of the olfactory receptor gene was amplified by PCR with the primer pair +Olf7-122 (5′-GGC CTA [C/T]GT GTT GGT G[T/C]T GAC-3′) and –OlfI7-916 (5′-CAG GTG CAG [C/A]GT GCG ACG TAG-3′), according to the mouse (Krautwurst et al. 1998) and rat (Buck and Axel 1991) *I7* genes. Numbers in the primer names designate the position of the 3′ end of the primer in the published rat *I7* sequence, and the prefixes + and – refer to the reading and complementary strands, respectively. A 0.8 kb portion of the fragment was then sequenced through the amplification of three segments with the following primer sets: R + SN-OlfI7-122 (5′-CAG GAA ACA GCT ATG ACC GGC CTA [C/T]GT GTT GGT G[T/C]T GAC-3′) and U-SN-OlfI7-533 (5′-TGT AAA ACG ACG GCC AGT CAC AGA AAA AGT GGT TGA TGG-3′); R + SN-OlfI7-524 (5′-CAG GAA ACA GCT ATG ACC TCT CGC CTG TCT TAC TGT GG-3′) and U-SN-OlfI7-916 (5′-TGT AAA ACG ACG GCC AGT CAG GTG CAG [C/A]GT GCG ACG TAG-3′). A 1.2 kb fragment of *vWF* was sequenced through the amplification of three segments with the following primer sets: *vWF*-F241-*Mus* (5′-TGT GAA CCT TAC GTG TGA AGC CTG-3′) and *vWF*-R843-rodent (5′-TCA TCC ACC CCA CTG AGC AGA AA-3′); *vWF*-F559-*Mus* (5′-CCC TCA GAG CTT CGG CGC AT-3′) and *vWF*-R1099-*musculus* (5′-ACG TCC ATG CGC TGG ATG ACC T-3′); *vWF*-F1030-*musculus* (5′-TCC ATG GTT CTG GAT GTG GT-3′) and *vWF*-R1507 (Huchon et al. 1999; in their paper, this primer was named W1). "F" and "R" in the primer names refer to forward and reverse, respectively. The numbers indicate the position of the 3′ ends in the published human sequence (Mancuso et al. 1989; accession no. M25851).

Sources for sequences obtained from databases are listed in Table 1. The new nucleotide sequences reported in this paper were deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession nos. AB285442-AB285452 and AB303224-AB303285.

Phylogenetic Analysis

Trees were constructed using the neighbor-joining (NJ; Saitou and Nei 1987), maximum parsimony (MP), and maximum likelihood (ML) methods using PAUP 4.0b10 (Swofford 2003). For the NJ analysis, we used Kimura's two-parameter distances (*d*; Kimura 1980) in single-gene datasets. For MP and ML analyses, ten heuristic searches were conducted with the tree-bisection reconnection option, in which the input order of taxa is randomized. The best-fit model of DNA evolution for ML analysis was chosen on the basis of Akaike's information criterion (AIC), according to the suggestion of Posada and Buckley (2004) that the AIC-based model selection is superior to the hierarchical likelihood ratio tests, using PAUP 4.0b10 (Swofford 2003) and Modeltest version 3.7 (Posada and Crandall 1998). Robustness of clades of phylogenetic trees was assessed using bootstrap analyses with 1,000, 1,000, and 100 resamplings for the NJ, MP, and ML analyses, respectively. The data were also analyzed by Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003), in which the appropriate model was selected with AIC in Modeltest, according to the suggestion of Posada and Buckley (2004).

Divergence times were estimated by the method first described by Thorne et al. (1998), in which the node dates were estimated in a Bayesian framework allowing changes in evolutionary rate over time, using PAML version 3.14 (Yang 1997), paml2modelinf, estbranches, and multidivtime according to the description by Rutschmann (2005). The superiority of using the combined dataset to estimate divergence time, as suggested by Yang and Yoder (2003), enabled us to date divergence times with the combined four sequences of rodents. First, we obtained single-gene trees by ML analysis with models and parameters determined by AIC (with Modeltest and PAUP). Model parameters [unequal nucleotide frequencies, transition/transversion rate, and rate heterogeneity among sites for the F84 + G model; F84, Felsenstein 84 implemented in Phylip (Felsenstein 1993)] were then estimated for each single-gene tree with PAML. The obtained model parameters and a single Bayesian tree from the combined dataset, as described above, were subsequently used for estimating branch lengths in this Bayesian tree and the variance–covariance matrix of the parameters (with estbranches). From the information above, Markov Chain Monte Carlo analyses were performed to approximate the posterior distributions of substitution rates and divergence times (with multidivtime).

