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Rapid communication

First record of Lineage IV of *Rattus tanezumi* (Rodentia: Muridae) from the southern Ryukyus, Japan

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Abstract. Based on mitochondrial cytochrome *b* gene sequences, we report representatives of the Lineage IV of *Rattus tanezumi* (Rodentia: Muridae) from Miyakojima, Taramajima, and Yonagunijima Islands in the southern Ryukyus as the first record of the lineage from Japan. The new record of Lineage IV provides an addition to the mammal fauna of Japan essentially at the species level. Accordingly, the genus *Rattus* in Japan consists of five species: *R. rattus*, Lineage II of *R. tanezumi*, Lineage IV of *R. tanezumi*, *R. norvegicus*, and *R. exulans*. The Miyakojima–Taramajima and Yonagunijima samples of the Lineage IV had discernible genetic differences, suggesting different origins. From Miyakojima Island and Kojima Island (an islet close to Miyakojima Island). We also found Lineage II of *R. tanezumi*, indicating that the two lineages coexist on Miyakojima Island. Morphological comparisons of these Lineage IV and Lineage II specimens suggest a greater molar length in Lineage IV than in Lineage II, and differences in the foramen magnum shape. Future detailed studies on genetic structures, morphological characteristics, and distribution of Lineage IV and Lineage II rats in the southern Ryukyus are needed to clarify their evolutionary history.

Key words: commensal species, Miyakojima Island, new record, Taramajima Island, Yonagunijima Island.

Rattus rattus and its relatives are commensal species and distributed worldwide, strongly related to human activities and movements (e.g., Aplin et al. 2011; Denys et al. 2017). Based on divergence in chromosome number and other features, Musser and Carleton (2005) separated Oceanian or Indian (2n = 38–40) and Asian (2n = 42) populations into *R. rattus* and *R. tanezumi*, respectively, although the species boundary remained unclear. Aplin et al. (2011) studied mitochondrial cytochrome *b* gene sequences of *R. rattus* sensu lato and recognized six distinct lineages. Of these, Lineage I, is attributable to *R. rattus* of Musser and Carleton (2005), supposedly originated on the Indian subcontinent and introduced worldwide (Aplin et al. 2011; Denys et al. 2017); Lineage II is thought to have originated in Asia and to have been introduced worldwide, with specimens from Asia (China, Vietnam, Laos, Myanmar, Bangladesh, Thailand, Indonesia, Papua New Guinea, the Philippines, Taiwan, and Japan), the USA, and South Africa (Aplin et al. 2011); Lineage III consists only of specimens from Nepal and Pakistan; Lineage IV is thought to have originated in Indochina and consists of specimens from Vietnam, Cambodia, Laos, Indonesia, Sri Lanka, and the Philippines (Aplin et al. 2011); Lineage V is known only from Thailand and Laos and is considered *R. sakeratensis*; and Lineage VI is known from Malaysia and Indonesia and corresponds to *R. tiomanicus* + *R. baluensis* (Aplin et al. 2011). Each of Lineages I–IV may represent a valid

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species itself, but the current taxonomy recognizes only two species: *R. rattus* for Lineage I and *R. tanezumi* for Lineages II–IV (Denys et al. 2017), although the taxonomy is still tentative and the three *R. tanezumi* lineages are paraphyletic (Aplin et al. 2011; Denys et al. 2017). When discussing the species taxonomy and phylogeography of *R. tanezumi*, it is necessary to clarify to which lineage or lineages of cytochrome *b* sequences a given argument are referred.

In Japan, Aplin et al. (2011) found Lineage I (*R. rattus*) from Otaru (Hokkaido), and Lineage II from Miyazaki (Kyushu), Tokyo (Honshu), and Amamioshima Island (central Ryukyus). Lineage II is thought to be widely distributed in Japan, while Lineage I is supposedly confined to areas near some ports because of its relatively recent introduction *via* ships (Chinen et al. 2005; Kambe et al. 2012). As mentioned above, Lineage II is usually referred to as *R. tanezumi*, but Iwasa (2015) considered both lineages the single species *R. rattus*.

