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Molecular phylogeny of East Asian moles inferred from the sequence variation of the mitochondrial cytochrome *b* gene

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Taxonomic analysis has previously revealed that the species of moles that inhabit Japan are characterized by exceptional species richness and a high level of endemism. Here, we focused on the evolutionary history of the four Japanese mole species of the genera *Euroscapte*r and *Mogera*, examining mitochondrial cytochrome *b* (cyt *b*) gene sequences and comparing them with those of continental *Mogera wogura* (Korean and Russian populations), *M. insularis* from Taiwan, and *Talpa europaea* and *T. altaica* from the western and central Eurasian continent, respectively. Our data support the idea that in a radiation center somewhere on the Eurasian continent, a parental stock evolved to modern mole-like morph and radiated several times intermittently during the course of the evolution, spreading its branches to other peripheral geographic domains at each stage of the radiation. Under this hypothesis, the four lineages of Japanese mole species, *E. mizura*, *M. tokudae*, *M. imaizumii*, and *M. wogura*, could be explained to have immigrated to Japan in this order. *Mogera wogura* and *M. imaizumii* showed substantial amounts of geographic variation and somewhat complicated distributions of the cyt *b* gene types. These intraspecific variations are likely to be associated with the expansion processes of moles in the Japanese Islands during the Pleistocene glacial ages.

INTRODUCTION

Mammalian fauna of the central domain of the Japanese Islands, which comprises three main islands, Honshu, Shikoku, and Kyushu, and numerous adjacent small islands, tends to show some peculiar features from the biogeographical viewpoint: richness in species number compared to the size of the land, a high level of endemism, and a high degree of geographic variation. The area is thought to have been connected with the Asian continent through the Korean Peninsula several times during the periods of Pleistocene glacial ages and isolated during the inter-glacial ages. In addition, the geographic domains are long from North to South and complicated in topographic configuration. These historical and geographic conditions of this area have been considered to be responsible for the origin of such unique fauna, but only a

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few attempts have been made to document the intrinsic features of this fauna on a molecular phylogenetic level. Moles and shrew-moles living in this area seem to be good examples for such a study. Species richness and a high level of endemism are the main features of the subfamily Talpinae members in the central domain of Japan. In addition, a high degree of geographic variation inferred from morphological characteristics is another trend of these Japanese mole species. Therefore, this animal group might provide an interesting subject for understanding the emergence of biodiversity along the Japanese Islands.

Moles are the most common small insectivores which live mainly in the grasslands and deciduous woodlands of the Temperate Zone of the Eurasian continent and Japanese Mainland, except Hokkaido (Abe, 1967, 1996; Imaizumi, 1970; Corbet, 1978). In the Palaearctic region, the subfamily can be classified into 14 genera and 36 species according to Hutterer (1993). Most of the genera are monotypic and only three genera, *Euroscaptor* (6 species), *Mogera* (7 species), and *Talpa* (11 species) are rich in species number (Hutterer, 1993). Recently, the species names of Japanese mole species have been revised, reflecting a reevaluation of species and generic status and correction of some mistakes in taxonomic operation (Abe, 1995; Motokawa and Abe, 1996); the current taxonomic classification puts Japanese moles into four species: *M. wogura* (= *M. kobeae* in Hutterer, 1993), *M. imaizumii* (= *M. minor* and *M. wogura* in Hutterer, 1993), *M. tokudae* (= *M. etigo* plus *M. tokudae* in Hutterer, 1993) and *E. mizura*. Together with two shrew-moles, *Urotrichus talpoides* and *Dymecodon pilirostris* (= *Urotrichus pilirostris* in Hutterer, 1993), the latter three species of moles are endemic to Japan (Abe, 1994; Hutterer, 1993). *Mogera wogura* is widely distributed in East Asia, including southern Japan, Korea, East China and Primorye, the far south-eastern part of Russia (Fig. 1). Its parapatric species *M. imaizumii* mainly inhabits northern Japan, and also mountainous areas in southern Japan, as fragmented populations in mountainous areas including the southern part of the Kii Peninsula (Fig. 1; Abe, 1996). The geographic distribution of *M. tokudae* is narrowly confined to Sado Island and a part of Echigo Plain where *M. imaizumii* surrounds the territory of *M. tokudae* parapatrically (Fig. 1; Abe, 1996). Populations of *E. mizura* are fragmented in subalpine areas of Honshu (Fig. 1; Abe, 1994).

