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Dietary Diversity of the Amami Rabbit Endemic to Insular Evergreen Forests

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The Amami rabbit (*Pentalagus furnessi*), endemic to the Amami-Oshima and Tokunoshima Islands, is an endangered species that faces habitat disturbance. This study used fecal DNA metabarcoding to analyze the dietary preferences of Amami rabbits. Fecal samples from six rabbits were collected and analyzed for plant DNA. The results revealed the presence of 85 operational taxonomic units (OTUs) representing different plant species, with individual rabbits consuming 17–38 plant species. The dietary composition varied significantly among individuals, with a notable preference for ferns in some cases. Diet diversity, assessed using Simpson's Diversity Index, ranged from 0.328 to 0.889. This study highlights the importance of a diverse plant diet for Amami rabbits and underscores the need for habitat preservation to ensure a stable food supply. Conservation efforts must focus on maintaining a unique subtropical moist forest ecosystem and mitigating human-wildlife conflicts to promote sustainable coexistence.

Key words: Amami rabbit, Fecal DNA metabarcoding, Dietary analysis, Endangered species, Subtropical moist forests

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BACKGROUND

The Ryukyu Islands, an extensive chain stretching approximately 1000 km southwest of the Japanese archipelago, are home to the Amami rabbit (*Pentalagus furnessi*). This nocturnal species, found exclusively on Amami-Oshima Island (land area: 712 km²) and Tokunoshima Island (248 km²), is relict endemic with no closely related species within its genus (Yamada and Cervantes 2005). It is currently listed as an endangered species on the IUCN Red List (Yamada and Smith 2016). In July 2021, these two islands were designated UNESCO World Natural Heritage Sites. Therefore, establishing a framework to promote sustainable coexistence between island residents and wildlife has become an urgent priority (Izawa 2022).

More than 80% of Amami-Oshima Island is covered by forests, with subtropical lucidophyllous forests prevalent in rugged mountainous areas (Matsumoto et al. 2020). Despite its location in the mid-latitude zone, the island features subtropical moist forests, which result in distinctive vegetation. The highest peak on Mt. Yuwan occurred at an altitude of 645 m. Notably, Amami rabbits are predominantly found in mountainous regions, including coastal areas, at sea level (Sugimura et al. 2000). In contrast, many rabbit species in subtropical and tropical regions are adapted to high altitudes exceeding 1000 m, inhabiting relatively cool environments despite low latitudes. For instance, the Sumatran striped rabbit (*Nesolagus netscheri*) in Indonesia occupies habitats at altitudes of 600–1600 m (McCarthy et al. 2019), the Annamite striped rabbit (*N. timminsi*) near the Vietnam-Laos border inhabits areas up to 1300 m above sea level (Tilker et al. 2019), and the Mexican volcano rabbit (*Romerolagus diazi*) is found at elevations of 3000–4000 m (Velázquez and Guerrero 2019). These observations suggest that the Amami rabbit, with its primitive traits, has adapted to survive in a unique island environment (Yamada et al. 2000).

Historically, logging activities in the Amami forests have been selective, resulting in limited human disturbance. However, the enactment of the Act on Special Measures for the Amami Islands Promotion and Developmen in 1954 led to repeated large-scale clear-cutting operations (Sugimura et al. 2003). Such extensive habitat disturbance is believed to have significantly affected the phenotype of Amami rabbits, particularly affecting vegetation and potential food resources. Ensuring the sustainability of this species necessitates preservation and enhancement of the feeding environment. Despite the importance of dietary studies, research on the diet of Amami rabbits is limited. Yamada and Cervantesu (2005) identified over 29 plant species consumed by rabbits, including herbaceous plants, such as grasses, and woody plants, such as *Castanopsis sieboldii* and *Melastoma candidum*, although most specific species have not been documented. More recently, Asari and Kimoto (2022) cataloged over 80 food plant species based on their feeding signs

throughout the year. However, reliance on feeding signs alone limits the assessment of the importance of individual plants to rabbits.

In this study, the potential of a non-invasive DNA metabarcoding approach for Amami rabbit feces was explored (Ando et al. 2020). We present preliminary findings from a limited sample illustrating the variation in dietary preferences among individual Amami rabbits.

