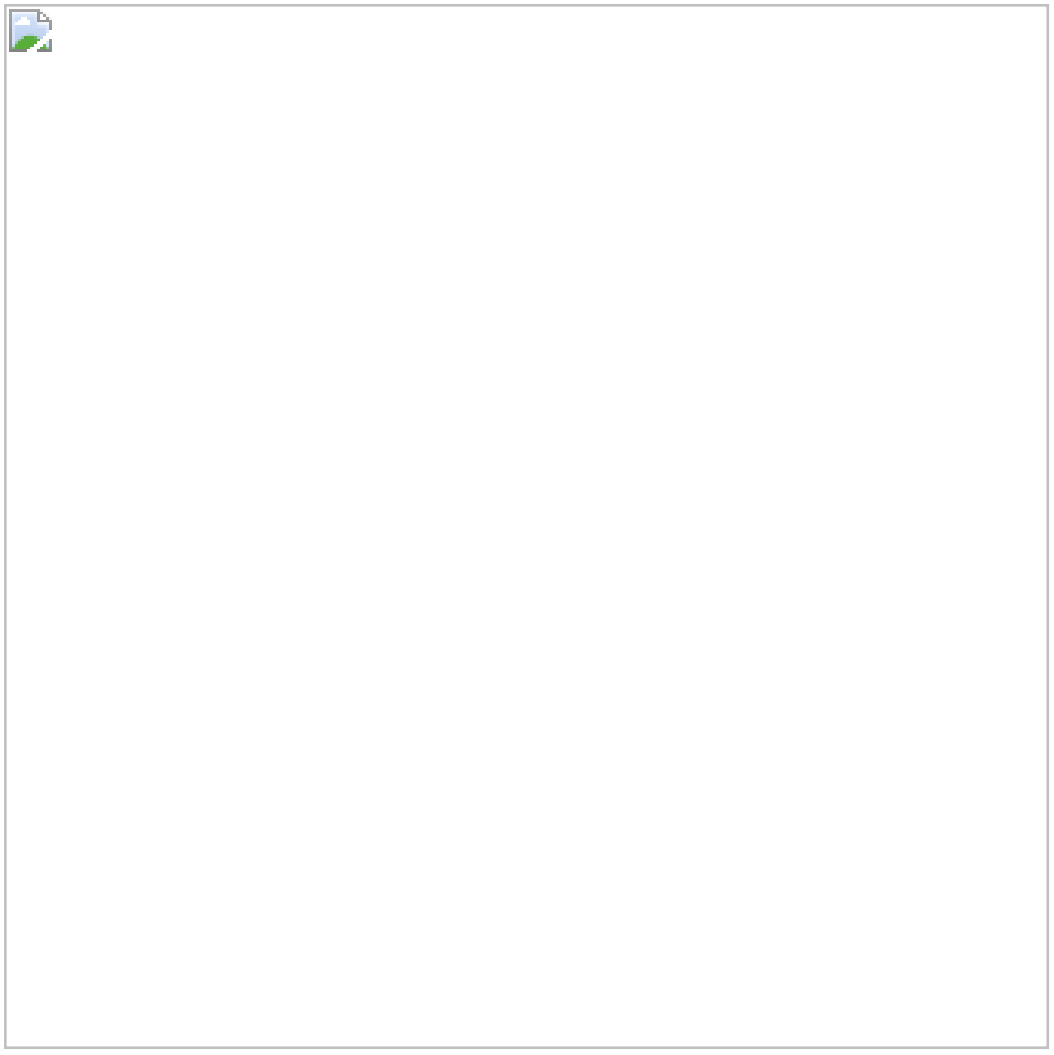

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Genetic Diversity and Population Structure of the Japanese Serow (*Capricornis crispus*) in Gunma Prefecture Based on Mitochondrial DNA Control Region Sequences

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The Japanese serow, *Capricornis crispus*, is a species endemic to Japan, residing in the mountainous forests of Honshu, Shikoku, and Kyushu. Gunma Prefecture, situated in central Honshu, is one of its habitats. To evaluate the genetic diversity and interrelationships among local populations in Gunma Prefecture, we examined the mitochondrial DNA control region sequences of 364 individuals. Our analysis, using all accessible Japanese serow sequences, revealed that they can be broadly categorized into four clades, labeled I to VI. Within Gunma Prefecture, we identified 15 distinct haplotypes, which can be classified into three haplogroups: G1, G2, and G3. G1, included in clade I, is associated with those reported in northeastern Honshu. G2, which forms clade IV on its own, is predominantly endemic to Gunma Prefecture, with a frequency of 90% in Showa Village at the western base of Mt. Akagi. In contrast, G3, included in clade V, forms a sister group to the haplotype discovered in the Japanese Alps. The haplotype composition exhibited a stark contrast between the regions on the west and east sides of the Kanto Plain, indicating that the Kanto Plain serves as a dividing line for Japanese serow populations. In Tsumagoi Village, 134 out of 144 animals shared the same haplotype, resulting in extremely low haplotype diversity, as indicated by a significant negative value in neutrality tests. This finding aligns with the observed rapid increase in serow in Tsumagoi.

Key words: genetic diversity, mitochondrial DNA control region sequences, haplotypes, Kanto Plain, Japanese serow (*Capricornis crispus*), phylogenetic relationships, haplogroups

INTRODUCTION

The Japanese serow (*Capricornis crispus*) is a species endemic to the Japanese archipelago, specifically residing in the mountain forests of Honshu, Shikoku, and Kyushu (Ohdachi et al., 2015). In the early 20th century, the serow population was severely threatened due to human hunting, leading to a significant decline in their numbers. Consequently, it has been protected as a special natural monument since 1955. Due to conservation efforts, the Japanese serow population in much of Honshu has recovered over the past 50 years and is no longer considered endangered. However, the populations in Shikoku, Kyushu, and the Kii Peninsula have not experienced similar recovery and were added to the threatened local population in the 2012, 2015, and 2020 Japanese Red Lists, respectively. Currently, Japanese serows in Honshu are broadly classified into 33 regional populations (Ministry of the Environment, 2010; Agency for Cultural Affairs, 2022). These classifications are

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based on topography and individual density but lack genetic support. Furthermore, the boundaries of each population are unclear and often overlap with neighboring populations. Thus, it remains uncertain whether the current regional population classifications are optimal for the conservation and management of Japanese serows. In 1978, licensed culling of the Japanese serow commenced due to significant damage to conifer plantations and crops in the 1970s. Since then, intermittent culling has been implemented in areas where the population density is high and the impact on agriculture and forestry is substantial.

Gunma Prefecture, spanning an area of 6362 km^2, is situated in the northwesternmost part of the Kanto region, less than 100 km from Tokyo. It borders the Chubu and Tohoku regions, positioning it almost at the center of the Japanese serow's distribution in Honshu (Fig. 1). Most of Gunma Prefecture is mountainous, with the exception of the central and southeastern parts, which form a portion of the Kanto Plain. The Tone River flows through the central part of this plain. The mountainous regions of Gunma Prefecture are densely populated by Japanese serows, which occasionally inflict substantial feeding damage on agriculture and forestry. In 2014, when the damage was at its peak, the total cost inflicted by the Japanese serows in Gunma Prefecture was recorded at approximately 250 million yen (Gunma Prefecture Government, 2021). Specimens from culled individuals, as well as animals found deceased due to traffic accidents, have been collected and preserved at the prefectural museum.

