Spatial and Temporal Aspects of Occurrence of *Mogera* Species in the Japanese Islands Inferred from Mitochondrial and Nuclear Gene Sequences

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We assessed dispersal and vicariant events in four species of Japanese moles in the genera *Mogera* and *Euroscaptor* to better understand the factors shaping intra- and interspecific differentiation in Japanese moles. We used the combined viewpoints of molecular phylogeny and historical geology using nucleotide sequences of mitochondrial (*cytochrome b*; *Cytb*) and nuclear (*A2ab*, *Bmp4*, *Tcf25*, *vWf*) genes. The divergence times estimated from the molecular data were verified with available geological data on the chronology of fluctuations in sea level in the Korea Strait, assuming sequential migration and speciation events. This produced possible migration times of 5.6, 3.5, 2.4, and 1.3 million years ago for four species of Japanese moles, *Euroscaptor mizura*, *Mogera tokudae*, *M. imaizumii*, and *M. wogura*, respectively. For the western Japanese mole *M. wogura*, *Cytb* sequences revealed four major phylogroups with strong geographic affinities in southwestern Central Honshu (I), western Honshu/Shikoku (II), Kyushu/westernmost Honshu (III), and Korea/Russian Primorye (IV). The nuclear gene sequences supported the distinctiveness of phylogroups I and IV, indicating long, independent evolutionary histories. In contrast, phylogroups II and III were merged into a single geographic group based on the nuclear gene data. Intraspecific divergences in *M. imaizumii* and *M. tokudae* were rather apparent in *Cytb* but not in nuclear gene sequences. The results suggest that repeated dispersal events have occurred between the Asian continent and the Japanese Islands, and intensive vicariant events associated with abiotic and biotic factors have created higher levels of species and genetic diversities in moles occurring on the Japanese Islands.

Key words: *Mogera*, the Japanese Islands, the Korea Strait landbridges, Late Tertiary, *cytochrome b*, nuclear genes

INTRODUCTION

The zoogeographic domain of the Japanese Islands, Honshu-Shikoku-Kyushu is known to have a high level of species diversity, harboring a large number of terrestrial vertebrate species including 110 mammals, 98 reptiles, and 64 amphibians (Abe, 2000, 2005; Suzuki, 2009; Biodiversity Center of Japan, 2010). These species lineages may have been established mainly through migration events across the Tsushima-Korea Strait (hereafter the Korea Strait),

which divides the Korean Peninsula and the Japanese Islands (Dobson, 1994; Millien-Parra and Jaeger, 1999). In mammals, half of Japanese lineages are now recognized as endemic at the species level, and several lineages at the genus level, such as dormice (*Glirulus*) and shrew moles (*Urotrichus* and *Dymecodon*), whereas the remaining portion is known as local populations conspecific to continental species (Suzuki, 2009). This in turn implies that the colonization events took place for a considerable geological time from the late Tertiary to Recent (Suzuki, 2009). However, the migration events through the Korea Strait have not been fully documented in the light of fossil evidence, except for large mammals, such as extinct proboscideans (Kawamura, 1998, 2007).

The Japanese Islands exhibit higher levels of intraspe-

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cific genetic diversity in a variety of terrestrial species, including mammals (e.g., Nagata et al., 1999) and insects (Sota and Hayashi, 2007), with several different substructures, although the factors shaping the genetic patterns remain under discussion. For example, a bimodal north–south structure in karyotype variation associated with topographic features of Honshu Island has been identified in the Japanese wood mouse *Apodemus speciosus* (Tsuchiya, 1974) and the large Japanese shrew mole *Urotrichus talpoides* (Kawada and Obara, 1999; Harada et al., 2001). Subspecies grouping characterized by pelage color in the Japanese hare (*Lepus brachyurus*) is reported to be maintained by natural selection operating in varying environments, i.e., different patterns of snowfall in winter (Nunome et al., 2010). Kawamoto et al. (2007) showed that the genetic structure of Japanese macaques was affected by their movement following climate fluctuations in the Quaternary ice ages, resulting in high and low genetic diversity in southern and northern areas, respectively.

Moles in the genus *Mogera* are the most common small insectivores in East Asia, occurring mainly in the grasslands and woodlands in this region, with their eastern peripheral distribution extending to the Japanese Islands and Taiwan (Hutterer, 2005). This group has attracted much attention from morphologists, cytogeneticists, and molecular phylogeneticists (e.g., Abe, 1995; Motokawa and Abe, 1996; Okamoto, 1999; Tsuchiya et al., 2000; Kawada et al., 2001; Motokawa, 2004; Shinohara et al., 2004, 2005; Yokohata, 2005) as it exhibits various phenomena associated with evolutionary episodes of Japanese terrestrial animals, including a substantial degree of species richness and genetic diversity. In particular, the Japanese Islands harbor three species of *Mogera* (Hutterer, 2005); two species with wider distributions, *M. imaizumii* and *M. wogura*, occur in the eastern and western parts of Japan, respectively, while *M. tokudae* is confined to tiny areas in Niigata Prefecture in the northern part of Honshu, including Sado Island and the Echigo Plain on the opposing coast (Fig. 1). The western mole *M. wogura* is also found in adjacent areas of the Asian continent, specifically the Korean Peninsula, East China, and the Maritime region of the Russian Far East (Primorye). In addition to these species, the Japanese Islands have a small mole, *Euroscaptor mizura*, with fragmented populations on mountainous areas in Honshu. Molecular phylogenetic analyses suggest that the talpid ancestors evolved on the continent and entered the Japanese Islands from the Korean Peninsula in the order *E. mizura*, *M. tokudae*, *M. imaizumii*, and *M. wogura* (Tsuchiya et al., 2000; Shinohara et al., 2003, 2004), limiting the more 'ancestral' *Mogera* lineages to restricted regions distant from their entryway, the Korea Strait. The earlier arrival of *E. mizura* in Japan was supported by our recent molecular study that showed ancient divergence between *E. mizura* and the continental congener *E. micrura*, which is equivalent to that between *E. mizura* and *Mogera* species (Shinohara et al., 2008). The concept emerging from these and earlier studies (Tsuchiya, 1990; Tsuchiya et al., 2000; Shinohara et al., 2003, 2004, 2005) is that the central domain of East Asia fostered the evolution of novel talpid phenotypes, and has been the cradle of multiple dispersion events to peripheral regions, including insular domains that were intermittently connected to the mainland by landbridges during the last several million years, although reliable dates have not yet been assigned to the dispersal events in each mole species.

The Japanese *Mogera* species, which show apparent interspecies competition in their ranges (Fig. 1), exhibit intraspecies geographic demarcation with rather complicated patterns. The mitochondrial sequences of *cytochrome oxidase subunit I* (*COI*; Okamoto, 1999) and *cytochrome b* (*Cytb*; Tsuchiya et al., 2000) have revealed four mutually exclusive geographic regions: Korea/Russian Primorye, Kyushu and the adjacent westernmost part of Honshu, Shikoku and adjacent western Honshu (i.e., the Chugoku District), and a southern part of Central Honshu (the Kinki and Tokai Districts and Shodo Island). Notably, the Russian population, which has a large body size (Okhotina, 1966), has non-divergent mitochondrial sequences from those of the small-body Korean moles (Tsuchiya et al., 2000). The mitochondrial data revealed the genetic structure of *M. imaizumii*, showing two (Tsuchiya et al., 2000) or four (Okamoto, 1999) major clades with high geographic affinity and dividing eastern Honshu into two parts: the coastal areas on the Sea of Japan and Pacific Ocean. Notably, several enclaves of *M. imaizumii* in western Honshu and Shikoku tend to possess non-divergent mitochondrial sequences with the Sea of Japan group (Tsuchiya et al., 2000; but see Okamoto, 1999). Recently, Iwasa et al. (2006) provided nuclear ribosomal DNA-RFLP data and confirmed the phylogeographic subdivision into the Sea of Japan and Pacific Ocean sides and showed possible involvement of postglacial geographic expansion from refugia of both sides and secondary contact followed by genetic exchange. However, intraspecies genetic structures in Japanese moles have not yet been documented with nuclear gene sequences, which have proven to be useful for phylogeographic inference in recent studies (e.g., Tomozawa and Suzuki, 2008).

To obtain a reliable picture of the evolutionary history of Japanese moles, we examined mitochondrial and nuclear gene sequences, conducted phylogenetic analyses, and gathered molecular data from the literature (Iwasa et al., 2006). Mitochondrial genome variation gives a finer genealogical relationship both within and between species, whereas nuclear gene sequencing is suitable for inferring deep interspecies phylogenies and the population genetic structure (e.g., Suzuki et al., 2008; Melo-Ferreira et al., 2009; Tomozawa and Suzuki, 2008; Yasuda et al., 2012). To capture temporal aspects, we addressed the issue of periodic changes in the depth of the Korea Strait during the last several million years using both global geological information (Haq et al., 1987; Woodruff, 2003) and a consideration of geological insights on the strait (Iijima and Tada, 1990; Kitamura et al., 2001; Kitamura and Kimoto, 2006). We focused on factors promoting vicariant events in moles in the Japanese Islands. This study will provide useful clues to better understand the genetic diversity of the Japanese moles and the richness of the terrestrial fauna of Japan.

