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Authors: Endo, Yu, Lin, Liang-Kong, Yamazaki, Koji, Pei, Kurtis Jai-Chyi, Chang, Shih-Wei, et al.

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Introduction and expansion history of the masked palm civet, *Paguma larvata*, in Japan, revealed by mitochondrial DNA control region and cytochrome *b* analysis

Yu Endo¹, Liang-Kong Lin², Koji Yamazaki³, Kurtis Jai-Chyi Pei⁴, Shih-Wei Chang⁵, Yen-Jean Chen⁶, Keiji Ochiai⁷, Shuuji Yachimori⁸, Tomoko Anezaki⁹, Yayoi Kaneko¹⁰ and Ryuichi Masuda^{1,11,*}

¹ Department of Natural History Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan

² Department of Life Science, Tunghai University, Taichung 407, Taiwan

³ Tokyo University of Agriculture, Tokyo 156-8502, Japan

⁴ National Pingtung University of Science and Technology, Pingtung 91201, Taiwan

⁵ Division of Zoology, Endemic Species Research Institute, Nantou 55244, Taiwan

⁶ Department of Zoology, National Museum of Natural Science, Taichung 404, Taiwan

⁷ Natural History Museum and Institute, Chiba 260-8682, Japan

⁸ Shikoku Institute of Natural History, Susaki 785-0023, Japan

⁹ Gunma Museum of Natural History, Tomioka 370-2345, Japan

¹⁰ Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

¹¹ Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

Abstract. The masked palm civet (*Paguma larvata*) in Japan is considered as an alien species; however, the details on introduction routes to Japan and the expansion history are still unclear. In the present study, to further solve these questions, we analyzed the mitochondrial DNA control region (523 base-pairs) of 226 individuals from Japan and Taiwan, and identified 13 haplotypes, which are phylogenetically closely related to each other. Then, we combined the present control region data with the previously reported cytochrome *b* sequences, and found multiple haplotypes in Shikoku, Gunma, and Tokyo. The population in Shikoku was formed by several genetic lineages, one of which was not found in other areas, indicating the differentiation of the Shikoku population from those in Central Honshu. In addition, animals with the major cytochrome *b* haplotype in Eastern Japan in the previous study were found to have three different haplotypes in control region. The results strongly show multiple introduced routes of *P. larvata* in Eastern Japan. Our study much contributes to further understanding of the expansion history of *P. larvata* on the Japanese islands, in conjunction with previous genetic data.

Key words: alien species, founder effect, haplotype, phylogenetics.

Many animal and plant species have been introduced into their non-native ranges through human activity, and such organisms are called ‘alien species’. Some alien species have adapted themselves to new environments and sometimes affected native ecosystems. To elucidate the population genetic structure and expansion history in new environments can contribute to evaluating the efficiency of ecosystem conservation activity by predicting new invasions (Sakai et al. 2001).

The masked palm civet *Paguma larvata* (Viverridae, Carnivora, Mammalia) is native to Southeast Asia, India, China, Taiwan, Nepal, and Pakistan (Torii 2015). In Japan, this species is thought to be one of the alien species because no fossil records on the species have been reported and their distribution is not continuous within Honshu Island (Torii 2015). Several studies on *P. larvata* in Japan have been published, about the food habit (Torii 1986), the litter size (Torii and Miyake 1986), the diploid

*To whom correspondence should be addressed. E-mail: masudary@sci.hokudai.ac.jp

chromosome number (Harada and Torii 1993), and conflict with native species (Matsuo et al. 2007). However, the origin of the Japanese population of *P. larvata* had long been unclear (Kuroda 1955; Nakamura 1994; Torii 2015).

Previously, researchers showed that *P. larvata* was introduced to Japan from foreign countries in 1930–40s for fur industry (Nawa 1965; Nakamura et al. 1989). A wild individual was first recorded in Shizuoka in 1943 (Nawa 1965), and then the inhabitation had been reported in Tohoku (upper Eastern Honshu, Tachibana 1955; Obara 1961), Central Honshu (Shimaoka 1953, 1955; Nawa 1965), and Shikoku (Shimizu 1958; Tateishi 1968) by 1980s (Suzuki 1985; Nakamura et al. 1989), whereas *P. larvata* was a rare animal in Gunma and Tokyo (Kanei 1989; Kabasawa 1998). At present, this species is widespread in Honshu and Shikoku (Torii 2015).