In phylogeographic studies of wild fauna, mitochondrial DNA (mtDNA) is a commonly employed molecular genetic marker. Its straightforward maternal inheritance and swift sequence divergence rate make it suitable for analyzing short-term evolutionary phenomena (Brown et al., 1986). Specifically, the control region, also known as the displacement-loop region (D-loop), is the primary noncoding regulatory region for mtDNA transcription and replication. It possesses the highest substitution rate across the entire mtDNA (Tamura and Nei, 1993). Therefore, polymorphisms of the mtDNA control region have been widely used to detect intraspecific variation and to investigate the genetic structure of populations (Bradley et al., 1996; Loehr et al., 2006; Moravčíková et al., 2020).

While there have been mtDNA studies on certain local populations of the Japanese serow, comprehensive phylogenetic analyses encompassing the entire habitat are lacking (Okumura, 2004; Iwahori et al., 2019). To understand the population structure of Japanese serows, studies employing nuclear markers in addition to mtDNA are essential. A study utilizing 13 autosomal microsatellite markers to investigate the genetic diversity of the population in the Kii Peninsula has been reported (Iwahori et al., 2019). Furthermore, Hori et al. (2024) analyzed a total of 23 microsatellite markers, including six newly developed loci, with the aim of individual identification and social structure analysis. However, only five loci are common between these two studies. Consequently, at present, the necessary reference information for other regions to position the serows in Gunma Prefecture is unavailable for microsatellite markers. Therefore, in this report, we have chosen to concentrate on the survey of mtDNA, for which reference information is readily accessible in databases.

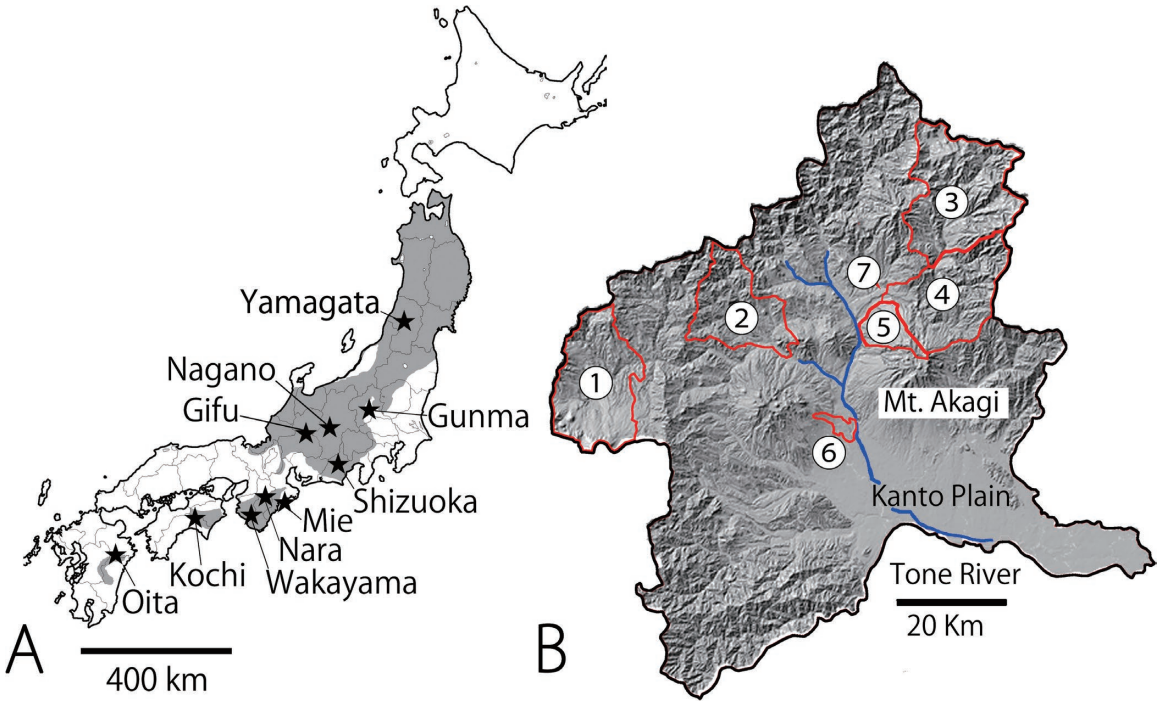

Fig. 1. The habitat of *Capricornis crispus* within the Japanese archipelago. It includes two maps: Map A (left) shows the prefectures where the haplotypes used in this study were detected, and Map B (right) displays the locations of local populations in Gunma Prefecture. The areas in Gunma Prefecture where the serow specimens were collected are indicated by circled numbers from 1 to 7 (1: Tsumagoi, 2: Nakanojo, 3: Katashina, 4: Tone, 5: Showa, 6: Tomioka, 7: Numata). Due to municipal merger, Tone is now Tone-cho, Numata City.

In this study, we sequenced the mtDNA control region of 364 serows in Gunma Prefecture. By examining a large number of individuals captured within a relatively brief period, we were able to elucidate the geographic structure of the Japanese serow's genetic diversity within Gunma Prefecture. Interestingly, despite the topographical continuity of the mountainous region in northern Gunma Prefecture, the maternal lineages of the Japanese serow exhibited significant variation based on location. We also investigated potential differences in the distribution of mtDNA haplotypes between males and females. The findings from this study offer valuable insights into the genetic variability and phylogeographic patterns of the Japanese serow. This information is crucial for devising effective conservation and management strategies.

MATERIALS AND METHODS

Sampling site, DNA extraction, and sequencing

Our analysis involved 364 Japanese serow samples preserved at the Gunma Museum of Natural History. These samples, small pieces of skeletal muscle, were stored at room temperature in 95% ethanol. The samples were collected from six municipalities in Gunma Prefecture between 2009 and 2022 (Fig. 1). The sample breakdown is as follows: 144 individuals (83 males and 61 females) from Tsumagoi (337.6 km^2), three individuals (two males and one female) from Nakanojo (439.3 km^2), 78 individuals (41 males and 37 females) from Katashina (391.8 km^2), 40 individuals (22 males and 18 females) from Tone (278.9 km^2), 97 individuals (48 males and 49 females) from Showa (64.14 km^2), one female from Tomioka, and one female from Numata. The individuals from Tomioka and Numata were found deceased in highway and railroad accidents, respectively, outside the typical Japanese serow habitat.

Approximately 50 mg of ethanol-fixed tissue was cut from each sample and treated overnight at 4°C in 1 ml of 10 mmol/L EDTA solution (pH 8.0). DNA was extracted using the QuickGene DNA tissue kit (FUJIFILM, Tokyo, Japan) as per the manufacturer's instructions. A segment of the mtDNA control region was amplified by PCR using the primer pair LS-1 (5′-AATATACTGGTCTTGTAAACC-3′) and HS-3 (5′-AGGCATTTTCAGTGCCTTGC-3′) (Okumura, 2004). PCR reactions were conducted using KAPATaq EXtra DNA polymerase (Kapa Biosystems, Woburn, MA, USA) under the following conditions: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 15 s, and extension at 72°C for 80 s, with a final extension at 72°C for 5 min. The PCR product was purified using a High Pure PCR Product Purification Kit (Roche, Mannheim, Germany). The purified PCR products were directly sequenced using the BigDye ™ Terminator v3.1 Cycle Sequencing Kit with LS-1 primers on all individuals and analyzed on the Applied Biosystems 3130xl or 3730xl Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). In many cases, an 842-bp partial sequence of the control region was obtained using only the LS-1 primer, from the start of the D-loop (5′-AATCACCACTAA-3′) to (5′-CAGGACATACTAT-3′). If sequencing with the LS-1 primer was unsuccessful, we performed alternative sequencing for the left side using the newly designed sequencing primer HS-SeqX4 (5′-AGGAGTACTGGGATGCATTG-3′). Subsequently, the full length of the control region (approximately 1031 bp) was determined by sequencing the right side using an additional sequencing primer, LS-Seq1 (5′-GAAACCAGCAACCCGCTAGA-3′). Among the 125 individuals exhibiting the Gunma-1 haplotype and 138 individuals exhibiting the Gunma-4 haplotype in the 842-bp sequence comparison on the left, 49 and 63 individuals, respectively, did not undergo additional sequencing by LS-Seq1.