Results

Nuclear Gene Sequences

We constructed single-gene NJ and MP trees for 13 species of *Apodemus* with data from *IRBP, RAG1, I7*, and *vWF* (Fig. 1). The number of variable sites for ingroup comparisons was 174, 125, 72, and 177, respectively. We did not detect any signals of gene duplication in our data, including those from *I7*, where gene duplication is known to occur. We also conducted phylogenetic inference with the combined *IRBP + RAG1 + I7 + vWF* data using the NJ, MP, ML, and BI methods (Fig. 2). In the ML and NJ analyses, we used a TrN + I + G model (-ln L = 12457.3728; A = 0.2253, C = 0.2818, G = 0.2696, T = 0.2254; gamma shape parameter = 0.8033; assumed proportion of invariable sites = 0.4695). There were 529 variable sites, and the resultant trees were likely to be more reliable than the single-gene trees.

Based on this dataset (Figs. 1, 2), we noted three groupings (designated here as species groups) of species with close phylogenetic relationships and low genetic distances: (1) *A. agrarius* and *A. chevrieri* (*agrarius* species group); (2) *A. draco, A. latronum*, and *A. semotus* (*draco* species group); and (3) *A. alpicola, A. sylvaticus, A. flavicollis, A. hermonensis, A. uralensis*, and *A. wardi* (*sylvaticus* species group, which corresponds to the subgenus *Sylvaemus*) (Musser et al. 1996). Together with four monospecific groups (*A. argenteus, A. gurkha, A. peninsulae*, and *A. speciosus*) and the *A. mystacinus* species group with two species (see Introduction), eight species groups were thus recognized in the *Apodemus* species examined.

Among the eight species groups, a monophyletic relationship with high support was recovered for four ancient lineages of the *A. agrarius* group (*agrarius, semotus, peninsulae*, and *speciosus*), which was suggested by Serizawa et al. (2000). The

a

IRBP

A. flavicollis Switzerland HS591
A. ponticus Georgia HS1101
A. flavicollis Ukraine HS1105
A. alpicola Austria HS596
A. sylvaticus Holland HS1290
A. sylvaticus Ukraine HS1104
A. hermonensis Israel Her102
A. hermonensis Israel Her103
A. hermonensis Armenia HS1102
A. uralensis Altai HS1106
A. uralensis Moldova HS1107
A. uralensis Kazakhstan HS1744
A. wardi Nepal HS1307
A. mystacinus Israel MAS2
A. gurkha Nepal HS1317
A. argenteus Japan HS361
A. agrarius Taiwan HS86
A. agrarius Shanghai HS328
A. agrarius Primorye HS1253
A. chevrieri Yunnan HS503
A. chevrieri Yunnan HS504
"*A. draco*" Yunnan HS1628
"*A. draco*" Yunnan HS2365
A. semotus Taiwan HS2
A. latronum Yunnan HS2515
A. latronum Yunnan HS2516
A. peninsulae Japan HS329
A. speciosus Japan HS240
Mus musculus molossinus
Mus musculus domesticus
Rattus norvegicus
Micromys minutus
Mesocricetus auratus
Spalax zemni

— 0.005 substitutions/site

b

RAG1

A. flavicollis Switzerland HS591
A. flavicollis Ukraine HS1105
A. ponticus Georgia HS1101
A. wardi Nepal HS1307
A. uralensis Altai HS1106
A. uralensis Moldova HS1107
A. uralensis Kazakhstan HS1744
A. sylvaticus Ukraine HS1104
A. sylvaticus Holland HS1290
A. hermonensis Israel Her102
A. hermonensis Israel Her103
A. hermonensis Armenia HS1102
A. alpicola Austria HS596
A. mystacinus Israel MAS2
A. agrarius Primorye HS1253
A. agrarius Korea HAN43
A. agrarius Yunnan HS1733
A. chevrieri Yunnan HS510
A. chevrieri Yunnan HS1729
A. chevrieri Yunnan HS503
"*A. draco*" Yunnan HS505
A. semotus Taiwan HS2
A. latronum Yunnan HS2366
A. latronum Yunnan HS2516
A. latronum Yunnan HS2515
A. peninsulae Japan HS329
A. speciosus Japan HS240
A. gurkha Nepal HS1317
A. argenteus Japan HS361
Mus musculus molossinus
Mus musculus domesticus
Mus macedonicus HS537
Micromys minutus
Rattus norvegicus
Mesocricetus auratus
Spalax ehrenbergi KBZ500