MATERIALS AND METHODS

Biological materials

The species examined in this study are listed in Table 1. We followed the nomenclature of species names commonly used in the

Fig. 1. Geographic map of the Japanese Islands and nearby continent, showing the distributions of the moles *Mogera imaizumii* (shaded area), *M. tokudae*, and *M. wogura*. Collection localities are indicated for intraspecies geographic groups based on the *cytochrome b* sequence in *M. imaizumii* (*Mim*-I, open squares; *Mim*-II, open circles; *Mim*-III, open triangles) and *M. wogura* (*Mwo*-I, closed stars; *Mwo*-II, closed triangles; *Mwo*-III, closed circles; *Mwo*-IV, closed squares). The administrative units of Japan (Tohoku, Kanto, Hokuriku, Koshinetsu, Tokai, Kinki, Chugoku, Shikoku, and Kyushu) are used to describe regionally cohesive sampling localities and phylogroups. The arrow indicates the direction of the Tsushima Warm Current.

literature for continental species (Hutterer, 1993, 2005), dividing the Japanese moles into three species, *M. tokudae*, *M. imaizumii*, and *M. wogura* (Fig. 1; Abe, 1995; Motokawa and Abe, 1996), although the taxonomic status of moles from Sado Island and the Echigo Plain is controversial. The Echigo Plain population is sometimes considered to be a separate species, *M. etigo*, distinct from the Sado Island population, *M. tokudae* (e.g., Yoshiyuki and Imaizumi, 1991; Kawada and Yokohata, 2009). Eighty-five specimens from three species of mole, namely, *M. tokudae*, *M. imaizumii*, and *M. wogura*, were collected from various localities in Japan (Honshu, Shikoku, Kyushu), Korea, and Russia (Table 1; Fig. 1). Specimens of *U. talpoides* (Mt. Tsurugi, Japan; DNA code: HS1841), *E. mizura* (Nara, Japan; HS1168), and *Talpa europaea* (Lübeck, Germany; HS564) were used in this study for the nuclear gene-sequence analyses. Voucher specimens collected by H. Abe (HA) are preserved in the Botanical Garden Museum of Hokkaido University.

Mitochondrial gene sequences: *Cytb*

Sequences of the complete mitochondrial *Cytb* (1140 bp) were determined with the primer sets and PCR conditions listed in Table 2. First, we amplified a large fragment using the universal primers L14724 and H15915 (Irwin et al., 1991). Two overlapping gene fragments were then amplified independently from the initial PCR product using species-specific primer sets and conditions (Table 2). The PCR reaction mixtures (20 μl) contained 2 mM Tris-HCl, 10 mM KCl, 2.5 mM MgCl₂, 0.1 mM dNTPs, 0.05 mM primers, and 0.5U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). Both DNA strands of the products of the secondary PCR were sequenced directly by an automated method using the BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems) and an automated sequencer (model 3100; Applied Biosystems). The sequence fragments obtained with different primers were assembled using DNASIS (Hitachi, Tokyo, Japan), and the sequences were aligned by eye. Eleven sequences of *Cytb* (1140 bp) for *M*.

Table 1. Specimens of East Asian moles examined in this study.

Species	Locality No	Collection Locality	Taxon Code	Specimen Code*	Group Cytb	Haplotype A2ab	Bmp4	Tcf25	vWf
Mogera tokudae	1	Sado I.	1_Mt1	HS3425	Mto-I	*a"*	*a"*	*a"*	*a"*
(Japan)	2	Mt. Kakuta	2_Mt2	HS667	Mto-II	*b"*	*a"*	*a"*	*a"*
Mogera imaizumii	3	Hachinohe	3_Mi1	HS499; AB037611	Mim-I	*b'*	*a'*	*a'*	–
(Japan)	4	Otsuchi	4_Mi2	HS659	Mim-IIa	*a'/e'*	*a'*	*a'*	*a'/d'*
			4_Mi3	HS1508; AB245948	Mim-I	*a'*	–	–	*a'*
			4_Mi4	AB245947	Mim-I	–	–	–	–
	5	Tono	5_Mi5	AB245952	Mim-IIa	–	–	–	–
	6	Ishinomaki	6_Mi6	AB245963	Mim-IIa	–	–	–	–
	7	Sendai	7_Mi7	HS582; AB037613	Mim-IIa	–	–	–	–
	8	Awashima I.	8_Mi8	HS1521	Mim-I	–	–	*a'*	–
	9	Niigata	9_Mi9	KT2795; AB270529	Mim-I	–	–	–	*a'/f'*
			9_Mi10	HS470	–	*a'*	*a'*	*a'*	*a'/e'*
	10	Noto	10_Mi11	HS3366	Mim-IIIa	*a'*	*a'*	*a'*	*a'*
	11	Karuizawa	11_Mi12	HS1778; AB245959	Mim-IIa	–	–	*a'*	–
	12	Yokohama	12_Mi13	HS365; AB037616	Mim-IIa	–	–	–	–
	13	Atsugi	13_Mi14	HS3896	Mim-IIa	*a'*	*a'/b'*	*b'*	*a'/c'*
			13_Mi15	HS3898	–	–	–	–	*a'*
	14	Aokigahara	14_Mi16	HS500	Mim-IIa	*a'/c'*	*a'*	*a'/d'*	–
			14_Mi17	HS3187	–	*a'/d'*	–	–	–
	15	Ito	15_Mi18	HS1022	Mim-IIb	*a'/d'*	*a'*	*a'*	–
			15_Mi19	HS1040	–	*a'/c'*	–	–	–
	16	Takashima, Shiga	16_Mi20	HS3367	Mim-IIIb	*a'/e'*	*a'*	*a'*	–
			16_Mi21	HS3368	Mim-IIIb	*a'*	–	–	–
	17	Iwakurahasemachi	17_Mi22	HS463; HS037619	Mim-IIIb	*a'*	*a'*	*a'/c'*	–
	18	Shirahama-cho	18_Mi23	HS315	Mim-IIIc	–	–	–	*a'/b'*
	19	Kumanogawa	19_Mi24	HS368	Mim-IIIc	*f'*	*a'*	*a'*	*a'*
Mogera wogura	20	Matto	20_Mw1	HS1416	Mwo-IIa	*o*	*e*	*d*	*f*
(Japan)			20_Mw2	HS3071	–	*o*	*e*	–	–
	21	Hakone	21_Mw3	HS3096	Mwo-Ia	–	–	*a*	–
	22	Fujinomiya	22_Mw4	HS3897	Mwo-Ia	*e*	*a*	*b*	*b*
	23	Mishima	23_Mw5	HS1023; AB037623	Mwo-Ia	*e*	*a*	*a*	*c*
	24	Kasugai	24_Mw6	HS581	Mwo-Ib	*g*	*a*	*a*	*b*
	25	Okazaki	25_Mw7	HS4429	Mwo-Ib	–	–	*a*	–
	26	Ashiu	26_Mw8	HS683	Mwo-IIa	–	–	–	–
			26_Mw9	HS810	Mwo-IIa	–	–	*c*	–
	27	Kawachinagano	27_Mw10	HS314	Mwo-Ib	–	–	–	–
			27_Mw11	HS316	Mwo-Ib	*a/c*	*a*	*a*	*b*
	28	Kanaya	28_Mw12	HS1073	Mwo-Ib	*a/b*	*a*	*a*	*a/c*
			28_Mw13	HS1074	–	*c/e*	–	–	*a/c*
	29	Tottori	29_Mw14	HA56105/HS4431	Mwo-IIa**	–	–	*c*	–
	30	Oki	30_Mw15	HA56101/HS4433	Mwo-IIb	–	–	*c*	–
	31	Yonago	31_Mw16	HA56115/HS4432	Mwo-IIa	–	–	*c*	–
	32	Tokushima	32_Mw17	MH5849/HS1034	Mwo-IIc	*h/m*	*c*	*c*	*c*
	33	Mt. Otaki	33_Mw18	KT2802/HS464	Mwo-IIc	*i/n*	*c/e*	*c*	*c*
	34	Hiwa	34_Mw19	HA55944/HS4430	Mwo-IIc	–	–	*c*	–
	35	Togouchi	35_Mw20	HA55940/HS4449	Mwo-III	–	–	*c*	–
			35_Mw21	HA55943/HS4450	Mwo-III	–	–	–	–
	36	Hohoku	36_Mw22	HA55939/HS4448	Mwo-III	–	–	*c*	–
	37	Tsushima Is.	37_Mw23	HS366	Mwo-III	–	–	–	–
			37_Mw24	HS409	–	*k*	–	–	–
			37_Mw25	HS2820	Mwo-III	*k/l*	*b*	*c*	*d*
			37_Mw26	HA55911/HS4434	Mwo-III	–	–	*c*	–
	38	Ukiha	38_Mw27	HA55915/HS4437	Mwo-III	–	–	*c/f*	–
			38_Mw28	HA55916/HS4438	Mwo-III	–	–	–	–
	39	Kurume	39_Mw29	HA55919/HS4439	Mwo-III	–	–	*f*	–
	40	Menda	40_Mw30	HS404	Mwo-III	*n*	*c/e*	*c*	*a/c*
			40_Mw31	HS414	Mwo-III	*j/n*	–	–	*c/d*
	41	Ashikita	41_Mw32	HA55924/HS4442	Mwo-III	–	–	*c*	–
	42	Amakusa I.	42_Mw33	HS4428	Mwo-III	–	–	*c*	–
	43	Yatsushiro	43_Mw34	HA55922/HS4441	Mwo-III	–	–	*c*	–
	44	Hitoyoshi	44_Mw35	HA55932/HS4446	Mwo-III	–	–	*c*	–
			44_Mw36	HS4447	Mwo-III	–	–	–	–
	45	Kiyotake	45_Mw37	HS3066	Mwo-III	–	–	*c/g*	–
	46	Makizono	46_Mw38	HS369	Mwo-III	*n*	*b/c*	*c/e*	–
	47	Uchinoura	47_Mw39	HS415	Mwo-III	–	–	–	*c*
			47_Mw40	HS416	Mwo-III	*i*	*d/e*	*c*	*c/e*
	48	Tanegashima I.	48_Mw41	KT2821/HS467	Mwo-III	*p*	*e*	*c*	*a*
			48_Mw42	HA55930/HS4445	Mwo-III	–	–	*c*	–
	49	Yakushima I.	49_Mw43	HS371	Mwo-III	*f*	*e*	*c*	*a*
			49_Mw44	HA55926/HS4443	Mwo-III	–	–	–	–
			49_Mw45	HA55928/HS4444	Mwo-III	–	–	–	–
M. wogura (Korea)	50	Pusan	50_Mw46	HS372	Mwo-VI	*d*	*f/g*	*h*	*g*
			50_Mw47	HS373	Mwo-VI	–	–	–	–
	51	Sukmodo I.	51_Mw48	HS1275	Mwo-VI	*n*	*f*	*h*	*g*
M. wogura (Russia)	52	Hasan	52_Mw49	AK707	Mwo-VI	–	–	–	–
	53	Kedrovaya Pad'	53_Mw50	AK002; AB037646	Mwo-VI	*n*	*h*	*h*	*g*
			53_Mw51	AK001/HS890	–	–	–	–	*g*
	54	Vladivostok	54_Mw52	AK411	Mwo-VI**	–	–	*h*	*g*
	55	Ussurijsky	55_Mw53	AK604/HS1261	–	*n*	*f*	*h*	*g*
			55_Mw54	AK820	Mwo-VI	–	–	–	*g*
			55_Mw55	AK821	Mwo-VI	–	–	–	–
	56	Sikhote-Alin	56_Mw56	AK727/HS1417	Mwo-VI	*n*	*f*	*h*	*g*
			56_Mw57	AK728/HS1418	Mwo-VI	–	–	–	–
	57	Krasnoarmeysky	57_Mw58	AK1225	–	–	–	*h*	*g*
			57_Mw59	AK884	Mwo-VI	–	–	–	–