MATERIALS AND METHODS

Sample collection

Fecal samples from Amami rabbits were collected from two locations on Amami-Oshima Island (Fig. 1). On August 18, 2022, two samples (sample ID: A-rab-1 and A-rab-2) were collected from the riverbank upstream of the Yakugachi River (28.22˚E, 129.34˚N). On October 28, 2023, four samples (A-rab-3, A-rab-4, A-rab-5, and A-rab-6) were collected from the roadside along the Asato Forest Road (28.32˚E, 129.49˚N). Preliminary surveys have indicated that fecal pellets exhibiting varying degrees of freshness are frequently observed concentrated within an area of several square meters. To prevent duplicate collection from the same individual, fecal masses in close proximity were avoided, maintaining a minimum interval of 20 m. Fecal pellets with a shiny and moist surface, indicating recent excretion, were collected. Six fecal pellets were collected from each fecal mass and were transported in a refrigerated state. In the laboratory, the samples were frozen and stored at -30°C.

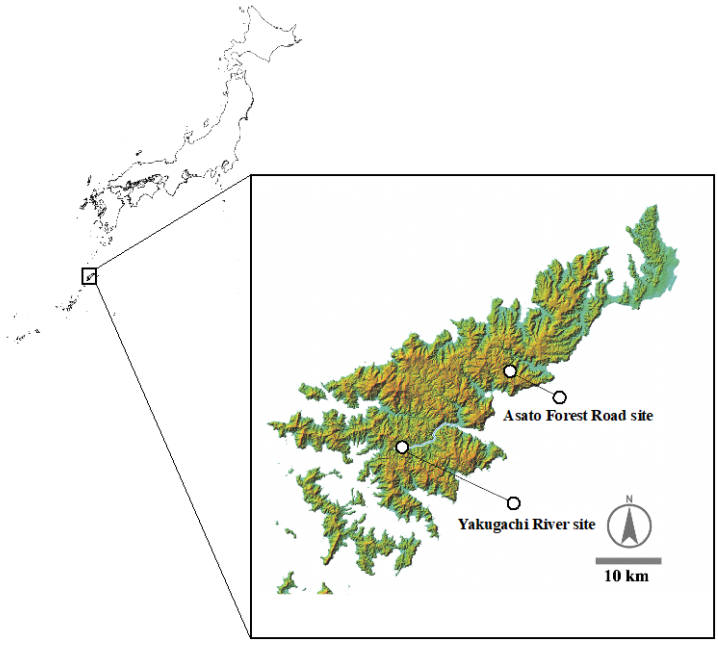

Fig. 1. Collecting sites of fecal samples from Amami rabbits (*Pentalagus furnessi*) on Amami-Oshima Island.

DNA metabarcoding

DNA extraction was performed using randomly selected a fecal pellet from each sample. DNA metabarcoding analysis was outsourced to the Bioengineering Lab. Co., Ltd. (https://gikenbio.com/, Kanagawa, Japan). First, freeze-drying was performed using a VD-250R freeze-dryer (TAITEC, Saitama, Japan). The dried samples were then ground in a Multi-beads Shocker® (YASUI KIKAI, Osaka, Japan) at 1500 rpm for 2 min. Subsequently, the samples were mixed with Lysis Solution F (NIPPON GENE, Tokyo, Japan) and incubated at 65°C for 10 min, followed by centrifugation at 12000 × g for 2 min to collect the supernatant. The supernatant was then subjected to a purification process involving stirring with a Purification Solution (NIPPON GENE) and chloroform, followed by centrifugation at 12000 × g for 15 min. The purified DNA supernatant was extracted using a Lab-Aid824s DNA Extraction Kit (ZEESAN, Xiamen, China). Finally, PCR inhibitors were removed using a DNeasy PowerClean Pro Cleanup Kit (Qiagen, Tokyo, Japan).

