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**Intraspecific Differentiation in the Lesser Japanese Mole in Eastern Honshu, Japan,  
Indicated by Nuclear and Mitochondrial Gene Analyses**

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The lesser Japanese mole, *Mogera imaizumii*, recognized by Motokawa and Abe (1996), occurs in eastern Honshu, western Honshu and Shikoku. Mitochondrial and nuclear DNA were analyzed for mole samples from eastern Honshu to elucidate intraspecific differentiation. Analyses of sequences of the mitochondrial cytochrome *b* gene (*Cytb*) and of a restriction fragment length polymorphism of the nuclear 28S ribosomal RNA gene spacer (rDNA-RFLP) revealed two genetic types, partially corresponding to Hutterer's (1993) taxa, *M. wogura* (= *M. imaizumii*) and *M. minor*. Most samples showed either of two combinations of mitochondrial / nuclear gene types. However, two specimens showed a different combination. This incongruent combination of mitochondrial and nuclear genes might have derived, in part, from an introgression event between genetically differentiated populations after secondary contact during the evolutionary history of the lesser Japanese mole in eastern Honshu.

Key words: Lesser Japanese mole, mitochondrial DNA, nuclear DNA, differentiation, secondary contact

## INTRODUCTION

Japan consists of four main islands: Hokkaido, Honshu, Shikoku, and Kyushu. Honshu, the largest, is narrow longitudinally and long latitudinally, with a high mountain chain (over 1,500 m) running north to south. This range is considered a geographic barrier between the eastern and western

distributions of fauna and flora in Honshu. Clearly, geographic factors during the Quaternary period influenced the intraspecific differentiation and speciation of ground animals, considering the number of endemic species in Honshu, for example, *Chimarrogale platycephala* and species of *Eothenomys*, (e.g., Iwasa and Suzuki, 2003; Iwasa and Abe, 2006).

According to Motokawa and Abe (1996), the lesser Japanese mole, *Mogera imaizumii* (Soricomorpha, Talpidae), occurs in eastern Honshu, the Kii Peninsula, and the mountainous ranges of western Honshu and Shikoku (Abe, 1999, 2005; Hutterer, 2005). Environmental conditions have brought about substantial variation in body size among local populations of this species (Imaizumi, 1960; Abe, 2001). Although *M. imaizumii* was recognized by Motokawa and Abe (1996), Abe (2005), and Hutterer (2005), Hutterer (1993) divided it into two valid species, *M. wogura* (= *M. imaizumii* at present; see Motokawa and Abe (1996) for its taxonomic chronology) and *M. minor*, on the basis of morphological characteristics, particularly cranial characters and body size (Kuroda, 1936; Imaizumi, 1960; Yoshiyuki, 1986). Based on its description, the former species (or subspecies in Imaizumi, 1960) is larger in size and is mainly distributed on the Pacific coast, while the latter species (or subspecies in Imaizumi, 1960) is smaller and is mainly distributed on the coast of the Sea of Japan. Abe (1967, 1998, 1999) noted that if the morphological variation considered by Imaizumi (1960) were the result of aging or environmental factors, such as soil hardness, the two species would be indistinguishable. Nevertheless, previous studies have recognized a certain degree of discontinuity in a morphological cline in areas of Iwate and Miyagi Prefectures on the Pacific coast (Abe, 1967, 1998, 1999).

Recently, genetic analyses of Motokawa and Abe's (1996) *M. imaizumii* have been conducted in order to understand intraspecific genetic variation. Okamoto (1999) analyzed local variation in the mitochondrial COI gene and found four clades, respectively distributed on western Honshu and Shikoku, the Kii Peninsula and central Honshu, the coast of the Sea of Japan, and the coast of the Pacific Ocean. The latter two COI-haplotype clades almost certainly correspond to the valid species *M. imaizumii* and *M. minor* of Yoshiyuki (1986) and Hutterer

(1993), with a boundary between them apparently lying on the Pacific coast in Iwate Prefecture (Okamoto, 1999). The location of the boundary was not well defined due to low sample sizes. In this study, samples of the lesser Japanese mole were collected in Iwate Prefecture, northeastern Honshu. Considering the evolutionary history of this species and its genetic variation, we analyzed mitochondrial and nuclear gene variation to reexamine the taxonomic status of Hutterer's (1993) *M. imaizumii* (formerly *M. wogura*, prior to Abe (1995)) and *M. minor*, and Motokawa and Abe's (1996) *M. imaizumii*.