*Cytb sequences (1140 bp) with accession number (DDBJ code) are those from Iwasa et al. (2006) and Tsuchiya et al. (2000).
Specimen codes of MH, KT, and AK are those of authors personal collections. See text and Okamoto (1999) for HA.
**Partial *Cytb* sequences were taken into accout for the phylogroup inference.

wogura and *M. imaizumii* were obtained from the database (Tsuchiya et al., 2000; Iwasa et al., 2006). The unique *Cytb* sequence reported from a Russian sample (Tsuchiya et al., 2000) was not reproducibly observed; it was not used in this study, as the sequence may be derived from pseudogenes incorporated in the nuclear genome. The sequences for *U. talpoides* (AB076833), *E. mizura* (AB037604), *Mogera insularis* (AB037606), *Talpa altaica* (AB037602), *Talpa caucasica* (FN640578), and *T. europaea* (AB037601) were obtained from nucleotide databases (Tsuchiya et al., 2000; Shinohara et al., 2003; Colangelo et al., 2010).

The divergence time of these mole species was estimated using the *Cytb* sequences and the software BEAST, ver. 1.6.2 (Drummond and Rambaut, 2007). The fossil evidence of Eurupean *Talpa* indicates a divergence at the Miocene–Pliocene transition (Loy et al., 2005; Colangelo et al., 2010). We set normally distributed calibration prior to the divergence between *T. europaea* and *T. caucasica* at 4.75 ± 0.5 mya and estimated the divergence times for the remaining nodes in the tree using the SRD06 model (Shapiro et al., 2006) and relaxed-clock model with an uncorrelated lognormal distribution. Analyses were run for 10 million generations, discarding the first 1 million generations as burn-in, and parameter values were sampled every 1000 generations. Parameter estimates and convergence were checked using Tracer version 1.4 (Rambaut and Drummond, 2007).

Since the confidence intervals for the inferred divergence time based on molecular data are rather broad, we referred to worldwide sea-level change to consider possible migration events of the ancestral lineages of the Japanese moles, assuming that historical short-term landbridge connections assisted the divergence events. According to Woodruff (2003; see also Haq et al., 1987), rapid, marked sea-level drops occurred approximately 5.6, 3.5, and 2.4 million years ago (mya) in the late Tertiary (Miocene and Pliocene) and 1.6, 1.3, and 0.9 mya in the early Quaternary (Haq et al., 1987; Woodruff, 2003).

Table 2. List of primers used in the study.

Gene and primer code	Sequence (reference)	Cycle condition (35 cycles)
*Cytb**		
L14724 (1st PCR)	5′-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3′ (Irwin et al., 1991)	**96°C (30 s), 50°C (30 s), and 60°C (30 s)
H15915 (1st PCR)	5′-AAC TGC AGT CAT CCT CCG GTT TAC AAG AC-3′ (Irwin et al., 1991)	
N-L14724 (2nd, upper half)	5′-CAG GAA ACA GCT ATG ACC GAT ATG AAA AAC CAT CGT TG-3′ (Suzuki et al., 1997)	96°C (30 s), 50°C (30 s), and 60°C (30 s)
SNH655A (2nd, upper half)	5′-TGT AAA ACG ACG GCC AGT TGT GTA GTA TG-3′	
SNL497A (2nd, lower half)	5′-CAG GAA ACA GCT ATG ACC CCT AGT AGA AT-3′	96°C (30 s), 50°C (30 s), and 60°C (30 s)
N-H15916 (2nd, lower half)	5′-TGT AAA ACG ACG GCC AGT GTC ATC TCC GGT TTA CAA GA-3′ (Suzuki et al., 1997)	
A2ab		
A2ab_F	5′-AGA ATC TGT TCC TGG TGT CGC TGG-3′	**95°C (30 s), 60°C (30 s), and 72°C (60 s)
A2ab_R	5′-ACG AAG GTA AAC CGC TTC TCG CG-3′	
Bmp4; exons 3, 4, and 5		
Bmp4_exon3_F	5′-TTA GGA GCC ATT CCG TAG TG-3′	**95°C (30 s), 55.5°C (30 s), and 72°C (60 s)
Bmp4_exon3_R	5′-GAA GAG GTG TCT ACT CAC TG-3′	
Bmp4_exon4_F	5′-TCC TGG TAA CCG AAT GCT GAT GG-3′	**95°C (30 s), 54°C (30 s), and 72°C (60 s)
Bmp4_exon4_R	5′-TGG AAG CTC CTC ACG GTG TTG G-3′	
Bmp4_exon5_F	5′-ATC TGG AGA ACA TCC CAG GGA CC-3′	** 95°C (30 s), 54°C (30 s), and 72°C (60 s)
Bmp4_exon5_R	5′-CCA CTC CCT TGA GGT AAC GAT C-3′	
Tcf25; intron 8		
Tcf25_F	5′-TCA GGA GGA TCA GGA GAT GG-3′	**95°C (30 s), 55°C (30 s), and 72°C (60 s)
Tcf25_R	5′-TCA GGT CTG CGG TAA TCC AG-3′	
vWf; exons 4 and 5		
vWf_F	5′-TTC CAA AAT GGC AAG AGA GTG AG-3′	**95°C (30 s), 51°C (30 s), and 72°C (60 s)
vWf_R	5′-CTG ACA GCA GGA CTT GAA AGT TG-3′	

*A semi-nested PCR method was taken. See Suzuki et al. (1997) for detail.
**AmpliTaq Gold polymerase (ABI) was used, with 10-min initial heating at 96°C prior to the cycle reaction.

Nuclear gene sequences: *A2ab*, *Bmp4*, *Tcf25*, and *vWf*

PCR amplifications were performed to determine three exon sequences (exon 3, 107 bp; exon 4, 315 bp; exon 5, 370 bp) in the *bone morphogenetic protein 4* (*Bmp4*) using the primer sets listed in Table 2, and combined sequences (792 bp) were used for phylogenetic inference. We determined the nucleotide sequences in the α-2b adrenergic receptor (*A2ab*, exon region, 852 bp), *von Willebrand factor* (*vWf*, 470 bp; exon 4, 75 bp; intron 4, 286 bp; exon 5, 109 bp), and *transcription factor 25* (*Tcf25*, intron region, 394 bp) using primer sets (Table 2). The PCR primer sequences were designed by seeking well-conserved regions among dogs, humans, and mice. All nuclear gene PCR reaction mixtures (20 µl) contained 2 mM Tris-HCl, 10 mM KCl, 2.5 mM MgCl$_2$, 0.1 mM dNTPs, 0.05 mM primers, and 0.5U AmpliTaq Gold (Applied Biosystems). The PCR conditions are listed in Table 2. Both DNA strands of the PCR products were sequenced directly using the method described above. No indels were seen in our sequences. When we found more than a single nucleotide polymorphism (SNP) for a given gene region, we conducted haplotype separation with a parsimony method (Karn et al., 2002) and used them to construct single gene trees. We dealt the SNPs as ambiguous sites when we constructed trees and networks with concatenated sequences. The nucleotide sequences reported here were stored in the DDBJ,

EMBL, and GenBank nucleotide sequence databases under accession numbers AB638494–AB638602.

Data analyses and tree building

We constructed maximum likelihood (ML), maximum parsimony (MP), and neighbor-joining (NJ) trees using the program MEGA5 (Tamura et al., 2011). In the ML analyses, substitution models that better describe the substitution patterns of the *Cytb* (HKY+G+I; Hasegawa et al., 1985), *A2ab* (T92+G; Tamura, 1992), *Bmp4* (T92), *Tcf25* (T92), and *vWf* (K2; Kimura, 1980) data were determined by MEGA5. In the ML analysis, the tree topology search was evaluated using simultaneous nearest-neighbor interchange (NNI). In the NJ analysis, the Kimura two-parameter model (K2) was used. The reliability of nodes was assessed using 1000 bootstrap replicates. Genetic distance between a given pair of taxon groups was calculated using MEGA5 with the K2 model. We combined the nuclear gene sequences in representative individuals in which the four nuclear gene sequence datasets were complete for each geographic group of the three species in *Mogera* examined and constructed phylogenetic trees with the ML, MP, and NJ methods. In the ML analysis, the Tamura three-parameter model (T92+G) selected by MEGA5 was used. Phylogenetic analysis was done using the software BEAST, version 1.6.1 (Drummond and

Rambaut, 2007), to perform esti-
mation of divergence times from
Cytb, as discussed below. To
address intraspecies groupings
in the three species of *Mogera*
(*M. tokudae*, *M. imaizumii*, and
M. wogura), a network tree was
constructed for the concatenated
nuclear sequences by the neigh-
bor-net method, as implemented
in SplitsTree4 (Huson and Bryant,
2006). Alignment files are avail-
able from the corresponding
author upon request.

