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Omnivorous food habits of the endangered Ryukyu long-furred rat *Diplothrix legata* (Muridae) estimated using the DNA metabarcoding method

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Abstract. The Ryukyu long-furred rat, *Diplothrix legata* (Muridae) is an endangered large arboreal species endemic to the central Ryukyus, Japan. Previous studies have recorded the food habits of this species through direct observations. However, its observation records are limited. Here, we aimed to clarify the food habits of *D. legata* using DNA metabarcoding of its stomach contents. The *ITS2* intergenic regions in plant nuclear genomes and the *COI* gene regions in animal mitochondrial genomes were used as DNA markers to identify the prey species of *D. legata* based on its stomach contents. We successfully identified 63 plant and 36 animal species as the prey species of *D. legata*. Finally, 84 plant and 46 animal species are listed as food menu in total. Thus, a greater number of its animal prey species were identified in this study than in previous observation-based studies. As this species is omnivorous, the conservation of a wide array of ecological habitats is necessary for maintaining its population. Regardless of the limited sample size and unknown parts consumed, slight differences in food items were observed between different sex- and age-based groups. Relatively more detailed DNA reference databases for the local fauna and flora are required for further analysis.

Key words: arboreal rats, diet, island biology, nocturnal mammals, Ryukyu Archipelago.

Knowledge of food habits is fundamental for understanding animal life and elucidating community structure and biodiversity through the food web. In addition, food habits need to be studied based on region to gain a comprehensive understanding of the ecological characteristics of the target species (Litvaitis et al. 1996). Information regarding food habits is essential for the conservation of endangered species (Rodríguez et al. 2007; Hejcmanová et al. 2013; Yamamoto-Ebina et al. 2016).

The Ryukyu long-furred rat, *Diplothrix legata* (Muridae), is a large rat endemic to the Okinawajima, Tokunoshima, and Amami-Oshima Islands in the central Ryukyus, Japan (Kaneko 2005). The population of this species has decreased owing to habitat loss, predation by invasive carnivores (Hamada 2007; Watari et al. 2007; Shionosaki et al. 2015; Kobayashi et al. 2020), and traffic accidents (Tamanaha et al. 2017). Therefore, this species is listed as "Endangered" in the IUCN Red List (Ishii 2016) and by the Ministry of the Environment, Japan (Ministry of the Environment 2020). Furthermore, as this species feeds heavily on fruits, it may play a crucial role as an effective seed disperser in the ecosystems of the central Ryukyus, where general seed dispersers found on continents such as arboreal primates, squirrels, carnivores, and large birds are absent (Nago et al. 2019).

Previous studies have reported 39 plant and 11 animal species used as food items by *D. legata*, based on the direct observations of their feeding behavior in the wild (Tokida 2001; Torikai and Ueda 2007; Takehara et al. 2015; Kudaka and Kudaka 2017; Kobayashi et al. 2018;

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Taniguchi et al. 2019). However, direct observations are difficult to acquire and insufficient for identifying prey species, because this rat species is nocturnal and arboreal, thus providing little information on its dietary habits. For example, direct observations are possible mainly in limited open environments such as roads; therefore, the degree of the utilization of food plants growing in the inner forest remained underestimated. In addition, the observational records of feeding on animals are limited (Takehara et al. 2015; Kudaka and Kudaka 2017; Tamanaha et al. 2017), and it is difficult to identify small prey species based on the direct observations of rats feeding on tall trees.

Fecal and stomach content analyses are used to identify the food menus of animals, whose feeding behaviors are challenging to record based on observations (Litvaitis et al. 1996). However, these methods are prone to be affected by the problem of digestion of food contents (Valentini et al. 2009; Ballari and García 2014). In addition, rodents have silt-like stomach contents because they grind their food. Thus, the identification of the content using microscopy and morphology is difficult. In contrast, the DNA metabarcoding method, which analyzes the DNA sequences of food items in the stomach and fecal contents, is an effective method for resolving bias due to the digestibility of foraged animals and plants (e.g., Arrizablaga-Escudero et al. 2018; da Silva et al. 2019; Ducotterd et al. 2021; Pereira et al. 2021; Ramirez et al. 2021). This method has been used to identify fecal content and the food habits of several rodents worldwide (e.g., Lopes et al. 2015; Petrosky et al. 2021; Pinho et al. 2022; Gabrielson et al. 2023). In Japan, although some studies have applied this method to identify dietary habits of mammals (e.g., Nakahara et al. 2015; Sato et al. 2018, 2023; Heim et al. 2021; Tobe et al. 2024), only a few studies have used both animal and plant primers (Sato et al. 2019b, 2022, 2023). In addition, no studies have applied this method to an endangered rodent endemic to Japan and examined its detailed dietary trends considering the variations in sexes and age.

This study aimed to identify the food menu of endangered *D. legata* using DNA metabarcoding method through animal and plant primers and to discuss the food habits of this species considering sexes and ages. We also discuss the effectiveness and limitations of this method for food habit analysis and its usability in rare and elusive animal species.

Materials and methods

Samples

Stomach contents were sampled from a total of 62 dead bodies of *D. legata* collected in the northern part of Okinawajima Island between 2009 and 2019 (Fig. 1). Dead *D. legata* were collected by the Ministry of the Environment. The causes of death were investigated under external conditions, and age was determined based on the hair condition of dead animals. The causes of deaths were roadkills and attacks by invasive domestic cats *Felis catus* and dogs *Canis lupus familiaris*. The age of the dead animals was divided into three categories by the rangers of the Ministry of the Environment, Japan, based on the hair condition of the individuals, i.e., juveniles had soft and gray hair; adults had stiff, stinging, and yellowish-brown hair; and subadults had mixed hair. However, these categories did not reflect sexual maturation. In this study, 34 adults (males, 15; females, 18; unknown, 1), eight subadults (male, 4; female, 3; unknown, 1), and 20 juveniles (male, 7; female, 12; unknown, 1) were used (Table 1). Although dead adults were found throughout the year, dead juveniles and subadults were found from November to May and February to July, respectively (Table 1).

An autopsy was conducted at the National Institute for Environmental Studies, and the stomach tissue was extracted. The stomachs were frozen under –20°C until their contents were extracted. After extraction, the contents were stored in 99% ethanol, and the bottles containing the extracts were stored in the laboratory. When stomach contents were retrieved, mollusks and feathers were observed visually; however, other contents were found to be silted, and no other organisms could be identified.

DNA extraction

DNA extraction experiments were conducted in 2019. Parts of the sample (approximately 1.0 mL) were placed into 5.0 mL tube with stainless-steel beads (approximately 3.0 mL) and 10x PBS buffer (0.5 mL) and homogenized for 3 min using a micro homogenizing system micro smash MS-100R (TOMY SEIKO, Tokyo, Japan). DNA was extracted using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was placed into a freezer at –20°C until the following experiments.