— 0.005 substitutions/site

c

I7

A. flavicollis Switzerland HS591
A. sylvaticus Ukraine HS1104
A. flavicollis Ukraine HS1105
A. ponticus Georgia HS1101
A. sylvaticus Holland HS1290
A. hermonensis Israel Her103
A. hermonensis Armenia HS1102
A. alpicola Austria HS596
A. uralensis Altai HS1106
A. wardi Nepal HS1307
A. uralensis Moldova HS1107
A. uralensis Kazakhstan HS1744
A. mystacinus Israel MAS2
A. gurkha Nepal HS1317
A. agrarius Italy 2F
A. agrarius Korea HAN43
A. agrarius Primorye HS1253
A. agrarius Germany HS133
A. chevrieri Yunnan HS1729
A. latronum Yunnan HS2515
A. latronum Yunnan HS2516
A. semotus Taiwan HS2
A. draco Yunnan HS505
A. draco Yunnan HS1628
A. peninsulae Japan HS329
A. speciosus Japan HS240
A. argenteus Japan HS361
Mus musculus molossinus
Mus musculus domesticus
Rattus norvegicus
Micromys minutus
Mesocricetus auratus

— 0.005 substitutions/site

d

vWF

A. flavicollis Switzerland HS591
A. ponticus Georgia HS1101
A. sylvaticus Holland HS1290
A. wardi Nepal HS1307
A. uralensis Kazakhstan HS1744
A. alpicola Austria HS596
A. hermonensis Armenia HS1102
A. mystacinus Israel MAS2
A. agrarius Primorye HS1253
A. chevrieri Yunnan HS503
A. peninsulae Primorye HS1390
"*A. draco*" Yunnan HS505
A. semotus Taiwan HS2
A. latronum Yunnan HS2516
A. speciosus Japan HS391
A. gurkha Nepal HS1317
A. argenteus Japan HS361
Mus musculus AJ238390
Rattus norvegicus AJ224673
Mesocricetus auratus
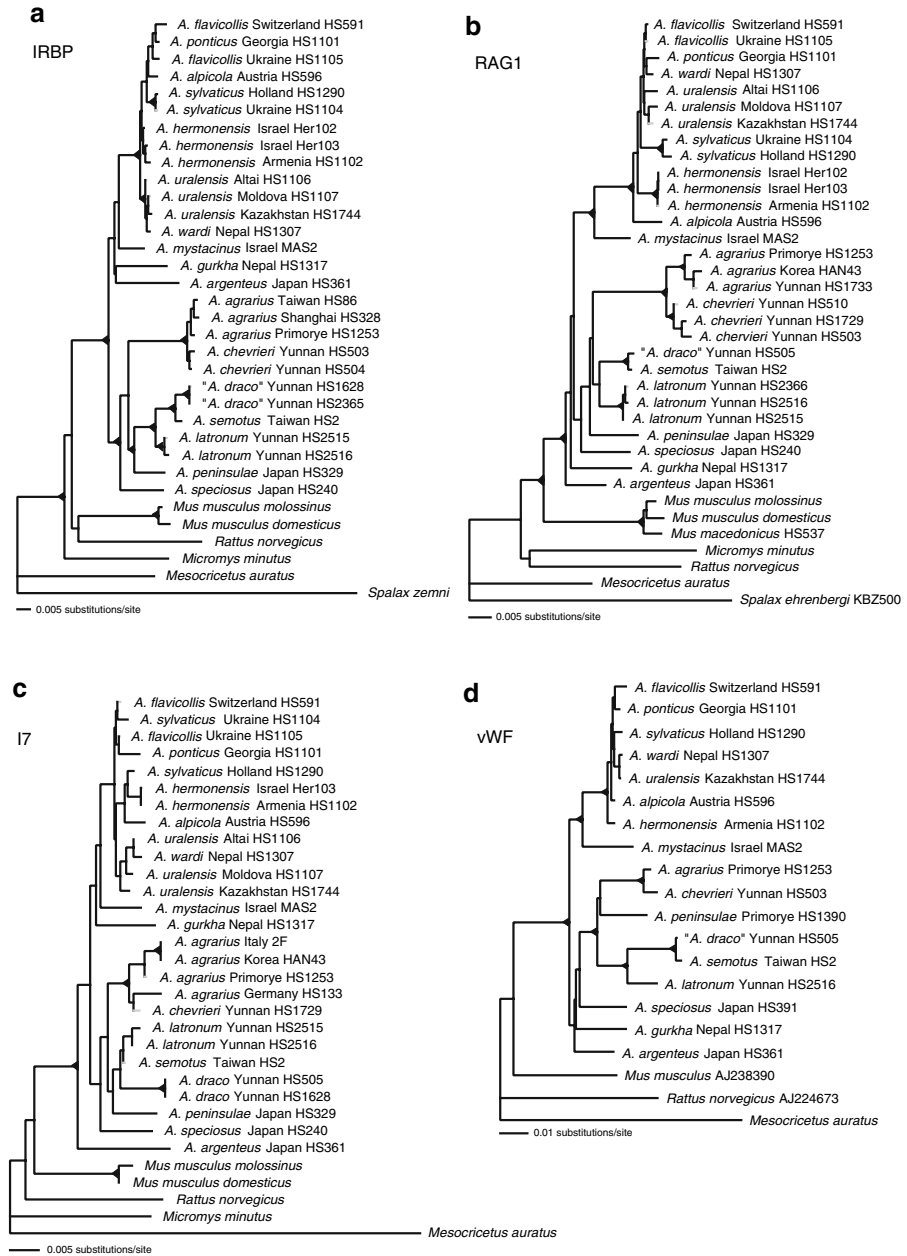
— 0.01 substitutions/site