## MATERIALS AND METHODS

### *Mole specimens*

A total of 32 specimens of the Japanese lesser mole were collected from 18 localities, as shown in Table 1 and Fig. 1. All voucher specimens are preserved in the authors' private collections (HA, HEG, and KT, Table 1).

### *Mitochondrial gene analysis*

Total DNA was extracted from liver tissues by the conventional phenol-chloroform method. PCR amplifications of a 1,140 bp length fragment of the mitochondrial cytochrome *b* (*Cytb*) gene were performed with a primer set, L14734/H15392 (Ohdachi et al., 2001; Iwasa and Abe, 2006). PCR reactions were carried out for 40 cycles, each consisting of 30 sec at 96°C for denaturation, 30 sec at 50°C for annealing, and 30 sec at 72°C for extension. Reaction mixtures (20  $\mu$  L) contained 2 mM Tris-HCl, 10 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.1 mM dNTPs, 0.05 mM primers and 0.5U Ex Taq™ polymerase (Takara). Both DNA strands of the products of the secondary PCR were directly sequenced by an automated method using the Big Dye Terminator Cycle Sequencing Kit (ABI) and an automated sequencer (model 310, ABI).

### *Nuclear gene analysis*

Total DNA was digested using nine restriction enzymes, *AatI* (A), *BamHI* (B), *BglII* (G), *DraI* (D), *EcoRI* (E), *PstI* (P), *PvuII* (V), *SacI* (S), and *XbaI* (X). The digested DNA was immobilized on a nylon membrane and allowed to hybridize with digoxigenin-labeled (Roche:

DIG DNA Labeling and Detection Kit) probes of rDNA (0.8  $\mu$ g/ml), namely, 28S, derived from BALB/c mouse (Suzuki et al., 1994; Iwasa et al., 2001; Iwasa and Suzuki, 2003). Detection of the probes on the membrane was carried out by using the kit supplier's instrument. Considering blotting band patterns, the restriction maps for the various types of rDNA repeating unit (repeats) were constructed with the restriction sites on the 28S coding regions that are known to be conservative among mammalian species (Suzuki et al., 1994; Iwasa et al., 2001; Iwasa and Suzuki, 2003). The restriction site maps were constructed based on restriction fragment length polymorphism (RFLP) blotting patterns for 28S external spacer regions.

#### *Data analysis*

We constructed a neighbor-joining tree (Saitou and Nei, 1987) for all transitions and transversions at all codon positions using *Cytb* sequence data on the basis of the Tajima-Nei distances (Tajima and Nei 1984), considering unequal nucleotide frequencies (A=0.319, T=0.288, G=0.132, C=0.261). Bootstrap analysis was performed (1,000 trials) using the MEGA ver. 2.1 program (Kumar et al., 2001), and higher values (> 90%) were described in the tree. We also used *Cytb* sequences for *M. wogura* and *M. imaizumii*, which had been previously studied (Tsuchiya et al., 2000). Analysis of molecular variance (AMOVA; Excoffier et al., 1992) was implemented in Arlequin (Schneider et al., 2000) to elucidate the extent of genetic variation among and within mitochondrial haplotype clades and nuclear rDNA repeat groups. Pairwise genetic distances using the estimator  $F_{st}(\theta)$  of Weir and Cockerham (1984) were also calculated for all the clades and groups. Nucleotide diversity ( $\pi$ ) within each haplotype clade and estimates of DNA net and raw divergence between clades ( $D_a$  and  $D_{xy}$ ) (Nei, 1987) were calculated using MEGA version 2.1, with the bootstrap estimates from 1,000 replicates serving to indicate standard error on the basis of the total number of substitutions. Finally, on the basis of current RFLP data, we constructed a parsimony network using PAUP\* 4.0b (Swofford, 1998) to understand the genetic relationships among the repeats.

## **RESULTS**

#### *Mitochondrial gene haplotypes*

We found a total of 17 haplotypes of *Cytb* among 32 mole individuals from eastern Honshu. Tajima-Nei distances among the haplotypes based on all substitutions at all codon positions ranged from 0.0000 to 0.0345 among the mole specimens from Iwate Prefecture. The NJ tree showed two clades with bootstrap values >90% that were tentatively defined by us as clades containing *imaizumii* haplotypes (*imaizumii*-1 to 5) and *minor* haplotypes (*minor*-1 to 12), taking into account Okamoto's (1999) results and Imaizumi's (1960) classification (Fig. 2). The *imaizumii* clade included only two specimens (Tohno-1 and Kanesawa-2) from Iwate Prefecture and more southeastern localities (Sendai, Ishinomaki, Kinkazan Is., Karuizawa, and Yokohama; Table 1 and Fig. 2). The *minor* clade included the remaining specimens from Iwate Prefecture (Fig. 2). Genetic distances between the *imaizumii* and *minor* clades ranged from 0.0280 to 0.0373.

