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Geographic Distribution and Genetic Structures of the Tideland Snails *Pirenella nipponica* and *P. asiatica* in Taiwan and Japan

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The tideland snails *Pirenella nipponica* and *Pirenella asiatica* are distributed north of the central Ryukyu Islands and in South Ryukyu, respectively, in Japan. To reveal their distribution and genetic characteristics in Taiwan, we sampled *Pirenella* snails along the western coast of Taiwan Island and analyzed the nucleotide sequences of their mitochondrial DNAs. *Pirenella nipponica* and *P. asiatica* inhabit the northern and southern parts of the western coast of Taiwan, respectively, and coexist only in the central part. Taiwanese and Japanese populations of *P. asiatica* showed significant genetic differentiation. The former showed higher genetic diversity and a larger effective population size than the latter. However, the Taiwanese population of *P. nipponica* was not genetically deviated from the local Japanese population on Kyushu Island. Both the Taiwan and Kyushu populations of *P. nipponica* showed significant genetic differences from local populations in other regions of Japan, namely, Honshu Island (the Japanese mainland) and Central Ryukyu. They also showed higher genetic diversity and a larger effective population size than the others. The Taiwanese populations of both species might be part of a large panmictic population with individuals from the Asian continent and Kyushu Island.

Key words: Tideland snail, *Pirenella*, Taiwan Island, Nansei Islands, Phylogeography

BACKGROUND

Taiwan Island is situated about 110 km west of the southernmost part of Japan, the Nansei (Ryukyu) Islands, which are situated between Taiwan and Kyushu Islands and consist of the south Ryukyu, central Ryukyu, and north Ryukyu regions (Fig. 1). The Taiwan and Kyushu Islands are separated from the Asian continent by a continental shelf with a depth corresponding approximately to the fall in the sea level

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during the last glacial maxima (Taiwan and Tsushima Straits; Saito 1998). That said, the last connection between Taiwan and the Nansei Islands is considered to have been completed by a much older event, namely the collapse of the Pleistocene land bridge around 0.4 Ma (Kizaki and Oshiro 1980). The divergence ages of amphibians and reptiles between Taiwan and South Ryukyu have also been estimated to be older than a million years (Kaito and Toda 2016). The deep Tokara Gap has isolated Central Ryukyu from North Ryukyu and Kyushu for a much longer time (Kizaki and Oshiro 1980; Osozawa et al. 2012).

Three snail species in the genus *Pirenella*, *P. nipponica*, *P. asiatica*, and *P. pupiformis*, dominate the tidelands of Taiwan and Japan (Reid and Ozawa 2016). The former two species were classified as a single species, *Cerithideopsilla cingulata*, of which a local population inhabiting the southern part of South Ryukyu (the Yaeyama insular group) was shown to have genetically deviated from other local Japanese populations (Kojima et al. 2006; Ozawa et al. 2015). Reid and Ozawa (2016) revised the genus *Pirenella* and described *C. cingulata*-like individuals distributed in the Yaeyama insular group, Taiwan, and southeastern Asia and those inhabiting Honshu (the Japanese mainland), Kyushu, the Korean Peninsula, continental China, and Taiwan as the new species *Pirenella asiatica* and *P. nipponica*, respectively.

To date, few studies have analyzed the phylogenetic relationships between Taiwanese and Japanese populations of tideland snails. In this article, we report the geographical distribution in Taiwan and the genetic characteristics of the Taiwanese and Japanese populations.

MATERIALS AND METHODS

A total of 79 individuals of *Pirenella nipponica* and *P. asiatica* were collected from five sites along the western coast of Taiwan Island during 4–7 Feb., 2020 (Fig. 1; Table 1). They were preserved in 99% ethanol in Taiwan and kept in a freezer (-30°C) after being transferred to Japan. Total DNA was extracted from the head-foot region of each individual using the DNeasy Blood & Tissue Extraction Kit (QIAGEN, Valencia, CA, USA) and purified using Gene Releaser (BioVentures Inc., Murfreesboro, TN, USA). A