Fig. 1 Phylogenetic relationships among *Apodemus* species, based on the nuclear genes of *IRBP* (1,152 bp; **a**), *RAG1* (1,002 bp; **b**), *I7* (792 bp; **c**), and *vWF* (1,192 bp; **d**). The phylogenetic trees were constructed using the NJ method, with various combinations of outgroup taxa, including *Micromys minutus* and *Rattus norvegicus*. Bootstrap analyses were performed with NJ and MP methods (1,000 replicates for both methods). The nodes supported by high-bootstrap values (>90% by both methods) are marked with closed triangles
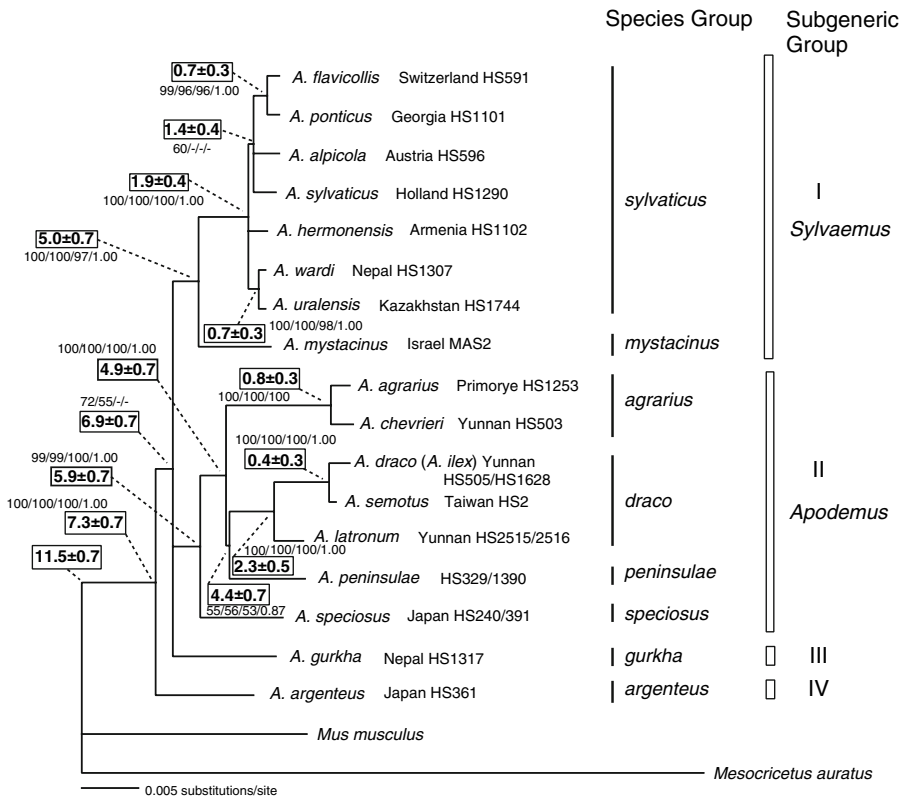
Fig. 2 Phylogenetic relationships among *Apodemus* species, based on the concatenate sequences of *IRBP* (1,152 bp), *RAG1* (1,002 bp), *I7* (792 bp), and *vWF* (1,192 bp). The phylogenetic trees were constructed using the NJ method. Bootstrap analyses were performed with NJ (1,000 replicates), MP (1,000 replicates), and ML (100 replicates) methods. The resultant values are shown at each node (NJ/MP/ML; as percentages, only if >50%). Bayesian inference posterior probability values were shown for the well-supported nodes. Divergence times estimated using the software Multidivtime (Thorne et al. 1998) are shown at each of the key nodes with 95% confidence intervals. Notably, the present subgeneric grouping system (I–IV) is in good agreement with the current taxonomic view of Musser and Carleton (2005), which is the modified version of Musser et al. (1996)

clustering of *A. mystacinus* with the *sylvaticus* species group had high bootstrap support in the *RAG1* (96–97%), *vWF* (72–93%), and *IRBP* + *RAG1* + *I7* + *vWF* (97–100%) analyses. Overall, as illustrated in Fig. 2, the *Apodemus* species examined can be grouped into four distinct lineages (Clades I–IV), those represented by *A. sylvaticus* (I) and *A. agrarius* (II), together with the two distinct lineages of *A. gurkha* (III) and *A. argenteus* (IV).