The results of AMOVA showed that there was significant genetic variation between and within the clades: 88.9% and 11.1%, respectively ( $P < 0.001$ ). The  $F_{st}$  index (0.8891;  $P < 0.001$ ) indicated that the *imaizumii*- and *minor*-haplotype clades were significantly differentiated. The differences in substitutions were  $6.3810 \pm 1.4740$  within the *imaizumii*-haplotype clade,  $3.1133 \pm 0.8478$  within the *minor*-haplotype clade, and  $34.8000 \pm 5.2305$  between the clades. Nucleotide diversity ( $\pi$ ) considering all codon positions in each haplotype clade was  $\pi = 0.002731 \pm 0.001634$  within the *minor*-haplotype clade and  $\pi = 0.005597 \pm 0.003459$  within the *imaizumii*-haplotype clade.  $D_a$  and  $D_{xy}$  indices between the haplotype clades were  $0.02047 \pm 0.00354$  S.D. and  $0.02789 \pm 0.00425$  S.D., respectively.

#### *Nuclear gene repetypes*

RFLP analysis of nuclear rDNA revealed only five polymorphic types among the specimens ( $n=20$ , Table 1), showing that the mitochondrial *imaizumii* and *minor* haplotypes examined in this study were relatively well discriminating. Here, the polymorphic types were tentatively defined by the RFLP data from nine enzymes as the *imaizumii* and *minor* repeating-unit types (repetypes) (Fig. 3a), according to Okamoto's (1999) genomic data and Imaizumi's (1960) classification. According to the restriction maps, the only *PvuII* site was variable among all individuals (Fig. 3a). The AMOVA results showed significant genetic variation between (70.4%) and within (29.6%) the replete groups ( $P < 0.001$ ). The  $F_{st}$  index (0.7038;  $P < 0.001$ ) indicated that the *imaizumii*- and *minor*-replete groups were significantly differentiated. All specimens from Iwate Prefecture were of the *minor* replete (Fig. 3a), and their discrimination by the *imaizumii* and *minor* haplotypes was not identical to that by the

*imaizumii* and *minor* repetypes based on restriction maps (Fig. 3a, b). That is, samples Tohno-1 and Kanesawa-2 with the *imaizumii* haplotype had the *minor* repetype and differed from the other specimens with *imaizumii* haplotypes (Table 1).

## DISCUSSION

Our present analysis agreed with known genetic variation in samples of the lesser Japanese mole from eastern Honshu, as well as with the previous data of Okamoto (1999) (Figs. 2, 3). In particular, two main genomic characteristics were similar to those considered in Hutterer's (1993) classification. Our current data revealed the same tendency based on several indices of genetic divergence between the two haplotype clades and also on the repetype groups. However, our present nuclear and mitochondrial data do not clearly show two types. That is, our current genetic data do not completely agree with Hutterer's (1993) classification. In addition, this genetic division seems to be unrelated to morphological differentiation in our samples, because our samples were not clearly divided using morphological criteria (Iwasa, unpublished data; Table 1). Thus, morphological and genetic differentiation are assumed to have occurred at other levels in the lesser Japanese mole. Considering the evidence for differentiation, our present results are relevant to the colonization history of the moles inhabiting eastern Honshu.

Our present data revealed complicated genetic structures in two individuals from Iwate Prefecture, eastern Honshu (specimens Tohno-1 and Kanesawa-2). These specimens had a combination of the mitochondrial *imaizumii* haplotype and the nuclear *minor* repetype that appeared for the first time as an incongruent gene combination in *Mogera* (Tsuchiya, 1990; Okamoto, 1999). Because the other specimens have alternative, typical gene combinations, the *imaizumii* haplotype / *imaizumii* repetype or the *minor* haplotype / *minor* repetype, most populations of the mole inhabiting eastern Honshu tend to have a distinctive genetic structure in the southern part of Iwate Prefecture (Figs. 1, 2, and 3). A previous study of the mitochondrial gene phylogeography of Japanese *Mogera* species (Okamoto, 1999) suggested that the distribution patterns of gene haplotypes were similar to those of morphotypes, such as indicated by Hutterer's (1993) classification, i.e., *M. imaizumii* and *M. minor* (Imaizumi, 1960, 1970; Yoshiyuki, 1986; Hutterer, 1993). Therefore, it was generally believed that there are two distinctive mole forms (or species) in eastern Honshu (Imaizumi, 1960, 1970; Yoshiyuki, 1986; Hutterer, 1993). Our data, however, are not in accordance with the results of previous studies,