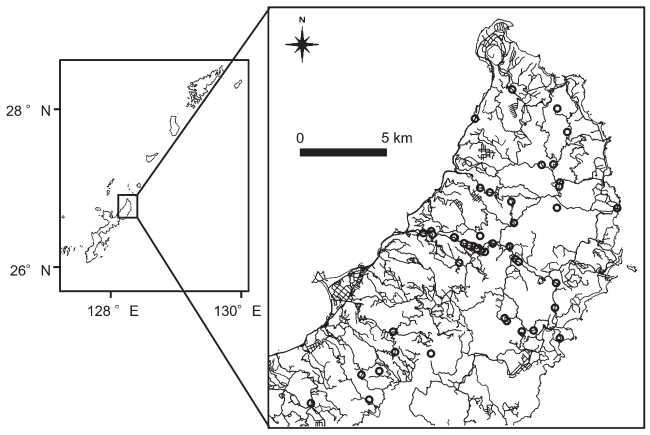

Fig. 1. Collection sites of used specimens (Circles in the map). Black lines in the map show roads.

Polymerase chain reaction amplification for the metabarcoding sequencing of plants

Two-step-tailed polymerase chain reactions (PCRs) were conducted to prepare libraries for next-generation sequencing (NGS) analysis. The first PCR amplified the target region, and the second PCR attached sequence adapters to connect to the flow cell of the Illumina MiSeq NGS platform (Illumina, San Diego, CA, USA). Each sample-specific dual index was used for sample identification (details in Sato et al. 2015). The PCR was performed using an automated thermal cycler (VeritiPro; Thermo Fisher Scientific, Waltham, MA, USA).

For amplifying the plant internal transcribed spacer 2 (*ITS2*) intergenic region, we used the universal primer pair UniPlantF and UniPlantR (Moorhouse-Gann et al. 2018) to assess plant materials. Each universal primer was combined with the priming region for second-round indexing PCR and sequencing and with random hexamer or dimer nucleotides for effective sequencing using a MiSeq platform (Illumina, San Diego, CA, USA). The complete sequences of forward and reverse primers are 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNTGTGAATTGCARRATYCMG-3' and 5'-GT GACTGGAGTTCAGACGTGTGCTCTTCCGATCTN NCCCGHYTGAYYTGRGGTCDC-3', respectively. The target length of the PCR products was expected to be 260–370 base pairs (bp). Two DNA polymerases, PrimeSTAR HS and ExTaq HS (Takara Bio, Shiga, Japan), were used to amplify relatively more diverse plant species. PCRs using each polymerase were adapted equally for all samples. Using two types of polymerases with different fidelity is expected to amplify relatively more diverse DNA types than possible when using only one of them, because of their different susceptibilities to DNA sequences, particularly to secondary structures. For PrimeSTAR HS, the PCR mix was prepared using 2.0 μL 5x PrimeSTAR Buffer, 0.8 μL of dNTP Mix, 0.6 μL of primer-F and primer-R (final concentration of 0.3 μM), 3.9 μL of RNase free water, and 0.1 μL of PrimeSTAR HS DNA Polymerase. Subsequently, 8.0 μL of mixture and 2.0 μL of the extracted DNA were mixed. The PCR conditions were as follows: 94°C for 3 min, followed by five cycles each at 98, 46, and 72°C for 10, 15, and 30 s, respectively, followed by 35 cycles each at 98, 55, and 72°C for 10, 15, and 30 s, respectively, and finally at 72°C for 5 min. For the enzyme ExTaq HS, the PCR mix was prepared using 1.0 μL of 10x PCR Buffer, 0.8 μL of dNTP Mix, 1.2 μL of primer-F and primer-R each (final concentration of 0.6 μM), 3.75 μL of RNase free water, and 0.05 μL of ExTaq HS. Subsequently, 8.0 μL mixture and 2.0 μL of the extracted DNA were mixed. The PCR conditions were as follows: 94°C for 1 min, followed by five cycles each at 94, 51, and 72°C for 60, 90, and 90 s, respectively, followed by 35 cycles each at 94, 55, and 72°C for 60, 90, and 90 s, respectively, and finally at 72°C for 5 min.

Polymerase chain reaction amplification for the metabarcoding sequencing of animals

The first and second PCRs were performed using the same protocols as those used for plants but with different primers and PCR conditions.

For amplifying the animal cytochrome *c* oxidase subunit-I (*COI*) gene, we used the dgLCO1490 and CO1-CFMRa primers (Geller et al. 2013; Jusino et al. 2019) to assess animal materials. Each primer was combined with the priming region for sequencing and second-round indexing PCR and with random hexamer or dimer nucleotides for effective sequencing using a MiSeq platform (Illumina, USA). The full sequences of forward and reverse primers are 5'-ACACTCTTTCCCTACACGAC GCTCTTCCGATCTNNNNNNAGATATTGGAACWT TATATTTTATTTTTGG-3' and 5'-GTGACTGGAGTTC AGACGTGTGCTCTTCCGATCTNNWACTAATCAA TTWCCAAATCCTCC-3', respectively. The target length of the amplified PCR product for the *COI* gene was 230 bp. The DNA polymerases, PrimeSTAR HS and ExTaq HS (Takara Bio, Shiga, Japan), were used to amplify relatively more diverse animal species. The PCRs using each polymerase were adapted equally for all samples. For using the PrimeSTAR HS enzyme, the PCR mix was prepared using 2.0 μL of 5x PrimeSTAR Buffer, 0.8 μL of

Table 1. Collected year and status of used samples

ID	Year	Month	Sex	Age
1	2009	Dec	M	Adult
2	2010	Dec	F	Juvenile
3	2011	Feb	F	Adult
4		Jul	M	Subadult
5		Nov	F	Juvenile
6		Dec	M	Adult
7			M	Juvenile
8	2012	Jan	F	Juvenile
9			F	Juvenile
10			F	Adult
11		May	M	Juvenile
12		Aug	M	Adult
13		Sep	M	Adult
14		Nov	F	Juvenile
15			M	Juvenile
16			F	Adult
17	2013	Jan	F	Adult
18		Mar	F	Juvenile
19		Apr	F	Juvenile
20		Sep	M	Adult
21	2014	Jan	F	Adult
22		Mar	M	Juvenile
23		Apr	F	Adult
24		May	F	Adult
25			M	Adult
26		Jun	F	Subadult
27			M	Subadult
28		Jul	M	Adult
29			F	Adult
30			M	Adult
31		Sep	M	Adult
32		Dec	M	Adult
33			M	Juvenile
34	2015	Jan	M	Adult
35		Oct	M	Adult
36	2016	Jan	F	Juvenile
37		Feb	F	Juvenile
38		Mar	M	Adult
39			M	Juvenile
40		Apr	F	Adult
41		Oct	M	Adult
42		Nov	F	Adult
43	2017	Mar	Unknown	Juvenile
44		May	F	Juvenile
45		Sep	F	Adult
46	2018	Feb	F	Subadult
47			Unknown	Adult
48		Mar	F	Juvenile
49		Apr	F	Subadult
50			M	Subadult
51			F	Adult
52		Jun	M	Subadult
53		Nov	F	Adult
54	2019	Jan	M	Adult
55			F	Adult
56		Feb	M	Juvenile
57			F	Adult
58			F	Adult
59		Mar	F	Adult
60			F	Juvenile
61			Unknown	Subadult
62			F	Adult