In the single-gene analyses, no substantial clustering patterns were observed for the members of Clade I (*sylvaticus* species group). In the concatenated trees (Fig. 2), however, five prominent lineages appeared: (1) *A. alpicola*, (2) *A. flavicollis/A. ponticus*, (3) *A. hermonensis*, (4) *A. sylvaticus*, and (5) *A. uralensis/A. wardi*. The relationships among the five lineages were entirely obscured with our current data on nuclear genes.

In Clade II, which roughly corresponds to the subgenus *Apodemus* (Musser et al. 1996), *A. speciosus* consistently had a basal position in the separate datasets, as well as in the concatenated dataset (85–100%). Relationships among the remaining three species groups are rather ambiguous. A close affinity was seen between the *draco* species group and *A. peninsulae* in the *IRBP* (Fig. 1) and concatenated (Fig. 2) datasets, although the supporting values were extremely low.

Cyt *b* Gene Sequences

The cyt *b* sequences were used to assess the phylogenetic relationships within the three species groups represented by *A. sylvaticus* (*sylvaticus* species group), *A. draco* (*draco* species group), and *A. agrarius* (*agrarius* species group), where the taxonomic situation is rather ambiguous. We abandoned the effort to solve the species group relationships with the cyt *b* sequences because of the heavy saturation in such a deep lineage comparison (e.g., $d > 0.12$; Suzuki et al. 2003; but see May-Collado and Agnarsson 2006). We constructed an NJ tree with our sequences and those from the literature and conducted bootstrap analyses with the NJ and MP methods (Fig. 3).

We added the *A. fulvipectus* sequence (AY179491; Reutter et al. 2003) to our analysis for the *sylvaticus* species group. Six distinct groups, consisting of (1) *A. alpicola*, (2) *A. flavicollis/A. ponticus*, (3) *A. hermonensis*, (4) *A. sylvaticus*, (5) *A. uralensis/A. wardi*, and (6) *A. fulvipectus*, were noted, with genetic distances between the groups ranging from 0.09 to 0.13 (Fig. 3). The sister species relationship between *A. flavicollis* and *A. ponticus* from Georgia was detected with weak support. *Apodemus wardi* from Nepal clustered with the *A. uralensis* clade, as in the nuclear gene analysis (see also Suzuki et al. 2003). The *uralensis* clade consisted of three lineages, (1) from eastern territories [Pakistan, Xinjiang (China), and Altai (Russia)], (2) *A. wardi* from Nepal, and (3) from western territories [Turkey, Kazakhstan, Moldova, Chelyabinsk (Russia)], with genetic distances ranging from 0.07 to 0.08.

The *A. draco* lineage from the Yunnan Plateau was distinct both from *A. semotus* from Taiwan and *A. draco* from other southern Chinese areas, with average genetic distances of 0.08–0.10 (Fig. 3), as noted by Liu et al. (2004).

We compared cyt *b* sequences to assess the phylogeographic episodes of *A. agrarius* from Asia and Europe and found that sequence variability was relatively low (0.01 between Italy and Germany) and high (up to 0.03 among China, Korea, and Primorye). The European sequences showed close relationships with a portion of the Asian sequences, namely those from Primorye, Far East Russia, with genetic distances of 0.01. In addition, individuals from Germany and Italy possessed similar cyt *b* sequences, which is somewhat concordant with a previous allozyme analysis (Filippucci 1992).