which suggested the presence of two species (Imaizumi, 1960, 1970; Yoshiyuki, 1986; Hutterer, 1993). Accordingly, the boundary that has been considered to divide the haplotypes and the repetypes appears to be absent, at least in the moles in Iwate Prefecture, eastern Honshu. Taking into account the genetic structure of the moles in eastern Honshu, we suggest a hypothesis for the formation of mitochondrial and nuclear gene-type combinations during the course of evolution. This hypothesis can be inferred from the secondary contact model, as previously seen in terrestrial wild mammals during the Quaternary (Ferris et al., 1983; Gyllensten and Wilson, 1987; Tegelström, 1987; Via, 2001). According to the results of previous studies and the present study, there are in eastern Honshu exactly two distinctive gene-type combinations in the moles, except in specimens taken in Iwate Prefecture (Abe, 1967, 1999; Imaizumi, 1960, 1970; Okamoto, 1999). Therefore, a boundary seems to exist in Iwate Prefecture between populations with the two gene-type combinations. We can conclude that two different populations exist there, each with its own genetic constitution.

As an explanation for our hypothesis involving the secondary contact model, the ancestor of the lesser Japanese mole evolved and expanded its distributional areas from southwestern to eastern Japan through two different routes: the coast of the Pacific Ocean and the coast of the Sea of Japan. These routes would be possible because of the presence of the long, high mountain chain running north to south on Honshu. According to Imaizumi (1960), there are two subspecies: a smaller form of the mole on the coast of the Sea of Japan and a larger form on the coast of the Pacific Ocean. Therefore, each form might have differentiated along each route during the expansion of its distribution, with genetic differentiation accumulating on each route. The high mountain range in eastern Honshu seems to be a factor in the geographic isolation between populations (e.g., Okamoto, 1999). One population turned along the coast of the Sea of Japan in northernmost Honshu, where the mountain chain ends, and invaded the coast of the Pacific Ocean (Fig. 4). Subsequently, the populations dispersing along the two routes might have come into secondary contact in southern Iwate Prefecture. After this contact, a few gene introgressions occurred in the parapatric zone (contact zone), as shown by the specimens from Ohtsuchi and Tohno (Figs. 1 and 4). Our evolutionary scenario supported at present by the incongruent genetic and morphological structures (Abe, 1999) in eastern Honshu, as we observed in the present study. However, there are no genetic data for moles in the high mountain ranges that cross from the coast of the Pacific Ocean to the coast of the Sea of Japan. Therefore, analysis of more samples will be needed to evaluate the proposed hypothesis.

From the current data, there appears to be no reproductive isolation at present between the two types. However, it is obvious that there are two genetic constitutions in Motokawa and Abe's (1996) *M. imaizumii*, such as Hutterer's (1993) *M. imaizumii* and *M. minor*, and that this mole may be an appropriate model to study speciation in an allopatrically diverged taxon by examination of the zone of secondary contact (Futuyma, 1993; Via, 2001). In addition, according to our present results on genomic differentiation, the classification should be reevaluated morphologically and biogeographically in the future.

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## Figure legends

Fig. 1. (a) Map of northern Japan showing the locations of Iwate Prefecture and of collection localities of the lesser Japanese moles examined in this study. Locality numbers are identical to those in Table 1; (b) map of Iwate Prefecture including place names; a lesser Japanese mole is illustrated at lower right.

Fig. 2. A neighbor-joining phylogenetic tree based on Tajima-Nei genetic distances (Tajima and Nei, 1984) among mitochondrial cytochrome *b* gene sequences.

Fig. 3. (a) Restriction maps of the rDNA repetypes for the downstream spacer region of the 28S coding gene; (b) parsimony network based on the restriction map data. Asterisks indicate restriction sites clearly distinguishing between imaizumii and minor repetypes. Solid triangles indicate polymorphic sites that appear as size variation within the genome of an individual. See text for abbreviated characters of enzymes.

Fig. 4. Map showing the colonization of two genetic types of the lesser Japanese mole in northeastern Honshu, Japan, according to our secondary-contact hypothesis.