dNTP Mix, 0.6 μL of primer-F and primer-R each (final concentration of 0.3 μM), 3.9 μL of RNase free water, and 0.1 μL of PrimeSTAR HS DNA Polymerase. Subsequently, 8.0 μL of mixture and 2.0 μL of the extracted DNA were mixed. The PCR conditions were as follows: 94°C for 3 min, followed by five cycles each at 98, 45, and 72°C for 10, 15, and 30 s, respectively, followed by 35 cycles each at 98, 55, and 72°C for 10, 15, and 30 s, respectively, and finally at 72°C for 5 min. For ExTaq HS, the PCR mix was prepared using 1.0 μL of 10x PCR Buffer, 0.8 μL of dNTP Mix, 1.2 μL of primer-F and primer-R each (final concentration of 0.6 μM), 3.75 μL of RNase free water, and 0.05 μL of ExTaq HS. Subsequently, 8.0 μL of mixture and 2.0 μL of extracted DNA were mixed. The PCR conditions were as follows: 94°C for 1 min, followed by five cycles each at 94, 52, and 72°C for 60, 90, and 90 s, respectively, followed by 35 cycles each at 94, 50, and 72°C for 60, 90, and 90 s, respectively, and finally at 72°C for 5 min.

Second-round indexing PCR

The products of the first-round PCR were amplified using the Illumina sequencing priming sites described above as the PCR priming sites by adding dual-index tag sequences (D5 and D7 series; Illumina) and flowcell binding sites of the Illumina adapter (Sato et al. 2015; Sato et al. 2019a). The forward and reverse primer sequences were 5'-AATGATACGGCGACCACCGAGATCTACACD {D501 to D508}ACACTCTTTCCCTACACGACGCTC TTCCGATCT-3' and 5'-CAAGCAGAAGACGGCATAC GAGAT{D701 to D712}GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCT-3', respectively. The second-round of PCR was conducted under the conditions described below. The PCR mix was prepared using 1.0 μL of 10x PCR Buffer, 0.8 μL of dNTP Mix, 1.5 μL of forward and reverse primers each (final concentration of 0.3 μM), 4.15 μL of RNase free water, and 1.0 μL of ExTaq HS. The prepared PCR mix (9.0 μL) was mixed with 1.0 μL of the first PCR product diluted 30-fold using RNase-free water (Thermo Fisher Scientific, USA) and used for the second PCR. The thermal cycle profile was as follows: 98°C for 30 s, followed by 15 cycles each at 98, 65, and 72°C for 40, 30, and 30 s, respectively, and finally at 72°C for 5 min.

DNA purification and preparation for sequencing

The products of the second PCR were purified by removing short DNA fragments (< 100 bp) using AMPure XP magnetic beads following the standard protocol

(Beckman Coulter, High Wycombe, UK). The sample concentration was quantified following the protocol of the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA) using a Qubit 3.0 Fluorometer and then diluted with Microbial DNA-Free water (Qiagen, Hilden, Germany) for DNA sequencing. The prepared 10 pM library was sequenced using the 500-cycle MiSeq reagent kit v2 (Illumina) following a standard paired-end sequencing protocol. The volume molarity of the libraries was estimated based on the average molecular weight of a DNA nucleotide (660 g/mol), quantified library concentration, and the length of the second PCR products. PhiX control DNA (Illumina) was incorporated into the sequencing system at a concentration of 0.2 pM (1% volume).

Metabarcoding data analysis

The DNA sequence data were filtered based on nucleotide quality scores and sequence lengths. The low-quality 3′-tail bases of each sequence ($> 10^{-1}$ error rate) were deleted using the program DynamicTrim (Cox et al. 2010). These tail-trimmed paired-end sequences were merged using the software FLASH (Magoč and Salzberg 2011) and filtered using a custom PERL script (Miya et al. 2015) to exclude sequences with atypical length compared with those of expected size described above: filter-pass ranges of the partial *ITS2* regions in plants and partial *COI* gene regions in animals were 230 to 400 bp and 145 to 250 bp, respectively. Primer sequences were removed using the TagCleaner program (Schmieder et al. 2010) with a maximum 5-base mismatch. Sequences lacking primers at either end were excluded from the analysis. The identical sequences with ≥ 2 count within each sample were merged into a de-replicated effective sequence while retaining its count information in its sequence name using UCLUST (Edgar 2010). Finally, the singleton sequences in each sample were aligned with effective sequences ≥ 2 count at ≥ 99% sequence similarity to remove a few nucleotide substitutions derived from PCR and sequencing errors. The number of aligned singletons was added to the count information of the matched effective sequence, and unaligned sequences were discarded.

The quality-filtered effective sequences of the partial *ITS2* and *COI* regions were subjected to similarity-based taxonomic assignments using the National Center for Biotechnology Information (NCBI) BLAST Plus program (Camacho et al. 2009) and the NCBI nucleotide (nt) database provided on its website. For species identification based on the sequence data, sequences with > 10 reads were selected, and species annotation was performed at a sequence similarity of > 99% at the species level, 98–99% at the genus level, and < 98% at the family level, following Pereira et al. (2019). However, when the similarity was > 99% and the species was not distributed in the study area, it was identified as belonging to a genus or family, whereas when the similarity was 98–99% and only one species was recorded in the study area, it was identified as that species. The sequence counts of the identified operational taxonomic units (OTUs) from two separate PCRs with different DNA polymerases were summed for each sample.