Masuda et al. (2008, 2010) examined the mitochondrial DNA (mtDNA) cytochrome *b* gene of *P. larvata* in Japan and Taiwan, and reported that ancestors of the Japanese population are originated from the Taiwan population, which is phylogenetically different from Southeast Asian populations. In addition, Masuda et al. (2010) found two dominant haplotypes, JA1 and JA4, in Eastern Honshu and Shikoku-Central Honshu of Japan, respectively. Interestingly, both of the two haplotypes appeared also in Gunma Prefecture, Eastern Japan. Inoue et al. (2012) analyzed microsatellite loci and showed population structures and the origin, in agreement with mtDNA analysis of Masuda et al. (2010).

Although Masuda et al. (2008, 2010) discussed routes of the introduction and expansion of *P. larvata* in Japan using the cytochrome *b* gene data, they insisted the necessity of more genetic information to confirm the migration history after introduction. In fact, Inoue et al. (2012) indicated that the Taiwanese population was not separated into two genetic clusters, although Masuda et al. (2010) reported two lineages of cytochrome *b*, Eastern Taiwan and Western Taiwan, separated by high mountains. Individuals in Taiwan are closely related to those in Japan, one of whose origins could be from Taiwan (Masuda et al. 2010). It is therefore important to examine the genetic information of Taiwan to know the expansion history in Japan. Inoue et al. (2012) also indicated that *P. larvata* in Shikoku has a different lineage from those in Honshu although Masuda et al. (2010) showed Shikoku and Central Honshu are in the same lineage of cytochrome *b*. Thus, we need to investigate the genetic information to indicate more precise expansion history of

P. larvata in Japan.

In the present study, we determined nucleotide sequences of the control region, which has the most rapid evolutionary rate among sequences of mtDNA (Hoelzel et al. 1991), for individuals of *P. larvata* from various regions in Japan, and examined them in conjunction with cytochrome *b* gene data previously reported by Masuda et al. (2010). Then, we discuss (1) the relationships between Japanese and Taiwanese populations and (2) the detailed expansion history on the Japanese islands, based on the geographical distribution of variable haplotypes.

Materials and methods

Sampling and DNA extraction

Muscle tissues, hairs, and feces from 226 individuals (206 in Japan and 20 in Taiwan) were collected from road kills, pest control specimens, ecological survey specimens, and zoos (Fig. 1). Many samples were common to those used in the previous studies (Masuda et al. 2010). Total DNA of the muscle or hair samples was extracted with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) or QIAamp DNA Investigator Kit (Qiagen, Hilden, Germany), and then they were stored at 4°C or –20°C until use.

Polymerase chain reaction (PCR) amplification and sequencing

The mtDNA control region (> 1500 base-pairs, bp, including tandem repeats) was amplified by PCR with a primer-pair: Cb-Z (Kurose et al. 1999) and Car-12S-R (Shimatani et al. 2008). For degraded DNA samples mainly from hairs and feces, the partial sequences (804 bp) of the mtDNA control region were amplified with Cb-Z and PLA-DL-R2 (5'-TCCTTGTGTCCGTTGAA CGTTAG-3'), which was newly designed in the present study, based on a sequence (5'-CTAACGTTCAACG GACA-3') in mtDNA control region. PCR amplification was performed in 20 µl of a reaction mixture containing 2.0 µl of 10× reaction buffer (Takara, Shiga, Japan), 1.6 µl of dNTP (2.5 mM each), 0.2 µl of each primer (25 pmol/µl), 0.1 µl of Takara *Taq* DNA polymerase (Takara, Shiga, Japan), 0.4 µl bovine serum albumin (20 mg/ml, Roche, Basel, Switzerland, for poor DNA) and 1.0 µl of the DNA extracts, and 14.9 µl of distilled water. Reactions were performed in the Takara TP350 PCR Thermal Cycler Dice (Takara, Shiga, Japan) with the following program: 94°C for 3 min; 30–40 cycles of 94°C for 1 min, 56.6°C for 0.5–1 min, and 72°C for 10 min. To check

