

Phylogeographic patterning of mtDNA in the widely distributed harvest mouse (*Micromys minutus*) suggests dramatic cycles of range contraction and expansion during the mid- to late Pleistocene

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Abstract: We examined sequence variation in the mitochondrial cytochrome *b* gene (1140 bp, $n = 73$) and control region (842–851 bp, $n = 74$) in the Eurasian harvest mouse (*Micromys minutus* (Pallas, 1771)), with samples drawn from across its range, from Western Europe to Japan. Phylogeographic analyses revealed region-specific haplotype groupings combined with overall low levels of inter-regional genetic divergence. Despite the enormous intervening distance, European and East Asian samples showed a net nucleotide divergence of only 0.36%. Based on an evolutionary rate for the cytochrome *b* gene of $2.4\% \cdot (\text{site} \cdot \text{lineage} \cdot \text{million years})^{-1}$, the initial divergence time of these populations is estimated at around 80 000 years before present. Our findings are consistent with available fossil evidence that has recorded repeated cycles of extinction and recolonization of Europe by *M. minutus* through the Quaternary. The molecular data further suggest that recolonization occurred from refugia in the Central to East Asian region. Japanese haplotypes of *M. minutus*, with the exception of those from Tsushima Is., show limited nucleotide diversity (0.15%) compared with those found on the adjacent Korean Peninsula. This finding suggests recent colonization of the Japanese Archipelago, probably around the last glacial period, followed by rapid population growth.

Résumé : Nous avons étudié la variation des séquences du gène mitochondrial du cytochrome *b* (1140 pb, $n = 73$) et de la région de contrôle (842–851 pb, $n = 74$) dans des échantillons de rats des moissons (*Micromys minutus* (Pallas, 1771)) répartis sur toute l'aire de répartition, de l'Europe de l'Ouest jusqu'au Japon. Des analyses phylogéographiques révèlent l'existence de regroupements régionaux d'haplotypes, ainsi que des niveaux globaux faibles de divergence génétique entre les régions. Malgré les distances considérables entre eux, les échantillons de l'Europe et de l'Asie de l'Est possèdent une divergence nette des nucléotides de seulement 0,36 %. Avec un taux d'évolution du gène du cytochrome *b* de $2,4 \text{ \%} \cdot (\text{site} \cdot \text{lignée} \cdot \text{million d'années})^{-1}$, la divergence initiale estimée de ces populations se situe à il y a environ 80 000 années. Nos résultats concordent avec les données disponibles sur les fossiles qui indiquent des cycles répétés d'extinction et de recolonisation de *M. minutus* en Europe durant le quaternaire. Les données moléculaires laissent aussi croire que la recolonisation s'est faite à partir de refuges des régions centrales et orientales de l'Asie. Les haplotypes de *M. minutus* du Japon ont une faible diversité des nucléotides (0,15 %), à l'exception de ceux des îles Tsushima, par comparaison à ce qui prévaut dans la péninsule adjacente de Corée. Cette information laisse croire à une colonisation récente de l'archipel japonais, probablement aux environs de la dernière période glaciaire, suivie d'une croissance rapide des populations.

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Introduction

Phylogeographic analysis using molecular markers is an effective tool for understanding the genetic structure and history of populations within a species, and a valuable adjunct

to more conventional studies of environmental history (Avice 2000). Small terrestrial mammals, such as shrews, voles, and mice, appear to be particularly good indicators of environmental change because they tend to have narrower habitat and climatic requirements than larger mammals and

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have better prospects for localized persistence under conditions of extreme environmental stress. For example, phylogeographic data from species of *Microtus* Schrank, 1798, *Dicrostonyx* Gloger, 1841, and *Lemmus* Link, 1795 suggest possible cycles of range contraction and expansion associated with late-Quaternary glacial cycles, with species persisting in scattered refugia in the Eurasian Arctic and European temperate regions (Fedorov et al. 1999a, 1999b; Jaarola and Searle 2002; Brunhoff et al. 2003; Haynes et al. 2003; Fink et al. 2004). To date, however, there have been few phylogeographic studies of widespread small mammals of the Eurasian temperate region.

The harvest mouse, *Micromys minutus* (Pallas, 1771), is the only member of the speciose subfamily Murinae (more than 450 species; Corbet and Hill 1991) that has its natural distribution spanning all of Eurasia, from Great Britain in the west to Japan and Taiwan in the east (Corbet 1978; some authors consider the population in Great Britain as a historical introduction, e.g., Yalden 1999). It is also unusual among Eurasian murines in its habitat preference, which includes various open communities such as grasslands, sedgeland, and agricultural fields (Trout 1978; Mitchell-Jones et al. 1999). In drier parts of its range the taxon is more closely linked to moist microhabitats such as wetlands and forest margins. Although there are many fossils of *M. minutus* and its extinct congeners from Europe and Asia (e.g., Storch and Dahlmann 1995; Kowalski 2001; Spitzenberger 2001), the relationships among the 10 or so described extinct species and their relationship to present day *M. minutus* remain uncertain.

In Japan, *M. minutus* is restricted to the islands of Honshu, Shikoku, and Kyushu and associated small islands (Corbet and Hill 1991; Abe et al. 2005); this distribution is bordered by the Watase and Blakiston lines (Abe et al. 2005). The broad extralimital distribution of *M. minutus* sets it apart from the majority of other Japanese small mammals, many of which are endemic at the species or even generic level (Corbet and Hill 1991; Abe et al. 2005). Nothing is yet known regarding the origin of the Japanese population of *M. minutus*, mainly owing to the complete absence of this taxon from the fossil record of Japan (Kowalski and Hasegawa 1976; Kawamura 1989). The lack of fossil evidence might even suggest that *M. minutus* is a recent historical introduction to Japan.