We recently found rats from Lineage IV in the southern Ryukyu Islands. This is the first record of this lineage from Japan. Those Lineage IV specimens were collected from Miyakojima and Taramajimna Islands of the Miyako Group, and Yonagunijima Island of the Yaeyama Group. Specimens representing Lineage II were also found on Miyakojima Island and Kojima Island (a small islet off Miyakojima Island). Although the taxonomy of *R. tanezumi* is still unclear, Lineage II and Lineage IV most likely represent distinct species from each other (Aplin et al. 2011; Denys et al. 2017). Therefore, the new record of Lineage IV is regarded as an addition to the mammal fauna of Japan at the species level. In this paper, we report these findings with reference to the species taxonomy of *R. tanezumi*.

Materials and methods

Rats were collected with cage or glue traps in 2015, 2016, and 2020. The 13 examined specimens from Miyakojima, Kojima, Taramajima, and Yonagunijima Islands are shown in Fig. 1 and Table 1. All specimens are deposited in the Zoological Collection, Kyoto University Museum, Kyoto University (KUZ). The following standard external measurements were made: body weight (BW) in grams, total length (TL), tail length (T), ear length (E), and hindfoot length without claw (HF) to 0.5 mm for TL and T, and 0.01 mm for E and HF. Head and body length was calculated by subtracting T from TL.

Genomic DNA was extracted from liver or muscle tissue following the method in Okamoto et al. (2006). The complete cytochrome *b* sequence and its adjacent region was amplified by polymerase chain reaction (PCR) using primers L14724 (5'-CGAAGCTTGATATGAAAAACC ATCGTTG-3') and H15915 (5'-AACTGCAGTCATC TCCGGTTTACAAGAC-3') (Irwin et al. 1991). PCR amplifications were performed in 12.5 μL reaction volumes using an Ex Taq Kit (TaKaRa Bio) and a PCR Thermal Cycler GeneAmp PCR System 2700 (Thermo Fisher Scientific), with an initial 5-min denaturing at 94°C; 30 cycles of denaturing at 94°C for 1 min, annealing at 45–47°C for 1 min, and extension at 72°C for 2 min; and a final 7-min extension at 72°C. The PCR products were purified following Okamoto and Hikida (2009). Macrogen Japan (Tokyo) sequenced both strands of each of the purified PCR products with the cytochrome *b* sequencing primers L14724, H15915 (Irwin et al. 1991), H15149 (5'-AAACTGCAGCCCCTCAGAATGATATT TGTCCTCA-3') (Kocher et al. 1989), and newly designed primer L15369 (5'-TCCACGAAACAGGAT CAAACAACCC-3'). The sequences were aligned using ClustalW (Thompson et al. 1994) in MEGA7 (Kumar et al. 2016). Of the 13 specimens, 11 could be sequenced (except KUZ M11865 from Taramajima Island and M17148 from Miyakojima Island). On alignment, we also incorporated most of the sequence data of Aplin et al. (2011) from GenBank in our dataset (see Table S1 of Aplin et al. [2011]). For the aligned sequences, lengths of the examined samples exceeding the data of Aplin et al. (2011) were trimmed. The original sequence data of the examined samples were deposited in DDBJ with accession numbers LC669419–LC669429.

Phylogenetic analysis was performed with Bayesian Inference (BI) using MrBayes v.3.2 (Ronquist et al. 2012). The nucleotide substitution model was selected by KAKUSAN4 (Tanabe 2011) under the Bayesian information criterion (Schwarz 1978), and models K80 + Γ, HKY85 + Γ, and GTR + Γ were used for the first to third triplet positions, respectively. Two independent runs of four Markov chains in Markov Chain Monte Carlo cycles were conducted for 5 000 000 generations, sampling a tree every 1000 generations. After confirming the parameter estimates and stationary distribution using Tracer v.1.7.1 (Rambaut et al. 2018a, 2018b), the first 10% of tree samples was discarded as burn-in, and a consensus topology and Bayesian posterior probabilities for the remaining tree samples were calculated.