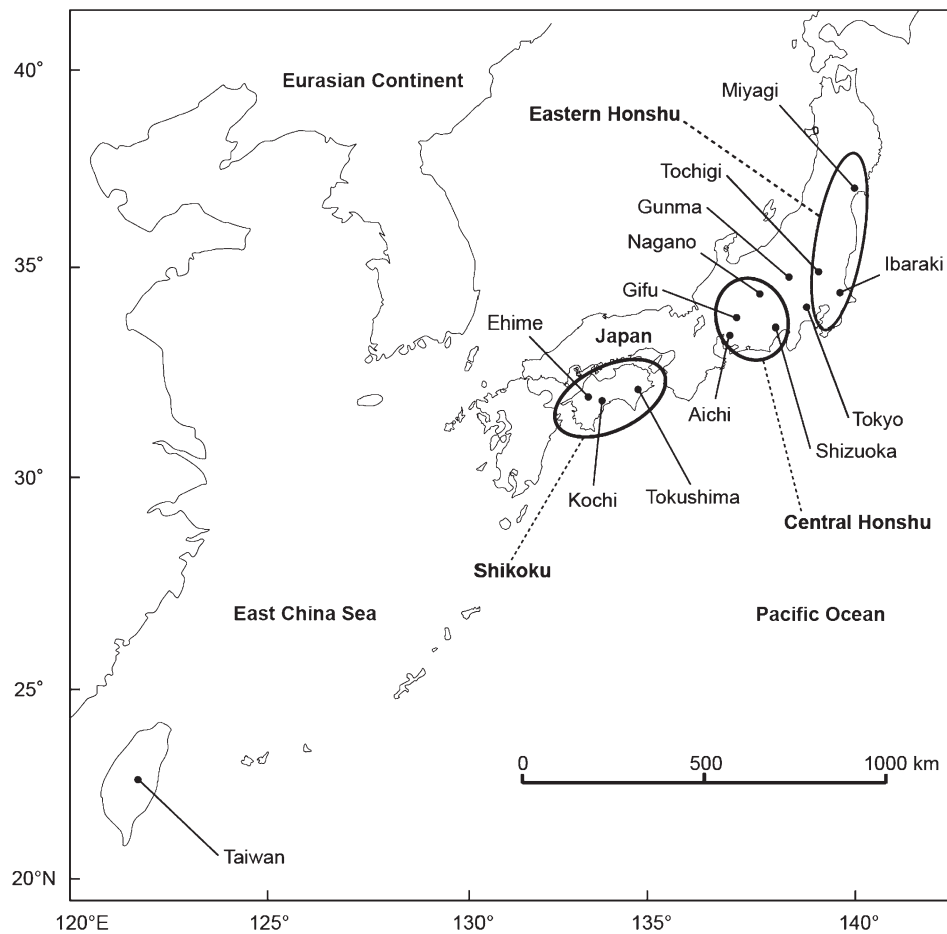


Fig. 1. Sampling locations of *Paguma larvata* in Japan and Taiwan in the present study. The three areas (Eastern Honshu, Central Honshu, and Shikoku) on the Japanese island are shown by circles.

amplification and contamination, we electrophoresed 5 μ l of each PCR product on a 2% agarose gel, and observed the amplicons under an ultraviolet illumination. The remaining PCR products (15 μ l) were purified by the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequence reaction was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Massachusetts, America) and Cb-Z/PLA-DL-R2 primers. DNA sequences were determined with an automated DNA sequencer (ABI 3730 Genetic analyzer, Applied Biosystems, California, America).

Data analyses

Sequences were aligned with Clustal W (Larkin et al. 2007) implemented in MEGA7 (Kumar et al. 2016), and trimmed between the 3' C-rich sequence and the 5' tandem repeats (523 bp in total). A haplotype network was constructed by TCS ver. 1.21 (Clement et al. 2000). Nucleotide diversity (Tajima 1983), haplotype diversity

(Nei 1987), and Watterson's theta (Watterson 1975) were calculated by ARLEQUIN ver. 3.5.2.2 (Excoffier and Lischer 2010).