The broad distributional range and strict environmental requirements of *M. minutus* make it a good candidate for phylogeographic investigation. To understand its genetic population structure and evolutionary history, we investigated sequence variation in mitochondrial cytochrome *b* gene and control region in specimens from Asia, Russia, and Europe.

Materials and methods

Sampling

We obtained tissue samples from a total of 75 individuals of *M. minutus* from 26 localities spanning the entire latitudinal range of this Palaearctic species (Table 1, Fig. 1). The eastern part of the range of *M. minutus* is fragmented, with several isolates in northern Indochina and southern China (Corbet and Hill 1992). One of the isolated Chinese popula-

tions is included in our sampling. We obtained cytochrome *b* sequences for two additional individuals from DDBJ/EMBL/GenBank (AB033697: Suzuki et al. 2000; AF159399: Martin et al. 2000).

DNA isolation, amplification, and sequencing

DNA was extracted from tissues preserved in ethanol using the conventional phenol–chloroform method. Two DNA fragments containing the cytochrome *b* coding region (1140 base pair (bp)) and control region (842–851 bp) were amplified using the polymerase chain reaction (PCR) with primers L14115 (5′-GACATGAAAAATCATCGTTG-3′) and H15300 (5′-GTTTACAAGACCAGAGTAAT-3′) for the cytochrome *b*, and L15399 (5′-GCACCCAAAGCTGATATTCT-3′) and H21 (5′-GCATTTTCAGTGCTTTTGCTT-3′) for the control region, respectively. The primers were designed from the complete mitochondrial sequences of *Mus musculus* L., 1758 (NC_011569; Bibb et al. 1981) and *Rattus norvegicus* (Berkenhout, 1769) (X14848; Gadaleta et al. 1989) from DDBJ/EMBL/GenBank. The letters L and H refer to the light and heavy strands, and the numbers refer to the position of the 3′ base of the primer in the complete sequence of *M. musculus* mitochondrial DNA (mtDNA) (Bibb et al. 1981). Each PCR mix contained 10 mmol/L of Tris (pH 8.3), 50 mmol/L of KCl, 0.01% gelatin, 0.1% Triton X-100, 2.5 mmol/L of MgCl₂, 0.2 mmol/L of dNTP mix, 0.5 μmol/L of each primer (10 pmol/reaction), 0.5 U (1 U ≈ 16.67 nkat) of AmpliTaq Gold DNA polymerase (ABI, Applied Biosystems), and 0.1–0.5 μg of template total genomic DNA in a total volume of 20 μL. The thermal cycling parameters for the PCR were as follows: 96 °C for 10 min and 35 cycles of 96 °C for 30 s, 50 °C for 60 s, and 72 °C for 60 s. The PCR product was sequenced according to the manufacturer instructions, using a Dye Terminator Cycle Sequencing Kit (ABI) and an ABI 3100 automated sequencer with the two PCR primers and two additional primers designed to anneal to the center of the cytochrome *b* gene and control region, named Mmin-cyt-L (5′-GCAACCCTAACA CGTTTCTT-3′) and Mmin-cyt-H (5′-GGTGGGAATGGGATTTTATCT-3′) for the cytochrome *b*, and Mmin-CR-L (5′-CAGGCATCTGGTTCTTACTT-3′) and Mmin-CR-H (5′-AGTGAGTCCGGCTACATGTT-3′) for the control region, respectively. Each primer was used to sequence the latter half of the L strand and the first half of the H strand.

Phylogenetic analysis

Interpopulational relationships were explored using the distance-based neighbor-joining (NJ) method (Saitou and Nei 1987), as implemented in PAUP* version 4.0b10 (Swofford 2003), and median-joining (MJ) networks (Bandelt et al. 1999), as implemented in Network[®] version 4.1 (Fluxus Technology Ltd. 2004). The NJ analysis employed matrices of genetic distances generated under the Kimura two-parameter method (Kimura 1980), ignoring gaps in the case of control region sequences. For the cytochrome *b* NJ tree, a sequence of *R. norvegicus* (AB033713; Serizawa et al. 2000) was used as the outgroup. For NJ analysis of control region and a concatenated data set we used one highly diverged individual of *M. minutus* from southern China as the outgroup. Bootstrap estimates (Felsenstein 1985) were obtained from 10 000 iterations of the NJ method and 100 iterations of the maximum-

Table 1. Sampling localities of the harvest mouse (*Micromys minutus*) and the haplotypes of cytochrome *b*, control region, and combined sequences.