As certain species, including those with no distribution data, were found in the trees of the *Ficus* spp., phylogenetic analysis was conducted using the *ITS2* region of the species listed in the Ryukyu Plant Database (Ryukyu Plant Research Group 2018 onward). The reference sequences of native species registered in GenBank were aligned with the *Ficus* sequences obtained in this study using MAFFT 7.310 (Katoh and Standley 2013). The *Ficus* dataset includes a relatively high number of closely related sequences within the same genus. Therefore, in the present study, we adopted a distance-based neighbor-joining (NJ) method for phylogenetic analysis. The NJ phylogenetic tree was constructed based on Kimura's 2-parameter model using MEGA 7 version 7.0.14 (Kumar et al. 2016). One hundred bootstrap replicates were used to evaluate the reliability of phylogenetic tree nodes. Based on the identified OTUs, the frequency of occurrence (FO) was calculated using the following formula:

$$FO = \frac{\textit{The number of samples the Item A appears}}{\textit{All analyzed all samples}}$$

Welch's *t*-test was used to compare the number of items between sexes, and the analysis of variance (ANOVA) was used to compare age. The statistical tests were performed using the *R* version 3.5.0 (R Core Team 2018). Non-metric multidimensional scaling (NMDS) was conducted based on the occurrence data and number of reads in each item to identify trends in food habits on sex and age. Stress values were calculated, with values around or above 0.2 deemed suspect and those equal to or below 0.1 considered fair. The analysis was performed using PAST ver. 4.06b (Hammer et al. 2001).

Results

A total of 26 orders, 38 families, and 63 species of plants (Table 2), and 12 orders, 35 families, and 36 species of animals (Table 3) were identified in the stomach contents of *D. legata*. The number of items showing OTUs was 83 for plants and 39 for animals. Seven *Ficus* species were identified using phylogenetic analysis (Appendix 1). Fifty-nine plant and 22 animal items, 35 plant and 14 animal items, and 46 plant and 18 animal items were identified in the adults, subadults, and juveniles, respectively. Birds whose feathers were observed during the sample treatment mentioned earlier were not detected in the present DNA analysis, although mollusks were detected. Fagaceae did not appear from samples which were collected six or more years earlier than the date of the DNA extraction experiments (Fig. 2).

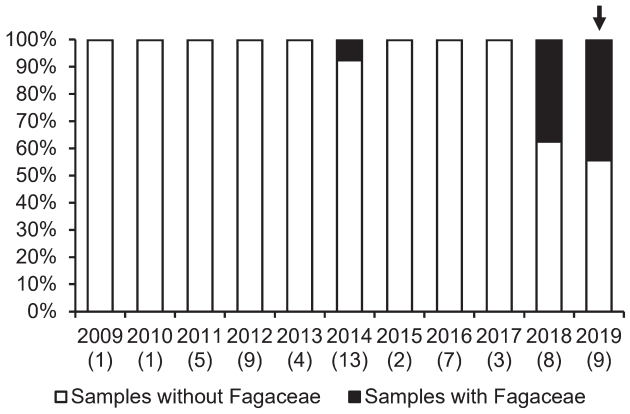

Fig. 2. Proportion of individuals in which Fagaceae were observed. Numbers in parentheses indicate number of analyzed individuals. An arrow indicates a year that the DNA extraction experiment was conducted.

The number of plant items that appeared in the contents of *D. legata* did not differ markedly between sexes, with 38 in adult males and 40 in adult females (Table 2). However, the number of animal items revealed marked sexual differences: six adult males and 19 females (Table 3). Among the plants, *Ficus* spp. and *Psychotria serpens* were found in more than 20% of the adult and juvenile samples. Certain species frequently appeared in the samples collected from the stomachs of *D. legata* adults but not in those collected from the stomachs of juveniles (e.g., *Pinus luchuensis*), whereas the opposite was also observed (e.g., *Bidens pilosa*) (Table 2). In animals, *Meghimatium* sp. frequently appeared in the samples collected from adult and subadult and was detected relatively more frequently in adult males than in adult females. Coleoptera were frequent in the stomach contents of adults, and Lepidoptera in those of subadults (Table 3). No invertebrate items were observed with an FO > 20% in the juveniles (Table 3).

The number of items observed in an adult stomach was 5.1 ± 3.6 (mean ± *SD*; plants: 4.0 ± 2.7 items, animals: 1.1 ± 1.9 items), those in the contents of a subadult stomach was 7.5 ± 3.8 (plants: 5.3 ± 3.3, animals: 2.3 ± 1.3), and those in a juvenile stomach was 4.8 ± 4.1 (plants: 4.4 ± 3.2, animals: 1.1 ± 1.7) (Table 4). Eleven items, which were the most abundant, were found in the stomach samples of adults, nine of which were Lepidoptera. There was no significant difference in the number of items between adult males and females (Welch's *t*-test; plant foods: $P = 0.484$, animal foods: $P = 0.412$, and all foods: $P = 0.933$). Due to the small sample size, we did not perform statistical tests for the other age groups. The number of items observed in the stomach contents of *D. legata* did not differ significantly among the age groups (ANOVA; plant foods: $P = 0.461$, animal foods: $P = 0.227$, all foods: $P = 0.207$). Moreover, the number of plant items observed in the stomach contents of all age groups was significantly higher than that of animal items (Welch's *t*-test; adults: $P < 0.05$, subadults: $P < 0.05$, and juveniles: $P < 0.05$).

The results of the NMDS plot indicated that the stomach contents of *D. legata* were not categorized based on sex or age, although the stress values were over 1.0, and the components of the stomach contents of a few females differed from those of the others (Fig. 3).

Discussion

Food habits of D. legata

Previous studies have shown that the main food source for *D. legata* is obtained from plants (Takehara et al. 2015; Kudaka and Kudaka 2017). In the present study, we identified 45 plant species not recorded in previous studies as food items. In total, 84 species are listed as food menu together with previous studies, which indicates that *D. legata* consume diverse plant species (Table 2). Plant species with > 20% varied in age (Table 2). Food habits have been suggested to differ among different age groups. However, because the sampling months of juveniles were limited to November–May, whereas adults were sampled throughout the year, the difference in FO among ages may reflect the sampled season. In addition, because annual and seasonal sampling biases were recognized (Table 1), the food habits of this species may vary depending on the year and season.