The evolutionary histories of these mole species are still uncertain even though several important findings have been revealed by morphological and karyological studies (Abe, 1996; Tsuchiya, 1988). Regarding the taxonomic status as well, there still exists some argument as to the generic and species status of these mole species, especially for the local or geographic populations. For example, the two geographic populations of *M. tokudae* are sometimes treated as distinct species, *M. tokudae* for the Sado Island population and *M. etigo* for the population of Echigo Plane, respectively (Yoshiyuki and Imaizumi, 1991; Hutterer, 1993).

As part of our molecular approach to understanding the evolutionary trends of Japanese mammals, here we analyzed these mole species on the basis of mitochondrial DNA (mtDNA) variation. This variation is a useful marker in this case, because the evolutionary rate is thought to be much higher than those of nuclear DNA markers (Avise, 1994). The lack of recombination events between mtDNA molecules makes it possible to trace the genealogy of these lineages. Here we examined the mitochondrial cytochrome *b* (cyt *b*) gene variation within and among seven species of moles from the Eurasian continent, Taiwan, and Japanese Islands in order to make an initial estimate of the evolutionary relationships among the species of Japanese moles and their related continental species.

MATERIALS AND METHODS

Samples The species examined in this study are listed in Table 1. We followed the nomenclature of the species names commonly used in the literature for continental species (Corbet and Hill, 1992; Huttler, 1993). Those revised recently by Abe (1995) and Motokawa and Abe (1996) were used for Japanese species. According to this revision, the names of the Japanese eastern mole and Japanese western mole have been changed from *M. wogura* and *M. kobeae* to *M. imaizumii* and *M. wogura*, respectively. Collection localities of samples of mole species examined and a schematic presentation of their geographic distributions are shown in Fig. 1.

Nucleotide sequencing Nested polymerase chain reactions (PCRs) and direct sequencing were performed according to the methods previously described (Suzuki et al., 1997, 1999). The nucleotide sequence of a 402-bp portion of the cyt *b* gene was determined in 49 specimens of moles using PCR fragments amplified with the primer pair R-L14724 and U- H15155 (Suzuki *et al.* 1997). In a representative individual of each species, the sequences of a 1140-bp portion was further determined using PCR fragments amplified with primer sets R-L15135 and U-H15599, and R-L15561 and U-H15916 (Suzuki et al., 1999), and other mole-specific primers. The sequence of the cyt *b* gene of the greater Japanese shrew-mole *Urotrichus talpoides* (Shinohara *et al.*, unpublished) was used as the outgroup.

Construction of phylogenetic trees Pairwise sequence divergences were calculated with the Kimura's two-parameter method (Kimura, 1980) using the computer software program DNADIST of the software package