Localities	<i>n</i>	Cytochrome <i>b</i> (<i>cytb</i>)	Control region (CR)	<i>cytb</i> + CR
Great Britain				
1. Avon	1	bU1	cU1	+U1
France				
2. Orne	1	bF1	cF1	+F1
Germany				
3. Osnabruck	2	bG1, bG2	cG1, cG2	+G1, +G2
4. Karlsruhe	3	bG3, bG4 ^a	cG3, cG4, cG5	+G3, +G4
5. Konstanz	1	bG5 (AF159399)		
Switzerland				
6. Estavayer	4	bS1(3), bS2	cS1(2), cS2 ^a	+S1(2), +S2
Russia				
7. Novosibirsk	7	bR1, bR2, bR3, bR4, bR5, bR6, bR7	cR1, cR2, cR3, cR4, cR5, cR6, cR7	+R1, +R2, +R3, +R4, +R5, +R6, +R7
8. Kyzyl	1	bR8	cR8	+R8
9. Khasan	1	bR9	cR9	+R9
Korea				
10. Chunchon	1	bK1	cK1	+K1
11. Gule	4	bK1(2), bK2, bK3	cK2, cK3(2), cK4	+K2, +K3(2), +K4
12. Kyongju	2	bK4 ^a	cK5, cK6	+K5
Japan				
13. Tatsunokuchi	1	bJ1	cJ1	+J1
14. Itoh	1	bJ2 (AB033697)	cJ2	+J2
15. Shitara	1	bJ3	cJ2	+J3
16. Kyotanabe	1	bJ4	cJ3	+J4
17. Hiroshima	11	bJ3(2), bJ5(8) ^a	cJ2, cJ4(7), cJ5(3)	+J5, +J6(7), +J7(2)
18. Iwakuni	3	bJ3(2), bJ6	cJ2(2), cJ6	+J3(2), +J8
19. Kawashima	2	bJ7, bJ8	cJ2, cJ7	+J9, +J10
20. Tsushima Is.	3	bJ9(3)	cJ8, cJ9(2)	+J11, +J12(2)
21. Fukuejima I.	2	bJ3(2)	cJ10(2)	+J13(2)
22. Miyazaki	7	bJ10, bJ11(6)	cJ11, cJ12(6)	+J14, +J15(6)
23. Kuchinoerabujima I.	3	bJ12(3)	cJ13(3)	+J16(3)
Taiwan				
24. Taipei	2	bT1, bT2	cT1, cT2	+T1, +T2
25. Tataka	6	bT3(6)	cT3(5), cT4	+T3(5), +T4
26. Taichung	4	bT2(2), bT4(2)	cT5(2), cT6(2)	+T5(2), +T6(2)
China				
27. Chengdu	1	bC1	cC1	+C1

Note: The numbers under Localities correspond with the numbers in Fig. 1.

^aDNA fragmentation was disturbed to analyse one individual from each locality.

Fig. 1. Maps showing the distribution of the harvest mouse (*Micromys minutus*) in Eurasia (Corbet 1978; Spitzenberger 1999). The numbers on the maps refer to those assigned to sampling localities in Table 1.



Fig. 2. Neighbor-joining (NJ) trees of the cytochrome *b* gene sequences (a), control region sequences (b), and combined sequences (c), based on the Kimura two-parameter model. The cytochrome *b* gene sequence of *Rattus norvegicus* was used as the outgroup for the cytochrome *b* tree. The bootstrap resampling support (NJ/MP) is listed on major nodes (>50%). The haplotype designations are the same as in Table 1. Except for the haplotype cK6 that was found in Korea, clade names present the regions where each haplotype was found. Subclades recognized in the Korea–Japan and Russia were named B1, B2, C1, and C2.

parsimony (MP) method (Swofford and Olsen 1990) using PAUP*.

Estimating divergence times

The estimation of lineage divergence times from molecular data rests on two primary assumptions, namely (1) that substitutions are accumulating at a relatively even rate across all lineages and (2) that the rate of molecular evolution is known with some degree of accuracy. The first of these assumptions (molecular clock hypothesis; Zuckerkandl and Pauling 1965) was tested for the data set of Murinae genera (*Apodemus* Kaup, 1829, *Micromys* Dehne, 1841, *Mus* L., 1758, *Tokudaia* Kuroda, 1943, and *Rattus* G. Fischer, 1803; Suzuki et al. 2003) using the branch-length test as implemented by the program LINTRE (Takezaki et al. 1995). The second assumption is usually defended by application of a general evolutionary rate derived from calibration against a fossil record. In the case of murine rodents, the common practice is to calibrate the rate of molecular evolution by using the genetic divergence between *Mus* and *Rattus* and assuming a divergence time of 12 million years ago as indicated by palaeontological evidence (see Michaux et al. 2003 and references therein; Suzuki et al. 2003). However, the validity of this practice is challenged by a growing recognition that the rate of molecular evolution is most likely time dependent even within a single gene lineage, perhaps owing to a combination of purifying selection and the presence of mutational hotspots (Howell et al. 1996; Lambert et al. 2002; Ho et al. 2005). One consequence is that it may be invalid to extrapolate molecular rates of change across different evolutionary timescales; and more specifically, that estimates of recent divergence times based on long-term values (such as that derived from the *Mus*–*Rattus* divergence) may seriously overestimate the true divergence times. In the absence of any independent estimate of the short-term rate of molecular change in *Micromys*, we used the conventional long-term evolutionary rate for all substitutions in the cytochrome *b* gene of 0.024 substitutions·(lineage·million years)⁻¹ (Suzuki et al. 2003) to establish a maximum divergence time for the primary mtDNA lineages within *M. minutus*. The net nucleotide divergence (*p* distance) (Nei 1987) between the two clades was calculated using MEGA version 2.1 (Kumar et al. 2001). The possibility of recent population expansion was explored using Fu's F_S statistics (Fu 1997), and the expansion time after a bottleneck was estimated from the mismatch distribution (Rogers 1995) and nucleotide diversity (Rogers and Jorje 1995). Each parameter was calculated using Arlequin version 2.000 (Schneider et al. 2000) and MEGA version 2.1.

Results

We recovered 39 distinct cytochrome *b* haplotypes from 73 individuals, with 169 variable sites and 174 substitutions in total. For the control region, we detected 44 haplotypes

from 74 individuals, with 141 variable sites, 154 substitutions, and 12 single-bp indels. We used 45 haplotypes from 71 concatenated sequences for phylogenetic inference. We failed to obtain sequences from some individuals, including one from Kyongju, Korea (locality 12; Fig. 1), possibly owing to fragmentation of the DNA samples. The new sequences have been deposited in the DNA databases DDBJ/EMBL/GenBank (accession Nos. AB125069–AB125100, AB201958–AB202070).