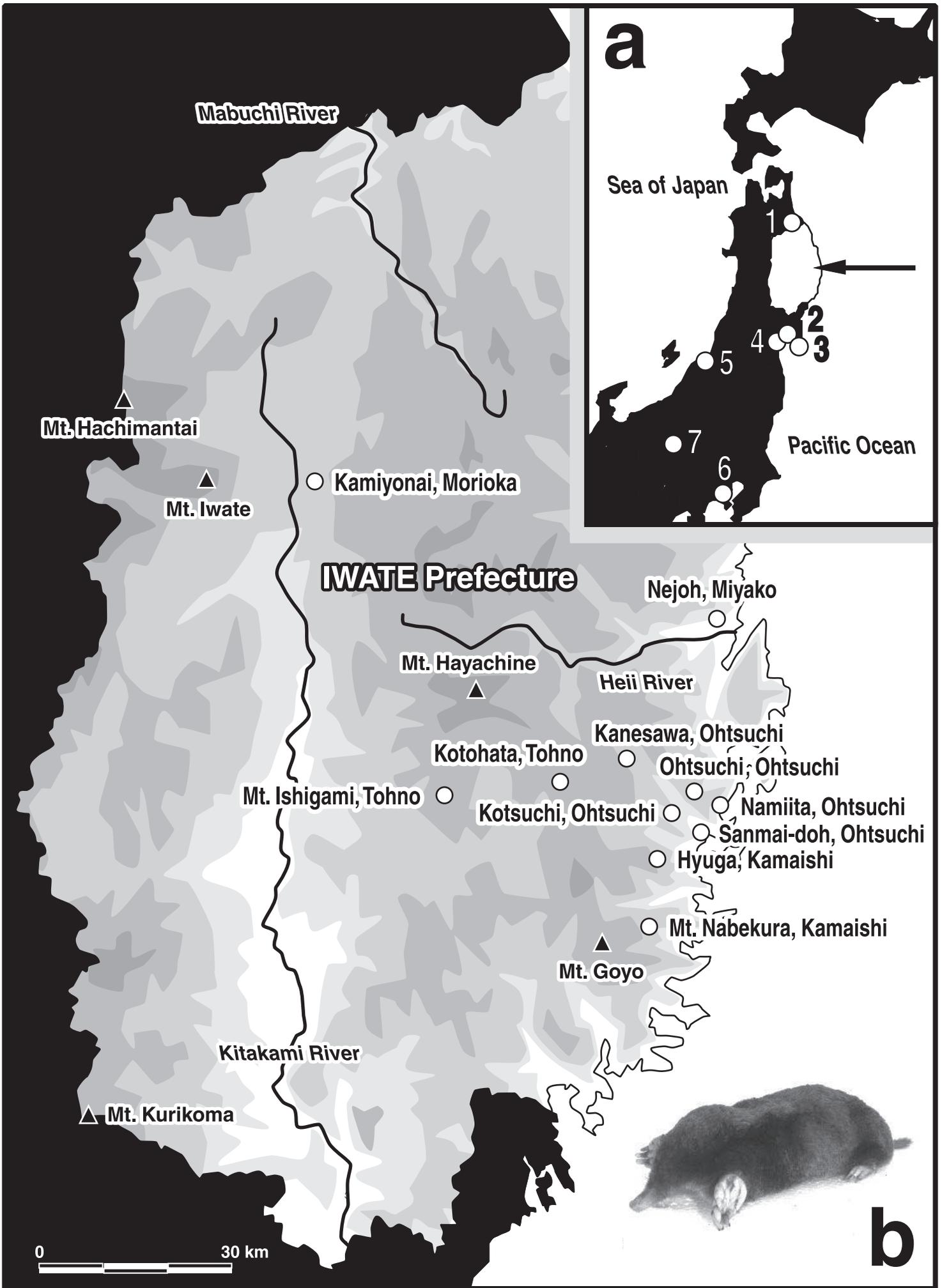
**Table 1. Specimens of *Mogera imaizumii* examined in this study.**