The individuals used for cytochrome *b* analysis were subjected to morphometric analyses if they had molar

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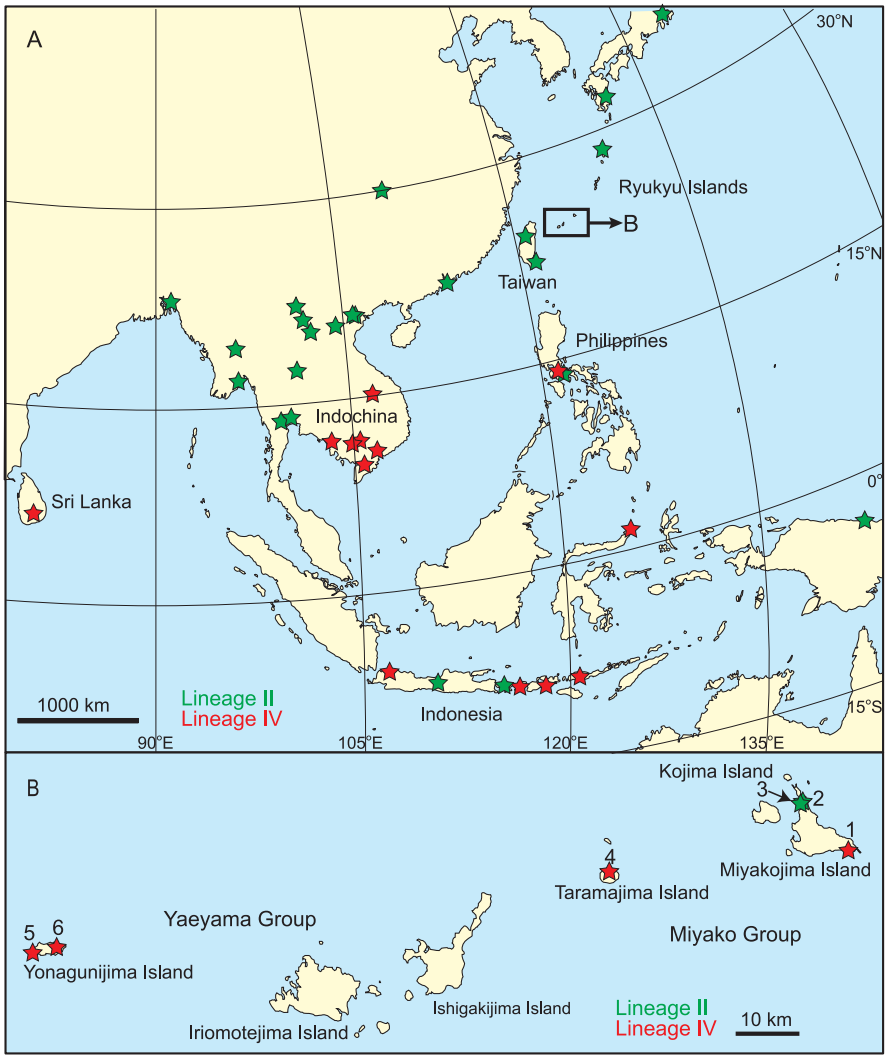


Fig. 1. Map showing localities of Lineage II (green stars) and Lineage IV (red stars) of *Rattus tanezumi* reported by Aplin et al. (2011) (A) and enlarged map of the southern Ryukyus showing the localities of examined specimens of *R. tanezumi* on Miyakojima, Kojima, Taramajima, and Yonagunijima Islands, as green and red stars for Lineages II and IV, respectively (B). Locality numbers correspond to those in Table 1.

wear and were regarded as adults and subadults; while individuals without molar wear (KUZ M11860 and M11867) were regarded as juveniles and excluded from morphometric analyses. Twenty skull measurements were made with digital calipers to the nearest 0.01 mm following Motokawa et al. (2004), with the addition of greatest width and length of the foramen magnum. Because the sample size was small, we made a preliminary comparison of the mean, minimum, and maximum values of selected measurements between Lineage II and Lineage IV samples.

Results

Figure 2 shows the BI phylogenetic tree of the cytochrome *b* sequences. The tree topology is concordant with Aplin et al. (2011) in consisting of the six lineages (Lineages I–VI). Of our specimens from the southern Ryukyus, M17154 and M17155 from Hirara-Karimata on Miyakojima Island, and M11860, M11862, M11863, and M11864 from Kojima Island clustered in Lineage II, whereas M11861 and M17149 from Bora on Miyakojima Island, M11867 from Taramajima Island, and M17138 and M17139 from Yonagunijima Island were in Lineage IV (Fig. 2). The genetic divergence among our Lineage II specimens was low, as was that between these specimens and other Lineage II samples in Aplin et al. (2011). Notably, one of the Miyakojima specimens (M17154) was closest to a sample from Amamioshima Island in the central Ryukyus (Aplin et al. 2011). Our Lineage IV