Phylogenetic trees constructed from separate cytochrome *b* (Fig. 2a) and control region (Fig. 2b) data sets, and from the concatenated data set (Fig. 2c), all are highly concordant with one another. In all trees, one haplotype from an isolated population of *M. minutus* in central China (locality 27, Chengdu; labelled E on Fig. 2) is highly divergent from all others. Most strikingly, for cytochrome *b*, this individual shows 12% raw nucleotide divergence from all other populations compared with a maximum divergence of 1.3% (Table 2) among all other samples of *M. minutus*. In comparison with a wider range of other Murinae, including regionally sympatric *Apodemus* spp., *Rattus* spp., and *Mus* spp., this haplotype is unambiguously monophyletic with *M. minutus*. Accordingly, its divergent position is unlikely to be due to incorrect taxonomic allocation.

In each of the analyses, the remaining populations are divided into four major clades (A–D in Fig. 2), each of which is supported by high to moderate levels of bootstrap support. The four clades are geographically meaningful entities, consisting of individuals from Europe (clade A, no. of specimens (*n*) = 10–11), Korean and Japan (B, *n* = 40–42), Russia (C, *n* = 9), and Taiwan (D, *n* = 12). In all analyses, clades A and B group together with low to high bootstrap support (NJ: 82%–99%; MP: 50%–95%), while clades C and D form a second group with lower support (NJ: 50%–77%; MP: <50%–74%). A striking feature of the results is the highly disjunct geographic groupings that do not follow a simple latitudinal progression.

In the European group (clade A), we found nine distinct cytochrome *b* haplotypes in 11 individuals from four countries (France, Germany, Switzerland, and Great Britain); each of the haplotypes differed from one another by 1–7 nucleotide substitutions (Fig. 2b). In all NJ trees, individuals from Great Britain (cU1) and France (cF1) group together, but these are embedded within the remaining haplotypes from Europe.

In clade B (Korea–Japan), most Japanese haplotypes form a discrete and very tight cluster (subclade B1), with low to moderate (NJ: 78%; MP: 61%) bootstrap support on the concatenated tree. In contrast, the Korean samples are genetically more diverse. Samples from Tsushima Is., located in the strait between Japan and Korea (locality 20; Fig. 1), also show a high diversity of haplotypes that are interspersed with respect to the Korean samples.

Within clade C, the cytochrome *b* tree distinguishes one haplotype from Far East Russia (Khasan, locality 9; Fig. 1)