Discussion

Taxonomic Status of Some Species

Various taxonomic views for *Apodemus* have been challenged by morphological and molecular phylogenetic approaches (e.g., Filippucci et al. 2002; Michaux et al.
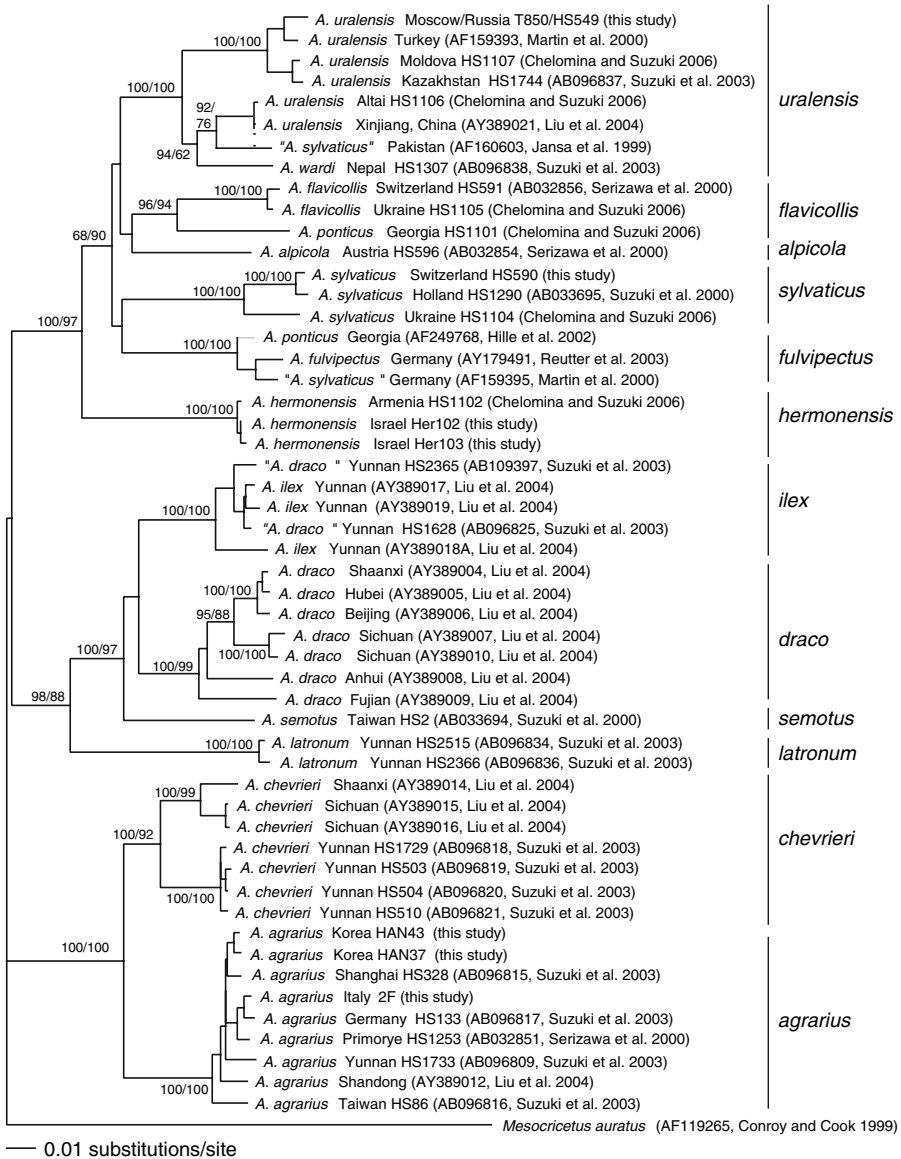
Fig. 3 Phylogenetic relationships focusing on three species groups of *agrarius*, *draco*, and *sylvaticus*, based on the mitochondrial cyt *b* gene sequences. The phylogenetic trees were constructed using the NJ method with distances inferred from Kimura's two-parameter distances (Kimura 1980). Bootstrap analyses were performed with NJ and MP methods (1,000 replicates for both methods). The resultant values are shown at each node (NJ/MP; as percentages, only if >50%). Taxonomic names of some species are likely to be unstable, and species names are temporally presented on the species clades. *Apodemus hermonensis*, for example, should be *A. whiterbyi* according to Musser and Carleton (2005)

2002; Musser et al. 1996; Serizawa et al. 2000; Suzuki et al. 1994, 2003) and have greatly improved to a new standard (Musser and Carleton 2005). The taxonomy may need further revision, however, in some species such as the southern Chinese "*A. ilex*" and the European "*A. fulvipectus.*"

Yunnan's *draco*-like taxon, *A. ilex*, possesses a distinct cyt *b* gene sequence (e.g., ∼8% genetic distance) from *A. draco* sequences from the other locations, making *A. ilex* likely to be a valid species (Liu et al. 2004). However, mitochondrial DNA sometimes shows high levels of genetic divergence among local populations, regardless of similarity in nuclear gene markers, partly due to female philopatry and partly due to random lineage sorting. Since each of the nuclear gene sequences examined here differed among *draco*, *ilex*, and *semotus*, these gene sequences and other single-copy genes should be useful markers for future phylogeographic studies to better understand the evolutionary history of species group *draco*.

The taxonomic situation in species group *sylvaticus* seems rather confusing. On the basis of the data presented here (Figs. 1, 2), there seem to be six distinct lineages, including one that represents *A. fulvipectus*. If we consider only the molecular data, *A. wardi* from Nepal is likely to be conspecific with *A. uralensis*. Thus, the species harbors three geographic populations, from the west (Turkey, Kazakhstan, Moldova), east (Pakistan, western China, Altai), and Nepal, on the basis of the mitochondrial dataset. A close relationship between Kazakhstan and Moldova individuals is seen in the nuclear gene sequences (NJ analysis in Fig. 1). The mitochondrial sequence of *A. ponticus* is distinct from but closely related to that of *A. flavicollis* (Fig. 3). This trend is also evident in the analysis of the combined nuclear gene sequences (Fig. 2). Overall, our current nuclear and mitochondrial gene data show differences in the major species lineages of the *sylvaticus* group, even though differentiation is low, providing reproducible patterns in both datasets. However, our datasets, especially for the nuclear genes, are likely insufficient to settle lower taxonomic problems (such as the status of *A. ilex*, *A. ponticus*, and *A. wardi*); further analyses with more nuclear gene markers are needed.