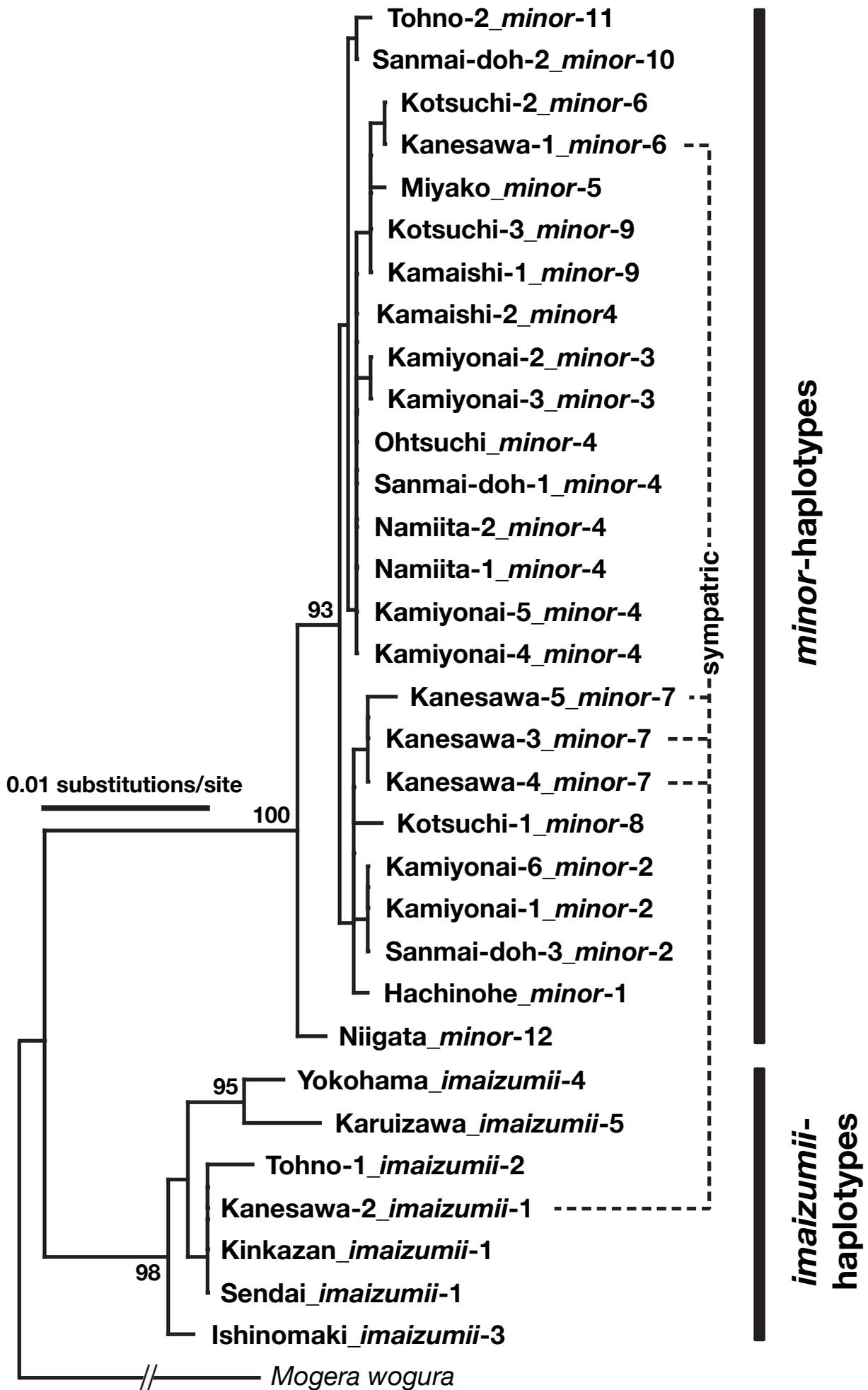
Collection Locality (code*)	Specimen No.	Individual code	Haplotype	Reptype	Accession No.	External morphological measurements**						
						TTL (mm)	TL (mm)	HBL (mm)	FFLsu (mm)	FFW (mm)	HFLsu (mm)	BW (g)
Hachinohe, Aomori Pref. (a-1)	KT2793*	Hachinohe	<i>minor-1</i>	----	AB037611	151.5	23.5	128.0	16.0	16.7	16.0	53.7
Kamionai, Morioka, Iwate Pref. (b)	HEG319-01	Kamionai-1	<i>minor-2</i>	<i>minor-I</i>	AB245936	153.0	15.0	138.0	17.5	18.0	17.3	74.0
	HEG320-01	Kamionai-2	<i>minor-3</i>	----	AB245958	144.0	15.5	128.5	18.0	18.5	18.0	71.0
	HEG321-01	Kamionai-3	<i>minor-3</i>	----	AB245937	144.0	15.0	129.0	18.5	18.0	17.3	72.0
	HEG322-01	Kamionai-4	<i>minor-4</i>	----	AB245938	139.0	14.0	125.0	17.6	18.5	16.7	52.0
	HEG323-01	Kamionai-5	<i>minor-4</i>	<i>minor-I</i>	AB245939	147.0	16.0	131.0	18.0	18.5	17.0	65.0
	HEG350-01	Kamionai-6	<i>minor-2</i>	----	AB245940	137.5	19.0	118.5	16.5	18.0	17.5	58.5
Nejoh, Miyako, Iwate Pref. (b)	HS1777	Miyako	<i>minor-5</i>	<i>minor-I</i>	AB245960	144.0	18.0	126.0	16.0	16.5	15.0	ND
Kanesawa, Ohtsuchi, Iwate Pref. (b)	HEG170-98	Kanesawa-1	<i>minor-6</i>	<i>minor-III</i>	AB245941	135.0	18.5	116.5	15.5	14.2	18.0	53.0
	HEG172-98	Kanesawa-2	<i>imaizumii-1</i>	<i>minor-I</i>	AB245942	ND	ND	122.0	ND	ND	16.0	55.8
	HEG352-01	Kanesawa-3	<i>minor-7</i>	<i>minor-I</i>	AB245943	112.5	12.0	100.5	17.0	17.5	16.0	46.8
	HEG353-01	Kanesawa-4	<i>minor-7</i>	<i>minor-I</i>	AB245944	127.0	16.0	111.0	16.5	17.5	17.0	38.4
	HEG354-02	Kanesawa-5	<i>minor-7</i>	----	AB245945	137.0	11.2	125.8	12.9	18.4	17.0	55.1
Namiita, Ohtsuchi, Iwate Pref. (b)	HEG197-98	Namiita-1	<i>minor-4</i>	----	AB245948	141.0	14.0	127.0	17.0	17.2	17.8	ND
	HEG200-98	Namiita-2	<i>minor-4</i>	<i>minor-II</i>	AB245949	ND	ND	ND	ND	ND	ND	ND
Kotsuchi, Ohtsuchi, Iwate Pref. (b)	HEG180-98	Kotsuchi-1	<i>minor-8</i>	<i>minor-I</i>	AB245946	131.0	16.0	115.0	17.0	16.5	16.3	49.9