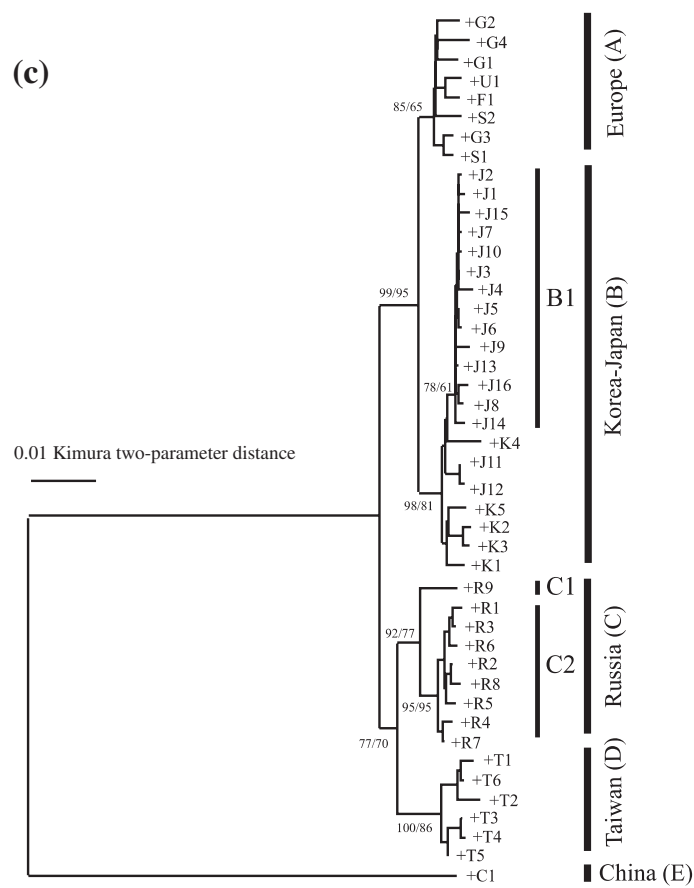
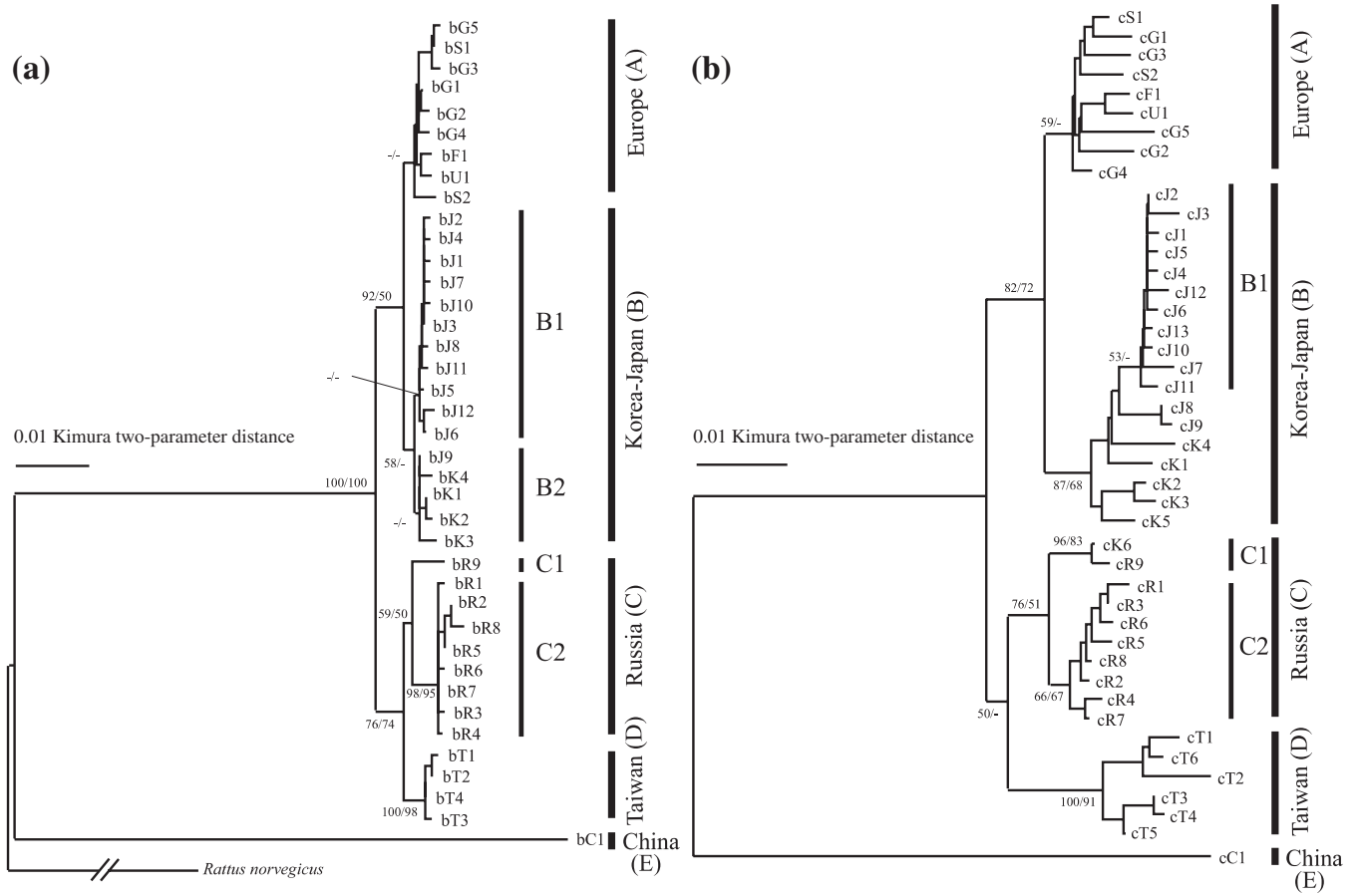


Table 2. Net nucleotide diversity of cytochrome *b* (above diagonal) and divergence (below diagonal).

	A (Europe)	B (Korea–Japan)	C (Russia)	D (Taiwan)
A (Europe)		0.004 (0.002–0.006)	0.013 (0.010–0.016)	0.012 (0.009–0.015)
B (Korea–Japan)	83 kya (42–130 kya)		0.013 (0.010–0.016)	0.012 (0.009–0.015)
C (Russia)	270 kya (210–330 kya)	270 kya (210–330 kya)		0.007 (0.005–0.009)
D (Taiwan)	250 kya (190–310 kya)	250 kya (190–310 kya)	150 kya (100–190 kya)	

Note: Values in parentheses are the 95% confidence intervals (CI). Divergence is measured in thousands of years ago (kya).

from those in Siberia (Novosibirsk and Kyzyl, localities 7 and 8, respectively). The level of divergence between these groups produced low bootstrap values for this node in the cytochrome *b* NJ tree. However, analysis of control region and of a concatenated data set produced much stronger bootstrap support for this clade (NJ: 76% and 92%, respectively; MP: 51% and 77%, respectively). Interestingly, in the control region data set, one individual from Korea (cK6) shows a close affinity with the Far East Russian haplotype (cR9; Fig. 2b); unfortunately, cytochrome *b* sequence data were not obtained from this individual.

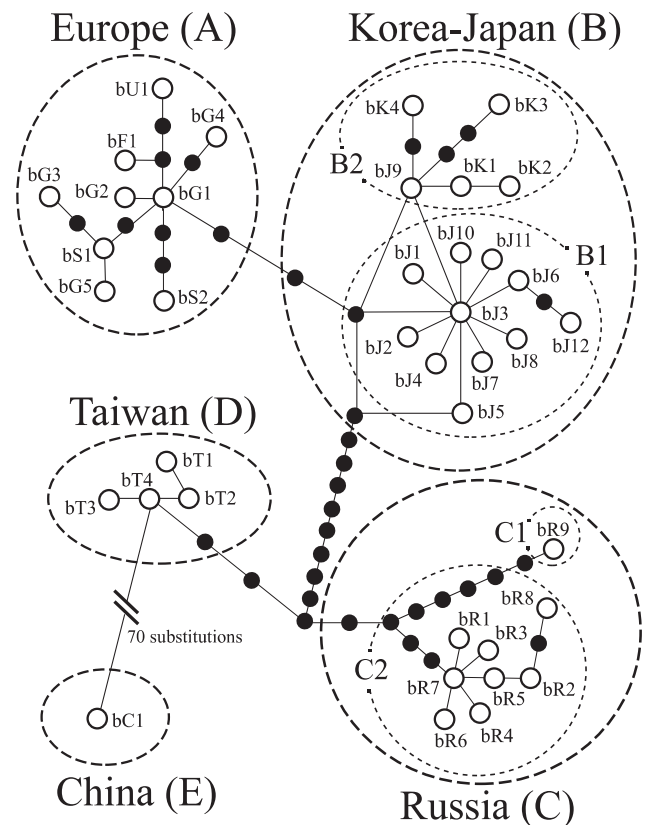
Clade D, consisting of samples from Taiwan, is very strongly supported on all trees (bootstrap values 85%–100%). Variability of haplotypes for the control region is high in the Taiwanese populations (6 haplotypes from 12 individuals from 3 localities) with relatively deep divergence among haplotypes.