RESULTS

Mitochondrial gene sequences

We constructed phyloge-
netic trees with *Cytb*
sequences (1140 bp) for
moles from Japan, Korea, and
Russia (Primorye) using the
newly determined sequences
and those obtained from the
databases for four species of
Mogera (*M. wogura*, *M.
imaizumii*, *M. tokudae*, and
M. insularis) and other mole
species (*E. mizura*, *T.
europaea*, *T. altaica*, and *U.
talpoides*) (Fig. 2). The ML,
MP, and NJ trees were topo-
logically identical to each
other.

In *M. tokudae*, the two
individuals from Sado Island
and the Echigo Plain showed
a relatively low level of
genetic divergence (*d* = 2%;
Table 3).

In our dataset, *M.
imaizumii* was shown to have
three major phylogroups with
apparent geographic subdivi-
sions and relatively high
genetic distances (*d*) of 3.6–
4.9% (Fig. 2; Table 3): those
occurring in coastal areas of
the Sea of Japan (*Mim*-I) and
the Pacific Ocean (*Mim*-II),
and central Honshu areas
covering the Hokuriku and
Kinki Districts (*Mim*-III). In
phylogroup *Mim*-II, an indi-
vidual from the Izu Peninsula
(15_Mi18, Locality 15 in Fig.
1; subclade *b* in Fig. 2), the
southern tip of the range,
was found to have haplo-
types distinct from others in
the area, with a *d* of 2%. In
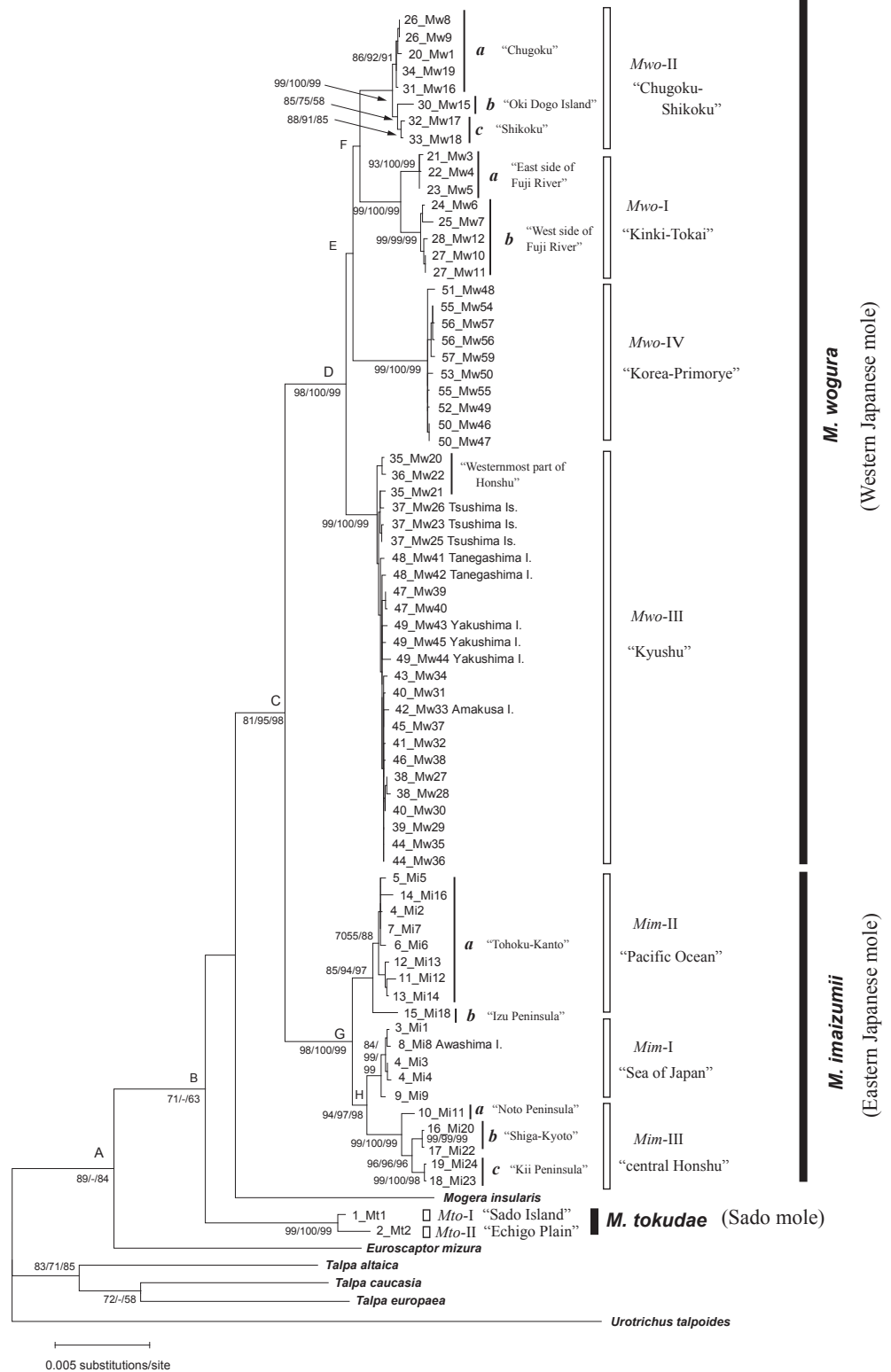
Fig. 2. Maximum-likelihood tree of 77 individual of moles based on variation in mitochondrial *cytochrome b* sequences (1140 bp). The HKY+G+I substitution model (Hasegawa et al., 1985) was applied considering all codon positions and all substitutions using MEGA5 (Tamura et al., 2011). Bootstrap scores with 1000 replicates (> 50%) are shown at the nodes of clades representing taxon and geographic groups. For codes of individuals specimens and selected key nodes (A–H), refer to Tables 1 and 3, respectively. In addition to the major phylogroups in *Mogera imaizumii*, *M. tokudae*, and *M. wogura*, further subdivisions with apparent geographic affinity are indicated.

Table 3. Estimated divergence times (D. T.) from the molecular and geological views.

Node*	Mole lineages focused	Cytochrome b (Cytb)**			Sea level drop***	
		D. T. (CI)	d	Evol. rate	D. T.	Evol. rate
A	Asian moles (*Euroscaptor mizura* and *Mogera*)	5.21 (4.23–5.81)	0.150	0.014	5.6	0.013
B	Japanese moles (three *Mogera* species)	3.64 (3.09–4.23)	0.115	0.016	3.5	0.016
C	*Mogera imaizumii* and *M. wogura*	2.31 (1.93–2.70)	0.088	0.019	2.4	0.018
D	*M. wogura*: clades I-IV	1.24 (1.01–1.46)	0.054	0.022	1.3 (1.6)	0.022 (0.018)
E	*M. wogura*: clades I+II and IV	1.23 (1.00–1.46)	0.059	0.024	–	–
F	*M. wogura*: clades I and II	1.00 (0.78–1.19)	0.047	0.024	–	–
G	*M. imaizumii*: clades I-III	0.94 (0.75–1.16)	0.049	0.026	–	–
H	*M. imaizumii*: clades I and III	0.74 (0.55–0.92)	0.036	0.024	–	–

* See Figure 2 for node codes.

** Mean divergence times (million years ago; mya) were obtained from the BEAST analysis. CI values in the parentheses are 95% highest posterior density intervall.

Divergence times were calculated with the *Cytb* sequences on the assumption of 4.75 mya for the divergence of the *Talpa caucasia* and *T. europaea* (Colangelo et al., 2005).

Average genetic distances (*d*) were calculated with the Kimura (1980) two parameter method, using MEGA5.

Evolutionary rate represents genetic distance (*d*) per estimated divergence time (D. T.) per lineage in *Cytb*.

*** The universal sea level drops (Woodruff, 2003) are assumed to have assisted the disperal events across the Korea Strait.

concordant overall with those obtained in the *Cytb* tree (Fig. 2). *Mogera wogura* was shown to have substantial geographic variation, whereas *M. imaizumii* and *M. tokudae* were less differentiated within species (Fig. 3; Table 1).