The amplicon library was prepared according to the following protocol: DNA concentration was determined using Synergy LX (Agilent Technologies, Santa Clara, USA) and QuantiFluor dsDNA System (Promega, Madison, USA). The library was constructed using a 2-step tailed PCR approach. In the first PCR, the target region of the plant was amplified, and an adapter sequence (homologous to the primer for the second PCR) was added using primers targeting the *psbA* region in the chloroplast DNA (*psbA*-597F: 5′-ACACTCTCTCCCTACGACGCTCTTCCGATCT-3′; *psbA*-927R: 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3′) designed by the Bioengineering Lab. Co., Ltd. For the second PCR, primers 2ndF (5′-AATGATACGGCGACCACCGAGATCTACAC-Index2-ACACTCTTTCCC- TACACGACGC-3′) and 2ndR (5′-CAAGCAGAAGACGGCATACGAGAT-Index1-GTGACTGGAGTTCAGACGTGTG-3′) were used. The concentration of the resulting library was assessed using Synergy H1 (Agilent Technologies) and the QuantiFluor dsDNA System. Library quality was verified using a Fragment Analyzer and a dsDNA 915 Reagent Kit (Agilent Technologies). Sequencing was performed on a MiSeq system using the MiSeq Reagent Kit v3 (Illumina, Tokyo, Japan) with 2 × 300 bp settings.

Bioinformatics and statistical analyses

Bioinformatic analysis was performed according to the following steps: amplicons that exactly matched the beginning of the specified index reads were extracted using the FASTX-Toolkit (ver. 0.0.14). The primer sequences in *psbA* were trimmed to 50 bp from the 3'-terminal end using the fastx-trimmer in FASTX-Toolkit. Sequences with a quality value of less than 20 were filtered out using a sickle (ver. 1.33). Sequences with a length of 40 bp or less, along with their paired sequences, were discarded. The paired-end reads were merged using FLASH (ver. 1.2.11)

with a minimum overlap of 10 bp. The chimera and noise sequences were removed using the Qiime2 plugin wrapped in DADA2 (ver. 2023.7). Plant species identification was primarily conducted using processed *psbA* sequences registered with NCBI through a BLASTN search (ver. 2.13.0). However, given that many of these sequences are from non-native plants, this method sometimes leads to misidentifications of plant species on Amami-Oshima Island. To address this issue, a database of plants that grew on Amami-Oshima Island was used for species identification in such cases (Tabata 2023).

In the metabarcoding analysis, data with low numbers of base sequences were deemed unreliable and consequently excluded from the analysis, even if samples were identified to the species level. The reliability threshold for the number of sequences varies and is affected by the total number of sequences (Alberdi et al. 2017). For this study, data representing less than 0.1% of the total number of sequences in each sample were defined as unreliable and were excluded from subsequent analyses. Metabarcoding provided detailed taxonomic data for the diet of each of these species, and the results were primarily examined as the relative sequence abundance of all identified taxa in the fecal content.

An unequal number of sequences can affect diversity estimates because of the positive relationship between the number of sequences and operational taxonomic units (OTUs) (Gotelli and Colwell 2001). OTU abundance is represented by the number of sequences. Richness was defined as the number of OTUs or taxa within a sample. Evenness was defined as the similarity between the frequencies of sequences in different operational taxonomic units (OTUs) within a sample. Simpson's Diversity Index（1 - λ）was subsequently applied to account for both the abundance and evenness. This index generates a single value between 0 and 1, representing the diversity of diet between individuals (Simpson 1949).

RESULTS

In the feces of the six Amami rabbits analyzed in this study, different 85 OTUs derived from photosynthetic plants were detected and taxonomically identified as the current food items on Amami-Oshima Island. Table 1 provides an overview of each sample. The top three plant species frequently found in a single feces grain varied among individuals. In individual ID A-rab-1, the top three were fern (*Blechnopsis orientalis*), fern (*Diplopterygium glaucu*), and fern (*Lindsaea chienii*). Similarly, in A-rab-2, the top three were fern (*Blechnopsis orientalis*), fern (*L. chienii*), and fern (*Cyathea hancocki*). In A-rab-3, the top three were fern (*Thelypteris torresiana*), herb (*Pouzolzia hirta*), and fern (*Pteris vittata*). In A-rab-4, the top three were tree (*Ficus oligodon*), tree (*F. erecta*), and tree (*F. pandurata*). In A-rab-5, the top three were tree (*Actinidia rufa*), fern (*Selaginella doederleinii*), and fern (*Thelypteris triphylla*). In A-rab-6, the top three were tree (*F. oligodon*), tree (*F. erecta*), and fern (*T. triphylla*). The richness of food items represented by the number of OTUs

within a single fecal pellet varied, ranging from a minimum of 17 in A-rab-2 to a maximum of 38 in A-rab-5. The feeding characteristics of individual rabbits were revealed by their diet diversity, as indicated by Simpson's diversity index ($1 - \lambda$), which was 0.33 for A-rab-2 and 0.89 for A-rab-5. The proportion of sequences for the most frequent OUT varied with a minimum of 26.2% in A-rab-5 and a maximum of 81.7% in A-rab-2. The proportion of total sequences of the top 3 OTUs also varied, with a minimum of 48.5% in A-rab-5 and a maximum of 92.2% in A-rab-2.