We also constructed a MJ network to better understand the relationships among haplotypes, using the cytochrome *b* sequence data set (Fig. 3). This tree features the same four groups as observed on the NJ tree, but it further clarifies the relationships within and between the groups. In clade B, the Japanese haplotypes form a discrete subclade, arranged in a star-like network with bJ3 as the central haplotype. This haplotype was found at four localities (Fukuejima I., Iwakuni, Hiroshima, and Shitara), further evidence that it represents the ancestral Japanese haplotype. A close relationship between the European (A) and the East Asian (B) clades is also portrayed in the MJ network, with a connection involving four base changes between bJ3 and bG1 (the centrally located haplotype in clade A). In contrast, these clades together are separated by a much longer branch involving 12 or more substitutions from the other clades of Central Russia (C) and Taiwan (D). Clades C and D are also quite widely separated, with 8 base changes between the centrally located haplotypes of the Taiwan cluster (bT4) and that of the Central Russian cluster (bR7), 11 base changes between bT4 and the East Russian sample bR9, and 9 base changes between bR7 and bR9. The wider haplotype variability within and between clades C and D further emphasizes the close relationship between the Korea–Japan and the European clades.

To estimate the divergence times among the clades (Figs. 2, 3), we calculated the net nucleotide divergence (Table 2). Assuming an evolutionary rate of $2.4\% \cdot (\text{site} \cdot \text{lineage} \cdot \text{million years})^{-1}$, the times for divergences between clades A and B, between clades C and D, and between the two groups of A/B and C/D would be 83 000 years before present (BP), 150 000 years BP, and 250 000 – 270 000 years BP, respectively (Table 2).

Fu's F_S statistics were calculated for the populations from the Japanese Archipelago, excluding Tsushima Is. A signifi-

Fig. 3. Median-joining network of the cytochrome *b* gene sequences (1140 bp) of 39 haplotypes of *M. minutus*. Open circles represent distinct haplotypes that were found in the network. Each branch represents a single nucleotide substitution and the solid circles indicate unobserved intermediate haplotypes. Each clade and subclade is denoted by broken polygons.



cantly large negative value (-5.058 ; $p = 0.003$) is suggestive of relatively recent population expansion. The timing of this population expansion was estimated using both the mismatch distribution ($\tau = 1.775$, 95% confidence interval (CI) = 0.503–2.506) and the nucleotide diversity ($\pi = 0.0015$, 95% CI = 0.0011–0.0019). Assuming an evolutionary rate of 2.4%, the mismatch distribution values suggest that population expansion occurred 32 000 years BP (95% CI = 9100–46 000 years BP), while the nucleotide diversity date this event to 31 000 years BP (95% CI = 23 000 – 40 000 years BP). Although the two sets of results are in close agreement, the latter has a smaller 95% CI.

Discussion

Micromys minutus shows an unusual phylogeographic structure that is coupled with unexpectedly low levels of

intraspecific variation across almost its entire range. These findings provide new insights into various issues including the taxonomy of *Micromys*, the response of Palaearctic small mammals to the Quaternary glacial cycles, and the origin of the Japanese populations of *M. minutus*.

A possible second species of *Micromys*

The extremely high level of genetic differentiation of the single individual from Chengdu in China from all other populations of *M. minutus* (average nucleotide divergence of cytochrome *b* = 12%) suggests the likely presence of a second species within *Micromys*. A sequence divergence of 12% indicates that divergence of the Chengdu lineage from that leading to typical *M. minutus* probably took place during the latest Pliocene or earliest Pleistocene, around 2–3 million years ago.

According to Corbet and Hill (1992) populations of *Micromys* from southern China, northeastern India, and northern Vietnam differ from those in western and northern Eurasia, Taiwan, and Japan in several aspects of fur coloration and body proportions. Several names have been proposed for the southern populations, including *Mus erythrotis* Blyth, 1856 (Cherrapunji, Assam, India), *Mus pygmaeus* Milne-Edwards, 1872 (Moupin, Sichuan, China), and *Micromys minutus berezowskii* Argyropulo, 1929 (Hotszihow, northern Sichuan, China). More extensive genetic sampling and parallel morphological studies are needed before any of these names can be associated with the divergent Chengdu lineage.

Phylogeographic structure within *M. minutus*

Excluding the highly divergent Chinese individual from further consideration, all of the remaining haplotypes show a maximum internal divergence of only 1.3% for cytochrome *b* (net nucleotide divergence = 0.012–0.013; Table 2). Despite this low level of genetic differentiation, the haplotypes segregate according to a well-defined geographic pattern, both on phylogenetic trees and on the MJ network, with discrete lineages represented by samples from each of Europe, Russia, Taiwan, and Korea–Japan. These lineages cluster into two larger groups, one consisting of samples from Russia and Taiwan, and the other, samples from Europe, Korea, and Japan. Surprisingly, given the huge geographic separation involved, the Europe and Korea–Japan group shows even less genetic differentiation for cytochrome *b* (net nucleotide divergence = 0.004; Table 2) than the Russia and Taiwan group (net nucleotide divergence = 0.007).