Table 2. Frequencies of occurrence of plant matters

Order	Family	Species	Adult			Subadult ($N = 8$)	Juvenile ($N = 20$)	Total ($N = 62$)	Previous studies*
			Male ($N = 15$)	Female ($N = 18$)	All ($N = 34$)				
Cyatheales	Cyatheaceae	*Cyathea lepifera*	–	–	–	–	–	–	3
Cycadales	Cycadaceae	*Cycas revoluta*	0.0	0.0	0.0	0.0	5.0	1.6	
Pinales	Pinaceae	*Pinus luchuensis*	13.3	22.2	26.5	0.0	0.0	9.7	3, 4, 6, **
Laurales	Lauraceae	*Machilus thunbergii*	–	–	–	–	–	–	3, 4
Poales	Poaceae	*Miscanthus floridulus*	0.0	5.6	2.9	0.0	0.0	1.6	
Poales	Poaceae	*Oplismenus undulatifolius*	0.0	5.6	2.9	0.0	5.0	3.2	
Poales	Poaceae	*Saccharum officinarum*	0.0	5.6	2.9	0.0	0.0	1.6	
Poales	Poaceae	Poaceae sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Poales	Cyperaceae	*Scirpus ternatanus*	–	–	–	–	–	–	2, 4
Poales	Cyperaceae	*Gahnia tristis*	–	–	–	–	–	–	3
Zingiberales	Zingiberaceae	*Alpinia zerumbet*	0.0	5.6	2.9	0.0	0.0	1.6	
Ranunculales	Menispermaceae	*Stephania japonica*	0.0	0.0	0.0	0.0	5.0	1.6	
Proteales	Sabiaceae	*Meliosma* sp.	0.0	0.0	0.0	12.5	0.0	1.6	
Malpighiale	Euphorbiaceae	*Macaranga tanarius*	0.0	0.0	0.0	12.5	0.0	1.6	
Malpighiale	Euphorbiaceae	*Mallotus japonicus*	–	–	–	–	–	–	4, 6, **
Malpighiale	Euphorbiaceae	*Vernicia montana*	–	–	–	–	–	–	3, 4
Malpighiale	Phyllanthaceae	*Glochidion zeylanicum*	0.0	0.0	0.0	12.5	0.0	1.6	
Malpighiale	Phyllanthaceae	*Glochidion* sp.	6.7	0.0	2.9	25.0	0.0	4.8	
Malpighiale	Salicaceae	*Idesia polycarpa*	–	–	–	–	–	–	3, 4
Fabales	Fabaceae	*Leucaena leucocephala*	6.7	11.1	8.8	12.5	0.0	6.5	3, 4
Fabales	Fabaceae	*Mucuna macrocarpa*	–	–	–	–	–	–	5
Fabales	Fabaceae	Fabaceae sp.	0.0	5.6	2.9	12.5	0.0	3.2	
Rosales	Cannabaceae	*Trema orientalis*	–	–	–	–	–	–	1
Rosales	Cannabaceae	Cannabaceae sp.	6.7	0.0	2.9	0.0	0.0	1.6	
Rosales	Moraceae	*Ficus ampelas*	13.3	0.0	2.9	12.5	10.0	9.7	3
Rosales	Moraceae	*Ficus benguetensis*	–	–	–	–	–	–	3
Rosales	Moraceae	*Ficus erecta*	20.0	22.2	23.5	25.0	10.0	17.7	3, 4, 6
Rosales	Moraceae	*Ficus irisana*	6.7	0.0	0.0	0.0	5.0	3.2	
Rosales	Moraceae	*Ficus pumila*	0.0	5.6	5.9	12.5	10.0	6.5	
Rosales	Moraceae	*Ficus thunbergii*	13.3	5.6	8.8	12.5	10.0	11.3	4
Rosales	Moraceae	*Ficus superba*	0.0	0.0	2.9	12.5	0.0	1.6	4
Rosales	Moraceae	*Ficus virgata*	13.3	11.1	11.8	25.0	0.0	9.7	3
Rosales	Moraceae	*Ficus* sp.	0.0	0.0	5.9	12.5	0.0	3.2	
Rosales	Moraceae	*Morus australis*	0.0	5.6	2.9	12.5	5.0	4.8	
Rosales	Moraceae	Moraceae sp.	13.3	11.1	23.5	12.5	10.0	11.3	
Rosales	Rosaceae	*Cerasus campanulata*	0.0	5.6	2.9	0.0	0.0	1.6	4
Rosales	Rosaceae	*Rubus grayanus*	–	–	–	–	–	–	4
Rosales	Rosaceae	*Rubus* sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Rosales	Rosaceae	Rosaceae sp.	0.0	5.6	2.9	12.5	0.0	3.2	
Rosales	Urticaceae	*Oreocnide pedunculata*	–	–	–	–	–	–	3
Cucurbitales	Cucurbitaceae	*Trichosanthes miyagii*	–	–	–	–	–	–	4, **
Cucurbitales	Cucurbitaceae	*Trichosanthes* sp.	6.7	0.0	2.9	12.5	0.0	3.2	
Fagales	Fagaceae	*Castanopsis sieboldii*	13.3	16.7	17.6	12.5	5.0	12.9	3, 4, 6, **
Fagales	Fagaceae	*Lithocarpus edulis*	–	–	–	–	–	–	4
Fagales	Fagaceae	*Quercus* sp.	6.7	16.7	14.7	12.5	5.0	11.3	
Fagales	Fagaceae	Fagaceae sp.	0.0	5.6	2.9	0.0	0.0	1.6	
Myricales	Myricaceae	*Morella rubra*	0.0	5.6	2.9	25.0	15.0	9.7	3, 4, 6, **
Myrtales	Myrtaceae	*Syzygium buxifolium*	0.0	0.0	0.0	0.0	5.0	1.6	
Myrtales	Melastomataceae	*Melastoma candidum*	–	–	–	–	–	–	4
Sapindales	Anacardiaceae	*Toxicodendron succedaneum*	6.7	0.0	2.9	0.0	0.0	1.6	3, 4, 6, **
Sapindales	Rutaceae	*Tetradium glabrifolium*	–	–	–	–	–	–	3, 4, 6, **
Sapindales	Rutaceae	*Zanthoxylum ailanthoides*	–	–	–	–	–	–	1
Sapindales	Staphyleaceae	*Staphylea japonica*	–	–	–	–	–	–	4
Brassicales	Brassicaceae	Brassicaceae sp.	0.0	5.6	2.9	0.0	0.0	1.6	

Table 2. (continued)