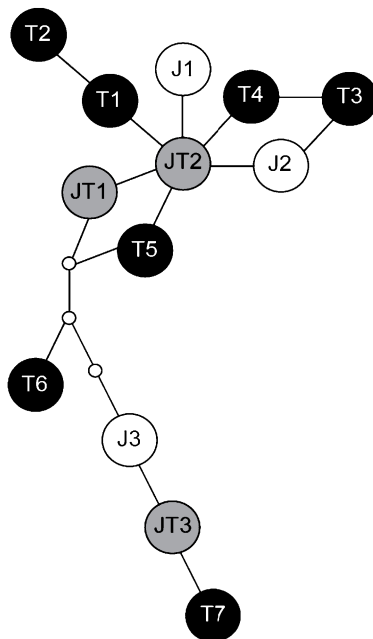
Results

From 226 *P. larvata* individuals from Japan and Taiwan, 13 haplotypes (JT1–3, J1–3, and T1–7) of the mtDNA control region (523 bp) were newly identified (Table 1). Of them, JT1–3 were found in both Japan and Taiwan, whereas three haplotypes (J1–3) were found only in Japan and seven haplotypes (T1–7) only in Taiwan. In the haplotype network (Fig. 2), there were 1–9 nucleotide substitutions between the haplotypes. The mtDNA control region sequences obtained in the present study appear at DDBJ/GenBank/EMBL with accession numbers LC514714–514726.

Table 2 shows the genetic diversities of the Japanese and Taiwanese populations. The genetic diversities of

Table 1. Frequencies of haplotypes of mtDNA control region of *Paguma larvata* from Japan and Taiwan

Country	Prefecture	Number of individuals	Haplotype												
			JT1	JT2	JT3	J1	J2	J3	T1	T2	T3	T4	T5	T6	T7
Japan	Miyagi	2	2	–	–	–	–	–	–	–	–	–	–	–	–
	Ibaraki	57	57	–	–	–	–	–	–	–	–	–	–	–	–
	Tochigi	15	15	–	–	–	–	–	–	–	–	–	–	–	–
	Gunma	45	26	2	4	1	12	–	–	–	–	–	–	–	–
	Tokyo	17	7	1	–	–	9	–	–	–	–	–	–	–	–
	Nagano	9	–	–	7	2	–	–	–	–	–	–	–	–	–
	Gifu	4	–	–	3	1	–	–	–	–	–	–	–	–	–
	Shizuoka	7	–	6	–	1	–	–	–	–	–	–	–	–	–
	Aichi	2	–	–	2	–	–	–	–	–	–	–	–	–	–
	Tokushima	15	–	–	12	–	–	3	–	–	–	–	–	–	–
	Kochi	27	–	–	25	–	–	2	–	–	–	–	–	–	–
	Ehime	6	–	–	6	–	–	–	–	–	–	–	–	–	–
	Taiwan	Western Taiwan	10	–	2	–	–	–	–	1	3	1	1	–	1
Eastern Taiwan		7	–	2	3	–	–	–	–	–	–	–	2	–	–
unknown origin in Taiwan		3	1	–	–	–	–	–	–	1	–	–	–	1	–
Total		226	108	13	62	5	21	5	1	4	1	1	2	2	1

**Fig. 2.** A haplotype network of the *Paguma larvata* mtDNA control region sequences found in Japan and Taiwan. Black and white circles indicate haplotypes found only in Taiwan and Japan, respectively. Gray circles show haplotypes found both in Taiwan and Japan. Small open circles mean missing haplotypes. A bar between haplotypes indicate one nucleotide substitution.

Eastern Honshu ($\pi = 0.0000$, $Hd = 0.00$, $\theta_w = 0.00$),
Gunma and Tokyo ($\pi = 0.0024$, $Hd = 0.61$, $\theta_w = 1.06$),

Central Honshu ($\pi = 0.0046$, $Hd = 0.62$, $\theta_w = 1.37$), and Shikoku populations ($\pi = 0.0000$, $Hd = 0.19$, $\theta_w = 0.00$) were lower than those of Eastern Taiwan ($\pi = 0.0060$, $Hd = 0.76$, $\theta_w = 2.45$) and Western Taiwan ($\pi = 0.0062$, $Hd = 0.91$, $\theta_w = 2.83$). In Japan, both the haplotype diversity and nucleotide diversity of the Central Honshu population were higher than those of the Eastern Honshu, Gunma and Tokyo, and Shikoku populations.

Figure 3 shows the haplotype distributions and frequencies of the Japanese population. In Eastern Honshu (Miyagi, Ibaraki, Chiba, and Tochigi Prefectures), JT1 was the major haplotype. Five haplotypes (JT1, JT2, JT3, J1, and J2) were identified from Gunma and Tokyo. In Central Honshu (Nagano, Gifu, Shizuoka, and Aichi) and Shikoku (Tokushima, Kochi, and Ehime), JT3 was found frequently. On the other hand, four haplotypes, JT2 and J1–3, were minor and limited to small areas: JT2 to Shizuoka and Gunma and Tokyo, J1 to Central Honshu, J2 to Gunma and Tokyo, and J3 to Shikoku.