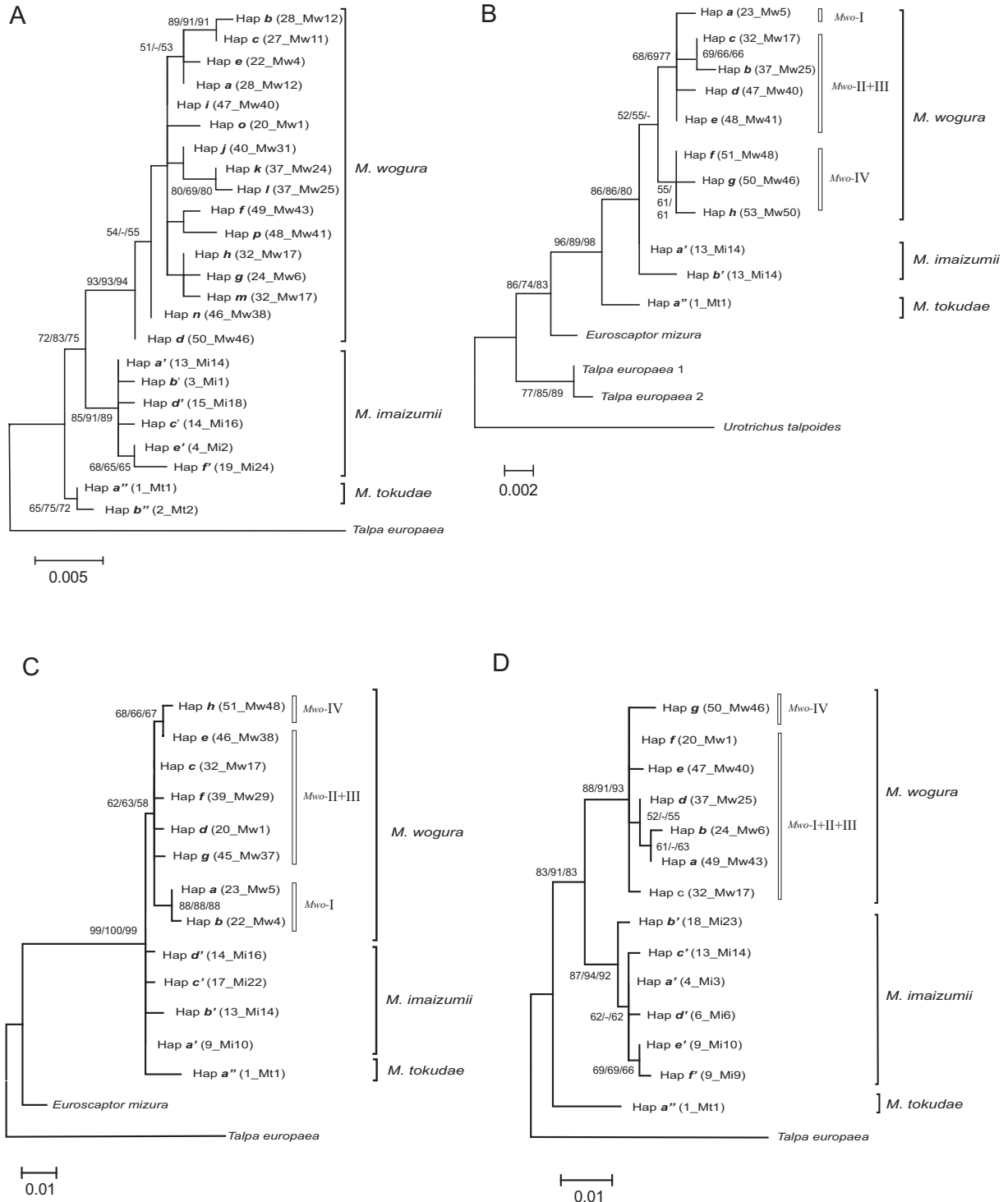In *M. wogura*, a trend to geographic subdivision was detected in the *A2ab* trees, revealing a haplotype group representing the members belonging to the Kinki-Tokai group *Mwo*-I. All the members belonging to the Korea-Primorye group *Mwo*-IV possessed a single haplotype *n*. In the *Bmp4* tree, clear geographic affinity was in good accord with the mtDNA phylogroups. Clades representing the two phylogroups of Kinki-Tokai (*Mwo*-I) and Korea-Primorye (*Mwo*-IV) were detected, although the remaining haplotypes were recognized as a single cluster, which we here designate Chugoku-Shikoku-Kyushu (*Mwo*-II+III). The *Tcf25* tree again supported the geographic subdivision of *M. wogura* into three groups: Kinki-Tokai (*Mwo*-I), Chugoku-Shikoku-Kyushu (*Mwo*-II+III), and Korea-Primorye (*Mwo*-IV). In the *vWf* tree, two major groups could be detected, Japan (*Mwo*-I+II+III) and Korea-Primorye (*Mwo*-IV), and no further spatial subdivision was seen.

No apparent geographic subdivision was recovered in *M. imaizumii*, despite the inclusion of individuals from the distantly separated population of the Kii Peninsula. The haplotype sequences of *M. tokudae* from Sado and Echigo were the same in *Bmp4*, *Tcf25*, and *vWf*, whereas slight variation in *A2ab*, differing by one substitution, was observed between Sado and Echigo.

To further define the genetic relationships among the mole species with finer resolution, we combined the four nuclear gene sequences (*A2ab*, *Bmp4*, *Tcf25*, and *vWf*; 2511 bp) in 26 representative individuals and constructed trees using the ML, MP, and NJ methods. In contrast to the apparent low levels of bootstrap scores in the single gene trees (Fig. 3), the concatenated gene trees had relatively higher support (Fig. 4A). In particular, the initial split of *M. tokudae* was strongly supported (BS = 100%). In the concatenated nuclear gene sequences, *M. wogura* was shown to have three distinct spatial lineages, namely Kinki-Tokai, Chugoku-Shikoku-Kyushu, and Korea-Primorye, whereas *M. imaizumii* and *M. tokudae* again showed no apparent geographic subdivision.

Phylogenetic trees based on concatenated sequences for the four genes (Fig. 4B) feature clear clusters for each of the three species of *Mogera*: *M. tokudae*, *M. imaizumii*, and *M. wogura*. In addition, the network yielded three divergent clusters in *M. wogura*, supporting the distinctiveness of the

phylogroup *Mim*-III, further geographic subdivision was observed among the populations of the Noto Peninsula (10_Mi11, subclade *a*), Shiga/Kyoto (16_Mi20, 16_Mi21, 17_Mi22; subclade *b*), and the Kii Peninsula (18_Mi23, 19_Mi24; subclade *c*), with *d* values of 1.3–1.9%.

From the trees constructed with the *Cytb* sequences (Fig. 2), the specimens of *M. wogura* collected from 30 localities (Fig. 1; Table 1) can be integrated into four mutually separated groups; namely, those distributed in the Kinki and Tokai Districts (Kinki-Tokai group; *Mwo*-I), the Chugoku and Shikoku Districts (Chugoku-Shikoku group; *Mwo*-II), Kyushu and the westernmost tip of Honshu (Kyushu group; *Mwo*-III), and Korea/Primorye (Korea-Primorye group; *Mwo*-IV). The genetic distances among the groups were relatively high, and the groups were equally distant from one another, with *d* values of 4.3% (*Mwo*-II vs. III) to 6.2% (*Mwo*-I vs. IV), showing no clear resolution of the phylogenetic relationships among the four phylogroups (nodes D and E in Fig. 2) in the ML, MP, and NJ analyses. By contrast, the genetic distances within the groups were quite low, ranging from 0.4% (*Mwo*-IV) to 1.4% (*Mwo*-I). Notably, *M. wogura* from the two distant collection localities in Korea ("*M. coreana*") and Russia ("*M. robusta*") were found to have few divergent sequences, with a *d* of 0.4%. A further spatial pattern was detected in *Mwo*-I; haplotypes from the easternmost part of the Fuji River (subclade I-*a*) differed from those from the rest of the range of the phylogroup (subclade I-*b*), showing a relatively high genetic distance of 1.4% on average (Fig. 2). In the Chugoku-Shikoku clade, *Mwo*-II, three geographic subgroups were apparent, with moderate support (85–88%): Chugoku (subclade II-*a*), Oki Dogo Islands (subclade II-*b*), and Shikoku (subclade II-*c*).