Table 1. Summary of DNA metabarcoding analysis for six fecal samples from Amami rabbit (*Pentalagus furnessi*) including Simpson's diversity index ($1 - \lambda$)

Sample ID	A-rab-1	A-rab-2	A-rab-3	A-rab-4	A-rab-5	A-rab-6
Number of OTUs	23	17	20	28	38	25
Sum of sequences	35159	30278	10513	37261	35812	40063
Simpson's diversity index	0.508	0.328	0.831	0.804	0.889	0.669
Top 3 OTUs	*Blechnopsis orientalis**	*Blechnopsis orientalis**	*Thelypteris torresiana**	*Ficus oligodon***	*Actinidia rufa***	*Ficus oligodon***
% total sequences	69.3%	81.7%	28.8%	34.6%	26.2%	51.6%
	*Diplopterygium glaucu**	*Lindsaea chienii**	*Pouzolzia hirta****	*Ficus erecta***	*Selaginella doederleinii**	*Ficus erecta***
	7.0%	9.0%	19.2%	20.2%	11.6%	22.5%
	*Lindsaea chienii**	*Cyathea hancocki**	*Pteris vittata**	*Ficus pandurata***	*Thelypteris triphylla**	*Thelypteris triphylla**
	4.7%	1.5%	13.7%	15.6%	10.7%	11.2%

*Fern. **Tree. ***Herb.

Based on the life forms of plants, the food items inferred from the sequence of OUT were categorized into three functional groups: trees, herbs, and ferns. The proportion of sequences corresponding to each type was compared between the samples (Fig. 2). Four species of moss were detected in one sample (A-rab-5), accounting for 0.8% of the total sequences, and were included with ferns in this analysis for convenience. The composition of food types differed significantly between the samples (Pearson's chi-square test, $\chi^2 = 86430.7$, $df = 10$, $P < 0.001$). A-rab-5, which exhibited the highest diversity of food items, differed from the other samples in that it contained nearly equal proportions of trees and ferns. Additionally, this specimen included small amounts of plants found near human settlements, such as the cultivated plum tree (*Prunus salicina*, 1.8%), the horticultural Madagascar periwinkle (*Catharanthus roseus*, 0.1%), and the coastal plant (*Lysimachia mauritiana*, 0.4%). Overall, herbs were a minority in all the samples, and ferns were detected in all the samples. Notably, samples collected from the riverside (A-rab-1 and A-rab-2) showed a diet biased towards ferns.

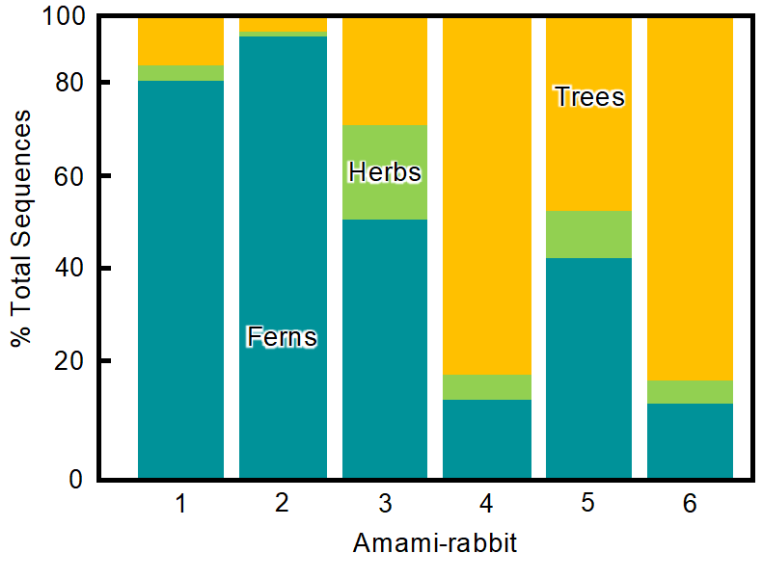

Fig. 2. Sequence compositions of OTUs categorized into trees, herbs and ferns based on DNA metabarcoding analysis for six fecal samples from Amami rabbit (*Pentalagus furnessi*).