Data analysis

The mtDNA control region sequences of the Japanese serow in Gunma Prefecture were organized alongside publicly available sequences reported from nine other prefectures (Fig. 1). The 52 cited Japanese serow haplotypes include three from Yamagata Prefecture (Yama-a–Yama-c), 11 from the Kiso region of Nagano Prefecture (Kiso-a–Kiso-k), 15 from Shizuoka Prefecture (Shizu-a–Shizu-o), seven from Gifu Prefecture (Gifu-1–Gifu-7), 11 from the Kii Peninsula spanning Mie, Nara, and Wakayama prefectures (Kii-1–Kii-11), four from Kochi Prefecture in Shikoku (Kochi-a–Kochi-d), and one from Oita Prefecture in Kyushu (Oita-a). Given that the habitats of the Japanese serow in Gifu, Nagano, and Shizuoka Prefectures fall within the Japanese Alps Mountain range, these prefectures are referred to as the Japanese Alps region in this paper. Details of these sequences, including accession numbers, are provided in Supplementary Table S1. Additionally, we utilized nine sequences of the Sumatran serow (*Capricornis sumatraensis*) (Csum1–Csum9: KC306696–KC306704), nine sequences of the Formosan serow (*Capricornis swinhoei*) (NT1: AF547433, NT3–NT4: AY149641–AY149642, TD1: AY139642, TD2–TD3: AY149644–AY149645, TD5: AY149643, HL1–HL3: AY149638–AY149640, and WL1:AY149646), and a sequence of the Himalayan goral (*Naemorhedus goral*: EU259133) for outgroup construction. The sequence alignment was performed using ClustalW (Thompson et al., 2002).

Phylogenetic relationships were analyzed using MEGA-X for both neighbor-joining (NJ) and maximum likelihood (ML) approaches (Kumar et al., 2018). For the NJ analysis, the Tamura three-parameter method (Tamura, 1992) was applied, incorporating a gamma distribution to model rate variation among sites. In the ML analysis, the "Find best DNA models" (ML) option tests in MEGA-X were employed to determine the most suitable nucleotide substitution pattern model. The HKY G+I model (Hasegawa et al., 1985) was identified as the best fit. In both NJ and ML analyses, sequence gaps were eliminated, and 1000 bootstrap replications were performed to evaluate reconstruction reliability. We also constructed phylogenetic networks as an alternative when a single phylogenetic tree was insufficient to explain the evolutionary history of a set of Japanese serow haplotypes. Unrooted phylogenetic network analysis was performed using SplitsTree CE 6.0.0 (Huson and Bryant, 2006).

The divergence time of the genus *Capricornis*, based on the control region of mitochondrial DNA, was previously reported by An et al. (2010) and Liu et al. (2013). However, their use of a substitution rate (10.62% per million years) derived from a 375-bp hypervariable region in the control region of cattle (Loftus et al., 1994) would significantly underestimate the divergence time, as noted by Okumura (2004). To re-estimate divergence times among mtDNA haplotypes of the Japanese serow, TimeTree was calculated using MEGA-X following the method of Mello (2018). The DNA sequence of the Himalayan goral served as a distinct outgroup for the genus *Capricornis*, and the ML phylogenetic tree was constructed using haplotypes of the Japanese, Sumatran, and Formosan serows. The ML phylogenetic tree was rooted by the Himalayan goral. Two calibration points were adopted: 2.81 million years ago (MYA) (Min 1.6, Max 7.6) between the Sumatran and Japanese serows, based on seven references (Brown and Yang, 2010; dos Reis et al., 2012; Hassanin et al., 2012; Bibi et al., 2013; Humphreys et al., 2014; Pérez et al., 2014; Toljagic et al., 2018), and 1.44 MYA (Min 1.1, Max 1.8) between the Sumatran and Formosan serows, based on two references (Hassanin et al., 2012; Humphreys and Barraclough, 2014). These values are derived from the median values provided by the TimeTree resource (Kumar et al., 2017).

A median-joining network (Bandelt et al., 1999) was constructed using POPART 1.7 (Leigh and Bryant, 2015) to summarize evolutionary relationships among haplotypes in Gunma Prefecture and their distribution among subpopulations. Arlequin version 3.5 (Excoffier and Lischer, 2010) was used to analyze the genetic

diversity of the Japanese serow in Gunma Prefecture. Haplotype diversity (*H*) and nucleotide diversity (*pi*) were computed for each subpopulation. Pairwise genetic distances between haplotypes were calculated using the Tamura and Nei model (Tamura and Nei, 1993). Genetic differentiation (fixation index F_{ST}) between subpopulations was calculated using 10,000 permutations. To test for gender differences in population structure, pairwise F_{ST} values were also calculated between all subpopulations, categorized by males and females. Population expansion was assessed by Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997) with 1000 simulations under a selective neutrality model using Arlequin 3.5.

RESULTS

A total of 15 haplotypes (Gunma-1–Gunma-15) of the mtDNA control region were identified in 364 Japanese serows in Gunma Prefecture (Table 1). The corresponding nucleotide sequences have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases, with the accession numbers: LC652839–LC652849 and LC730806–LC730809. These sequences consist of 1087 bp, but the first 56 bp represent a partial sequence of tRNA-Pro, making the full length of the d-loop region 1031 bp. In the analyses for constructing phylogenetic trees, we similarly removed adjacent sequences such as tRNA from the sequences cited from databases and used only the full length of the D-loop. A comparison of these 15 haplotypes with all reported Japanese serow sequences (see Supplementary Table S1) revealed no perfect matches. The frequencies of Gunma haplotypes, based on an 842 bp sequence, are detailed in Table 1. This sequence spans from the first to the 842nd base of the left side of the control region (D-loop). While the results by sex are also presented in Table 1, the overall results are discussed here as no significant difference was observed between males and females. Excluding sites where the number of analyzed animals was three or less, multiple haplotypes were present within the population. Specifically, five haplotypes were detected in Tsumagoi, eight in Katashina, seven in Tone, and five in Showa. Notably, there was no significant correlation between the number of samples and haplotypes, as indicated by the Spearman's rank correlation test (P = 0.262). In Tsumagoi and Showa, one haplotype constituted more than 85% of each population. Haplotype Gunma-4 was predominant in Tsumagoi, accounting for 93.1%, while in Showa, haplotype Gunma-1 was the most frequent, comprising 86.6%. Conversely, in Katashina and Tone, four haplotypes (Gunma-1, 6, 7, 9) had frequencies exceeding 10%, with none surpassing 50% (Table 1). Gunma-1, dominant in Showa, was also the most frequent haplotype in Katashina (28.2%) and Tone (47.5%), but was absent in Tsumagoi. Conversely, Gunma-4, the prevailing haplotype in Tsumagoi, was not found in Katashina, Showa, or Tone.