Nuclear gene sequences

We determined exon sequences of the *A2ab* (852 bp), *Bmp4* (792 bp), and *vWf* (470 bp), as well as an intron region in *Tcf25* (394 bp), with special interest in the intraspecies genetic variation of representative individuals from three species: *M. wogura* (*n* = 20–37), *M. imaizumii* (*n* = 9–14), and *M. tokudae* (*n* = 2). The interspecies relationships that appeared in the ML, MP, and NJ trees (Fig. 3) were
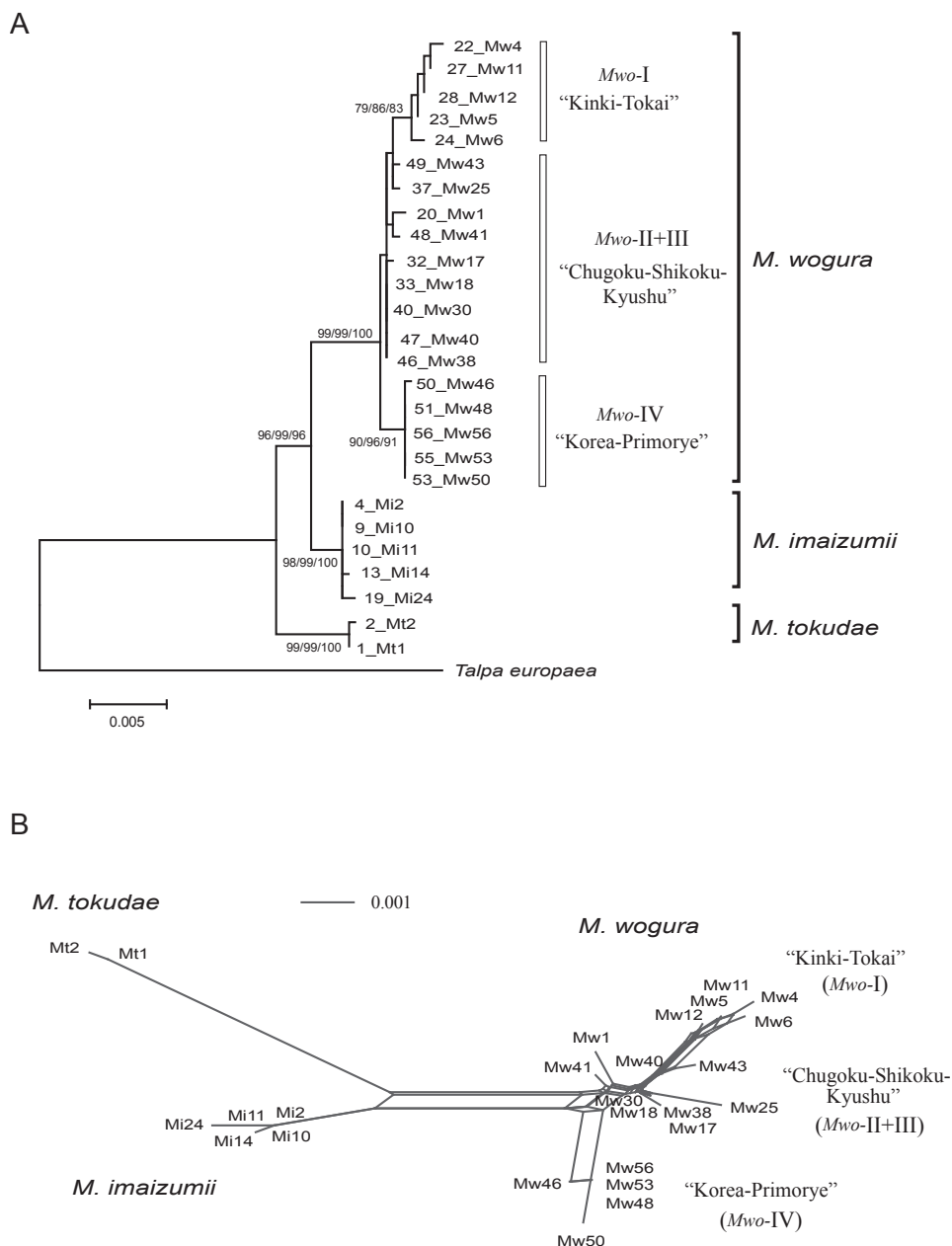
Fig. 3. Maximum-likelihood trees based on sequences of four nuclear genes using MEGA5 (Tamura et al., 2011), considering all codon positions and substitutions. Substitution models selected by MEGA5 were used in the datasets for *A2ab* (A, 852 bp; T92+G), *Bmp4* (B, 792 bp; T92), *Tcf25*(C, 394 bp; T92), and *vWf* (D, 470 bp; K2). Bootstrap scores (ML/MP/NJ) with 1000 replicates (> 50%) associated with each node are given. For individual codes, refer to Table 1.

two mtDNA phylogroups of Kinki-Tokai (*Mwo*-I) and Korea-Primorye (*Mwo*-IV) and again exemplifying the close relationships of haplotypes of the two western Japanese phylogroups of Chugoku-Shikoku (*Mwo*-II) and Kyushu (*Mwo*-III).

Divergence times

Divergence times were calculated using the *Cytb* sequences on the assumption of 4.75 mya for the divergence of *T. caucasica* and *T. europaea* (Colangelo et al., 2010) using BEAST (Table 3). The common ancestor of *M.*

A

B

Fig. 4. **(A)** Maximum-likelihood phylogenetic tree based on concatenated sequences of four nuclear genes (2511 bp; *A2ab*, *Bmp4*, *Tcf25*, and *vWf*) in *Mogera imaizumii*, *M. wogura*, and *M. tokudae* with the outgroup taxon of *Talpa europaea* using Tamura's (1992) three-parameter method. Bootstrap values (ML/MP/NJ), expressed as a percentage of 1000 replications (> 50%), are given at each node. For codes of individual specimens, refer to Table 1. **(B)** A network tree was constructed with the concatenated dataset using the neighbor-net method. Assessment of the three geographic groups of *M. wogura* is indicated.

tokudae could be traced back to 3.64 mya (95% CI, 3.09–4.23 mya). The species divergence of *M. imaizumii* from *M. wogura* was calculated to be 2.31 mya (CI, 1.93–2.70 mya). In *M. imaizumii*, the estimated divergence times for I+III vs. II and I vs. III were 0.94 and 0.74 mya, respectively. In *M. wogura*, the estimated divergence times between the phylogroups (I–IV) ranged from 1.24 to 1.00 mya.

DISCUSSION

In the present study, we performed phylogenetic inference using both mitochondrial and nuclear gene sequences, focusing mainly on the three Japanese mole species: *M. wogura*, *M. imaizumii*, and *M. tokudae*. The resultant datasets, together with those in the literature (Okamoto, 1999; Tsuchiya et al., 2000; Shinohara et al., 2003, 2004, 2005; Iwasa et al., 2006), generated reliable phylogenetic relationships among the mole species (Figs. 2 and 3) and clarified the spatial genetic structures of the two dominant species, *M. imaizumii* and *M. wogura*, in both mitochondrial DNA (Fig. 5A) and nuclear genes (Fig. 5B). Based on these molecular inferences and paleontological evidence (e.g., Haq et al., 1987; Kawamura, 1998, 2007; Woodruff, 2003; Kitamura and Kimoto, 2006; Yoshikawa et al., 2007), we are now ready to reconstruct the evolutionary history of Japanese moles, which show remarkably high levels of species and genetic diversity. This in turn may offer new insights into causative factors that have promoted both colonization events across the Korea Strait and genetic structuring in terrestrial animals inhabiting the major zoogeographic zone of Honshu, Shikoku, and Kyushu.

Evolutionary episodes in the speciation of the Japanese moles

Our data for mitochondrial *Cytb* (1140 bp) and the combined nuclear gene sequences (2511 bp) of *A2ab*, *Bmp4*, *Tcf25*, and *vWf* provide robust evidence for species relationships; *M. wogura* is distant from the other Japanese moles, *M. imaizumii*, *M. tokudae*, and *E. mizura*, in that order. A divergence time for *T. caucasica* and *T. europaea* of 4.75 mya was considered based on the fossil evidence (Colangelo et al., 2010). Based on molecular phylogenetic inference using *Talpa Cytb* sequences, Colangelo et al. (2010) provided a mean clock rate of 1.4% per million years per lineage, which is in good accord with our current estimation based on a comparison of relatively deeper lineages,
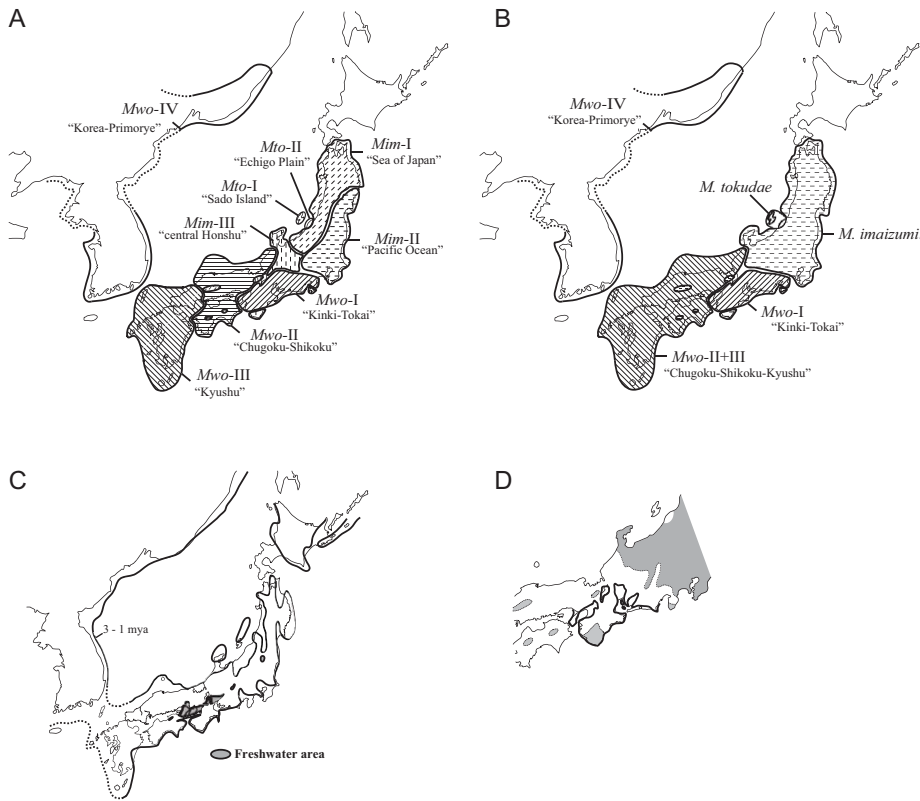
Fig. 5. Distributions of genetically characterized subpopulations of *Mogera imaizumii* and *M. wogura* based on the sequence variation of the mitochondrial DNA **(A)** and nuclear genes **(B)**. **(C)** Estimated coastline of historical landbridges across the Korea Strait 1–3 million years ago (mya) (Iijima and Tada, 1990; Yoshida, 1992). In that period, an inland sea is thought to have separated the Kii Peninsula from the rest of Honshu and Shikoku (Okada, 1980; Yoshida, 1992). **(D)** The bold lines in central Honshu are the predicted coastlines around 1 mya (Japan Association for Quaternary Research, 1987). The uplift of mountains that divide the Second Setouchi Basin is thought to have begun during the last 1 million years (Yoshida, 1992); these mountains connected the Kii Peninsula with central Honshu via mountain lines and may have contributed to the isolation of the Kinki-Tokai group from the rest of the range of *M. wogura*.

namely between *E. mizura* and *Mogera* species (Table 3).