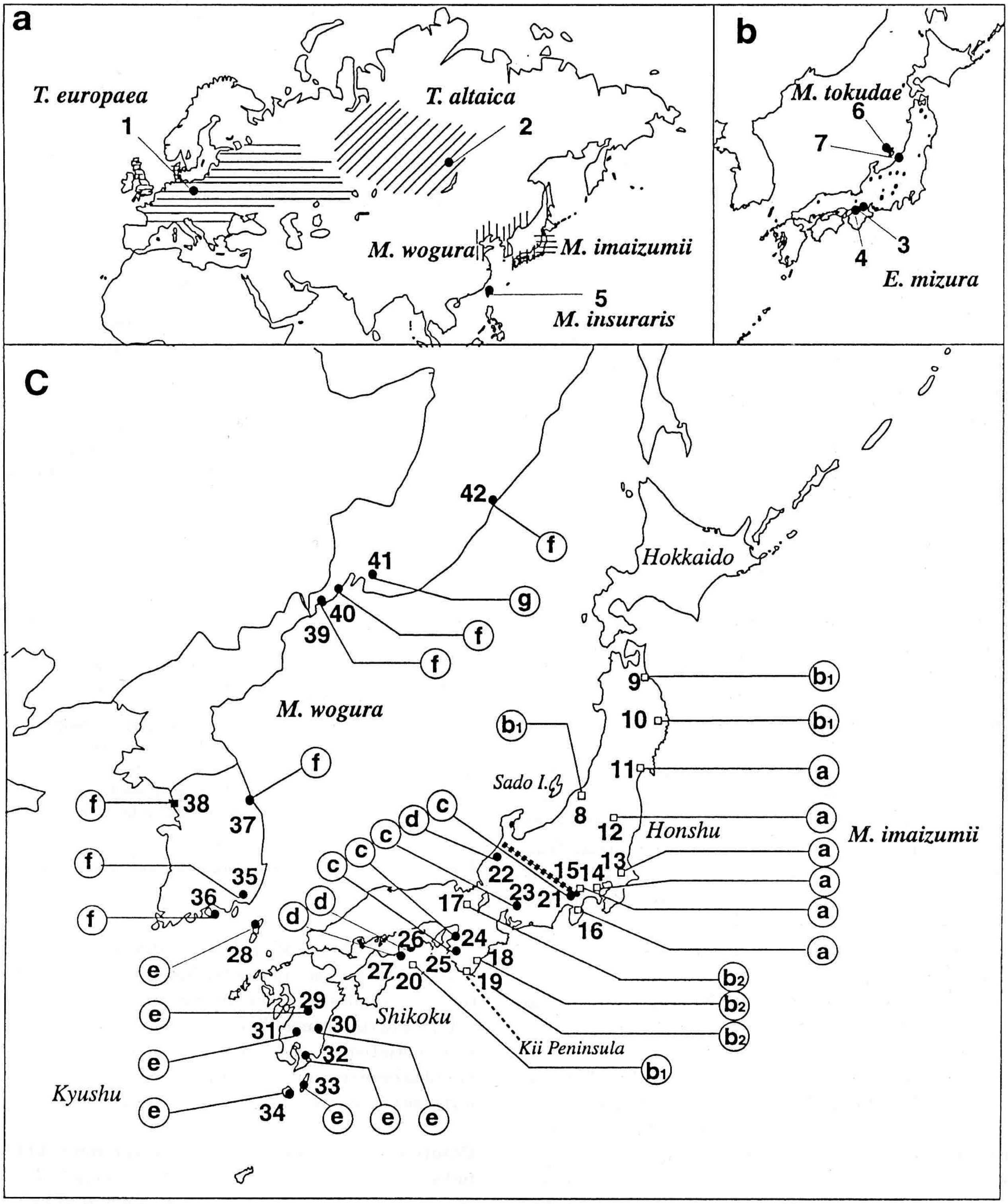

Fig. 1. Geographic distribution and collection localities of moles examined. (a) The distribution of *Talpa europaea*, *T. altaica*, *Mogera insularis*, *M. imaizumii*, and *M. wogura*, according to Abe (1994) and Corbet (1978). (b) Collection site of *Euroscaptor mizura* and *M. tokudae*. The distribution of *E. mizura* is fragmented in mountainous regions in Honshu. The distribution of *M. tokudae* is confined to Sado Island and a small area opposite on Honshu. (c) Collection sites and haplotypes of *M. imaizumii* and *M. wogura*. The dotted line at the center of Honshu schematically represents the northern most limit of *M. wogura* (Abe 1996) (c). Alphabetical letters associated with the collection localities of *M. wogura* and *W. imaizumii* represent the clusters to which each haplotype belongs to (see Fig. 2). Locality numbers refer to the locations listed in Table 1.

PHYLIP V (Felsenstein, 1993). Trees were constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) and unweighted pair-group method (UPGMA, Sokal and Micherner 1953) using NEIGHBOR in PHYLIP.

RESULTS

Molecular phylogeny with partial cyt *b* sequences (402 bp) To illustrate inter- and intra-species phylogenetic relationships, we determined sequences of the cyt *b* gene region (402 bp) in 49 moles including individuals from one species of *Euroscaptor*, four species of *Mogera* and two species of *Talpa* (Table 1). A total of 38 haplotypes were then observed and an NJ tree was constructed using sequence divergences calculated by the Kimura's (1980) two-parameter method taking into account all substitutions (*d*) at all codon positions (Fig. 2). The phylogenetic tree clearly indicated that these haplotypes can be integrated into seven groups, *altaica*, *europaea*, *mizura*, *insularis*, *tokudae*, *imaizumii*, and *wogura*, with high interclade divergences ($d > 0.08$; Fig. 2), which showed a good consistency with the taxonomic system of the species that we used here. These clustering patterns were also seen in NJ trees constructed based on pairwise sequence divergences calculated by transversional substitutions or substitutions at first and second codon positions (trees not shown). The groups with a large sample size, such as *wogura* and *imaizumii*, consisted of several distinct clades that tended to reflect the region-specific geographic distribution (Fig. 2).