Figure 2 illustrates a phylogenetic tree of the Japanese serow mtDNA control region (full-length), generated using the ML method. While there is not robust statistical support for the clades, the Japanese serow haplotypes appear to be divided into six clades: I, II, III, IV, V, and VI. However, haplotype Kii-11 was isolated from the other haplotypes and could not be placed within any of these clades. The NJ method also produced a phylogenetic tree (see Supplemen-

Table 1. Summary of the mitochondrial DNA control region haplotype information for the Japanese serow found in Gunma Prefecture.

Location	Sex	N	Haplotype of mitochondrial DNA control region														
			Gun-1*	Gun-2	Gun-3	Gun-4*	Gun-5	Gun-6	Gun-7	Gun-8	Gun-9	Gun-10	Gun-11	Gun-12	Gun-13	Gun-14	Gun-15
Tsumagoi	Male	83	0	0	1	79	0	0	0	0	0	0	3	0	0	0	0
	Female	61	0	0	0	55	0	0	0	2	0	0	2	0	0	2	0
	Subtotal	144	0	0	1	134	0	0	0	2	0	0	5	0	0	2	0
Nakanojo	Male	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
	Female	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	Subtotal	3	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
Katashina	Male	41	10	0	0	0	2	10	7	0	7	0	0	4	0	0	1
	Female	37	12	0	0	0	0	9	9	0	6	1	0	0	0	0	0
	Subtotal	78	22	0	0	0	2	19	16	0	13	1	0	4	0	0	1
Tone	Male	22	10	0	0	0	1	4	4	0	3	0	0	0	0	0	0
	Female	18	9	0	0	0	0	3	2	0	2	0	0	0	1	1	0
	Subtotal	40	19	0	0	0	1	7	6	0	5	0	0	0	1	1	0
Showa	Male	48	43	2	0	0	2	1	0	0	0	0	0	0	0	0	0
	Female	49	41	1	0	0	4	2	0	0	1	0	0	0	0	0	0
	Subtotal	97	84	3	0	0	6	3	0	0	1	0	0	0	0	0	0
Tomioka	Female	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Numata	Female	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Sum total of Gunma prefecture	Male	196	63	2	1	81	5	15	11	0	10	0	3	4	0	0	1
	Female	168	62	2	0	57	4	14	11	2	9	1	2	0	1	3	0
	Total	364	125	4	1	138	9	29	22	2	19	1	5	4	1	3	1

*Among Gunma-1, 28 males and 21 females from Showa, and among Gunma-4, 36 males and 27 females from Tsumagoi have been sequenced only left side 842-bp.

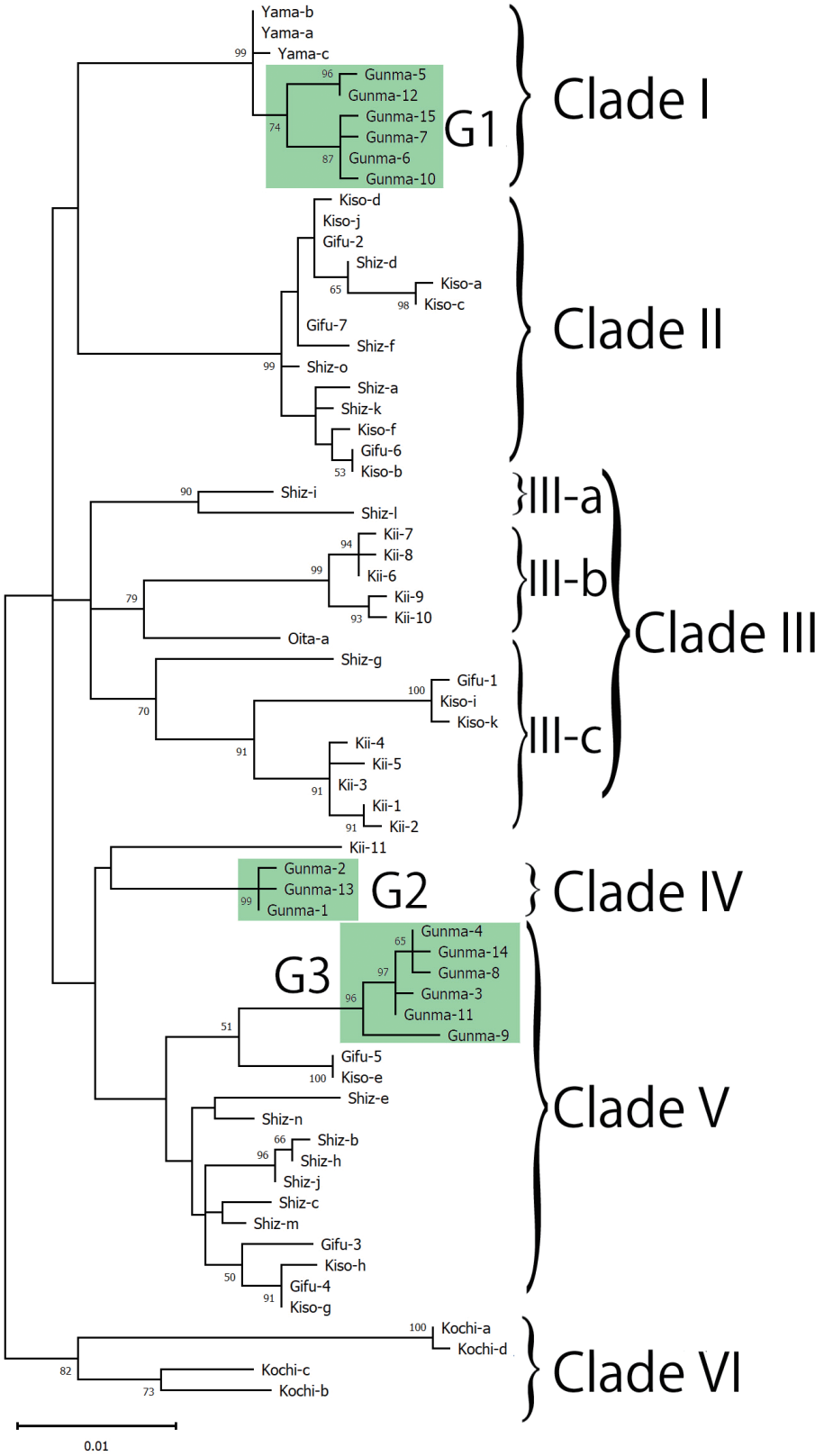

Fig. 2. The Maximum Likelihood tree of the mitochondrial DNA haplotypes of the Japanese serow. The haplotypes identified in this study, labeled as Gunma-1 through Gunma-15, are highlighted in green. The labels G1 to G3 indicate the haplogrouping in Gunma Prefecture. The abbreviations for other haplotypes are based on their respective GenBank registration details, which are further elaborated in Supplementary Table S1. The tree includes bootstrap probabilities, calculated from 1000 replicates, for the major lineages, and these are displayed above the nodes. Values below 50% are not shown. The tree is drawn to scale, with the lengths of the branches representing the number of substitutions per site.

tary Figure S1) with relationships similar to those of the ML method. Of the six clades, clades I, II, IV, and VI were statistically well supported. Clade I includes three haplotypes from Yamagata Prefecture in the Tohoku region and six haplotypes from Gunma Prefecture. Clade II includes 14 haplotypes from the Japanese Alps (Shizuoka, Nagano, and Gifu Prefectures). Clade IV consists of three haplotypes found around Mount Akagi in Gunma Prefecture. Clade VI is composed exclusively of haplotypes from Shikoku Island (Kochi Prefecture). Although clade III lacks strong statistical support, it is primarily composed of haplotypes from the Kii Peninsula (10 out of 11) and includes a few haplotypes from Kyushu and the Japanese Alps. Clade III can also be further divided into three deeply rooted subclades: III-A, III-B, and III-C. Clade V consists of 13 haplotypes from the Japanese Alps region and six haplotypes from Gunma. Although Clade V has weak statistical support within the phylogenetic tree, the haplotypes it contains are highly homologous to each other. The analysis reveals that all haplogroups except clade I, IV, and VI were detected in the Japanese Alps region. The 15 haplotypes in Gunma Prefecture can be classified into three haplogroups: G1, G2, and G3. Haplogroup G1 includes Gunma-5, 6, 7, 10, 12, and 15, forming a sister group with the Tohoku region haplotypes. Haplogroup G2 comprises Gunma-1, 2, and 13, which form Clade IV. Haplogroup G3 consists of Gunma-3, 4, 8, 9, 11, and 14, which are closely related to the Japanese Alps haplotypes within Clade V.