The inference obtained here implies that multiple speciation events in Eurasian moles were associated with the global environmental changes of 5–7 mya (Cerling et al., 1997, 1998), which is thought to have promoted the evolutionary development of temperate mammals in Eurasia, as is the wood mice (*Apodemus*: Suzuki et al., 2008) and leporids (*Lepus*: Matthee et al., 2004; Wu et al., 2005). To provide a temporal perspective on mole evolution, here we consider divergence times using the *Cytb* data (1140 bp), which provides a more complete set of mole taxa for discussion. From the analysis with the *Cytb* dataset, the divergence times between the continental lineage of *M. wogura* and Japanese lineages of *E. mizura*, *M. tokudae*, *M. imaizumii*, and *M. wogura* were estimated to be 5.2, 3.6, 2.3, and 1.2 mya, respectively. However, the confidence intervals are quite large, making the divergence times rather broad, e.g., 3.09–4.23 mya for *M. tokudae* (Fig. 6A; Table 3). We next referred to geological data on sea-level changes to find patterns that concurred with the molecular data, assuming that the dispersal/vicariant events of the moles were essentially affected by the appearance and disappearance of landbridges across the Korea Strait, where historical

landbridges arose periodically during the past 7 million years (Iijima and Tada, 1990; Yoshida, 1992).

The Japanese mole fauna is thought to have been structured by frequent dispersal events from the continent to Japan (Tsuchiya et al., 2000; Shinohara et al., 2004). If so, it would be worthwhile to consider the chronology of fluctuating sea levels from a geological perspective (Haq et al., 1987; Woodruff, 2003; Kitamura and Kimoto, 2006); rapid, marked sea-level drops happened ca. 5.6, 3.5, and 2.4 mya in the late Tertiary (Miocene and Pliocene) and 1.6, 1.3, and 0.9 mya in the early Quaternary (Fig. 6B). The rapid drops in sea levels would have created land routes that connected Korea and Japan, enabling the dispersal of ancestral lineages of Japanese moles (Table 3). Given the range of the estimated divergence times from the *Cytb* data mentioned above, it is reasonable to assume that ancestors of *E. mizura*, *M. tokudae*, *M. imaizumii*, and *M. wogura* came to Japan and diverged from their continental counterparts 5.6, 3.5, 2.4, and 1.3 (or 1.6) mya, respectively. In this study we included a single taxon of Taiwanese moles *M. insularis* (Fig. 2). The resultant phylogenetic trees may imply that the dispersal event of *M. insularis* is possibly coincident with *M. tokudae*. This issue should be addressed in future study using sufficient number of phylogenetically close taxa from the Asian continent.

Supporting this view, landbridge formation has been inferred from the proboscidean biostratigraphy, and it seems worthwhile to consider this view. Three land bridge stages are suggested from robust fossil evidence for three proboscidean species: Marine Isotope Stage 36 (MIS36; 1.2 mya), MIS16 (0.65 mya), and MIS12 (0.43 mya) (Fig. 6C; Yoshikawa et al., 2007). In addition, *Stegodon miensis* and *S. aurorae*, both considered to be endemic to Japan, first appeared around 4 and 2.6 mya, respectively (Saegusa et al., 2004; Yoshikawa et al., 2007), and the speciation processes could be explained by landbridge formation at that geological time. However, an alternative possibility is that *S. aurorae* is an evolved form of *S. miensis*, in which case the speciation event would have occurred in situ due to reduction of terrestrial space in the Japanese Islands during marine transgression (Aiba et al., 2010). This alternative view of the fossil data of *S. aurorae* encourages us to consider alternative hypotheses regarding the evolutionary scenario for the differentiation of the species lineages of the Japanese *Mogera* in addition to the well-known idea of sequential colonization and isolation via the Korea Strait.
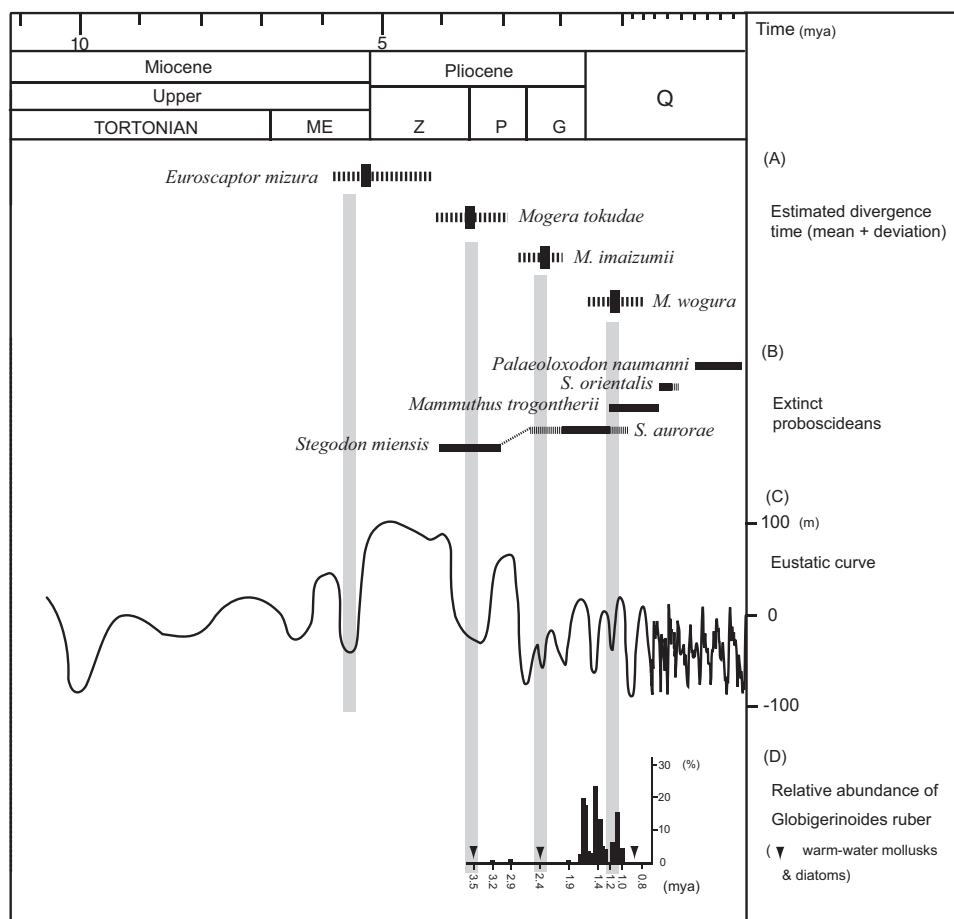
Fig. 6. Comparison of four pieces of geological information related to dispersal and vicariant events across the Korea Strait in Japanese moles. **(A)** Estimated divergence time of the four Japanese moles; means (solid line) and confidence intervals (dotted line). **(B)** Fossil record of Japanese extinct proboscideans (Dobson and Kawamura, 1998; Kawamura, 2007). **(C)** Eustatic changes in the global sea level during the last 10 million years (Woodruff, 2003). Gray vertical columns indicate the marked sea drops 5.6, 3.5, 2.4, and 1.3 million years ago. The timescale is from Berggren et al. (1995). **(D)** Stratigraphic distribution of maximum relative abundance of the foraminiferan *Globigerinoides ruber*, an indicator of the inflow of the Tsushima Warm Current into the Sea of Japan, at each interglacial stage (Kitamura and Kimoto, 2006). Arrowheads indicate periods of short-term, non-intensive inflow of the warm current predicted by warm-water mollusks and diatoms (Kitamura and Kimoto, 2006).

mediate speciation events, we need to seek other causative factors to explain the lineage divergence of *M. tokudae* around 3.5 mya and that of *M. imaizumii* around 2.6 mya. The Japanese Islands are an area in which lineage differentiation events occurred rather intensively via vicariant events. Ancient-onset lineage differentiation, such as in the late Pliocene and early Pleistocene, is known in a variety of terrestrial animals, such as the Japanese dormouse (Yasuda et al., 2007, 2012) and Japanese shrew mole (Shinohara et al., unpublished data). It is possible to presume that physical landscape barriers such as mountains and rivers have promoted speciation events. Alternatively, marine transgressive events associated with glacial terminations (Haq et al., 1987; Woodruff, 2003) may have mediated the vicariance of the mole lineages.

Overall, the lineage differentiation of the Japanese moles would have been associated with either dispersal or vicariant events mediated by specific geological changes, which may have been triggered by a drop in sea level (appearance of a landbridge) or in elevation (destruction of a landbridge). In either case, the appearance and disappearance of the landbridges across

In contrast to the above-mentioned consideration related to migration events from the continent to Japan, the ancient condition of the Korea Strait predicted from recent geological studies may draw another picture of mole evolution, especially for *M. tokudae*, *M. imaizumii*, and *M. wogura*. Based on fossil evidence of mollusks and planktonic foraminiferans from the Sea of Japan, it is inferred that the present-day Korea Strait was substantially shallower in the Tertiary era, 1.7–3.5 mya (Fig. 6D; Kitamura et al., 2001; Kitamura and Kimoto, 2006); indeed, even the Tsushima Current (see Fig. 1) periodically entered the southern part of the Sea of Japan from the East China Sea via the strait (3.2, 2.9, 2.4, and 1.9 mya; Kitamura and Kimoto, 2006). According to Kitamura and Kimoto (2006), the present-day southern channel (= the Korea Strait) was formed by MIS59 (1.71 mya). This suggests that the Japanese Islands were not an insular landmass, but an extension of the continental landmass, like a peninsula, during the late Pliocene, 1.7–3.5 mya (Fig. 5C). Therefore, if the Korea Strait did not

the Korea Strait may have played an important role in promoting the speciation process of the Japanese mole species. Note that the zoogeographic zone of Honshu/Shikoku/Kyushu, perhaps due to habitat richness, now harbors six talpid lineages, namely two shrew moles (*U. talpoides* and *Dymecodon pilirostris*) and a mountain-dwelling mole, *E. mizura*, in addition to the three *Mogera* species (Shinohara et al., 2004). This supports the previous notion that from a radiation center somewhere on the continent, a parental stock of *Mogera* dispersed several times intermittently during the course of evolution, reaching peripheral geographic domains, including Japan and Taiwan (Tsuchiya et al., 2000; Shinohara et al., 2003, 2004, 2005). Further investigation is needed for a full understanding of this evolutionary history.