DISSCUSSION

DNA metabarcoding analysis of fecal samples from six Amami rabbits revealed a diet breadth of 17–38 plant species per individual, encompassing a total of 85 distinct plant taxa. Given the seasonal fluctuations and habitat-specific variation in plant availability, the potential dietary range of this species may expand beyond the previously documented food plant species (Yamada and Cervantes 2005; Asari and Kimoto 2022) if its distribution extends year-round across the island. The overlap between the plant species identified in this survey and those reported in Arai and Kimoto (2020) includes frequently occurring species such as a fern (*T. interrupta*), a herb (*P. hirta*), and trees (*F. erecta*, *Mussaenda densiflora*, *Oreocnide pedunculate*, and *Helwingia japonica*). Comparative analysis of hare (*Lepus brachyurus*) feces from different habitats in temperate Japan (Matsuki et al. 2008) has demonstrated that dietary variation is linked to environmental factors. Beech forests supported a diet of 13 plant species, including trees and bamboo, whereas logged areas primarily featured 12 herbaceous species. Cedar forests exhibited the highest plant diversity, with 26 taxa, encompassing both woody and herbaceous components. Notably, ferns were absent in all hare diets. In contrast, some Amami rabbits displayed a pronounced preference for ferns thriving in humid environments. This dietary specialization, coupled with the diverse plant composition of Amami–Oshima's subtropical moist forests, suggests that this species has adapted to survive by exploiting a broad spectrum of food resources rather than relying on specific plant taxa. The Amami rabbit's observed dietary plasticity, characterized by the utilization of readily available plant species, may play a significant role in mitigating intraspecific

competition for resources. This dietary flexibility is hypothesized to facilitate the coexistence of multiple individuals within a limited habitat.

In addition, differences in diet composition were observed among individuals sharing the same defecation site on the same day. In the forests of Amami-Oshima, the Habu snake (*Trimeresurus flavoviridis*), a venomous snake, is an apex terrestrial predator. It preys mainly on brown rats (*Rattus rattus*), and with regard to Amami rabbits, with the exception of small young rabbits, it is understood that adult Amami rabbits, which exceed the swallowing capacity of a habu snake, rarely become prey for the snake (Mishima 1966). The nocturnal Habu snake uses infrared receptors or pit organs to detect and attack warm-blooded mammals (Minakami 2004). For nocturnal Amami rabbits, expanding their home range increases the chance of encountering Habu snakes. Once bitten, the Amami rabbit inevitably dies from the venom. Amami rabbits are known to forage within a limited home range (Yamada et al., 2000). This restricted foraging behavior may contribute to a reduced risk of snake envenomation. To survive within the limited space on the island, Amami rabbits may have developed a feeding strategy that maximizes the use of nearby food resources.

A stable supply of diverse plant groups, including ferns, is crucial to meet the dietary needs of Amami rabbits. Furthermore, where their home range overlaps with human living areas, measures are needed to prevent conflicts caused by crop damage. Successful conservation based on human coexistence requires comprehensive management that considers the entire ecosystem.

CONCLUSIONS

This study presents preliminary findings on the dietary preferences of Amami rabbits using a non-invasive DNA metabarcoding approach. The results demonstrate the potential of this technique to elucidate the dietary composition of this endangered species. While limited by sample size, the analysis revealed variations in dietary preferences among individual rabbits, suggesting a diverse and flexible diet.

Further research with a larger sample size and a more comprehensive DNA barcode library is necessary to fully understand the dietary ecology of Amami rabbits and its implications for conservation efforts. This information can contribute to effective habitat management and the development of targeted conservation strategies to protect this unique species.

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Authors' contributions: Study design: KI. Sample collection: KI, ST and SA. Diet identification: KI, MS and MT. Manuscript writing: KI, ST, MS and SA.

Competing interests: All authors declare that they have no conflict of intrest.

Availability of data and materials: A file containing a list of highly homologous species based on fecal analysis of the Amami rabbit will be made available upon request.

Consent for publication: Not applicable.

Ethics approval consent to participate: Not applicable.

REFERENCES

Alberdi A, Aizupurua O, Gilbert MTP, Bohmann K. 2017. Scrutinizing key steps for reliable metabarcoding of environmental samples. Methods Ecol Evol **9:**134–147. doi:10.1111/2041-210X.12849.