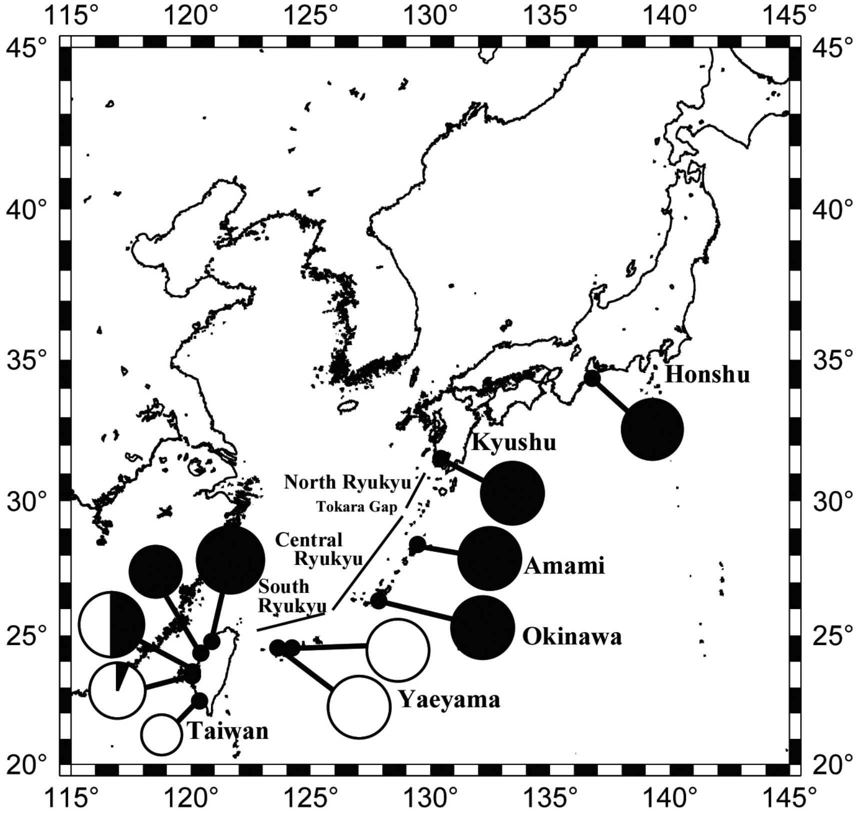

Fig. 1. Sampling sites of *Pirenella nipponica* and *P. asiatica*. Sampling at Japanese sites was carried out in the previous study (Kojima et al. 2006). Areas shown in the pie graphs reflect the number of collected individuals. Black and white sectors in the pie graphs indicate relative frequencies of *P. nipponica* and *P. asiatica*.

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part of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene was amplified by polymerase chain reaction (PCR) using the primer sets either LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3'; Folmer et al. 1994), PniCOIf (5'-GATCWGGYTTAGTYGGRACTGCTYTRAG-3'), or COI-B (5'-GGATGAACNGTNTAYCCNCC-3'; Hasegawa et al. 1996) and either PniCOIr (5'-GTYARTCCYCCWACTGTRAAYAGGAAA-3') or COI-6 (5'-GGRTARTCNSWRTANCGNCGNGGYAT-3'; Shimayama et al. 1990). Primers PniCOIf and PniCOIr were newly designed based on the sequences of these species reported by Kojima et al. (2006). The PCR conditions were as follows: an initial denaturation at 94°C for 2 min, followed by 30–40 cycles of denaturation at 94°C for 40 s, annealing at 50°C for 1 min, and extension at 72°C for 90 s. The PCR products were purified using Exo-SAP-IT (United States Biochemical, Cleveland, OH, USA) for use in cycle-sequencing reactions. Nucleotide sequences of the same region (516 bp) in the *COI* gene as used in the previous study (Kojima et al. 2006) were determined using the BigDye Terminator Cycle Sequencing Kit, version 3.1 (Applied Biosystems, Foster City, CA, USA) with primers PniCOIf, COI-B, PniCOIr, or COI-6.