The *imaizumii* group consisted of two distinct clades with a high level of interclade divergence ($d > 0.05$; Fig. 2). Here, we designated them as "Eastern"-clade and "Western"-clade. This demarcation had a certain accord with Y. Imaizumi's claim that *M. imaizumii* comprised two forms, the so-called "Azuma-mogura" and "Ko-mogura" with substantial morphological differences (e.g. head and body lengthes are 128.3 ± 3.0 mm and 136.1 ± 6.4 mm, respectively; Imaizumi, 1998). The Eastern-clade is restricted to the Pacific part of eastern Honshu, whereas the Western-clade covers northern Honshu, the Kii Peninsula and Shikoku (Fig. 1c).

Using the same criteria ($d > 0.05$) as those used for *M. imaizumii,* the *wogura* group appeared to have five distinct clades, Honshu-clade, Shikoku-clade, Kyushu-clade, Continent-clade 1, and Continent-clade 2 (Fig. 2). These five clades were likely to have diverged within a short period of evolutionary time, as evidenced by short branches between the nodes. The three Japanese clades showed distinct geographic distributions whereas the continental clades seemed to exist with a polymorphic status in Russian Primorye, although the sample numbers were limited. Continental populations consist of common type Continent-1 or rare type Continent-2, which show considerable sequence divergence.

Table 1. Samples used in this study

Species	Locality collected (Locality No[a], Sample No.)
1. *Talpa europaea*	Germany: Leubechk(1, HS564*).
2. *Talpa altaica*	Russia: Novosibirsk (2, MH6804*, MH6664).
3. *Euroscaptor mizura*	Japan: Ashiu, Kyoto (3, KT3032HS624*); Oodaigahara, Nara (4, HS1169).
4. *Mogera insularis*	Taiwan: Pingdong,(5, HS1036*).
5. *Mogera tokudae*	Japan: Sawada, Sado I. (6, KT2672HS317*); Maki, Niigata (7, KT2675HS312)
6. *Mogera imaizumii*	Japan: Niigata (8, KT2795HS364*, HS470), Hachinohe (9, KT2793HS499), Ootsuchi (10, HS659), Sendai (11, HS582), Kooriyama (12, HS575), Chiba (13, HS580), Yokohama (14, KT2697HS365), Aokigahara (15, KT2923HS500), Ito (16, HS1022), Iwakura-hasemachi, Kyoto (17, KT2971HS463), Kumanogawa (18, KT2707HS368), Shirahama (19, KT2770HS315), Mt. Tsurugi (20, MH6848).
7. *Mogera wogura*	Japan: Mishima (21, KT3201HS1023*), Mattoh (22, KT3095HS1416), Kasugai (23, HS581), Kawachinagano (24, KT2771HS316), Kanaya (25, HS1073 and HS1074), Funashi, Tokushima (26, MH5849), Mt. Ootaki (27, KT2802HS464), Tsushima Is. (28, KT2717HS367), Menda (29, KT2955HS404), Miyazaki (30, KT2706HS370), Makizono (31, KT2699HS369), Uchinoura (32, KT2956HS415, KT2957HS416), Nishinoomote, Tanegashima I. (33, KT2821HS467), Yaku, Yakushima I. (34, KT2731HS371). Korea: Pusan (35, KT2754HS372), Namhae (36, HS929). Mt. Solak (37, HS930), Sukmo I. (38, HS1275). Russia: Khasan (39, AK605, AK606), Kedorovaya (40, AK001HS890, AK002HS1170*), Ussurijsky (41, AK604HS1261), Shikhoto-Alin (42, AK727HS1417, AK728HS1418).

[a] Locality numbers are as shown in Fig. 1

* Individual in which the whole sequence for the cytchrome *b* gene (1140 bp) was determined.

The Sado mole, *M. tokudae*, showed considerable sequence divergence in the cyt *b* gene sequences between the two samples from the allopatric populations in Sado Island and the Echigo Plain (see Fig. 1b), with *d* of 0.020. Differentiation between the two geographic populations has also been documented based on morphological characteristics (Yoshiyuki and Imaizumi, 1991).

