Noninvasive Genetic Methods for Species Identification and Dietary Profiling of the Japanese Dormouse Glirulus japonicus from Fecal Samples

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Original paper

Noninvasive genetic methods for species identification and dietary profiling of the Japanese dormouse *Glirulus japonicus* from fecal samples

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Abstract. Noninvasive methods for species identification and dietary profiling from fecal samples in an artificial nest box were developed for the Japanese dormouse *Glirulus japonicus*. The species is a natural monument in Japan protected by domestic regulations since 1975. We assessed the mitochondrial cytochrome *b* gene for species identification and obtained location-specific sequences for Oki and Yamanashi, Japan. This marker was able to identify the dormouse species from feces. We also performed DNA metabarcoding analyses to clarify the invertebrate and plant diets of the Japanese dormouse. Various invertebrates and plants were detected, supporting the omnivorous nature of this species. Furthermore, almost all dietary items were identified to the genus level (82.1% and 99.5% for invertebrates and plants, respectively). The dietary components in Yamanashi Prefecture suggested that the Japanese dormouse mainly consumed Lepidoptera and various plants in summer, and Diptera and Hemiptera (aphids) invertebrates and *Actinidia* sp. plants in autumn. The latter plants produce fruit in autumn, enabling the dormouse to accumulate fat before hibernation. We discuss the potential and pitfalls of the noninvasive method, including the necessity of local DNA databases, reliability of the global DNA database, sampling procedure to avoid contamination, and individual identification.

Key words: DNA barcoding, DNA metabarcoding, DNA scatology, dormice, next-generation sequencing.

Noninvasive genetic (NIG) methods are used to assess wildlife ecology and evolution (Ferreira et al. 2018). Understanding basic ecology noninvasively is especially important when dealing with protected or endangered animals on a background of accelerated biodiversity loss and high extinction rate (Rockström et al. 2009; Ceballos et al. 2020). Although previous studies mainly used the NIG method for species identification (Rodrigues et al. 2020), population genetic diversity (Ferreira et al. 2018), or phylogeography (Querejeta and Castresana 2018), next-generation sequencing (NGS) has enabled noninvasive dietary profiling (Buglione et al. 2018).

The Japanese dormouse, *Glirulus japonicus* (Rodentia, Gliridae), is an arboreal species inhabiting Honshu, Shikoku, and Kyushu Islands of the Japanese archipelago (Ohdachi et al. 2015). It has been protected as a natural monument in Japan since 1975. This species is the most ancient mammal in Japan (Nunome et al. 2007; Sato 2017), and each local population is highly differentiated (3–5 million years of divergence: Yasuda et al. 2007, 2012). Although its conservation status is currently Least Concern in the International Union for Conservation of Nature Red List, the basic ecology of this species has not been fully examined, and the population trend in the Red List is unknown. Considering the important roles of this species in the forest ecosystem, ecological insight into this strictly arboreal species is needed for sustainable forest management aiming at biodiversity conservation.

The diet of the Japanese dormouse has been assessed by direct observation of their feeding behavior or microscopic morphological analyses of stomach or fecal contents (Minato et al. 1997; Iwabuchi 2008; Aoki and

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Moriya 2009; Ochiai et al. 2015; Minato 2018; Takatsuki and Suzuki 2022). However, direct observation of feeding behavior is difficult because of the nocturnal nature of the dormouse. Also, tiny fragments of dietary species in the stomach or feces are difficult to identify taxonomically without special expertise. Therefore, discussions of dietary components in previous studies were typically based on broad taxonomic units (e.g., Ochiai et al. 2015). Furthermore, Aoki and Moriya (2009) used a sample that died in an accident, which is required for analysis of stomach contents. However, dissection is not recommended for such protected animals. Fecal analysis therefore has promise for noninvasive assessment because feces are left behind in artificial nest boxes (Ochiai et al. 2015). A novel noninvasive technique based on fecal analysis is needed to clarify the diet of the dormouse.