	HEG344-01	Kotsuchi-2	<i>minor-6</i>	<i>minor-I</i>	AB245947	132.0	14.0	118.0	16.0	17.5	16.5	46.2
	HEG347-01	Kotsuchi-3	<i>minor-9</i>	<i>minor-II</i>	AB245951	136.0	15.0	121.0	16.5	15.0	16.0	55.5
Sanmai-doh, Ohtsuchi, Iwate Pref. (b)	HEG325-01	Sanmaidoh-1	<i>minor-4</i>	<i>minor-I</i>	AB245962	144.0	17.0	127.0	18.5	18.5	18.0	70.5
	HEG326-01	Sanmaidoh-2	<i>minor-10</i>	----	AB245955	122.0	14.5	107.5	17.0	17.0	18.5	51.0
	HEG327-01	Sanmaidoh-3	<i>minor-2</i>	<i>minor-I</i>	AB245954	132.0	15.5	116.5	19.0	18.5	19.0	68.5
Sakota, Ohtsuchi, Iwate Pref. (b)	HEG348-01	Sakota	<i>minor-4</i>	<i>minor-III</i>	AB245950	137.0	14.0	123.0	15.4	16.1	15.0	45.8
Tsuchibuchi-cho, Tono, Iwate Pref. (b)	HEG346-01	Tohno-1	<i>imaizumii-2</i>	<i>minor-I</i>	AB245952	129.0	15.0	114.0	17.5	17.5	17.0	44.4
Mt. Ishigami, Tono, Iwate Pref. (b)	HS2315	Tohno-2	<i>minor-11</i>	----	AB245961	111.2	16.2	95.0	18.0	15.5	19.9	30.8
Fuefuki-Pass, Kamaishi, Iwate Pref. (b)	HEG181-98	Kamaishi-1	<i>minor-9</i>	<i>minor-I</i>	AB245956	141.0	16.5	124.5	16.5	----	17.2	62.2
Hyuga-Dam, Kamaishi, Iwate Pref. (b)	HEG328-01	Kamaishi-2	<i>minor-4</i>	<i>minor-I</i>	AB245953	132.0	14.0	118.0	16.5	17.0	16.5	65.0
Minami-Sakai, Ishinomaki, Miyagi Pref. (a-2)	HEG343-01	Ishinomaki	<i>imaizumii-3</i>	----	AB245963	ND	ND	ND	ND	ND	ND	ND
Kinkazan-Is., Oshika, Miyagi Pref. (a-3)	HEG175-98	Kinkazan	<i>imaizumii-1</i>	----	AB245957	149.5	16.0	133.5	19.0	ND	19.0	86.0
Rokuchonome, Sendai, Miyagi Pref. (a-4)	HS582*	Sendai	<i>imaizumii-1</i>	<i>imaizumii-II</i>	AB037613	ND	ND	ND	ND	ND	ND	ND
Niigata, Niigata Pref. (a-5)	KT2795*	Niigata	<i>minor-12</i>	<i>minor-I</i>	AB106236	146.0	18.5	127.5	17.0	18.5	17.0	49.5
Yokohama, Kanagaw Pref. (a-6)	KT2697*	Yokohama	<i>imaizumii-4</i>	<i>imaizumii-I</i>	AB037616	159.0	18.0	141.0	17.0	18.0	17.0	76.5
Karuizawa, Nagano Pref. (a-7)	HS1778	Karuizawa	<i>imaizumii-5</i>	----	AB245959	131.5	17.6	113.9	17.0	17.2	16.4	41.2