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Table 1. Specimens of *Rattus tanezumi* from the southern Ryukyus examined in this study

Specimen	Sex	Locality (Numbers correspond to those in Fig. 1)	Date	Latitude	Longitude	Cytochrome *b*	Accession No.
KUZ M11861	M	1. Bora, Miyakojima Island (Miyakojima C., Okinawa Pref., Japan)	Mar. 5, 2016	N24.729°	E125.426°	Lineage IV	LC669420
KUZ M17148	M	1. Bora, Miyakojima Island (Miyakojima C., Okinawa Pref., Japan)	Mar. 6, 2020	N24.729°	E125.426°	–	–
KUZ M17149	F	1. Bora, Miyakojima Island (Miyakojima C., Okinawa Pref., Japan)	Mar. 6, 2020	N24.729°	E125.426°	Lineage IV	LC669427
KUZ M17154	M	2. Hirara-Karimata, Miyakojima Island (Miyakojima C., Okinawa Pref., Japan)	Mar. 7, 2020	N24.860°	E125.285°	Lineage II	LC669428
KUZ M17155	F	2. Hirara-Karimata, Miyakojima Island (Miyakojima C., Okinawa Pref., Japan)	Mar. 7, 2020	N24.860°	E125.285°	Lineage II	LC669429
KUZ M11860	M	3. Kojima Island (Miyakojima C., Okinawa Pref., Japan)	Dec. 23, 2015	–	–	Lineage II	LC669419
KUZ M11862	M	3. Kojima Island (Miyakojima C., Okinawa Pref., Japan)	Mar. 8, 2016	–	–	Lineage II	LC669421
KUZ M11863	M	3. Kojima Island (Miyakojima C., Okinawa Pref., Japan)	Mar. 8, 2016	–	–	Lineage II	LC669422
KUZ M11864	M	3. Kojima Island (Miyakojima C., Okinawa Pref., Japan)	Mar. 8, 2016	–	–	Lineage II	LC669423
KUZ M11865	M	4. Taramajima Island (Tarama V., Okinawa Pref., Japan)	Feb. 28, 2016	–	–	–	–
KUZ M11867	F	4. Taramajima Island (Tarama V., Okinawa Pref., Japan)	Apr. 16, 2016	–	–	Lineage IV	LC669424
KUZ M17138	M	5. Yonagunijima Island (Yonaguni T., Okinawa Pref., Japan)	Mar. 2, 2020	N24.450°	E122.936°	Lineage IV	LC669425
KUZ M17139	M	6. Yonagunijima Island (Yonaguni T., Okinawa Pref., Japan)	Mar. 3, 2020	N24.459°	E123.017°	Lineage IV	LC669426

specimens from Miyakojima and Taramajima Islands showed little divergence from each other. By contrast, the two Lineage IV specimens from Yonagunijima Island were very close to each other as well as to samples from Indonesia, Sri Lanka, and Luzon Island in the Philippines, but were distinctly divergent from the Miyakojima and Taramajima specimens and from the Indochinese Lineage IV samples of Aplin et al. (2011) (Fig. 2).

Figure 3 shows the external features of the Lineage IV rats from Yonagunijima and Miyakojima Islands. Figure 4 shows the skulls of Lineage IV from these two islands and of Lineage II from Miyakojima Island. Pelage color (Fig. 3) showed individual or geographic variation, but we could not make a sufficient comparison due to the small number of specimens. Table 2 gives selected external and cranial measurements. Alveolar length of the maxillary toothrow, which reportedly has less effect of age variation (Motokawa et al. 2004), was greater in Lineage IV than in Lineage II without overlap. For most other measurements, Lineage IV had greater mean values than Lineage II, but with variable range overlap. By contrast, the greatest height of the foramen magnum was greater in Lineage II than in Lineage IV without overlap. In qualitative comparison of skulls (Fig. 4), we could not find any characters that distinguished Lineages IV and Lineage II, except that the foramen magnum was wide and oval in Lineage IV, but narrow, high, and pentagonal in Lineage II (Fig. 4).