Comparison of whole cyt *b* gene sequences (1140 bp) between species To further define the genetic relationships among the mole species with finer resolution, we determined the 1140-bp sequences of cyt *b* in representative individuals from all seven species groups and two clades of *M. wogura* from the continent (Primorye) and Japan (Honshu). We then calculated the sequence divergence among these sequences as well as that of *U. talpoides* (Shinohara *et al.*, unpublished), which was used

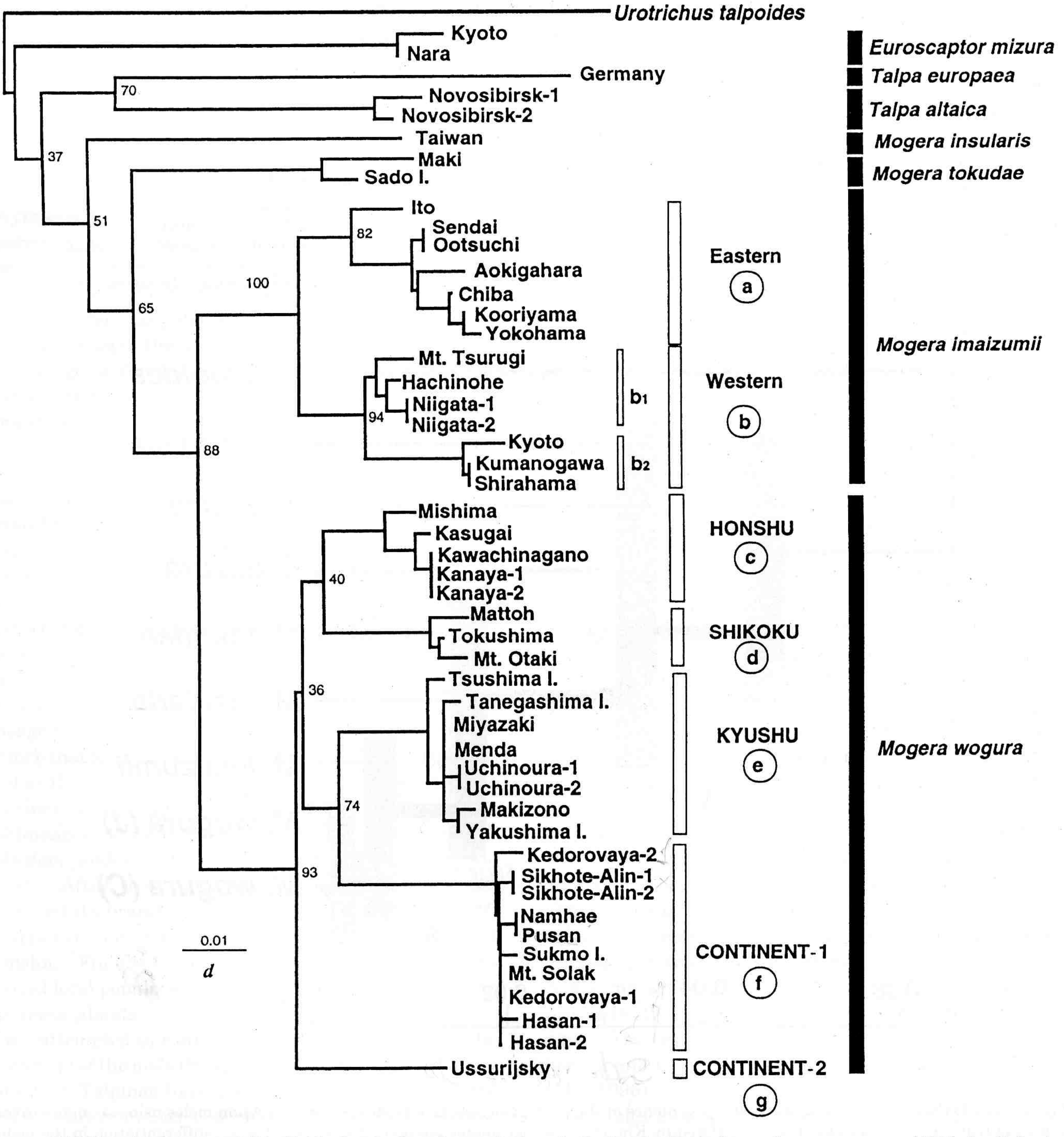

Fig. 2. Neighbor-joining tree of 49 individual moles based on variation of mitochondrial cytochrome *b* gene sequences (402 bp). Pairwise sequence divergences were calculated by Kimura's (1980) two-parameter methodconsidering all codon positions and all substitutions. Bootstrap scores with 1000 replicates are given associated with each node. The cluster names of *M. wogura* and *M. imaizumii* and their abbreviations are the same as in Fig. 1c.