Figure 3 illustrates a phylogenetic network (SplitsTree) of the Japanese serow mtDNA control region (full-length), generated using the Neighbor-Net algorithm (Bryant and Moulton 2004, Bryant and Huson 2023). The classification of Clades I to VI was also reproduced by SplitsTree analysis. In addition, SplitsTree analysis supported that Clades III and V,

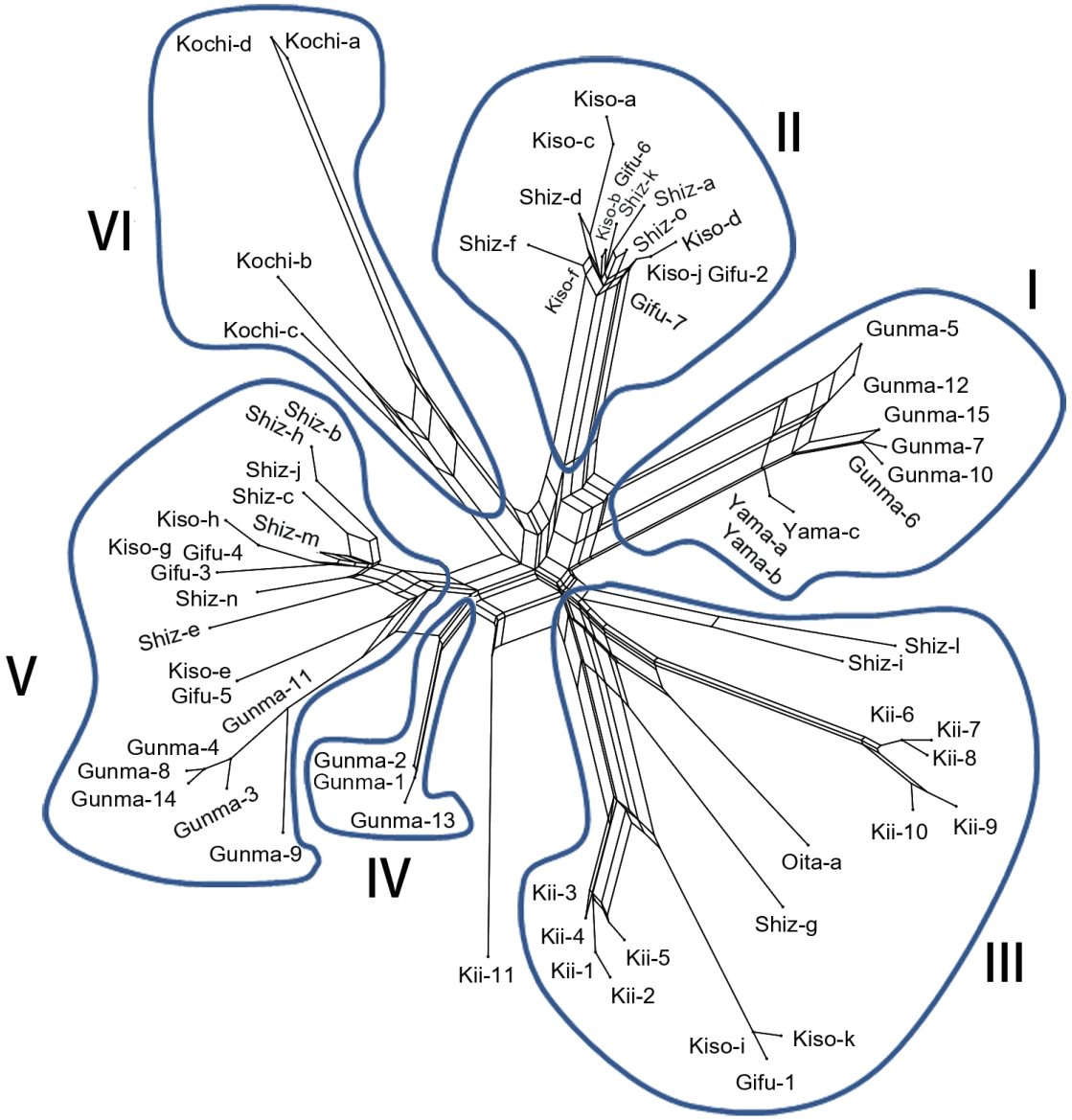

Fig. 3. SplitsTree phylogenetic network (Huson and Bryant, 2006) of Japanese serow mitochondrial DNA control region haplotypes (see Materials and Methods; Supplementary Table S1). Phylogenetic clades from I to VI, enclosed in blue lines, correspond to those used in Fig. 2.

which had weak statistical support for the clade roots in the ML and NJ trees, are independent lineages. However, SplitsTree showed that the separation of Clades IV and V was not clear and could be interpreted as forming one large group. Furthermore, the solitary haplotype Kii-11 was shown to branch from the midpoint between Clades III and IV in the SplitsTree analysis.

The ML phylogenetic tree, constructed to estimate divergence time, is displayed in Supplementary Figure S2. By removing sequence gaps among the outgroups, the number of informative sites decreased, and the junctions with the outgroups became ambiguous. As a result, the tree structure differed from that shown in Fig. 2. A significant difference was observed in Clade III, where subclades III-a, III-b, and III-c were formed, but they did not combine into a monophyletic group (see Supplementary Figure S2). Using this tree, the deepest divergence time among the Japanese serow haplotypes was estimated to be 494,767 years ago (95% confidence interval: 386,784 to 632,895 years). Within the six clades of the Japanese serow, the time to the Most Recent Common Ancestor (tMRCA) was approximately 9.4 kiloyears ago (KYA) (3.9–22.4 KYA) for Clade I, 6.4 KYA (3.1–13.6 KYA) for Clade II, and 3.4 KYA (1.45–8.1 KYA) for Clade IV. For Clade V and Clade VI, the tMRCA was 394.3 KYA (291.8–532.6 KYA) and 226.0 KYA (118.1–432.7 KYA), respectively. The tMRCA within subclades III-a, III-b, and III-c was 196.4 KYA (123.0–313.5 KYA), 309.9 KYA (210.3–456.7 KYA), and 494.7 KYA (378.8–632.9 KYA), respectively (see Supplementary Table S2).