Figure 4 shows the distribution and frequencies of the combination of cytochrome *b* and control region haplotypes in the Japanese and Taiwanese populations. In Japan, the individuals with JA1 had two or three haplotypes of the control region in the present study. Individuals with JA1 were separated to those with JA1/JT1, JA1/JT2, and JA1/J2. JA1/JT2 and JA1/J2 were found only in Gunma and Tokyo. Individuals having JA4 was also

Table 2. Genetic diversity of *Paguma larvata* in Japan and Taiwan

Region group	<i>N</i>	<i>h</i>	π (<i>SD</i>)	<i>Hd</i> (<i>SD</i>)	θ_w (<i>SD</i>)
Japan	206	6	0.0032 (0.0021)	0.64 (0.02)	0.85 (0.41)
All Honshu	158	5	0.0022 (0.0016)	0.51 (0.04)	0.89 (0.43)
– Eastern Honshu	74	1	0.0000 (0.0000)	0.00 (0.00)	0.00 (0.00)
– Gunma and Tokyo	62	5	0.0024 (0.0017)	0.61 (0.04)	1.06 (0.54)
– Central Honshu	22	3	0.0046 (0.0029)	0.62 (0.07)	1.37 (0.73)
Shikoku	48	2	0.0000 (0.0000)	0.19 (0.07)	0.00 (0.00)
Taiwan	20	10	0.0071 (0.0042)	0.91 (0.04)	2.54 (1.17)
Eastern Taiwan	7	7	0.0060 (0.0041)	0.76 (0.11)	2.45 (1.41)
Western Taiwan	10	3	0.0062 (0.0039)	0.91 (0.08)	2.83 (1.46)

N, number of animals; *h*, number of haplotypes; *Hd*, haplotype diversity; π , nucleotide diversity; θ_w , Watterson estimator; and *SD*, standard deviation.

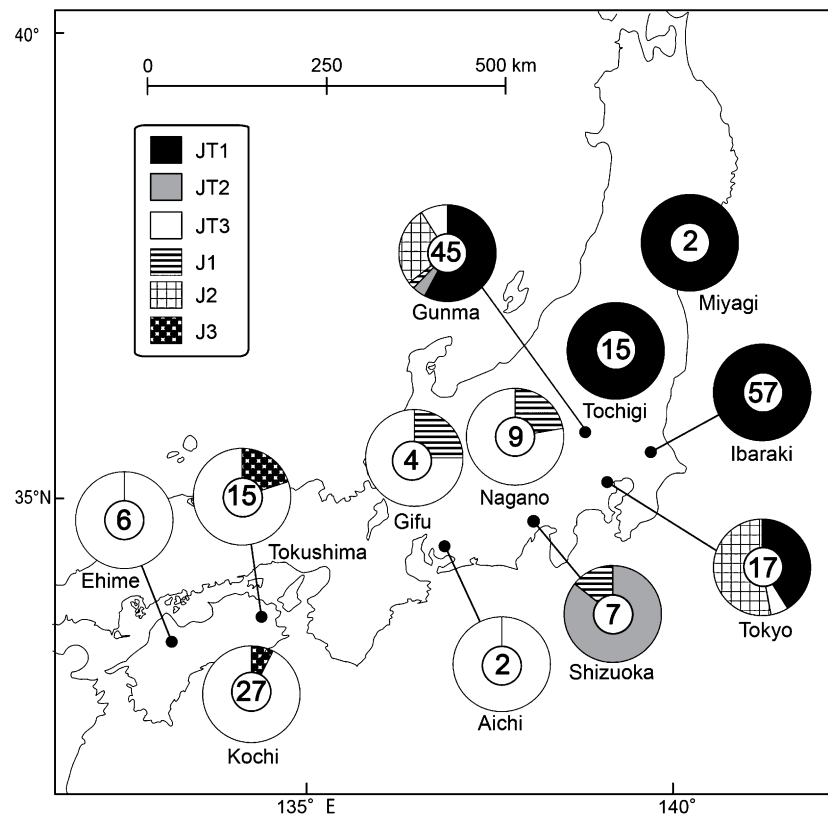


Fig. 3. Distribution and frequencies of the *Paguma larvata* mtDNA control region haplotypes in Japan. The numbers in circles show the sample sizes.

separated to some haplotypes with JA4/JT4 and JA4/J3, both of which were found only in Shikoku. On the other hands, JT2 was connected with different cytochrome *b* haplotypes.