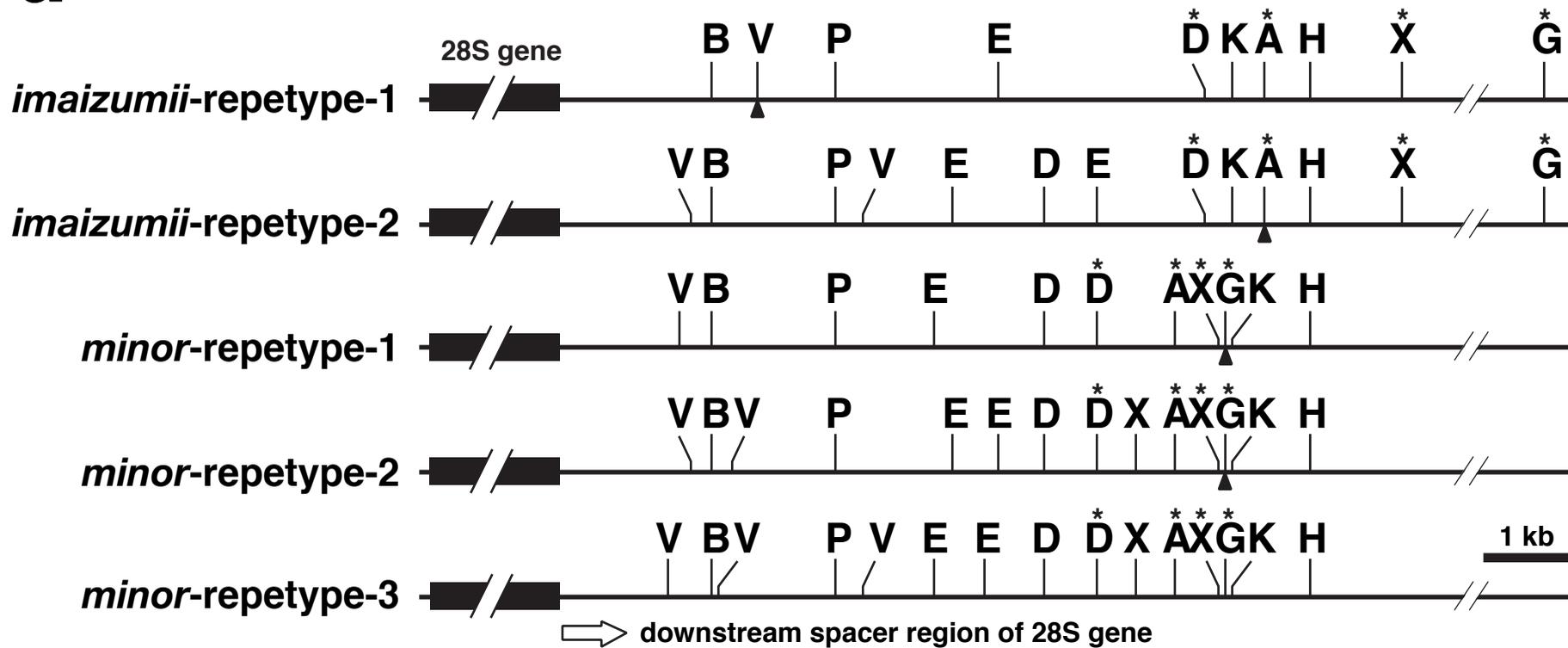
ND, no data.

\*Locality codes are shown in Fig. 1.

\*\*TTL, total length; TL, tail length; HBL, head and body length; FFL, fore foot length cum-unguis; FFW, fore foot width; HFLsu, hind foot length sine-unguis; BW, body weight.





**a****b**