as the outgroup, using Kimura's (1980) two-parameter method with consideration only of transversional substitutions (*d*v) (Table 2). Transitional substitutions were neglected because of their saturation bias in comparisons of such higher taxa (Irwin et al., 1991; Suzuki et al., 1999).

We then constructed phylogenetic trees using these pairwise sequence divergences (*d*v) by the NJ (tree not shown) and UPGMA (Fig. 3) methods. Both trees showed the same topological relationships among the taxa. The extent of average sequence divergence between moles and shrew-moles was 0.064 (*d*v; Table 2), which was similar to those between several distinct genera of the subfamily

Table 2. Sequence divergenes among eight taxa of moles in the mitochondrial cytochrome *b* gene region (1140 bp)

Species	*Uta*	*Teu*	*Tal*	*Emi*	*Min*	*Mto*	*Mim*	*Mwo* (J)
U. talpoides	–							
T. europaea	0.0595	–						
T. altaica	0.0655	0.0345	–					
E. mizura	0.0695	0.0440	0.0498	–				
M. insularis	0.0615	0.0421	0.0459	0.0345	–			
M. tokudae	0.0625	0.0411	0.0488	0.0336	0.0206	–		
M. imaizumii	0.0635	0.0459	0.0498	0.0289	0.0106	0.0133	–	
M. wogura (Japan)	0.0625	0.0449	0.0488	0.0298	0.0151	0.0142	0.0044	–
M. wogura (Continent)	0.0645	0.0488	0.0527	0.0317	0.0188	0.0179	0.0080	0.0035

Sequence divergences were caluculated by Kimura's two-parameter method (Kimura, 1980) taking into account only transverional substitution.