Figure 5 shows the network of the concatenated sequences of mtDNA control region and cytochrome *b*. JA4/J3 was derived from JA4/JT3, and JA1/J2 was from JA1/JT2.

Discussion

Relationship between P. larvata in Japan and Taiwan

In the present study, three haplotypes of the control region (JT1, JT2, and JT3) are identified both from Japan and Taiwan. Of the combination of cytochrome *b* and control region haplotypes, JA1/JT1 and JA4/JT3 were found in both Japan and Taiwan. Therefore, the relation-

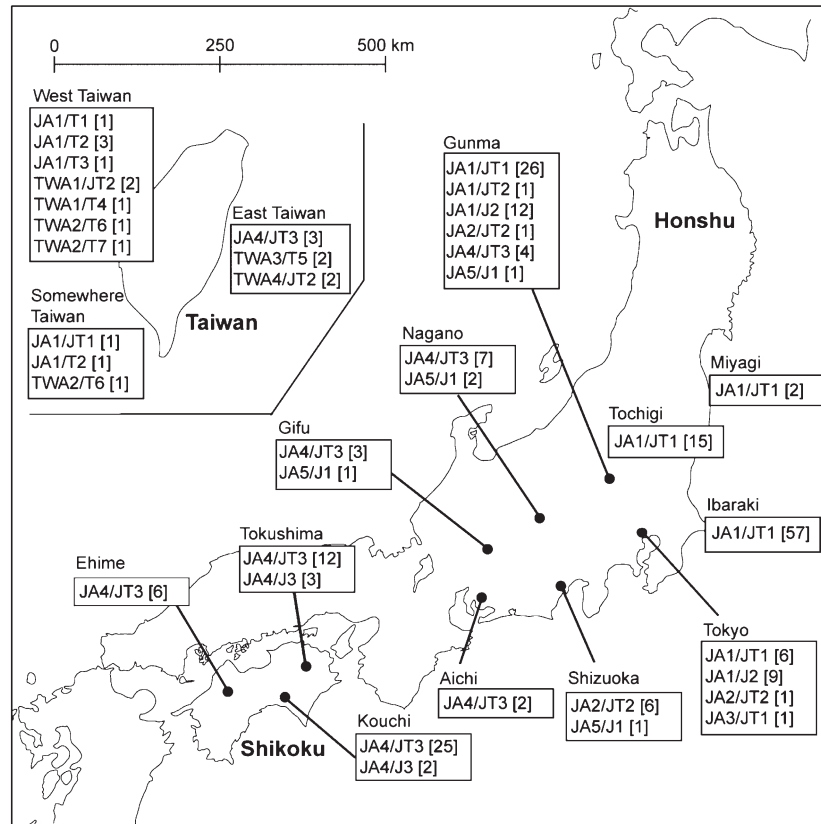


Fig. 4. Distribution and frequencies of combination of *Paguma larvata* mtDNA cytochrome *b* haplotype (Masuda et al. 2010) and control region haplotypes obtained in the present study. The combinations show the cytochrome *b* haplotype/control region haplotype. Numbers in brackets show the frequencies of individuals having those combinations.

ship between individuals in Japan and Taiwan is closely related. Masuda et al. (2010) reported that the origin of *P. larvata* in Japan is Taiwan, although there might be multiple origins to be clarified. Inoue et al. (2012) examined nuclear microsatellite loci and also showed some genetic evidence of the close relatedness between *P. larvata* of Japan and Taiwan. Patou et al. (2009) mentioned the possibility that *P. larvata* of Japan came from areas other than Taiwan while Masuda et al. (2008, 2010) reported that cytochrome *b* haplotypes of Japan were phylogenetically different from those of Southeast Asia (Thailand and somewhere of the Malay Peninsula). Further study is needed for understanding the precise origin of *P. larvata* in Japan.