A notable feature of the phylogeographic pattern is the fact that three of the four regional clades can be resolved into fairly simple star-like or weakly branching MJ networks. Such patterns are suggestive of local differentiation from single original haplotypes (Fig. 3). The one exception is the Russian clade, which in reality includes populations from two widely separated regions of Siberia (C2; Fig. 3) and Far East Russia (C1). In this case, the one haplotype of clade C1 is set well apart from the star-like cluster of clade C2 and is only slightly closer to the hypothetical central haplotype C1 (9 substitutions) than it is to the central haplotype of clade D (10 substitutions). As noted earlier, bootstrap support for clade C is weak (59%) in the NJ analysis of cytochrome *b* alone but rises to 92% when a concatenated cytochrome *b* and control region data set is used.

As noted above, the most unusual feature of the phylogeographic pattern is the close affinity of European and Far East Asian populations of *M. minutus*. Although each of these groups forms a discrete, star-like cluster in the cytochrome *b* MJ network (Fig. 3), the hypothetical ancestral nodes of the two groups are separated by only four substitutions. This pattern contrasts strikingly with the more usual phylogeographic pattern in Eurasian small mammals, where levels of genetic divergence between regional clades are broadly consistent with the expectations of divergence by isolation (Fedorov et al. 1999a, 1999b; Iwasa et al. 2000; Jaarola and Searle 2002; Brunhoff et al. 2003; Haynes et al. 2003; Fink et al. 2004). For example, the root vole, *Microtus oeconomus* (Pallas, 1776), a species of higher latitudes with a Holarctic distribution, shows a stepwise increase in genetic divergence between regional clades as one moves from west to east or vice versa (Brunhoff et al. 2003). The low level of genetic diversity within *M. minutus* might be explained either in terms of positive selection for the contemporary mitochondrial lineages or as a by-product of extreme population fluctuations with loss of genetic diversity perhaps amplified by lineage sorting during periods of population recovery. Among other Palaearctic mammals, low levels of genetic diversity usually are attributed to a combination of bottlenecks and lineage sorting caused by repeated cycles of range contraction and expansion under the influence of Quaternary glacial cycles (e.g., Hewitt 1996). We favor a similar interpretation in the case of *M. minutus*, and note in support of this view both the strong phylogeographic pattern in this species and the fossil evidence for recent recolonization of Europe by *Micromys* (see below).

The phylogeographic pattern in *M. minutus* suggests a history of extreme and recent population fluctuations across the entire geographic range of the species. As noted above, the fairly simple star-like structure of each clade in the MJ network suggests a process of local radiations from single, regionally distinctive haplotypes. In each region, genetic diversity thus appears to have passed through a severe bottleneck, with only limited recovery of diversity since that time. The most likely explanation of this pattern is that *M. minutus* experienced one or more phases of extreme range fragmentation and contraction into refugia across most or all of its range, probably within the time frame of the late Quaternary.

Some direct evidence on the nature and timing of the environmental events that shaped genetic diversity in *M. minutus* comes from the fossil record of Europe. Fossils attributable to *M. minutus* are known since the late Villanyian (late Pliocene) of Europe (Kowalski 2001). Within the Pleistocene, the species is recorded from various time slices including the Early, Middle, and Late Pleistocene up until around 0.2 million years ago (G. Storch, personal communication). No fossils of *M. minutus* are known from the latest part of Quaternary, and the species is thought to have been absent from Europe during the last two glacial periods (Spitzenberger 2001). Subsequent to the last glaciation, *M. minutus* is recorded first in Poland at about 10 000 – 14 000 years BP, and only in the late Holocene in Germany. No fossils are known for this period from Italy, France, and Spain (Spitzenberger 2001). The fossil record thus indicates a regional extinction of *M. minutus* in Europe at around 0.2 million years BP, with subsequent recolonization from an

eastern refugium completed only during recent millennia. Unfortunately, the fossil record from elsewhere in Eurasia is not sufficiently complete to test the generality of this explanation. However, based on the genetic evidence, we suspect that the populations of *M. minutus* underwent similar regional-scale extinctions elsewhere in Eurasia over the same general time period. In particular, we note that while much of Russia and East Asia remained ice-free through the most recent Quaternary glacial cycles, large areas were exposed to conditions of extreme cold and aridity (Schirmer et al. 2002). Such conditions are unlikely to have supported populations of a small mammal with preference for moist microhabitats, other than perhaps in localized refugia.

The apparent phylogeographic link between the European and the Korean–Japanese populations is perplexing given the presence of a more distinct haplotype group in the intervening area of Siberia. One possible explanation of this pattern is that European and East Asian populations were derived from a refugium, perhaps via a corridor situated to the north of a simultaneously expanding central Siberian population. To discuss the population dynamics, we would have to collect more specimens from Russia in a future study.