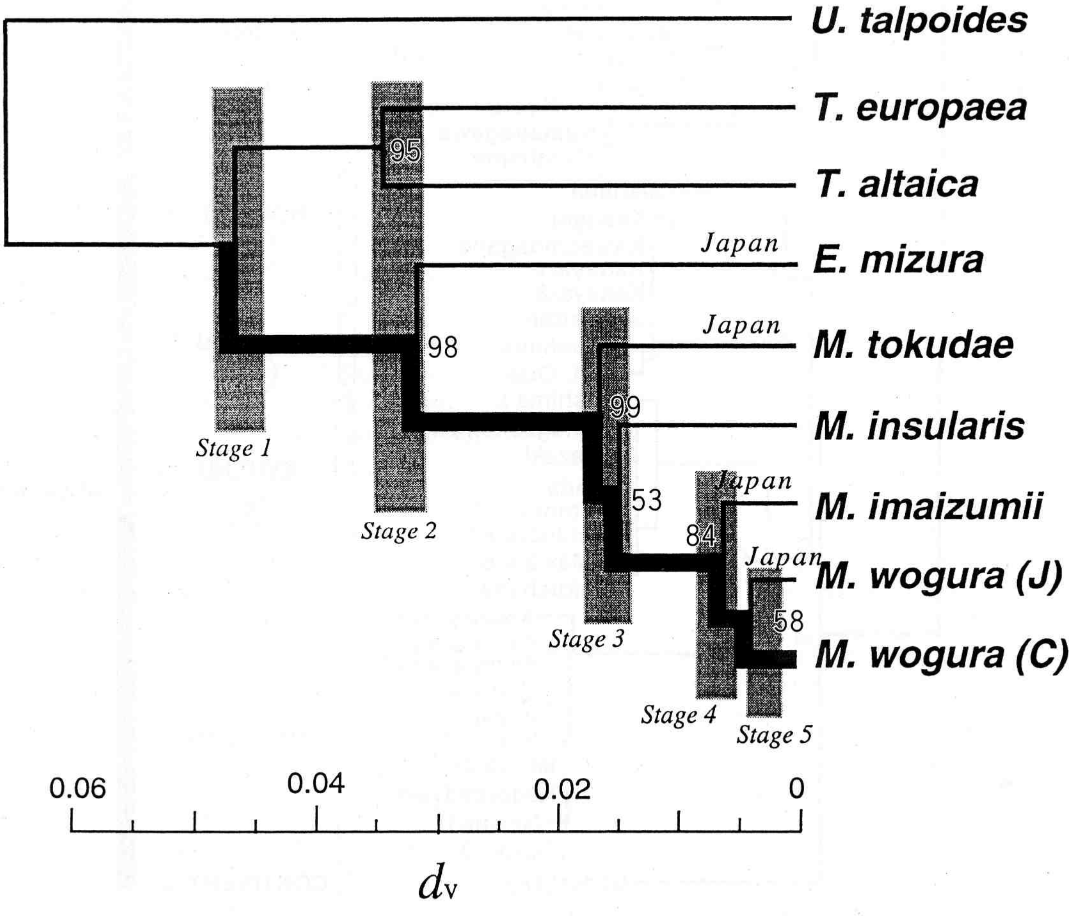

Fig. 3. A UPGMA tree based on the 1140-bp sequence of the cyt *b* gene region of eight taxa of East Asian moles using sequence divergences of transversional substitutions calculated by Kimura's two-parameter method (1980). The lineage differentiation in the moles can be explained by five consecutive divergence events. Positions of nodes that associated with the events are marked as stages 1–5. In *M. wogura*, two geographic groups from the continent (C) and Japan (J) were examined. Bootstrap scores with 1000 replicates are given associated with each node.

Murinae (Suzuki et al., 1999). After the split from the shrew moles, the lineage of moles appears to have two distinct lineages of European (*Talpa*) and Asian (*Mogera-Euroscapter*) groups, with 0.047 divergence (*d*v) on average. These two groups differentiated into *T. europaea* and *T. altaica* (*d*v = 0.034), and the *Mogera* lineage and *E. mizura* (*d*v = 0.032 on average), respectively. The *Mogera* group then further diverged into three groups within a short period of evolutionary time (*d*v among the three ranged from 0.011–0.021), the Taiwanese mole *M. insuralis*, the Sado mole *M. tokudae* and the remaining *Mogera* lineage, which was further differentiated into two species, *M. imaizumii* and *M. wogura*. The amount of sequence divergence between the two species was calculated to be 0.0062 on average (*d*v). The divergence of the cyt *b* gene among the races of *M. wogura* was only about half as great as that of the species (Fig. 3, *d*v = 0.0035).

DISCUSSION

Phylogenetic framework of the East Asian moles

Species of moles are distributed widely in Palaearctic regions and they have now established their status as the most common subterranian mammals. This raises the question of how they developed their lineages, especially for those living in the Asian Far East, after splitting from the lineage of shrew moles. One of our main findings here was the presence of periodic divergent events, which appear to have occurred at least five times in the mole lineages (Fig. 3). This notion supports the idea that in a radiation center, somewhere in the continent, a parental stock evolved to a modern mole-like morph and radiated several times intermittently during the course of evolution, spreading its branches to peripheral geographic domains at each stage of the radiation. In the context of this hypothesis, the first stage of the evolution of moles that we detected was the split of the European and Asian lineages. Then in the second stage, the former split into *Talpa europaea* and *T. altaica*, and the latter split into two groups: one consisting of the main *Mogera* lineage (the lineage that led to *M. wogura*; Fig. 3) and the other of a branch that led to *E. mizura*, which can thus be characterized as the oldest lineage that now exists in Japan. The continental parental lineage then produced two sublineages: *M. insularis*, endemic to Taiwan, and *M. tokudae*, endemic to Sado Island and the Echigo Plain. After that, the continental parental lineage extended its branch to Japan and its descendants, *M. imaizumii*, now found mostly in the eastern part of Honshu. Finally, the ancestral *M. wogura* had split into several local populations, and the population invaded the Japanese islands.