Order	Family	Species	Adult			Subadult ($N = 8$)	Juvenile ($N = 20$)	Total ($N = 62$)	Previous studies*
			Male ($N = 15$)	Female ($N = 18$)	All ($N = 34$)				
Malvales	Elaeocarpaceae	*Elaeocarpus* sp.	0.0	0.0	0.0	0.0	5.0	1.6	4, **
Malvales	Malvaceae	*Hibiscus tiliaceus*	0.0	5.6	2.9	0.0	0.0	1.6	
Malvales	Malvaceae	*Hibiscus mutabilis*	0.0	0.0	0.0	0.0	5.0	1.6	
Malvales	Malvaceae	*Hibiscus* sp.	0.0	0.0	0.0	12.5	0.0	1.6	
Saxifragales	Daphniphyllaceae	*Daphniphyllum macropodum*	13.3	0.0	5.9	0.0	10.0	6.5	
Saxifragales	Daphniphyllaceae	*Daphniphyllum teijsmannii*	13.3	5.6	11.8	12.5	10.0	11.3	
Santalales	Santalaceae	*Korthalsella japonica*	6.7	0.0	2.9	25.0	0.0	4.8	
Caryophyllales	Polygonaceae	*Persicaria chinensis*	0.0	5.6	2.9	0.0	5.0	3.2	
Caryophyllales	Polygonaceae	*Persicaria* sp.	0.0	5.6	2.9	0.0	15.0	6.5	
Cornales	Hydrangeaceae	*Hydrangea liukiuensis*	0.0	0.0	0.0	0.0	5.0	1.6	
Cornales	Hydrangeaceae	*Hydrangea viburnoides*	0.0	0.0	0.0	12.5	0.0	1.6	
Ericales	Actinidiaceae	*Actinidia rufa*	6.7	11.1	8.8	12.5	5.0	8.1	3, 4
Ericales	Primulaceae	*Ardisia crenata*	0.0	0.0	0.0	0.0	5.0	1.6	
Ericales	Primulaceae	*Ardisia* sp.	6.7	0.0	2.9	0.0	0.0	1.6	
Ericales	Styracaceae	*Styrax japonica*	0.0	0.0	0.0	0.0	10.0	3.2	4
Ericales	Ebenaceae	*Diospyros japonica*	–	–	–	–	–	–	4
Ericales	Symplocaceae	*Symplocos okinawensis*	13.3	0.0	5.9	0.0	0.0	3.2	
Ericales	Symplocaceae	*Symplocos glauca*	0.0	0.0	0.0	0.0	5.0	1.6	
Ericales	Symplocaceae	*Symplocos liukiuensis*	0.0	11.1	8.8	0.0	0.0	4.8	
Ericales	Symplocaceae	*Symplocos prunifolia*	0.0	5.6	5.9	0.0	10.0	6.5	6
Ericales	Symplocaceae	*Symplocos* sp.	13.3	11.1	14.7	0.0	5.0	9.7	
Ericales	Symplocaceae	Symplocaceae sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Ericales	Ternstroemiaceae	*Eurya japonica*	13.3	11.1	11.8	0.0	5.0	8.1	
Ericales	Ternstroemiaceae	*Eurya* sp.	13.3	5.6	8.8	0.0	0.0	4.8	
Ericales	Ternstroemiaceae	*Ternstroemia gymnanthera*	6.7	5.6	5.9	0.0	0.0	3.2	
Ericales	Ternstroemiaceae	Ternstroemiaceae sp.	6.7	5.6	5.9	0.0	0.0	3.2	
Ericales	Theaceae	*Camellia japonica*	–	–	–	–	–	–	4
Ericales	Theaceae	*Schima wallichii*	13.3	11.1	11.8	25.0	15.0	14.5	3, 4, **
Gentianales	Apocynaceae	*Anodendron affine*	13.3	0.0	5.9	12.5	0.0	4.8	
Gentianales	Apocynaceae	*Trachelospermum gracilipes*	0.0	5.6	2.9	12.5	15.0	8.1	4
Gentianales	Apocynaceae	Apocynaceae sp.	0.0	0.0	0.0	12.5	0.0	1.6	
Gentianales	Rubiaceae	*Coptosapelta diffusa*	6.7	0.0	2.9	0.0	5.0	3.2	
Gentianales	Rubiaceae	*Gardenia jasminoides*	–	–	–	–	–	–	4
Gentianales	Rubiaceae	*Psychotria serpens*	33.3	16.7	26.5	12.5	25.0	24.2	4
Gentianales	Rubiaceae	Rubiaceae sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Lamiales	Oleaceae	Oleaceae sp.	0.0	0.0	0.0	12.5	0.0	1.6	
Aquifoliales	Aquifoliaceae	*Ilex integra*	0.0	0.0	0.0	0.0	5.0	1.6	
Aquifoliales	Aquifoliaceae	*Ilex maximowicziana*	13.3	5.6	11.8	12.5	5.0	9.7	
Aquifoliales	Aquifoliaceae	*Ilex warburgii*	6.7	0.0	2.9	0.0	0.0	1.6	
Aquifoliales	Aquifoliaceae	*Ilex* sp.	20.0	0.0	11.8	0.0	15.0	11.3	
Aquifoliales	Aquifoliaceae	Aquifoliaceae sp.	6.7	0.0	2.9	0.0	0.0	1.6	
Apiales	Araliaceae	*Aralia ryukyuensis*	0.0	0.0	0.0	0.0	5.0	1.6	
Apiales	Araliaceae	*Dendropanax trifidus*	6.7	0.0	2.9	12.5	5.0	4.8	
Apiales	Araliaceae	*Schefflera heptaphylla*	13.3	5.6	8.8	0.0	10.0	8.1	
Apiales	Pittosporaceae	*Pittosporum boninense*	0.0	5.6	2.9	25.0	0.0	4.8	
Asterales	Asteraceae	*Ainsliaea macroclinidioides*	6.7	0.0	2.9	0.0	5.0	3.2	
Asterales	Asteraceae	*Ainsliaea* sp.	6.7	0.0	2.9	0.0	5.0	3.2	
Asterales	Asteraceae	*Bidens pilosa*	6.7	16.7	11.8	12.5	20.0	14.5	
Asterales	Asteraceae	*Youngia japonica*	0.0	0.0	0.0	0.0	5.0	1.6	
Asterales	Campanulaceae	*Cyclocodon lancifolius*	0.0	5.6	2.9	0.0	0.0	1.6	

* References of previous studies; 1: Tokida (2001), 2: Torikai and Ueda (2007), 3: Takehara et al. (2015), 4: Kudaka and Kudaka (2017), 5: Kobayashi et al. (2018), 6: Taniguchi et al. (2019).