The values for divergence time in turn allow us to assess the evolutionary rate of *Cytb* in moles: a rate of approximately 2–3% per million years per lineage for relatively shallower lineage divergences (nodes E–H; Table 3).

This provides a convenient measurement for assessing time with mitochondrial DNA data. For example, the calculated divergence time for the sequences of *M. tokudae* from Sado Island and the Echigo Plain (*d* = 2%) is approximately 0.5–0.3 mya. This would give further insight into the evolutionary history of the fauna of Sado Island, which harbors other mammals such as shrews (Ohdachi et al., 2001), wild boars (Watanobe et al., 2004), wood mice (Tomozawa and Suzuki, 2008; Tomozawa et al., 2010), hares (Nunome et al., 2010), and insects (Nakamine and Takeda, 2008), suggesting that different and intermittent colonization events from Honshu to Sado Island occurred during the past half million years.

A phylogeographic view on intraspecies genetic differentiation in *Mogera wogura*

From our mitochondrial data, the three species, *M. wogura*, *M. imaizumii*, and *M. tokudae*, exhibit geographic structures, with four (*Mwo*-I–IV), three (*Mim*-I–III), and two (*Mto*-I, II) phylogroups, respectively (Fig. 5). By contrast, our nuclear gene data provide new insights into the geographic grouping in *M. wogura*, supporting the view of mitochondrial gene data, but uniting groups *Mwo*-II and III, whereas no substantial geographic subdivision was seen in *M. imaizumii* and *M. tokudae* (Fig. 5). Note that two genetically distinct groups exist in the western part of Japan, *Mwo*-I (Kinki-Tokai) and *Mwo*-II+III (Shikoku, Chugoku, and Kyushu), which are relevant for future taxonomic revision of the Japanese *M. wogura*.

In *M. wogura*, the mitochondrial divergence pattern of the four major mitochondrial lineages is polytomous (Fig. 2), suggesting rapid lineage differentiation within a short period of evolutionary time. It has been noted that the Japanese Islands are effective in causing lineage differentiation events compared with the neighboring areas of the continent. The Korean Peninsula and Maritime region of Russia (Primorye) show low levels of population genetic divergence of less than 0.6% in the *Cytb* data, despite their large geographic area, although some apparent genetic subdivisions between Korean and Russian populations are seen in rodents (e.g., wood mice, Serizawa et al., 2002; harvest mice, Yasuda et al., 2005; chipmunks *Tamias sibiricus*, Lee et al., 2008). Efficient vicariant events are visible in a variety of small mammals occurring in the landmass of Honshu, Shikoku, and Kyushu, such as shrews (Ohdachi et al., 2001), shrew moles (Shinohara et al., unpublished), water shrews (Iwasa and Abe, 2006), voles (Suzuki et al., 1999; Iwasa et al., 2002), and dormice (Yasuda et al., 2007, 2012).

The mitochondrial data indicate that the basic structuring of the mitochondrial phylogroups of *M. wogura* appears to be associated with the fundamental features of the geographic domain, namely, the three islands of Honshu (*Mwo*-I), Shikoku (*Mwo*-II), and Kyushu (*Mwo*-III). The exact borders of these phylogroups, however, lie somewhere in western Honshu. The border that demarcated mitochondrial phylogroups *Mwo*-II and III likely lies near a pair of rivers, the Takatsu and Ota, in Hiroshima Prefecture, Chugoku District (Fig. 1). This geographic area is known to function as a border for a variety of animals, including harvestmen (Tsurusaki, 2006), spiders (Ihara, 2007), and salamanders (Matsui et al., 2006). The borders for the ranges of *Mwo*-I and II in Honshu are ambiguous; no apparent physical borders exist near the area.

One of the remaining questions here is the incongruence between mtDNA and the nuclear genes in the intraspecies phylogenetic relationships. *Mogera wogura* from Shikoku and Kyushu had distinct mitochondrial haplotypes, but shared similar genetic components in the nuclear gene markers (Fig. 4A). Such discrepancies have been seen in a variety of organisms, such as red-backed voles (Suzuki et al., 1999; Iwasa et al., 2002) and dormice (Suzuki et al., 1997; Yasuda et al., 2007, 2012), and is explained either by different resolution among the gene regions (lineage sorting) or by sex-biased migration events. In our study, *M. imaizumii* had the least differentiated nuclear gene sequences among the three phylogroups, suggesting the presence of efficient genetic exchange across the borders of the phylogroups during the course of evolution. The admixture of nuclear genes between the *Mwo*-II and III of *M. wogura* could be explained in a similar way; the phylogroups may have been separated during ice ages and expanded their ranges in warm periods, followed by the exchange of nuclear genetic components in contact zones. In the last glacial age, about 20,000 years ago, most of the Seto Inland Sea, which now separates the ranges of *Mwo*-II and III, became land due to the sea-level reduction (Japan Association for Quaternary Research, 1987).

Possible factors underlying the distinctiveness of the Kinki-Tokai group

In contrast to the inter-phylogroup genetic exchanges in *Mwo*-II and III of *M. wogura* and *Mim*-I, II, and III of *M. imaizumii*, it is rather puzzling to see the discrete differentiation between the groups *Mwo*-I and *Mwo*-II (Fig. 5A, B). The border between the two groups lies somewhere in Kinki and Chugoku Districts, where there is no apparent geographic barrier, as previously mentioned. Abe (1996) noted the distinctiveness of the Kinki-Tokai group of *M. wogura*, reporting that the skull and body of this group were large compared with those from southern Honshu, Shikoku, and Kyushu. Why did the Kinki-Tokai group (*Mwo*-I) retain its independence from the Kyushu-Shikoku group (*Mwo*-II+III)? There are two possible reasons: a specific geological history associated with the Kii Peninsula and the presence of periodic expansion of the competing species, *M. imaizumii*.

First, the western part of Honshu and Shikoku regions experienced dramatic changes in their configuration during the geological history of the Japanese Islands. About 5 mya, geological movements started to form an inland sea (the Second Setouchi Basin: Yoshida, 1992; Yonekura et al., 2001). By about 1–3 mya, the inland sea separated the Kii Peninsula from the rest of Honshu (Fig. 5C; Okada, 1980; Iijima and Tada, 1990; Yoshida, 1992), thereby divided the geographic areas represented by *Mwo*-I and *Mwo*-II groups. Then, 1 mya, an uplift of mountains (Mt. Suzuka) connected the Kii Peninsula and Honshu block (Fig. 5D; Yoshida, 1992). Yoshikawa et al. (2008) adopted the theory of Iijima and Tada (1990) to explain the distinctiveness of a mitochondrial clade of the Kii Peninsula in their phylogeographic study of Japanese clawed salamanders. Consequently, the discrete differentiation of group *Mwo*-I from group *Mwo*-II may be attributable to the geological episodes in the surrounding area of the Kii Peninsula.

Second, it is reasonable to consider the impact of the competing species, *M. imaizumii*, on the genetic structuring of *M. wogura*. In fact, the borders between them are continually changing, even at present; *M. wogura* is thought to be moving northward in the central part of Honshu, pushing back *M. imaizumii* and leaving several small relic populations of *M. imaizumii* within the range of *M. wogura* (Abe, 2001, 2005). This suggests that the boundary changed from time to time during the Quaternary ice ages, as has been demonstrated in a variety of temperate species (Hewitt, 2000, 2011). The historical genetic relationship between the Kii Peninsula and nearby central Honshu is evident in our present study (see Fig. 2 for clade III of *M. imaizumii*) and a variety of other animals, including voles (Iwasa et al., 2002) and dormice (Yasuda et al., 2007, 2012). In the vole *Eothenomys andersoni*, isolation and reunion are predicted by the mitochondrial and Y-specific gene data, in which the Kii Peninsula population has two or three distinct haplotypes, one of which is specific to Kii, and the other of which is associated with Central Honshu (Iwasa et al., 2002). According to Iwasa et al. (2002), when the climate became warmer during interglacial periods, the southern species *E. smithii* expanded its distribution to the northern districts of Honshu, whereas *E. andersoni* retreated to northern areas and alpine regions. Such repeated distribution changes in two species with niche competition during the Quaternary has been documented in a variety of animals occurring in Europe (Hewitt, 2000, 2011). *Mogera imaizumii* and *M. wogura* may have experienced such complicated range alterations in responding to the fluctuations in the Quaternary climate. If *M. imaizumii* extended its distribution southward during the cooling periods, this may have prevented genetic exchanges between groups *Mwo*-I and *Mwo*-II in *M. wogura*.

In conclusion, as discussed above, our data are in good agreement with the general notion that both multiple migration events from the continent to Japan and efficient vicariant events along with important topographic features led to the evolution of moles in the Japanese Islands (Shinohara et al., 2004, 2005), establishing the Japanese Islands as a hot spot of mole diversity, with at least six extant talpid species. Our study using mitochondrial and nuclear markers revealed apparent distinctions within the western Japanese mole, dividing it into three geographic groups, of which the continental group is sometimes regarded as a valid species (Abe, 2005), namely, *Mogera robusta* (e.g., Yokohata, 2005) or *Mogera coreana* (Ohdachi et al., 2009). By contrast, the division between the Shikoku-Kyushu and Kinki-Tokai groups implies the existence of cryptic species and demonstrates the need for taxonomic reconsideration of the western Japanese mole. The zoogeographic perspective derived from this work provides important clues not only for better understanding the evolutionary episodes of East Asian moles, but also for clarifying the fundamental bases of the higher levels of species and genetic diversity among Japanese indigenous animals.

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