A median-joining network tree was generated using the 15 Gunma haplotypes (using an 842 bp sequence) detected in this study (Fig. 4). The network tree supported the division of the Gunma haplotypes into three haplogroups, G1, G2, and G3, as indicated by the ML tree (Fig. 2). Haplogroup G1 was the most abundant in Katashina, accounting for 55.1%, with all six haplotypes detected. It also accounted for 37.8% in Tone (three haplotypes) and 9.4% in Showa (two haplotypes), but was not detected in Tsumagoi (Fig. 4, Table 1). Haplogroup G2 is centered on Gunma-1, which accounts for 121 of 126 (96%), and is accompanied by two derived haplotypes that differ by one-step substitution. Haplogroup G2 accounted for 89.6% in Showa, located at the western foot of Mt. Akagi. The frequency of G2 was 45.9% in Tone, 28.2% in Katashina, and was not detected in Tsumagoi. The appearance frequency of G2 decreased with increasing distance from Mt. Akagi, suggesting that the G2 haplogroup characterizes the Mt. Akagi population. Haplogroup G3, which consists of six haplotypes, was divided into Gunma-9 and others. Gunma-9 was detected in Katashina, Tone, and Showa but was not found in Tsumagoi, and there was a difference of seven substitutions from Gunma-4. The other five haplotypes are distributed mainly in Tsumagoi and its surrounding area on the west side of the Tone River and are conjoined with the dominant haplotype, Gunma-4, within two steps. This indicates that Gunma-4 characterizes the Tsumagoi population.

The study found that the haplotypic and nucleotide diversity within populations based on an 842 bp sequence, varied from 0.1334 ± 0.0384 in Tsumagoi to 0.7979 ± 0.0179 in Katashina. Similarly, the nucleotide diversity ranged from

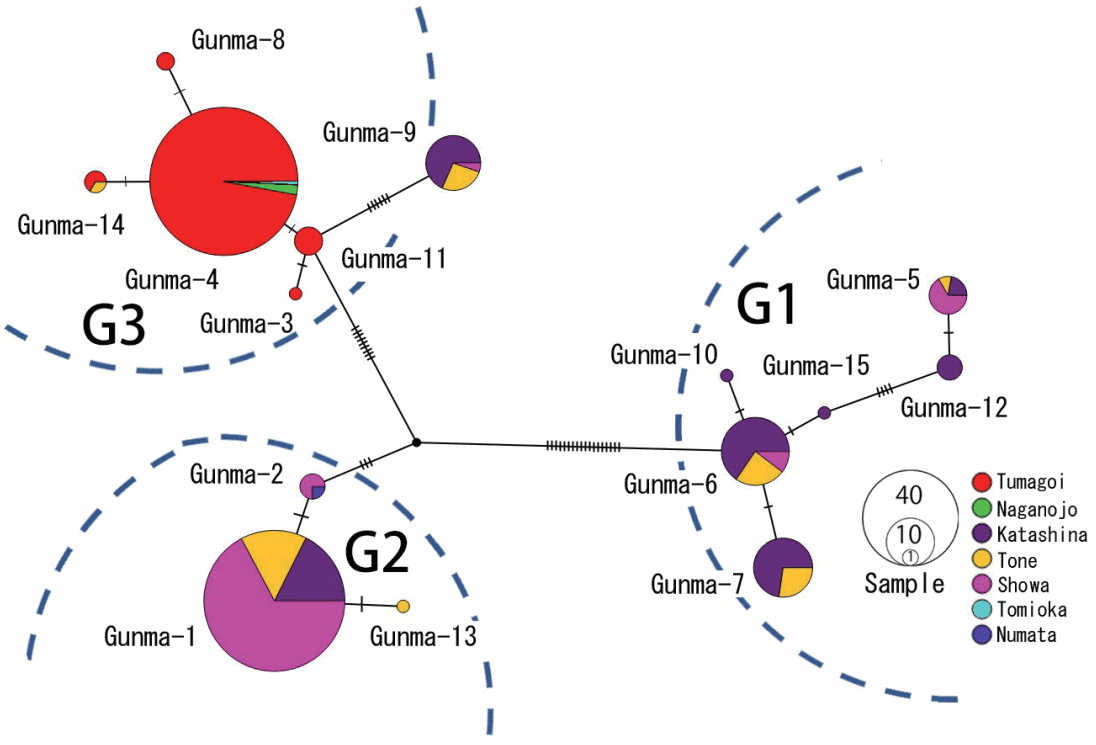

Fig. 4. The median-joining network of 15 mitochondrial DNA control region haplotypes, each based on 842 bp, discovered in Gunma Prefecture. The network uses various color codes to represent different sampled areas. The size of each circle within the network is proportional to the number of samples from that area. Tick marks along the lines between the circles indicate the number of nucleotide substitutions between haplotypes. The labels G1 to G3, surrounded by dashed lines, indicate the haplogrouping in Gunma Prefecture.

Table 2. Genetic diversity and neutrality tests conducted on *Capricornis crispus* in Gunma Prefecture.

Population	Haplotype diversity	Nucleotide diversity	Tajima's *D*	Fu's *Fs*
Tsumagoi	0.1334 ± 0.0384	0.000178 ± 0.000280	**–1.48094***	**–4.89383****
Katashina	0.7979 ± 0.0179	0.017308 ± 0.008681	2.6989	19.68025
Tone	0.7218 ± 0.0566	0.016793 ± 0.008543	1.77044	13.72418
Showa	0.2468 ± 0.0570	0.005228 ± 0.002884	–1.20743	8.87518
Sum total of Gunma prefecture	0.7264 ± 0.0149	0.015302 ± 0.007643	2.62542	21.00099

* indicates $P > 0.05$, ** indicates $P > 0.005$.

Table 3. Population differentiation of the Japanese serow in Gunma Prefecture.

	Tumagoi	Katashina	Tone	Showa
Tumagoi		**0.53439**	**0.57208**	**0.80993**
Katashina	**0.59444**		0.01132	**0.22259**
Tone	**0.68998**	0.01386		**0.09613**
Showa	**0.81910**	**0.31213**	**0.20602**	

Above the diagonal is the corrected average pairwise difference (PiXY-(PiX + PiY)/2), and below the diagonal is the population pairwise F_{ST}s. Bold letters indicate statistically significant difference ($P < 0.05$).

0.000178 ± 0.000280 in Tsumagoi to 0.017308 ± 0.008681 in Katashina (Table 2). These findings suggest that the genetic diversity within the population is relatively low in Tsumagoi and Showa, while it is high in Katashina and Tone. In Tsumagoi, both Tajima's *D* and Fu's *F*s statistics for neutrality tests were negative (Tajima's $D = -1.48094$, $P < 0.05$; Fu's $Fs = -4.89383$, $P < 0.01$). This indicates a historical population expansion following a population bottleneck in Tsumagoi (Table 2). Although Tajima's *D* was also negative in Showa, the neutrality test did not reveal any significant indices in the three populations located east of the Tone River (Table 2).

Table 3 in the study presents the values of the pairwise F_{ST} and corrected average pairwise differences between populations. A similar analysis was conducted with different sexes, the results of which are shown in Supplementary Table S3. The study found no significant differences between the sexes within the same local populations. However, among the populations, significant differences were observed in the pairwise F_{ST} values and average pairwise differences when comparing pairs between the west (Tsumagoi) and east (Katashina, Tone, and Showa) sides of the Tone River. This indicates that the population of the Japanese serow is largely divided by the Kanto Plain in the Tone River basin. Among the three populations located east of the Tone River, significant differences were detected between Showa and the other two populations.