We attempted to calibrate the divergence time for the branching of the mole lineages. The earliest fossils of the subfamily Talpinae have been recovered from Oligocene and possibly from Eocene deposits, while both the earliest fossils of the tribes Urotriquini (shrew-moles) and Talpini (moles) have been recovered from Oligocene (McKenna and Bell, 1979). Thus, here we can tentatively regard the time of the split between moles and shrew-moles to be 25–35 million years ago (Mya) (K. Koyasu, personal communication). The divergence rate of the cyt *b* gene for *d*v is then calculated to be 0.0018–0.0025 per site per million years. Based on this assumption, the splitting of the lineages of *E. mizura*, *M. tokudae*, and *M. imaizumii* can be estimated to be17.6–12.6, 9.2–6.6– and 3.4–2.5 Mya, respectively. Due to various uncertain factors, including the uncertainty of the rate constancy of nucleotide substitution, this is not a definite time estimation, and it needs careful verification in the future. However, it is probable that the times of establishment of the three old Japanese lineages of *E. mizura*, *M. tokudae*, and *M. imaizumii* can go back to the latter part of theTertiary epock. Thus, it is conceivable that the Japanese Islands (Honshu, Shikoku, and Kyushu) now retain mole lineages of such ancient origin with a high degree of genetic endemism.

Evolutionary history of *M. imaizumii* and *M. wogura*

Mogera imaizumii and *M. wogura*, especially in the Japanese populations, showed substantial amounts of geographic variation and somewhat complicated distribution in the cyt *b* gene types (Fig. 2, Table 2). Although mtDNA data are not sufficient to discuss their evolutionary history, the diversity of mtDNA in *M. imaizumii* and *M. wogura* moles would provide us several important clues for understanding their evolutionary history.

About halfway after the divergence of *M. imaizumii* and *M. wogura*, the mtDNA lineage of *M. imaizumii* split into two groups that are comparable to the two geographic groups that have sometimes been treated as distinct species based on morphological differences (Imaizumi 1960, 1998; Hutterer 1993). Moreover, the *Mogera wogura* lineage split into three groups, the Kyushu, Shikoku, and Honshu groups (Fig. 2). Such divergent Japanese populations of *M. wogura* are likely to have emerged in the Japanese Islands soon after immigration from the continent. The high magnitude of divergence among the geographic races suggests that the time of migration of *M. wogura* was considerably longer ago (probably a few Mya; Fig. 3) than our initial expectation, which was that the ancestor came to Japan at the time of the last glacial age, about 0.1–0.01 Mya. Thus, our data suggest that *M. wogura* has coexisted in Japan with *M. imaizumii* for a considerable time, and that these two species have been competing with each other for their distribution ranges, probably throughout the entire Pleistocene glacial ages. The two mole species now show parapatric distribution, and the main border is located in central Honshu (Abe, 1996).

Our data also suggest that *M. imaizumii* in southern Japan are relic poplations. The haplotype in Shikoku (Mt. Tsurugi) showed close affinity with those of the populations in the northern part of Honshu (Niigata and Hachinohe), even though these areas are widely separated. Similarly, in *M. wogura*, a rare mtDNA type from Mattoh, Honshu (locality 22 in Fig. 1c), showed affinity with those from Shikoku (Tokushima and Otaki). These data can be explained by the occurrence of recent geographic distribution in Shikoku and Honshu in each species. On the other hand, there exist considerable amount of intraspecific sequence divergences in the western Honshu in both species and it may imply that old habitation of these species there. It is therefore probable that both populations of *M. imaizumii* and *M. wogura* inhabited the western part of Japan, including Shikoku, during considerable time of the ice ages. Consequently, it is suggested that the environmental changes, including generation of land bridges, during the Quaternary glacial ages played an important role in generating such complicated geographic distributions of mtDNA in the two species.

In conclusion, the Japanese Islands constitute a peripheral domain that has a great capability of retaining various descendent lineages for considerably long times, although the area of the islands is not so large. It can be postulated that the various evolutionary branches of moles moved into the Japanese domain stepwise in each radiation step, which resulted in the unique fauna of moles with species richness and a high degree of endemism. In addition, along with findings regarding other Japanese mammalian species, the present findings in moles emphasize that the zoogeographical domain of Honshu-Shikoku-Kyushu has provided an important area for the generation of biodiversity, and this domain as well as the domain of Ryukyu (Suzuki et al., 1999) has played an important role in conservation of lineages of Tertiary origin.

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