Ando H, Mukai H, Komura T, Dewi T, Ando M, Isagi Y. 2020. Methodological trends and perspectives of animal dietary studies by noninvasive fecal DNA metabarcoding. Environ DNA **2:**391–406. doi:10.1002/endn3.117.

Asari Y, Kimoto Y. 2022. *Pentalagus furnessi* food plants at two sites on the western Amami-Oshima Island, Japan. Wildl Hum Soci **10:**17–19. doi:10.20798/awhswhs.10.0_17. (in Japanese with an English abstract

Gotelli NJ, Colwell RK. 2001. Quantifying biodiversity: Procedures and pitfalls in the measurement and comparison species richness. Ecol Lett **4:**379–391. doi:10.1046/j.1461-0248.2001.00230.x.

Izawa M. 2022. Characteristics of island natural world heritage "Amami-Oshima Island, Tokunoshima Island, the northern part of Okinawa Island, and Iriomote Island. Jpn Inst Landsc Archit **84:**96–99. doi:10.5632/jila.86.96. (in Japanese)

Matsuki R, Abe S, Shimano K, Takeuchi T, Nashimoto M. 2008. Construction of plant *rbcL* gene database and its application to analysis of food habits of herbivorous animals. Jps J Ecol **58:**105–112. doi:10.18960/seitai.58.2_105. (in Japanese)

Matsumoto H, Inoue N, Washitani I. 2020. Historical transition of Crown Size Index on Amami Oshima Island after the 1960s: correspondence to important forest areas. Jps J Conserv Ecol **25:**25–41. doi:10.18960/hozen.1902. (in Japanese with an English abstract)

McCarthy J, Holden J, Martyr D, McCarthy K. 2019. Smatran striped rabbit, *Nesolagus netscheri*. The IUCN Red List of Threatened Species 2019. www.iucnredlist.org/ja/species/14662/45178557.

Minakami K. 2004. Physiology and ecology of *Trimesurus* snakes in the Amami Islands. Bul Herpetol Soc Jpn **2004:**11–17. doi:10.14880/hrghsj1999.2004.11. (in Japanese)

Mishima S. 1966. Studies on the poisonous snake "Habu", *Trimeresurus flavoviridis flavoviridis*, I. Food habit of *Trimeresurus flavoviridis flavoviridis* on the Amami Islands. Sanit Zool **17(1):**1–21. doi:10.7601/mez.17.1. (in Japanese)

Simpson E. 1949. Measurement of Diversity. Nature **163:**688. doi:10.1038/163688a0.

Sugimura K, Sato S, Yamada F, Abe S, Hirakawa H, Handa Y. 2000. Distribution and abundance of the Amami rabbit *Pentalagus furnessi* in the Amami and Tokuno Island, Japan. Oryx **34:**198–206. doi:10.1046/j.1365-3008.2000.00119.x.

Sugimura K, Yamada F, Miyamoto A. 2003. Population trend, habitat change and conservation of the unique wildlife species on Amami Island, Japan. Glob Environ Res **7:**79–90.

Tabata M. 2023. The catalogue of plants in the Amami Islands. Nanpo-shin-sya, Kagoshima, Japan.

Tilker A, Timmins RJ, Nguyen TTA, Coudrat CNZ, Gray T, Le TT, Willcox DHA, Abramov AV, Wilkinson N, Steinmetz R. 2019. Annamite striped rabbit, *Nesolagus timminsi*. The IUCN Red List of Threatened Species 2019. Available at: www.iucnredlist.org/species/41209/45181925.

Velázquez A, Guerrero JA. 2019. Volcano rabbit, *Romerolagus diazi*. The IUCN Red List of Threatened Species 2019. Available at: www.iucnredlist.org/species/19742/45180356.

Yamada F, Cervantes FA. 2005. *Pentalagus furnessi*. Mann Species **782:**1–5. doi:10.1644/782.1.

Yamada F, Smith AT. 2016. Amami rabbit, *Pentalagus furnessi*. The IUCN Red List of Threatened Species 2016. www.iucnredlist.org/species/16559/45180151.

Yamada F, Sugimura K, Abe S, Handa Y. 2000. Present status and conservation of the endangered Amami rabbit Pantalagus frunessi. Tropics **10:**87–92. doi:10.3759/tropics.10.87.