DISCUSSION

Phylogeography of the Japanese serow based on mitochondrial DNA analysis

In this investigation, our aim was to elucidate the genetic diversity and regional population structure of the Japanese serow in Gunma Prefecture. A comprehensive analysis was conducted on 364 individuals, leading to the discovery of 15 previously unidentified haplotypes. Despite numerous studies on the Japanese serow, a holistic phylogenetic analysis utilizing all publicly available mtDNA sequences covering the entire distribution area of the Japanese archipelago has not been documented (Okumura, 2004; Iwahori et al., 2019). Thus, we commence our discussion by addressing the mtDNA diversity and phylogeography of the Japanese serow across the Japanese archipelago, providing a broader context before delving into a detailed examination of Gunma Prefecture. As illustrated in Fig. 2, the mtDNA control regions of Japanese serow sequences were classified into six distinct clades. Of the six clades, clades I and VI have clear regional distribution: clade I has been found only in the area east of the Tone River in Gunma Prefecture and the Tohoku region, and only clade VI has been reported in Shikoku. While the frequency of occurrence remains undisclosed, it is noteworthy that many haplotypes have been reported in the Japanese Alps region. Specifically, all Kiso haplotypes were sourced from Kiso-Fukushima (Kisomachi, Nagano Prefecture), covering an area of 476.1 km^2. Additionally, all Shiz haplotypes were collected from the former towns of Misukubo (271 km^2) and Honkawane (375 km^2) in Shizuoka Prefecture, prior to the municipal merger in 2005. A survey in Gero City (851 km^2), Gifu Prefecture, identified seven distinct Gifu haplotypes with varying frequencies. This diversity indicates that serows in the Japanese Alps region exhibit a wide range of haplotypes within the confined areas, spanning clades II, III, and V. A separate investigation involving 19 individuals in the Kii Peninsula, the westernmost habitat of the Japanese serow in Honshu, identified 11 Kii haplotypes. Interestingly, except for Kii-11, the remaining 10 Kii haplotypes were all categorized under clade III. The frequencies of these haplotypes varied, with notable occurrences of Kii-6 (six instances), Kii-1 and Kii-3 (three instances each), and singular instances for the remaining eight Kii haplotypes (Board of Education of Mie, Nara, and Wakayama prefectures 2018). Intriguingly, no haplotype belonging to Clade III has been observed in Gunma Prefecture or the Tohoku region, suggesting a distinctive regional distribution pattern. Notably, the haplotype Oita-a, reported from Kyushu at the western edge of the Japanese serow distribution, was classified into Clade III. This observation implies a tendency for Clade III to be prevalent in the Japanese Alps and the adjoining western areas. On the other hand, Clades II and V have not been found in the Kii Peninsula or the Tohoku region and are distributed mainly in the Japanese Alps region. In Gunma Prefecture, Clade V was frequently detected, but Clade II was never detected, suggesting that the distribution area of Clade II is narrower than that of Clade V. Furthermore, Clade VI is composed of four Kochi haplotypes found in Japanese serows living in Shikoku, but it has never been found in either Kyushu or Honshu. Intriguingly, no haplotype belonging to Clade III has been observed in Gunma Prefecture or the Tohoku region, suggesting a distinctive regional distribution pattern. Notably, the haplotype Oita-a, reported from Kyushu at the

western edge of the Japanese serow distribution, was classified into Clade III. This observation implies a tendency for Clade III to be prevalent in the Japanese Alps and the adjoining western areas. On the other hand, Clades II and V have not been found in the Kii Peninsula or the Tohoku region and are distributed mainly in the Japanese Alps region. In Gunma Prefecture, Clade V was frequently detected, but Clade II was never detected, suggesting that the distribution area of Clade II is narrower than that of Clade V. Furthermore, clade VI is composed of four Kochi haplotypes found in Japanese serows living in Shikoku, but it has never been found in either Kyushu or Honshu.

mtDNA haplotype diversity and paleogeography and paleoenvironment in the Japanese serow

The genus *Capricornis* has never been found in Hokkaido, as evidenced by both the current distribution of species and the fossil record. The ancestors of the Japanese serow are believed to have migrated from the continent to the western part of the Japanese archipelago via a land bridge that formed in the East China Sea, according to extant species distribution and fossil records (Okumura, 2003). Marine isotope stages (MIS) and mammalian fossils suggest that land bridges in the East China Sea formed twice during the Middle Pleistocene, approximately 630 and 430 KYA (Yoshikawa et al., 2007). However, in the Japanese archipelago, fossils of *Capricornis* spp. have only been discovered dating back to the Late Pleistocene (130 to 10 KYA) (Shikama and Hayakawa, 1962; Taruno et al., 2018). This leaves the exact timing of the serows' first appearance in the Japanese archipelago unclear. In this study, we used a molecular clock with mtDNA to estimate the deepest divergence time among Japanese serow haplotypes to be approximately 494.8 KYA, with a range of 632.9 to 386.8 KYA. This estimation represents the time to the most recent common ancestor (tMRCA) of Japanese serows, indicating when the current lineages began to diversify within the Japanese Archipelago. Due to genetic drift and demographic events, the tMRCA may appear more recent than the actual time of colonization (Kinoshita et al., 2012; Wu et al., 2015). Therefore, it is challenging to determine the precise time when the ancestral population of the Japanese serow colonized the Japanese Archipelago. However, the tMRCA calculated in this study suggests that the ancestral population of the Japanese serow colonized the Japanese Archipelago much earlier compared to the fossil records discovered so far. Specifically, this colonization likely occurred around the last formation of the East China Sea land bridge (Marine Isotope Stage 12, approximately 430 KYA) or even earlier.

From the beginning of the Middle Pleistocene (770 KYA) to the present, four ice ages (MIS2-5a: 70–10 KAY, MIS6: 191–123 KYA, MIS12: 478–424 KYA, MIS16: 676–621 KAY) have been recorded (Lisiecki and Raymo, 2005). During these periods, serows likely experienced severe climate changes due to glacial–interglacial cycles (Yoshikawa et al., 2007). The Japanese serow, which primarily feeds on shrubs, leaves, and shoots in deciduous broad-leaved and coniferous forests, as well as in adjacent shrub zones formed under cool temperate climate conditions (Ochiai, 2015; Takada and Minami, 2022), now inhabits cool temperate forests distributed over 1000 m above sea level in Honshu, Shikoku, and Kyushu. However, during the Ice Age, glaciers formed in the high mountains and cold temperate forests spread in the lowlands (Momohara, 2016). These changes in vegetation likely forced the Japanese serow's habitat to move to lower elevations during the Ice Age. Conversely, during interglacial periods, rising temperatures caused the distribution of cool temperate forests to shift from continuous lowlands to discontinuous mountainous areas. As a result, the Japanese serow would have been divided into isolated regional populations by mountain ranges during these interglacial periods, leading to genetic differentiation. Considering the large-scale climate changes that affected the habitat of the Japanese serow, this explains the diverse and highly divergent mtDNA lineages found in the Japanese Alps. The Japanese Alps region, consisting of the Hida, Kiso, and Akaishi mountain ranges, reaching altitudes of up to 3000 meters, experienced both isolation and reintegration due to climate change. The basins and adjacent plains of the Japanese Alps region may have served as refugia for the Japanese serow during the Ice Age.