** Feeding behavior was frequently observed.

Table 3. Frequencies of occurrence of animal matters

Order	Family	Species	Adult			Subadult ($N = 8$)	Juvenile ($N = 20$)	Total ($N = 62$)	Previous studies*
			Male ($N = 15$)	Female ($N = 18$)	All ($N = 34$)				
Orthoptera	Gryllidae	*Velarifictorus* sp.	0.0	0.0	0.0	12.5	0.0	1.6	
Orthoptera	Tettigoniidae	*Mecopoda elongata*	–	–	–	–	–	–	3
Diptera	Culicidae	*Aedes flavopictus*	0.0	0.0	0.0	12.5	0.0	1.6	
Diptera	Drosophilidae	*Amiota* sp.	0.0	5.6	2.9	0.0	0.0	1.6	
Diptera	Calliphoridae	*Lucilia* sp.	0.0	5.6	2.9	0.0	0.0	1.6	
Diptera	Lonchaeidae	Lonchaeidae sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Diptera	Cecidomyiidae	Cecidomyiidae sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Diptera	Scatopsidae	Scatopsidae sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Diptera	Anthomyiidae	Anthomyiidae sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Diptera	Psychodidae	Psychodidae sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Diptera	Xylophagidae	Xylophagidae sp.	0.0	0.0	0.0	12.5	0.0	1.6	
Diptera	Tipulidae	Tipulidae sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Diptera	–	Diptera sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Psocoptera	Psocidae	Psocidae sp.	0.0	0.0	0.0	12.5	5.0	3.2	
Hemiptera	Pyrrhocoridae	Pyrrhocoridae sp.	0.0	0.0	0.0	12.5	0.0	1.6	
Lepidoptera	Erebidae	Erebidae sp.	0.0	5.6	2.9	0.0	5.0	3.2	
Lepidoptera	Neoblastobasis	Neoblastobasis sp.	6.7	0.0	2.9	0.0	0.0	1.6	
Lepidoptera	Yponomeutidae	*Plutella xylostella*	0.0	5.6	2.9	0.0	0.0	1.6	
Lepidoptera	Lasiocampidae	Lasiocampidae sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Lepidoptera	Gelechiidae	Gelechiidae sp.	0.0	5.6	2.9	0.0	0.0	1.6	
Lepidoptera	Geometridae	Geometridae sp.	0.0	11.1	5.9	0.0	10.0	6.5	
Lepidoptera	Notodontidae	Notodontidae sp.	0.0	5.6	2.9	0.0	0.0	1.6	
Lepidoptera	Tortricidae	Tortricidae sp.	0.0	11.1	5.9	37.5	5.0	9.7	
Lepidoptera	Thyrididae	Thyrididae sp.	0.0	5.6	2.9	0.0	0.0	1.6	
Lepidoptera	Oecophoridae	Oecophoridae sp.	0.0	0.0	0.0	0.0	5.0	1.6	
Lepidoptera	Noctuidae	Noctuidae sp.	0.0	5.6	2.9	25.0	15.0	9.7	
Lepidoptera	–	Lepidoptera sp.	0.0	11.1	5.9	12.5	0.0	4.8	
Hymenoptera	Braconidae	*Apanteles cypris*	0.0	0.0	0.0	12.5	0.0	1.6	
Hymenoptera	Formicidae	*Technomyrmex albipes*	0.0	0.0	0.0	12.5	5.0	3.2	
Coleoptera	Cerambycidae	*Pterolophia formosana*	0.0	5.6	2.9	0.0	0.0	1.6	
Coleoptera	Curculionidae	Curculionidae sp.	6.7	16.7	20.6	12.5	5.0	9.7	
Coleoptera	Elateridae	Elateridae sp.	–	–	–	–	–	–	1, 2
Acari	Triophtydeidae	Triophtydeus sp.	6.7	5.6	5.9	0.0	0.0	3.2	
Trombidiformes	–	Trombidiformes sp.	6.7	0.0	2.9	0.0	0.0	1.6	
Polydesmida	Paradoxosomatidae	*Chamberlinius hualienensis*	0.0	5.6	2.9	0.0	5.0	3.2	
Polydesmida	Xystodesmidae	*Riukiaria* sp.	–	–	–	–	–	–	2
Decapoda	Potamidae	*Candidiopotamon okinawense*	–	–	–	–	–	–	2
Decapoda	Potamidae	Potamidae sp.	0.0	0.0	2.9	0.0	0.0	1.6	
Stylommatophora	Philomycidae	*Meghimatium* sp.	33.3	5.6	26.5	25.0	5.0	14.5	1, 2
Stylommatophora	Bradybaenidae	*Bradybaena circulus*	0.0	0.0	0.0	12.5	0.0	1.6	
Stylommatophora	Camaenidae	*Satsuma eucosmia*	20.0	5.6	11.8	12.5	0.0	8.1	
Stylommatophora	Camaenidae	*Coniglobus mercatorius*	0.0	5.6	2.9	0.0	0.0	1.6	
Stylommatophora	Clausliidae	Clausliidae sp.	–	–	–	–	–	–	2
Architaeniolossa	Cyclophoridae	*Cyclophorus turgidus angulatus*	–	–	–	–	–	–	2
Opisthopora	Megascolecidae	Megascolecidae sp.	–	–	–	–	–	–	1, 2, 3
Anura	Rhacophoridae	*Buergeria japonica*	–	–	–	–	–	–	2
Anura	Rhacophoridae	*Zhangixalus viridis*	–	–	–	–	–	–	2
Caudata	Salamandridae	*Cynops ensicauda popei*	–	–	–	–	–	–	2
Squamata	Lacertidae	*Takydromus smaragdinus*	0.0	5.6	2.9	0.0	0.0	1.6	

* References of previous studies; 1: Takehara et al. (2015), 2: Kudaka and Kudaka (2017), 3: Tamanaha et al. (2017).

Table 4. Mean (± *SD*) of number of items in a stomach

	Adult ($N = 34$)		Subadult ($N = 8$)		Juvenile ($N = 20$)	
	Mean ± *SD*	Min–Max	Mean ± *SD*	Min–Max	Mean ± *SD*	Min–Max
Plants	4.0 ± 2.7	1–11	5.3 ± 3.3	0–10	4.4 ± 3.2	0–10
Animals	1.1 ± 1.9	0–11	2.3 ± 1.3	0–4	1.1 ± 1.7	0–6
Total	5.1 ± 3.6	0–18	7.5 ± 3.8	0–12	4.8 ± 4.1	0–11

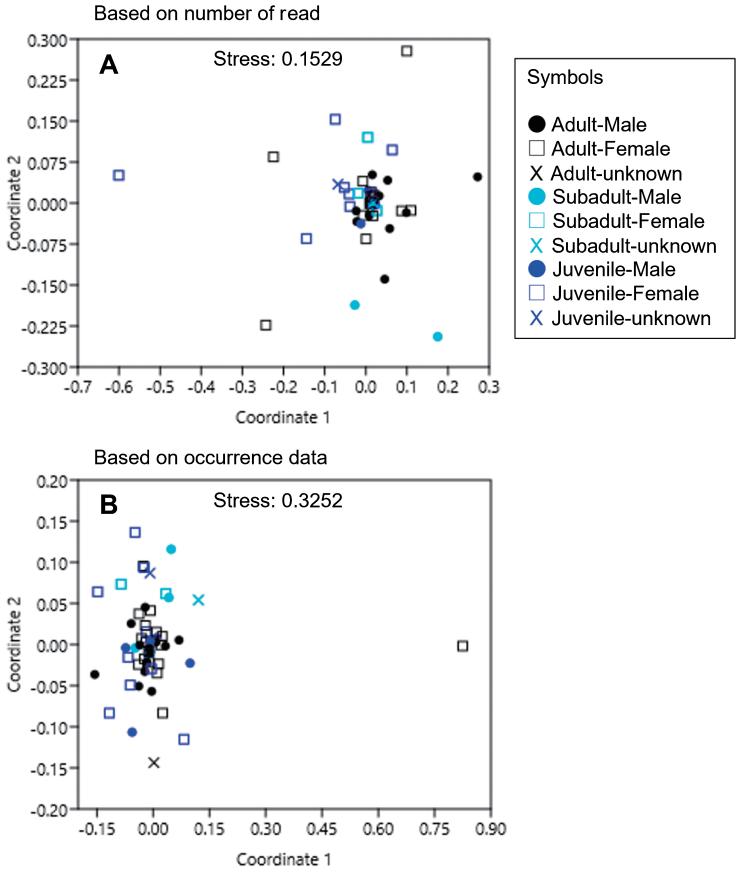

Fig. 3. Plots of non-metric multidimensional scaling (NMDS) based on the data of number of reads (A) and occurrence (B).