To clarify the phylogenetic relationships among haplotypes, haplotype networks were constructed using the TCS method (Clement et al. 2000) in the PopART software (Leigh and Bryant 2015). The genetic diversity of populations on the basis of haplotype (gene) diversity (*h*), which is the probability that two randomly chosen haplotypes are different (Nei 1987), and nucleotide diversity (π), which is the probability that two randomly chosen homologous nucleotides are different (Tajima 1983; Nei 1987) were estimated using Arlequin version 3.5.2.2. (Excoffier and Lischer 2010). Neutral tests, Tajima's D (Tajima 1989a b) and Fu's F_S (Fu 1997), and mismatch distribution analysis (Rogers and Harpending 1992) were conducted using Arlequin. The significance of the neutrality tests was assessed by 1,000 permutations. Relative effective population size was estimated as the value at the present ($t = 0$) on the Bayesian skyline plot using BEAST version 1.6.2. (Suchard et al. 2018). Runs were recorded every 1,000 steps out of a total of 10,000,000 steps with a burn-in of 1,000,000 steps. The differences in haplotype frequencies between populations were examined using the exact test of population differentiation (Raymond and Rousset 1995) performed with Arlequin. The unbiased fixation index, F_{ST} (Weir and Cockerham 1984), was estimated, and its significance was tested using a nonparametric permutation approach (10,000 permutations) performed with Arlequin.

RESULTS

Based on the nucleotide sequences of the *COI* gene, 79 individuals were identified as 47 *Pirenella nipponica* and 32 *P. asiatica* individuals. The former was obtained from the northern (Xiangshan and Sinkang) and central (Budai and Beimen) parts of the western coast of Taiwan Island, and the latter was sampled from the central and southern (Dapeng Bay) parts (Table 1; Fig. 1). Despite the short distance between Budai and Beimen (approximately 12 km), the compositions of the two species were markedly different.

The nucleotide sequences obtained from *P. nipponica* and *P. asiatica* were classified into 33 and 19 haplotypes, respectively (Table 2). Three *P. nipponica* and two *P. asiatica* haplotypes have been reported in Japanese individuals (Kojima et al. 2006). The haplotype networks of both the species are shown in figure 2. For *P. nipponica*, all individuals collected from Central Ryukyu formed an exclusive group with the exception of two individuals from Amami that shared a haplotype (haplotype 24) with individuals from Kyushu and Taiwan. All Honshu individuals formed a group with a few individuals from Kyushu. A haplotype situated in the center of the star-shaped partial network (haplotype 24) was dominant in Kyushu and Taiwan. For *P. asiatica*, the dominant haplotypes differed between the Taiwanese and Japanese populations and

Table 1. List of sampling sites

Site	Location	N	
		Pirenella nipponica	*P. asiatica*
Xiangshan	24º46'53.8"N, 120º54'50.9"E	21	
Sinkang	24º09'31.7"N, 120º27'33.2"E	14	
Budai	23º21'47.2"N, 120º07'52.1"E	11	11
Beimen	23º16'54.0"N, 120º05'58.7"E	1	15
Dapeng Bay	22º26'51.3"N, 120º27'37.0"E		6

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relatively rare haplotypes in the Japanese population were shared with the Taiwanese population.

The genetic diversity, Tajima's D, Fu's F_S, τ value of mismatch distribution, and relative effective population size were estimated for local populations from which more than 10 individuals were obtained (Table 3). Taiwanese populations of both species and the Kyushu population of *P. nipponica* showed higher genetic diversity and a larger effective population size than other Japanese populations. Most local populations of both species showed recent sudden expansion. Based on the nucleotide divergence rate of the *COI* gene of marine gastropods in the genus *Tegula* (2.4% per million years; Hellberg and Vacquier 1999), their expansion was estimated to have occurred between 660,000 and 860,000 years ago, except for the Honshu and Okinawa populations of *P. nipponica* (240,000 and 82,000 years ago, respectively). Only the Honshu and Okinawa populations showed significant negative values for both Tajima's D and Fu's F_S, which suggests demographic expansion after past population bottleneck (Table 3). These results suggest that drastic changes in the population size of both species did not occur during and after the last glacial period.