Phylogenetic Framework of the *Apodemus* Species

The distinctness of these eight species groups is prominent in the sequence analyses of all nuclear genes and their concatenated sequences (Fig. 2). Given previous phylogenetic studies and the present one, using various markers including morphological and molecular characters (e.g., Filippucci et al. 2002; Liu et al. 2004; Michaux et al. 2002; Musser et al. 1996; Serizawa et al. 2000; Suzuki et al. 2003), the genus *Apodemus* consists of eight distinct lineages: (1) the *agrarius* species group, (2) *A. argenteus*, (3) the *draco* species group, (4) *A. gurkha*, (5) the *mystacinus* species group, (6) *A. peninsulae*, (7) *A. speciosus*, and (8) the *sylvaticus* species group.

Among the eight major lineages, the two lineages confined to Europe and western Asia, *A. mystacinus* and *A. sylvaticus*, were sister groups with high support, as previously denoted by Michaux et al. (2002; cyt *b* + 12S rRNA) and Filippucci et al. (2002; 34 allozyme loci), but not in accord with Liu et al. (2004; cyt *b*), who

suggested that *A. mystacinus* is closely related to East Asian lineages such as *A. agrarius*. Contrary to the ambiguous phylogenetic inference from the mitochondrial gene sequences, our combined sequence dataset firmly supports (100–97%) the monophyly of *A. mystacinus* and the *sylvaticus* species group.

Considering the Europe/Central Asia and *agrarius/draco/peninsulae/speciosus* groups together with the two remaining Asian lineages, *A. gurkha* and *A. argenteus*, we recognize four anciently diverged lineages. The concatenated dataset suggests monophyly of the former three lineages and the basal position of *A. argenteus* with low support, which may imply a geographic affinity in the diversification of the four ancient lineages. Notably, this view supports the subgeneric grouping of Musser et al. (1996), with a minor revision excluding *A. gurkha* from the *Apodemus* group and making it monospecific (Fig. 2; Musser and Carleton 2005). Given that a weak trend was seen for monophyly of *A. argenteus* and *A. gurkha* in our previous study (Suzuki et al. 2003), this issue needs to be addressed further in future studies.

Evolutionary Views of *Apodemus*

The phylogenetic data obtained here (Figs. 1–3) provide insight into several evolutionary topics related to *Apodemus*, including multiple radiation events, factors determining body size, and the recent westward expansion of *A. agrarius*.

From our current phylogenetic study (Fig. 2), the first radiation of the *Apodemus* species is itself separated in three steps: the first one, appearing around 6 million years ago, and two others, around 5 million years ago, which gave rise to *agrarius*, *draco*, *peninsulae*, and *speciosus* and to *mystacinus* and *sylvaticus*. Around 2 million years ago, a second evolutionary event gave rise to more recent species, and most recently, *agrarius*, which colonized the whole Palearctic region. These remarkable radiation events were probably primed by global changes that occurred 6–7 million and 2–3 million years ago (e.g., Cerling et al. 1997). The existence of old lineages in Asia implies that the forest ecosystem has been maintained for a long evolutionary period and that wood mice accommodated the ecosystem at the initial stage of radiation.

Excluding the broad range of *mystacinus/sylvaticus*, three geographic regions can be considered for *Apodemus* evolution, i.e., China, Nepal, and the Japanese archipelago, which hosted three ancient lineages (II, III, and IV, respectively; Fig. 2), as noted previously (Serizawa et al. 2000; Suzuki et al. 2003). It is currently unknown why the second step of the radiation occurred in East Asia around 1 million years after the first step. In the second radiation, the four old lineages of *A. agrarius*, *A. draco*, *A. peninsulae*, and *A. speciosus* emerged (Fig. 3), probably in China and Japan. From an ecological perspective, this would be correlated with habitat capacity, which is associated with species richness in small mammals (McShea et al. 2003). In the case of *Apodemus*, two species could co-occur in the same general habitat. A possible scenario is encompassed by two processes: the first one is allopatric speciation, and the second is range overlapping. From the genetic perspective, it is possible to presume that it takes 1–2 million years for speciation to occur in the first process, which allowed range overlapping without erosion by