In addition, although previous studies have reported a few animals as food items (Takehara et al. 2015; Kudaka and Kudaka 2017; Tamanaha et al. 2017), in the present study, we demonstrated that *D. legata* fed on 36 animal species and totally 46 animal species are listed as food menu together with previous studies (Table 3). That is relatively more frequent than previously described. Furthermore, the higher FO of animals found in the stomach contents of *D. legata* adults than in the stomach contents of juveniles suggests a greater importance of animal foods for adults. The likely reasons for the differences in the food menus of adults and juveniles include differences in their experiences (Zhang and Wang 2011), seasonal differences in food availability and preferences, with the breeding season of *D. legata* being from September to February (Okano et al. 2015), and the limited season available for the appearance of juveniles (Puig et al. 1999). Both adults and juveniles frequently feed on *Ficus* spp. and *P. serpens*; thus, these plants are important food sources for *D. legata*. The problem with the DNA metabarcoding method that the plant parts cannot be identified (Tercel et al. 2021). However, this rat species is known to frequently feed on fruits, nuts, and seeds (Kudaka and Kudaka 2017); therefore, a few plant foods that appear in the stomachs of these animals in this study could be fruits, nuts, and seeds. Muridae species fre-

quently utilize acorns (nuts of Fagaceae) in general (Sunyer et al. 2016; Bonacchi et al. 2017; Onodera et al. 2017). If the Fagaceae detected in this study were nuts, they would be more than 10% in FO at all ages. Fruit mass has largely changed over the years in Fagaceae, and *Castanopsis sieboldii*, the dominant tree species in the forest studied here, shows large annual fluctuations in fruiting (Takashima et al. 2021). Thus, although nuts may be an important food for *D. legata*, evaluating the importance of nuts in the Fagaceae is difficult because the fruiting season is limited. This indicates that this species cannot forage for sufficient nuts of Fagaceae every year, and this study did not address seasonal changes in food habits owing to the small sample size.

Among the animal items, Gastropoda appeared frequently in both adults and juveniles, although the FO of juveniles was lower than that of the adults. Males tended to feed on specific animals, such as *Meghimatium* sp., whereas females tended to feed on various animals, including insects. Females generally try to reduce the cost of searching for food by using animals that can be easily captured (Puig et al. 1999). This is one possible explanation for the differences between the sexes found in the food items of *D. legata*. However, the seasonal differences could not be analyzed in this study because of the small sample size. The composition of the stomach contents of several females was different from that of the others (Fig. 3), which may reflect female reproductive conditions. Possible seasonal changes in the food habits of this species should be investigated in future studies with large sample sizes.

Species related to *D. legata* include *Rattus* spp. (Fabre et al. 2013; Thomson et al. 2018) and *D. legata* is endemic to the Ryukyu Archipelago. Most rodents are omnivorous (Landry 1970), and species related to *Rattus rattus* exhibit flexible food habits depending on their habitats (Gales 1982; Caut et al. 2008; Shiels et al. 2013, 2014). Most native *Rattus* species are distributed in large islands and continents. In contrast, although the distribution of *D. legata* is limited to relatively large islands in the Ryukyu Archipelago, the area of the distributed islands ranges from 105–1199 km^2, which is not large. In general, the environmental conditions on small islands are simpler than those on large islands, resulting in a relatively small number of species and populations of plants and animals that can inhabit small islands. Surviving in such a limited environment on a small island carries risks, particularly if one relies solely on foraging for a specific species. *Diplothrix legata*, found on small islands, has been observed to utilize a wide variety of species as food, with each animal feeding on a diverse range of species, indicating that it forages for various food items in a short time because multiple food items are found in its stomach. This diversity in food menus may be one of the reasons that the species has survived on these small islands.

The limitations of DNA metabarcoding for identifying food habits

Although, in this study, we identified various food items detected in the stomach contents of *D. legata*, certain limitations of this methodology need to be noted. The limitations of DNA metabarcoding have been described in several studies. Species that were not distributed at the study site were identified. This may be due to the incompleteness of the reference DNA database, erroneous sequences, or misassigned data (e.g., Nakahara et al. 2015; Alberdi et al. 2018; Ando et al. 2020; Tedersoo et al. 2022; Wanniarachchi et al. 2022). Considering that the identification of animal species is insufficient, the identification seems to be accurate at the family level. However, the identification of species may be difficult when multiple species of the same genus are distributed. In addition, both the *COI* and *ITS2* regions have limited detection abilities, and their sensitivities generally differ among taxa. Although using multiple primers reduces taxonomic bias (Alberdi et al. 2018), in this study, we used only one primer set each for plants and animals, and mosses and ferns were not detected. The UniPlant primers target Metaphyta, but the main target is Spermatophyta (seed plants) (Moorhouse-Gann et al. 2018). Therefore, one of the possible reasons why mosses and ferns did not appear was the selection of specific primers. In addition, our results suggest that the deterioration speed may differ among food item species because Fagaceae did not appear in old samples and that PCR primers, particularly the *COI*-specific primers, did not cover taxonomic groups evenly (Clarke et al. 2014; Deagle et al. 2014). Furthermore, feeding parts such as leaves, fruits, and seeds were not identified, and it was not known whether *D. legata* forages for certain foods such as small insects. Several issues above need to be addressed in future studies.

Conclusion

Diplothrix legata feeds on various plants, including the newly recorded items in this study, as well as on animals such as invertebrates, which have rarely been observed at a high frequency in the stomach contents of this species.

These food habits may have been acquired for adaptation to island habitats with limited biomass. From this point of view, various environments with diverse plants and animals are needed for the conservation of *D. legata.* DNA metabarcoding is the preferred tool for research on the food habits of rare, arboreal, omnivorous, and nocturnal mammals, regardless of its limitations.

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Appendix 1.

Phylogenetic tree of *Ficus* spp. found in stomach contents.