The significance of genetic differentiation was tested between the local populations from which more than 10 individuals were obtained. For *P. nipponica*, no significant differentiation was detected among the Kyushu and Taiwanese populations ($p > 0.05$), while the Honshu, Amami, and Okinawa populations showed significant differences from other populations ($p < 0.01$ or $p < 0.05$; Table 4). For *P. asiatica*, significant genetic differentiation was observed between the Japanese and Taiwanese populations by the exact test ($p < 0.01$), whereas the difference was significant only between local populations of Ishigaki Island and Beimen by the F_{ST} test ($p < 0.05$; Table 5). However, when each of the Japanese and Taiwanese local populations was treated as a single group, significant genetic differentiation was observed between them by both the F_{ST} test ($F_{ST} = 0.102$;

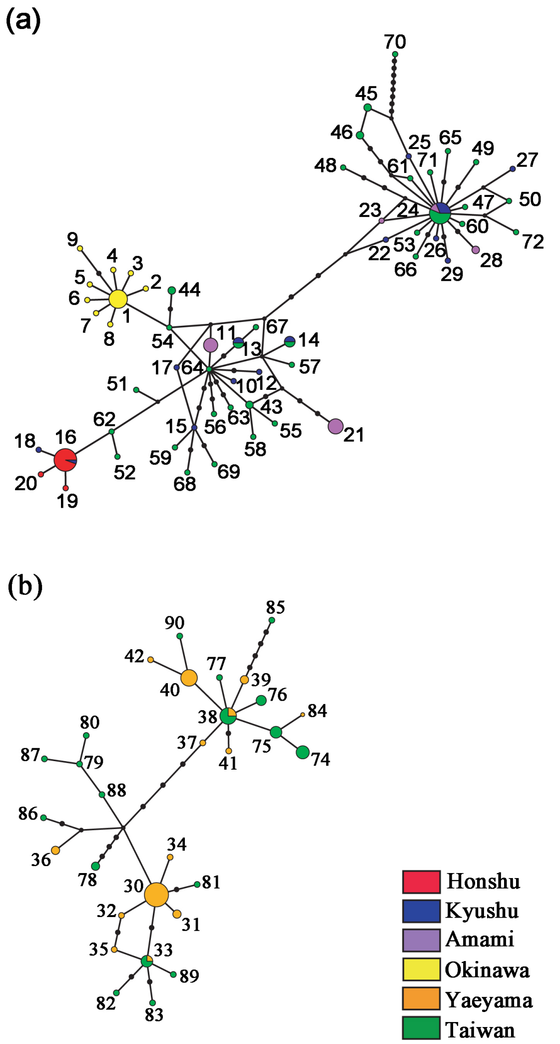

Fig. 2. A statistical parsimony haplotype network of *Pirenella nipponica* (a) and *P. asiatica* (b) based on nucleotide sequences of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene. The areas of the circles are proportional to the frequency of the occurrence of the haplotypes. Numbers of haplotypes are same as those in table 2 and Kojima et al. (2006). Colors of sections denote the relative occurrence frequency of individuals collected in each area. The haplotypes that were not detected in the sample are indicated by small black circles.

Table 2. Haplotype composition of *Pirenella nipponica* and *P. asiatica*

Spacies		Haplotype																		
	Sampling site	13*	14*	24*	33*	38*	43	44	45	46	47–59	60–66	67–71	72	73	74	75	76–82	83–88	89
Pirenella nipponica	Xiangshan		2	3			1			1	each 1									
	Sinkang			4			1	2				each 1								
	Budai	2		2					2	1			each 1							
	Beimen													1						
P. asiatica	Budai					1									3			each 1		
	Beimen				2	3										4			each 1	
	Dapeng Bay					1									1		3			1

*Haplotypes reported in Kojima et al. (2006)

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$p < 0.01$) and the exact test ($p < 0.01$).

DISCUSSION

Pirenella nipponica and *P. asiatica* have not yet been discovered to coexist in Japan; the present study showed for the first time that they coexist at two tidelands in the central parts of the Taiwanese western coast. Thus, their exclusive distribution in Japan might be attributable to environmental and/or historical factors rather than competitive exclusion. They were not discovered on Miyakojima Island, which is located between the Yaeyama insular group and Central Ryukyu, during the sampling by Kojima et al. (2006), and Yonagunijima Island, which is located between Taiwan and the Yaeyama insular group during the recent survey (Kano, personal communication). However, the

Table 3. The genetic diversity, results of neutrality tests, τ value, and relative effective population size of local populations of *Pirenella nipponic*a and *P. asiatica*