genetic hybridization in the second process. One or two million years after the first colonization event of *A. argenteus*, the second colonization event of *A. speciosus* could have occurred in Japan. In the case of the three ancient lineages of *A. agrarius*, *A. draco*, and *A. peninsulae*, which are considered to have diverged simultaneously (Fig. 2), they would have been in three different geographic areas somewhere in the continental part of East Asia, although we cannot specify the exact locations, followed by range overlapping with one another. In Asia, thus, we currently see the following consequences: *A. agrarius* and *A. peninsulae* in North China, Korea, and Far East Russia; *A. agrarius* and *A. draco* in southern China; *A. draco* (or *A. ilex*) and *A. latronum* in West Yunnan; *A. chervieri* and *A. draco* (or *A. ilex*) in East Yunnan; and *A. argenteus* and *A. speciosus* in Japan. The idea that the subsequent events can be explained by a range overlap (or coexistence of two species) due to habitat capacity applies to the Yunnan wood mice *A. latronum* and *A. draco* (or *A. ilex*), where two closely related lineages overlapped; *A. latronum* and *A. draco* were allopatrically or parapatrically speciated, and the latter expanded into the range of the former. This trend can also be seen in the evolutionary patterns of subtropical Eurasian *Mus* (Suzuki et al. 2004), in which lineage differentiation occurred in the subgenus *Mus* during a short period of evolutionary time at the initial stage of the radiation.

Body size change within an evolutionary lineage is a major topic in mammalogy (e.g., Adler 1995; Millien 2004; Musser et al. 1996). A finely resolved phylogenetic tree for the Asian species (Fig. 2) shows the evolutionary changes of body size in *Apodemus*. In Asia, where *Apodemus* species have long evolutionary histories, thereby showing evolutionary consequences for stationary states, there appears to be a difference in body size in the two sympatric species in southern mainland China (i.e., *A. agrarius* and *A. draco*) and the Japanese archipelago (*A. speciosus* and *A. argenteus*; Musser et al. 1996). Since the lineages with large (*agrarius*, *speciosus*) and small (*draco*, *argenteus*) body sizes are not monophyletic (Fig. 2), body size divergence may have occurred independently in southern China and Japan. This parallel trend can be explained by diversifying selection through niche segregation between the sympatric taxa, in which ecotype segregation of ground and arboreal dwellers (Sekijima and Sone 1994) and/or preferences for different acorn sizes (Shimada 2001) may be involved in selection. Additionally, in Yunnan, *A. latronum* and *A. draco* co-occur, and these taxa appear to differ in body size (Musser et al. 1996) despite their recent divergence from a common ancestor (Fig. 2). Interestingly, the Taiwanese endemic *A. semotus* is larger than its closely related taxon *A. draco*, which occurs solely at higher elevations and thus departs from the range of *A. agrarius* residents at lower elevations (Adler 1996; Musser et al. 1996). It is possible that *A. semotus* evolved a larger body size without niche segregation, although we must consider other factors such as insular syndrome. In contrast, the situation in North China, Korea, and Far East Russia is likely to be different; the coexisting species *A. agrarius* and *A. peninsulae* do not show a substantial difference in body size. This may be due to their habitat preferences; *A. agrarius* is often regarded as a generalist meadow species (e.g., Bryja and Rehák 1998; Kostenko 2000), and *A. peninsulae* is a typical forest species in Korea and Far East Russia. The European *Apodemus* (*sylvaticus* species group) may provide

information to better understand the evolutionary factors determining body size when there is a clearer phylogeny.

We analyzed the phylogenetic relationships among local populations of *A. agrarius*, hoping to learn about the factors shaping dispersal patterns in wood mice. We compared individuals from Europe (Italy) to those from China, Russia, and Korea and found a close relationship among those from Far Eastern Russia and Europe. Since the *A. agrarius* lineage appears to have originated in Asia, the European lineage is likely the descendant of recent colonizers from Asia. Our data suggest that some genetic elements localized in northern Asia contributed to the mitochondrial DNA in Europe. The question remains as to why *A. agrarius* was selected for further radiation events in the last part of the biogeographic development of *Apodemus*, namely the transcontinental dispersal of *A. agrarius* from Asia to Europe. It is possible that this occurred because (1) an omnivorous species has more chances for dispersal, (2) an ancestral *A. agrarius* population was situated in a locality accessible to Europe at the time of dispersal, (3) *A. agrarius* adapted to a special habitat that was newly established, or (4) *A. agrarius* is always dispersing into new areas. In our opinion, a plausible explanation is that *A. agrarius* inhabits shrub habitats rather than forests, at least in Far East Russia, which may be a source area of European *A. agrarius* (Fig. 3). Considering the low genetic distance of 0.01 between Europe and Far East Russia, the time of the migration from the east to the west may have been around 200,000 years ago, assuming the evolutionary rate of the cyt *b* in *Apodemus* to be 0.024 per site per million years (Suzuki et al. 2003). The Quaternary ice ages, when shrub fields developed across the continent, would have been a good time for *A. agrarius* to migrate to Europe.

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