Discussion

Our samples from Miyakojima (Bora), Taramajima, and Yonagunijima Islands in the southern Ryukyus constitute the first record of Lineage IV of *R. tanezumi* from Japan. This finding provides an addition to the mammal fauna of Japan essentially at the species level, and the genus *Rattus* in Japan consists of five species involving taxa waiting for clarification of species names: *R. rattus*,

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Fig. 2. Bayesian inference phylogenetic tree of *Rattus tanezumi* and other related species constructed using cytochrome *b*. Numbers on nodes are Bayesian posterior probabilities. New sequences from the southern Ryukyus that clustered in Lineage II and Lineage IV are shown with rectangles.

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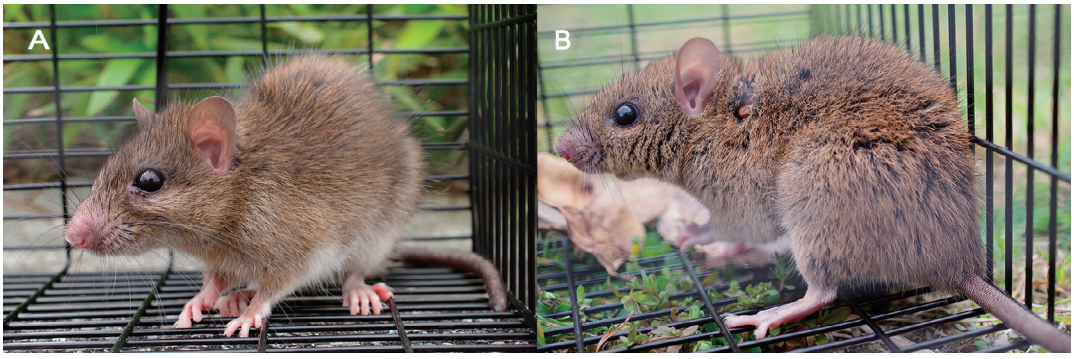


Fig. 3. External features of Lineage IV of *Rattus tanezumi* from Yonagunijima (A: KUZ M17139) and Miyakojima (B: KUZ M17149) Islands.



Fig. 4. Dorsal, ventral, and left lateral views of the skull and foramen magnum of Lineage IV of *R. tanezumi* from Yonagunijima (A: KUZ M17138) and Miyakojima (B: KUZ M17149) Islands and Lineage II of *R. tanezumi* from Miyakojima Island (C: KUZ M17154). The scale bar represents 10 mm for skulls, while no scale is given for the foramen magnum.

Lineage II of *R. tanezumi*, Lineage IV of *R. tanezumi*, *R. norvegicus*, and *R. exulans* (this study; Iwasa 2015; Denys et al. 2017). For scientific research in various fields and other practical issues such as rat control and risk management of introduced species, that concern rats in Japan, accurate recognition of Lineage IV as distinct species from Lineage II is becoming indispensable. Identification among Lineage IV, Lineage II, and *R. rattus* (or Lineage I) is currently possible only by referring to cytochrome *b* gene sequences.

Lineage IV has been reported from Indochina (Laos, Vietnam, and Cambodia), Sri Lanka, the Philippines, and Indonesia (Fig. 1; Aplin et al. 2011; Denys et al. 2017). The cytochrome *b* sequences of the Lineage IV Miyakojima and Taramajima specimens were almost identical, but they collectively differed substantially from the other Lineage IV sequences, including the two Yonagunijima specimens. By contrast, the haplotypes of the two Yonagunijima specimens were almost identical to those of samples from a broad insular region of tropical Asia (Fig. 2). These results suggest that the current Lineage IV populations of *R. tanezumi* in the southern Ryukyus may result from separated colonizing events from different origins. Considering the geographic genetic patterns illustrated in Fig. 2, we conclude that the current Yonagunijima Island population seems to

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Table 2. External and cranial measurements between Lineage II and Lineage IV of *Rattus tanezumi* from the southern Ryukyus