Species	Area	Site	N	Gene diversity (h)	Nucleotide diversity (π)	Tajima's D	Fu's F_S	τ	Relative effective population size
Pirenella nipponica	Honshu	Mie[a]	19	0.2047 ± 0.2291	0.000408 ± 0.000578	-1.51077*	-1.80438*	3.000***	0.0004050
	Kyushu	Kagoshima[a]	20	0.9368 ± 0.0427	0.011873 ± 0.006584	-0.50554	-3.95037*	8.938***	0.0033292
	Amami	Amai-Ohshima Island[a]	20	0.7316 ± 0.0644	0.009894 ± 0.005593	1.82839	4.07574	8.150	0.0001834
	Okinawa	Okinawajima Island[a]	20	0.6526 ± 0.1225	0.001744 ± 0.001413	-2.21889**	-7.31547**	1.010***	0.0006390
	Taiwan	Xiangshan	21	0.9810 ± 0.0225	0.012763 ± 0.007010	-1.01066	-9.28902**	9.176***	0.0173161
		Sinkang	14	0.9231 ± 0.0604	0.011543 ± 0.006577	-0.22134	-1.92647	10.268***	0.0038922
		Budai	11	0.955 ± 0.0535	0.016068 ± 0.009103	-0.13155	-0.18615	10.585***	0.0020846
		Beimen	1						
		Total	47	0.9630 ± 0.0202	0.013216 ± 0.007033	-1.50751*	-23.92969**	9.445***	0.0158787
	Taiwan + Kyushu		67	0.9516 ± 0.0193	0.012750 ± 0.006762	-1.60096*	-25.12283**	9.277***	0.0246237
P. asiatica	Yaeyama	Ishigaki Island[a]	20	0.7105 ± 0.0889	0.007354 ± 0.004316	0.43757	0.72932	8.393	0.0006693
		Iriomote Island[a]	20	0.8684 ± 0.0640	0.008966 ± 0.005127	-0.12917	-2.01369	8.266***	0.0023031
		Total	40	0.7846 ± 0.0563	0.008005 ± 0.004521	-0.07795	-1.53346	8.205***	0.0021455
	Taiwan	Budai	11	0.9455 ± 0.0659	0.014165 ± 0.008106	0.08832	-1.67099	9.723***	0.0022279
		Beimen	15	0.9048 ± 0.0544	0.011406 ± 0.006471	-0.36461	-0.62009	8.742***	0.0018706
		Dapeng Bay	6						
		Total	32	0.9476 ± 0.0213	0.011515 ± 0.006275	-0.98898	-5.98138*	9.348***	0.0085035

[a]Refferd from Kojima et al. (2006); *$p < 0.05$; **$p < 0.01$; ***Mismatch distribution not significantly deviated from the expansion model ($p > 0.05$).

Table 4. Pairwise F_{ST} values between local populations of *Pirenella nipponica*

	Honshu	Kyusyu	Amami	Okinawa	Xiangshan	Sinkang
Kyusyu	0.571**					
Amami	0.635**	0.117*				
Okinawa	0.899**	0.495**	0.538**			
Xiangshan	0.550**	-0.025	0.093**	0.446**		
Sinkang	0.656**	-0.022	0.132*	0.538**	-0.026	
Budai	0.641**	0.027	0.157**	0.532**	0.025	0.026

*$p < 0.05$; **$p < 0.01$.

Table 5. Pairwise F_{ST} values between local populations of *Pirenella asiatica*

	Ishigaki Island	Iriomote Island	Budai
Iriomote Island	-0.038		
Budai	0.068	0.032	
Beimen	0.100*	0.053	-0.028

*$p < 0.05$.

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possibility that they coexist on other islands around these two islands cannot be ruled out. As reproductive isolation between related species can be tested by comparing nuclear and mitochondrial DNA markers between individuals of each species inhabiting the same habitat (Hirose et al. 2022), the present finding is very important for further taxonomic studies.