Comparison with phylogeographic patterns in other Palaeartic mammals

The relatively low level of divergence in *M. minutus* across such a large geographic area is unique in the context of similar investigations of Palaeartic small mammals. For example, net nucleotide divergence is relatively high (2.0%–3.3% in cytochrome *b* sequences) among the Eurasian populations of the root vole (*M. oeconomus*) and the gray red-backed vole (*Clethrionomys rufocanus* (Sundevall, 1846)), two inhabitants of the Eurasian Arctic (Iwasa et al. 2000; Brunhoff et al. 2003), and higher again (up to 6.3%) in the ecologically similar field vole, *Microtus agrestis* (L., 1761) (Jaarola and Searle 2002). Similarly, the forest-dwelling Korean wood mouse (*Apodemus peninsulae* (Thomas, 1907)) shows 1.0%–2.2% divergence among clades, representing the geographic regions of Far East Russia, Korea, and Siberia (Serizawa et al. 2002), and similar levels of divergence are reported for European species of *Apodemus* (Michaux et al. 2003). The unusually low level of genetic diversity in *M. minutus* might reflect a particular susceptibility of its preferred temperate moist grassland habitat to the pronounced and long-lasting climatic perturbations of the mid- to late-Quaternary glacial cycles. This possibility is of course testable by examination of genetic diversity either for other animals that are ecologically tethered to these communities or, even more directly, for key floristic elements in comparison with those of other major biotic communities.

Origin of the Japanese population

Our results are also relevant to determining the status of *M. minutus* in the Japanese Archipelago — whether native or introduced — and to the wider issue of the history of faunal interchange between Korea and Japan. Populations of *M. minutus* in Korea and Japan together constitute one of the four discrete lineages identified thus far (Figs. 2, 3). This close relationship is exceptional within the small-mammal fauna of Honshu, Shikoku, Kyushu, and the peripheral islands that otherwise constitute species showing high levels

of genetic divergence from related species on the continent (e.g., Suzuki et al. 1997; Serizawa et al. 2000; Tsuchiya et al. 2000).

Although the Japanese populations of *M. minutus* are most closely related to those from Korea, with the exception of haplotypes from some individuals from Tsushima Is., all Japanese cytochrome *b* haplotypes form a monophyletic clade that is embedded within a Korean assemblage. For control region, all Japanese haplotypes are monophyletic and similarly embedded with the Korean clade. This shared mitochondrial pattern is most readily explained by postulating that diversification among the Japanese haplotypes has occurred subsequent to their arrival in Japan. As explained earlier, estimates of divergence times for such closely related lineages are fraught with uncertainty. Nevertheless, we feel reasonably confident in postulating that divergence among the Japanese cytochrome *b* haplotypes, involving up to three substitutions, equates to thousands or even tens of thousands of years, rather than hundreds of years.

The possibility that *M. minutus* reached Japan during the last glaciation raises the vexed issue of late-Quaternary land-bridge connections between the Korean Peninsula and the Japanese Islands (Park et al. 2000; Kitamura and Kimoto 2004). Fossil evidence suggests that the most recent period of dispersal of land animals occurred around 300 000 years BP (Dobson and Kawamura 1998). Bathymetric data for the intervening Tsushima Strait (Japan Association for Quaternary Research 1987), coupled with the latest estimates of sea-level depression during the last glacial cycle (Shackleton 1987; Chappell et al. 1996; Yokoyama et al. 2001), likewise suggest that the strait remained open through the last glacial maximum (LGM: 30 000 – 15 000 years BP), albeit with a greatly narrowed channel, probably no more 20 km wide. Analysis of marine fossils from the Japan Sea by Ishiwatari et al. (1999) suggests that cold water flow through the Tsushima Strait was impeded during the LGM and only recommenced at 11 600 – 11 100 years BP. However, this evidence does not necessarily contradict the notion that the strait persisted as a narrow and shallow channel through the LGM, impeding water flow and providing a filter barrier for a highly selective faunal interchange between Korea and Japan.

The suggestion that *M. minutus* entered Japan from Korea during the late Pleistocene is seemingly at odds with two other pieces of evidence, namely (1) the lack of evidence for other mammalian interchange at the same time and (2) the lack of fossil evidence for *M. minutus* in Japan. On the first point, it is reasonable to view even a narrowed Tsushima Strait as a significant barrier to dispersal of many groups of mammals. Why it should be *M. minutus*, alone among the small mammals, that effected a crossing of the strait is not a question that we can answer at this point. However, this scenario is clearly possible and worthy of further consideration. The lack of any fossil evidence for *M. minutus* in Japan is more problematic. However, it is possible that the remains of this diminutive species have been overlooked, or that the species was relatively scarce in Japan through the late Quaternary, perhaps only becoming more widespread during the mid- to late Holocene as a consequence of the creation of more open environments through human forest clearance for agriculture and general habitat disturbance. This hypothesis

can be tested through more vigorous pursuit of small mammal remains representing the late Pleistocene and Holocene from archaeological and other subfossil contexts.

Conclusion

The mitochondrial data set for *M. minutus* contains a strong and well-structured phylogeographic signal. A major feature of the results is the evidence for transcontinental dispersal of this species, almost certainly from east to west, during the past 80 000 years or less. The proposed direction of recolonization of Europe by *M. minutus* following deglaciation is consistent with the regional fossil record of this taxon, but contrasts markedly with the postulated mode of dispersal of most other organisms, from south to north, out of refugia in Spain, Italy, and the Balkans. Although the sampling remains incomplete across central Asia and Russia, sufficient detail is available to propose several hypothetical scenarios to account for the distribution of haplotypes in Europe and East Asia; these hypotheses can be tested through analysis of other markers including nuclear genes. Another significant outcome of the study is the suggestion that *M. minutus* entered Japan around the last glacial period, presumably across a land-bridge connection. Our findings highlight the potential value of *M. minutus* as an indicator species for Quaternary environmental changes in temperate grassland regions of Eurasia.

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