The genetic diversity of *P. larvata* in Japan was lower than that of Taiwan, even though the sample size for the Taiwanese population was much lower. In general, the genetic diversity of introduced populations is lower than that of natural populations (Dlugosch and Parker 2008). The results indicate that the Japanese population

of *P. larvata* is an introduced population, in agreement with Masuda et al. (2010).

Of the six haplotypes of control region identified from the Japanese *P. larvata* in the present study, three were common between Japan and Taiwan, but the other three were not found in Taiwan. Based on the distribution pattern of cytochrome *b* haplotypes, Masuda et al. (2010) showed that the founder of the eastern Japanese population came from western Taiwan and that that of the western Japanese population was from eastern Taiwan. In the present study, the major haplotype (JT3) in Central Honshu and Shikoku was found in Eastern Taiwan. On the other hand, the major haplotype (JT1) in Eastern Honshu was not seen in Western Taiwan although JT1 was found with unknown origin in Taiwan. Thus, the present study did not reveal further precise original localities for the founder of the Japanese *P. larvata*. Such a result might be responsible for the small sample size in Taiwan: that is, it is possible to find these three types if more efforts on sampling in Taiwan are paid.

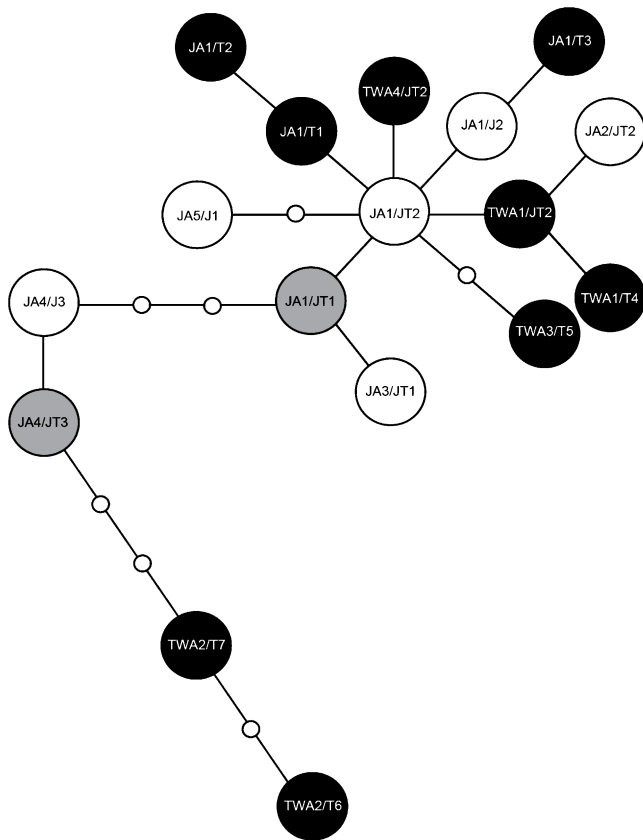


Fig. 5. A haplotype network of the *Paguma larvata* mtDNA cytochrome *b* gene (Masuda et al. 2010) and control region sequences obtained in the present study, in Japan and Taiwan. Black and white circles indicate haplotypes found only in Taiwan and Japan, respectively. Gray circles show haplotypes found both in Taiwan and Japan. Small open circles mean missing haplotypes. A bar between haplotypes indicates one nucleotide substitution.

Expansion of *P. larvata* in Japan

The genetic population structure of *P. larvata* was different between the eastern (Eastern Honshu and Gunma and Tokyo) and western (Central Honshu and Shikoku) areas in Japan. Especially, the combination of cytochrome *b* and control region haplotypes JA1/JT1 was exclusively distributed in Eastern Honshu. Masuda et al. (2010) showed that cytochrome *b* haplotype JA1 was distributed in Eastern Japan, but not found in the other areas. This suggests a low genetic diversity as founder effect in Eastern Honshu. On the other hand, JA4/JT3 was frequently found in Central Honshu and Shikoku, but not seen in Eastern Japan. Haplotype combination JA1/JT1 was not found in Central Honshu nor Shikoku. Thus, founders of Central Honshu and Shikoku could be different from that of Eastern Japan.