Taiwan Island is the southern limit of the distribution of *P. nipponica*, whereas the northern limit of *P. asiatica* is the Yaeyama insular group (Reid and Ozawa 2016). The average lowest and highest temperatures from 1991 to 2020 were, respectively, 17.4°C and 29.4°C at Naha, the Okinawajima Island, 18.8°C and 29.8°C at Ishigaki, the Yaeyama insular group, 16.6°C and 30.6°C at Taipei, the northern part of Taiwan, and 18.7°C and 28.6°C at Tainan, the southern part of Taiwan (Pegasusweb; https://www.climatestotravel.com/). The lowest temperature of approximately 18.0°C possibly limits their geographical distribution. *Pirenella nipponica* and *P. asiatica* form a monophyletic group with *P. retifera*, which is distributed in the Philippines (Reid and Ozawa 2016). As *P. asiatica* is more closely related to *P. retifera* than to *P. nipponica* (Reid and Ozawa 2016), *P. asiatica* might be more sensitive to low temperature than *P. nipponica*.

The Taiwanese population of *P. nipponica* showed no genetic deviation from the Kyushu population despite significant deviations from other Japanese populations. Taiwan and Kyushu, adjoining the Asian continent, interpose the continental shelf, most of which became land during the glacial periods (Saito 1998). Although *P. nipponica*, which has a planktonic larval period of approximately 12 days (Kimura et al. 2002; Kimura, personal communication) may be able to disperse between Kyushu and the Asian continent, the rise in sea level after the glacial periods possibly accelerated the dispersal. The present results suggest that *P. nipponica* individuals from Taiwan and Kyushu may be part of a single large panmictic population with continental populations. Indeed, the Taiwanese and Kyushu populations, as well as a population combining both, showed higher genetic diversity and a larger effective population size than the others (Table 3). Kamimura et al. (2010) reported that local populations on the western coast of Kyushu Island (Kumamoto) and the southernmost part of the Korean Peninsula (Suncheon) shared a dominant haplotype. Unfortunately, their data could not be used in the present analyses because of the marginal overlap in the sequences between the two studies. For *P. asiatica*, the Taiwanese population showed higher genetic diversity and a larger effective population size than the Japanese population. The former may also compose a large panmictic population with individuals from the Asian continent. To confirm this hypothesis, analyses using a dataset containing continental populations are required. That said, the Taiwanese population of the neon damselfish *Pomacentrus coelestiis* genetically deviated from both the Okinawa and Kyushu populations (Liu et al. 2008) and genetic deviation between the Japanese and continental populations was shown for the mudskipper *Periophthalmus modestus* (He et al. 2015). Genetic connectivity via the Asian continent might not be a general phenomenon among Taiwanese and Japanese coastal species.

The Japanese *Cerithideopsilla cingulata*-like species (*P. nipponica* + *P. asiatica*) was categorized as a near-threatened species (Japanese Association of Benthology 2012). The present results show genetic characteristics and, therefore, conservative values were different among local populations. For example, the local population of *P. nipponica* in the Okinawa area is the most genetically deviated among the Taiwanese and Japanese conspecific populations (Fig. 2a; Table 4). Significant negative Tajima's D and Fu's F_S values (Table 3) suggest past population bottleneck promoted the genetic deviation. The genetic uniqueness of populations in South and Central Ryukyus has been revealed for the tideland crab *Macrophthalmus banzai* (Aoki et al. 2012; Kobayashi et al. 2023) and the soft shore barnacle *Fistulobalanus albicostatus* (Chang et al. 2017). Only one of two clades of the tideland crab *Tubuca arcuata* is distributed in Central Ryukyus while both clades inhabit Taiwan Island (Shih et al. 2022). Tideland species in Taiwan and Japan need to be conserved considering their population structures.

CONCLUSIONS

Pirenella nipponica and *P. asiatica* inhabit the northern and southern parts, respectively, and coexist only in the central part along the western coast of Taiwan. Their Taiwanese populations are genetically deviated from and show higher genetic diversity and larger effective population size than the Japanese populations, except for the local population of *P. nipponica* on Kyushu Island. These Taiwanese populations might be part of a large panmictic population with individuals from the Asian continent and Kyushu Island.

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Authors' contributions: Specimen collection: MO, TH, TC, and SKo. Molecular analysis: Ska, HF, and SKo. Manuscript writing: SKo.

Competing interests: All authors declare that they have no conflicts of interest.

Availability of data and materials: The material analyzed is available at Atmosphere and Ocean Research Institute, the University of Tokyo. The nucleotide sequences reported in the present study will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under the accession numbers LC754205–754251. (haplotypes 43–89)

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