Measurements	Lineage II			Lineage IV		
	Mean	Min–Max	*n*	Mean	Min–Max	*n*
Body weight (in gram)	98.8	82.0–126.0	5	112.5	103.7–128.0	3
Head and body length	153.0	131.0–170.0	5	172.3	169.0–178.0	3
Tail length	157.5	144.0–165.0	4	–	–	–
Ear length	20.08	17.43–22.32	4	20.99	20.91–21.19	4
Hindfoot length	29.40	29.04–30.32	5	30.71	28.64–32.27	4
Occipitonasal length	38.81	36.89–41.22	5	40.58	38.70–42.65	4
Greatest zygomatic breadth	19.00	18.06–20.05	5	19.76	18.59–20.91	4
Breadth of the braincase	15.60	15.11–16.72	5	16.26	15.96–16.65	4
Alveolar length of the maxillary toothrow	6.40	6.23–6.72	4	7.20	6.81–7.55	4
Coronal width of the first upper molar	1.96	1.91–2.04	5	2.08	1.94–2.26	4
Greatest height of foramen magnum	4.71	4.49–4.92	5	4.21	4.06–4.32	4
Greatest width of foramen magnum	5.40	5.14–5.76	5	5.56	5.23–6.02	4

have come from the Philippines or other tropical Asian islands relatively recently. This view is concordant with Nishioka et al. (2016), who could not find a single confirmed *Rattus* specimen in Holocene skeletal remains of rodents from Yonagunijima Island. On the other hand, the origin and date of colonization of the Miyakojima and Taramajima Lineage IV populations remain open questions. One may argue that the apparent sequence differences of these samples from other Lineage IV samples reflect their long isolation on the two islands, but this view is not concordant with absence of the fossil record or skeletal remains of the genus *Rattus* from Miyakojima and adjacent islands despite relatively intense paleontological and archaeological surveys of these islands (Ota 2003; Kawamura 2016; Kawamura et al. 2016). We suspect that the absence of a haplotype close to those of the Miyakojima–Taramajima samples is simply attributable to insufficient genetic surveys of relevant *Rattus* populations.

In comparison, Lineage II rats were found on Miyakojima and Kojima Islands and their haplotypes did not differ substantially from the Lineage II haplotypes from other regions, including the Philippines, Japan (including Amamioshima in the central Ryukyus), and Taiwan (Fig. 2). Pagès et al. (2013) found that Lineage IV (R3 mt lineages in their nomenclature) and Lineage II (*R. tanezumi* therein) in mitochondrial genes occurred in northern and southern Indochina, respectively, and both lineages are sympatric in several localities; while those two lineages showed no difference in nuclear genes. Miyakojima Island is intriguing that both Lineages II and IV were sympatric in such isolated small island (158.9 km^2) and found in different localities within the island (Hirara–Karimata and Bora, respectively), raising the possibility of habitat segregation between two lineages. In this regard, presence or absence of hybridization between Lineages II and IV in Miyakojima Island is considered interesting topic. Future studies of genetic structures and spatial distribution based on mitochondrial and nuclear genes in the populations from Miyakojima and adjacent islands should clarify the complicated history of rats in the southern Ryukyus and the evolutionary mystery of Lineage IV within and outside Indochina.

The morphological comparison did not find characters that explicitly distinguished between Lineages II and IV in the southern Ryukyus, partly due to the small number of examined specimens. However, molar size and foramen magnum shape might be different between Lineages II and IV. Molar size shows less effect of growth, making it less necessary to consider age variation when analyzed. Our results suggest that Lineage IV has more developed molars than Lineage II. In addition, the foramen magnum was wide and oval in Lineage IV, but narrow, high, and pentagonal in Lineage II. These differences may deserve morphological diagnostic characters between Lineage II and Lineage IV, and are necessary for further verification based on skull specimens covering whole distribution ranges of the two lineages.

Uchida (1964) reported rats from Iriomotejima Island in the Yaeyama Group as two aberrant forms of *R. rattus*. One of the forms attributable to *R. exulans* was thought to be a juvenile of *R. rattus* sensu lato (Motokawa et al. 2001). Based on Uchida (1964), another form differed

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from *R. rattus* on Okinawajima Island in the shape of the foramen magnum and bulla size. Although those two specimens are no longer available for detailed examination, their photos in Uchida (1964) suggest their similarities to our Lineage IV specimens in the foramen magnum shape. Considering the geographic location of Iriomotejima Island between Miyakojima and Yonagunijima Islands (Fig. 1), the *Rattus* population of this island is an interesting subject for future genetic studies.

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