Occurrence of haplotype combination JA4/J3 specific

in Shikoku indicates that the Shikoku population is different from the Central Honshu population. JA4/J3 has only single nucleotide difference from JA4/JT3, and thus it is possible that JA4/J3 derived from JA4/JT3 after it was introduced to Shikoku. The origin of the Shikoku population is still unknown. Although JA4/JT3 was highly common in both Shikoku and Central Honshu, Inoue et al. (2012) suggested that the origin of the Shikoku population was different from that of Central Honshu. In addition, they also suggested the possibility that the origin of the Shikoku population was not Taiwan, so more genetic information including the individuals from areas other than Taiwan is needed to clarify the origin.

All individuals with JA5 found only in Central Honshu in the previous study (Masuda et al. 2010) had J1 for the control region haplotype in the present study. In addition, there were no haplotypes derived from JA5/J1. Therefore, haplotype combination JA5/J1 might have been derived from JA1/JT2 with two nucleotide substitution and expanded in Shizuoka, Gifu, Nagano, and Gunma. Inoue et al. (2012) reported that only one genetic cluster was assigned in Chubu District by microsatellite analysis, while multiple haplotypes of the mtDNA control region were found in the present study. Thus, the resultant difference between biparentally and maternally inherited genes were seen. The wider dispersal of males than females could reflect the result by biparentally inherited microsatellites, whereas the data of maternally inherited mtDNA reflects female philopatry.

The genetic constitution in Shizuoka is unique compared with that in other prefectures in Central Honshu, because JA2/JT2 (which was not found in Central Honshu) was the main haplotype combination in Shizuoka. The first capture record of wild individuals in Japan was in Shizuoka (Nawa 1965). Another study based on witness recodes (Furuya 1973) suggested multiple introductions into different locations in Shizuoka because of patchy distribution in Shizuoka. Therefore, in Shizuoka multiple introductions could have been done. It is required to analyze this area using more samples.

In each of Gunma and Tokyo and Central Honshu, more than one haplotype were found, although the haplotype frequencies in each prefecture were different. The genetic population structures based on haplotype distribution in these areas were more complex than that in Eastern Honshu population, where only JA1/JT1 was seen. There are three reasons why the genetic structures of Gunma and Tokyo are caused.

First, JA4/JT3 was not a major but a minor haplotype combination in Gunma. JA1/J2 had the second highest frequency. This indicates that Gunma is a contact zone between Eastern and Central Honshu by population expansion from east to west on Honshu, which agrees with the previous studies of cytochrome *b* gene (Masuda et al. 2010) and microsatellites (Inoue et al. 2012).

Second, Tokyo is also the contact zone between two lineages, because both JA1/JT1 (found mainly in Eastern Honshu) and JA2/JT2 (found most frequently in Shizuoka) were sympatric in Tokyo. It suggests that *P. larvata* with JA2/JT2 was first introduced around Shizuoka and then the descendants expanded their habitats from Shizuoka to eastern Gunma and Tokyo. Such expansion is supported by the previous witness reports, which mentioned that *P. larvata* in Tokyo originated from Shizuoka (Suzuki 1985) through Kanagawa (Nakamura et al. 1989). Inoue et al. (2012) also showed individuals from Eastern Japan and Central Honshu had similar genetic features.

Finally, 21 individuals in Gunma and Tokyo had JA1/J2, which was not found in any other areas, although they were not distinguished from other individuals in Eastern Honshu in the previous studies of cytochrome *b* haplotypes (Masuda et al. 2010) reporting at least two introduction points, Eastern Japan and Western Japan, where haplotypes JA1 and JA4 are dominant, respectively. Inoue et al. (2012) also showed that individuals of Eastern Japan were genetically clustered, whereas there were at least two routes in Western Japan, Central Honshu and Shikoku. However, individuals having JA1 were separated to those with three haplotypes (JA1/JT1, JA1/JT2, and JA1/J2), which two of them (JA1/JT2 and JA1/J2) were found only in Gunma and Tokyo. Two haplotype combinations, JA1/JT2 and JA1/J2, were not found in Eastern Honshu. Therefore, JA1/JT2, from which JA1/J2 were derived, were introduced around Gunma and Tokyo, which is a different introduction point from that of JA1/JT1 introduced around northern areas of Gunma and Tokyo, and expanded.

Cytochrome *b* haplotype JA3 found only in Tokyo (Masuda et al. 2010) was connected with a major control region haplotype in Japan, JT1. Figure 4 shows that JA3/JT1 derived from JA1/JT1, and there are no haplotypes, which derived from JA3/JT1; therefore, JA3/JT1 might have derived from JA1/JT1 after introduced to Japan.

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