While the number of animals surveyed was not extensive, it is intriguing that the Kochi haplotypes of the Japanese serow in Shikoku form an independent clade VI, distinct from clade III in Kyushu and the Kii Peninsula. In many mammals, such as Japanese macaques (*Macaca fuscata*), sika deer (*Cervus nippon*), and Asiatic black bears (*Ursus thibetanus japonicus*), the mtDNA lineage of the Shikoku population closely aligns with that of either Kyushu or the Kii Peninsula, or both (Yamada et al., 2006, 2007; Kawamoto et al., 2007; Yasukochi et al., 2009; Wu et al., 2015). However, for a few species, such as Smith's red vole (*Eothenomys smithii*) and Japanese dormouse (*Glirulus japonicus*), the mtDNA lineage of Shikoku populations differs from that in both Kyushu and the Kii Peninsulas (Suzuki et al., 1999; Yasuda et al., 2012). A significant geological event that may have impacted land animals in Kyushu is the massive caldera eruption (ASO-4) that occurred approximately 88,000 years ago at Mt. Aso in the center of the island (Takarada and Hoshizumi, 2020). Pyroclastic flows from the ASO-4 eruption completely covered the mountainous region of central Kyushu to a depth of more than 10 m, likely leading to the local extinction of some mammals. While the potential impact on Japanese serows in Kyushu remains a hypothesis, volcanic activity has been shown to influence genetic diversity and population structure in other species, such as moles, where eruptions caused genetic bottlenecks and population shifts (Nakamoto et al., 2021). Conducting detailed surveys of Japanese serows in the Kyushu and Shikoku regions will aid in understanding the effects of large-scale eruptions on the western Japanese archipelago.

On the Japanese serow population in Gunma Prefecture

In this study, we delve into the population structure of Japanese serows within Gunma Prefecture. Despite a mere 50 km separation across the Kanto Plain, where the Tone River flows, the mtDNA haplotypes of Tsumagoi and other areas were entirely distinct, barring one individual. This aligns with observations indicating that Japanese serows are predominantly sedentary and do not undertake extensive migrations (Takada and Minami, 2022). Interestingly,

the current topography of the Kanto Plain and its surrounding mountains does not appear to significantly hinder serow migration. However, only one individual, Gunma-14, which is derived from Tsumagoi's dominant haplotype Gunma-4, was found in Tone (Table 1). In contrast, the distribution of clade I exclusively in areas east of the Kanto Plain strongly suggests that the Japanese serow habitat was once segregated here. Late Pleistocene and Holocene sea-level changes transformed the Kanto Plain into a geographical barrier to the distribution of the Japanese serow. During the warm period of approximately 125 KYA (MIS-5e), sea levels rose several tens of meters above present levels, causing the sea to extend into the inland areas of the Kanto Plain (Tam and Yokoyama, 2021). Although not as large-scale as MIS-5e, seawater also invaded the interior of the Kanto Plain during the warm period about 6000 years ago (Umitsu, 1991).

The current conservation plan for the Japanese serow, as outlined by the Gunma Prefecture Government, (2021), treats the four areas of Tsumagoi, Katashina, Showa, and Tone as a single regional population (Joshinetsu/Minamiaizu population). However, our mtDNA survey revealed that the Japanese serows in Gunma Prefecture can be broadly divided into three distinct populations: Tsumagoi, Showa, and Katashina-Tone (Table 3). Notably, there was minimal migration between groups across the Tone River, underscoring the need to revise the Japanese serow conservation unit in Gunma Prefecture.

In Tsumagoi, situated to the west of the Kanto Plain, only haplotype Gunma-4 and its derivatives within haplogroup G3 were identified (refer to Table 1 and Fig. 4). Notably, Gunma-9, a member of haplogroup G3 located seven steps away from Gunma-4, was conspicuously absent in Tsumagoi. Haplogroup G3, categorized in clade V, displays a close relationship with counterparts distributed in the Japanese Alps region (Figs. 2, 3). However, in contrast to the Japanese Alps region, Tsumagoi lacked clade II and clade III haplotypes, reflecting a notably low genetic diversity (Table 2). Within Gunma Prefecture, the Tsumagoi population stood out with a significant value in the neutrality test (Tajima's $D = -1.48094$, $P < 0.05$; Fu's $Fs = -4.89383$, $P < 0.01$), indicative of rapid population expansion. This aligns with observation records in Tsumagoi, where Japanese serows caused substantial crop damage, particularly to cabbage, in the 2010s, leading to population control measures.

Moving to Showa, positioned at the western foot of Mt. Akagi on the northern edge of the Kanto Plain, haplogroup G2, consisting of Gunma-1 and its derivatives (Gunma-2 and -13), constituted a dominant 90.7% of the total (Fig. 4). Haplogroup G2 forms a distinct clade IV not found in other regions. While haplogroup G2 is also present in Katashina and Tone, its frequency is considerably lower than in Showa, hinting that the haplotype Gunma-1 may have originated from Mt. Akagi (Table 1). This suggests a historical isolation of the Japanese serow habitat on Mt. Akagi. However, in the Showa population, minor haplotypes Gunma-5 and -6 of haplogroup G1, along with Gunma-9 of haplogroup G3, were also present (Fig. 4, Table 2). Interestingly, these minor haplotypes were more prevalent in Katashina and Tone, implying some level of gene flow among neighboring areas of Showa. No significant difference was observed between Katashina and Tone, indicating that they can be considered as one continuous population (Table 3). In the Katashina-Tone area, haplogroups G1, G2, and G3 were evenly distributed, reflecting an extraordinarily large genetic diversity within the population. Particularly noteworthy is the prevalence of haplogroup G1 (included in clade I and related to the Tohoku region) in Katashina within Gunma Prefecture (Fig. 4). The presence of mountain ranges extending north of this area toward the Tohoku region suggests a mixture of multiple Japanese serow populations in the Katashina-Tone area.

In Gunma Prefecture, the distribution of mtDNA haplotypes exhibited substantial variation among local populations. Consequently, through the analysis of mtDNA, it became feasible to ascertain the origin of an individual that had ventured into a location distinct from its original habitat. For instance, the Japanese serow involved in a fatal accident on a highway in Tomioka City was identified with haplotype Gunma-4, indicating its origin in the Tsumagoi area. Conversely, the Japanese serow involved in a train accident in Numata possessed haplotype Gunma-2, strongly suggesting its origin in the Showa region. Conducting an in-depth analysis, as exemplified in this study, requires the accumulation of a substantial volume of data. Under the Japanese government's Cultural Properties Protection Act, prefectures are mandated to document all instances of Japanese serow discoveries. Despite this requirement, the specimens collected have not undergone thorough analysis. A comprehensive examination of all serow specimens collected under this act nationwide, employing a methodology akin to the one employed in this study, holds the potential to unveil the intricate structure of the entire local Japanese serow population. Such an approach is extremely important for developing effective conservation strategies for serows.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

KT, KN, and TA designed the research project and obtained funding for this study. TA collected tissue samples of *C. crispus* from Gunma Prefecture. KT, MN, and RO performed DNA extraction and biochemical and genetic analyses. MM examined the consistency of the ecology of the Japanese serow and the results of DNA analysis. KN contributed to the population genetic analysis. SK, YI, and JK contributed to filling in the gaps in the study area by providing data on serows in the Kii Peninsula and Gifu prefecture. HN provided important information and encouraged this study through NGS analysis of the Japanese serow genome. All authors have discussed the results and contributed to the final manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://doi.org/10.2108/zs240006)

Supplementary Figure S1. Neighbor-joining tree of mitochondrial DNA haplotypes of the Japanese serow.

Supplementary Figure S2. Maximum Likelihood phylogenetic tree. This tree represents the mtDNA control regions of all currently existing species within the *Capricornis* genus.

Supplementary Table S1. A comprehensive list of mitochondrial control region sequences for the Japanese serow.

Supplementary Table S2. List of branch times that were determined through a time tree analysis conducted using MEGA-X.

Supplementary Table S3. Population differentiation of Japanese serows in the Gunma Prefecture. The data is categorized by sex, providing insights into the genetic differentiation among populations as well as between the sexes within the populations.

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