Recently, DNA metabarcoding analysis has been applied for dietary profiling of various mammals. This method is based on high-throughput DNA sequencing of target PCR amplicons from fecal or gastrointestinal samples (de Sousa et al. 2019; Ando et al. 2020). Some rodent species have also been examined, e.g., *Alexandromys* (*Microtus*) *montebelli* and *Apodemus* sp. in Japan (Sato et al. 2018, 2019, 2022; Murano et al. 2023) and *Ctenomys* sp. in Brazil (Lopes et al. 2020). The technique enables assessment of seasonal variations, interspecific competition, and geographic variations in dietary components. This method was also applied to an endangered vole in the United States (*Microtus californicus scirpensis*) to explore its diet for the aim of conservation management (Castle et al. 2020). In addition to the stable-isotope analysis to assess the trophic levels of species (e.g., Shiozuka et al. 2023), the DNA metabarcoding method provides a useful non-invasive dietary analysis.

In this study, we developed a noninvasive method to identify the host species and to profile the dietary components of the Japanese dormouse from fecal samples collected from artificial nest boxes. Specifically, we established DNA barcoding and metabarcoding methods for species identification and dietary profiling, respectively. We also discussed the potential and pitfalls of the method to examine the dietary ecology of animals.

Materials and methods

Samples and study area

We collected feces from artificial nest boxes placed in forests of Kiyosato (N35.94128°, E138.44830°; Hokuto City, Yamanashi Prefecture; Yamanashi hereafter) and Okinoshima Islands (N36.25166°, E133.31559°; Oki Island, Shimane Prefecture; Oki, hereafter) (Fig. 1; Table 1) in August, September, and October of 2020 and June, July, August, September, and October of 2021 (Yamanashi), and November in 2019 (Oki). We focused on these two sites since efficiency of our non-invasive genetic methods could be tested with geographically distant habitats harboring genetically distinct Japanese dormice. These nest boxes are placed for the survey of basic ecology and conservation of this elusive species. We have placed 370 nest boxes in Yamanashi and 82 in Oki and collected samples from the nest boxes shown in Table 1. The study was mostly conducted in autumn, which is the season prior to dormice hibernation (Iwabuchi et al. 2017). Soon after hibernation, dormice in Yamanashi for example usually start breeding and give birth in spring although geographic variation is observed in season for parturition (Minato, 2018). Each nest box had a basal area of approximately 12 cm × 12 cm, and front (with a hole) and back (no hole) heights of approximately 17 cm and 20 cm, respectively. Each box was attached to a tree trunk 1.2–1.5 m above ground level (Fig. 2A). Nothing was placed within the nest boxes before dormice entered. Dormice use nest boxes for their resting and breeding, not for hibernation. Dormice typically bring moss (bryophytes) and tree bark inside nest boxes (Shibata 2000; Shibata et al. 2004; Minato 2018) and leave their feces on this plant matter (Fig. 2B). We used tweezers to collect feces in the nest box and transfer them to sampling tubes. Collecting feces from the nest box may cause stress on the dormice if they are present in the nest box. In this study, we found the dormice in eight of 24 nest boxes at the sampling of feces (Table 1). We took special care not to be invasive on the dormice when sampling. Feces were transported to the experimental room as soon as possible and preserved

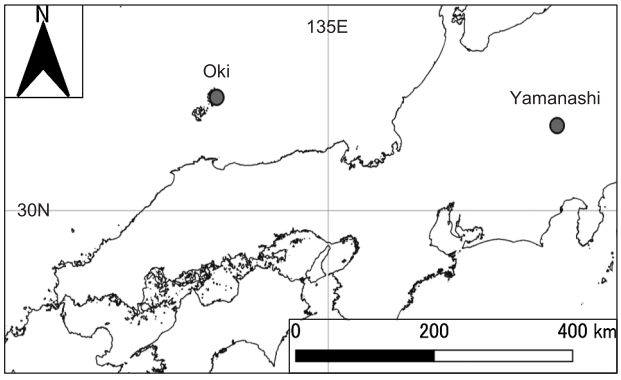

Fig. 1. Sampling locality in the Japanese archipelago (Oki and Yamanashi). See Table 1 for detailed information on the study sites.

Table 1. Sample information

Sample ID	Nest box	Date of sampling	Location	DNA[1]	*Cytb* Haplotypes[2]	Obtained reads[3]	
						COI	*ITS2*
Dor1_2019	O2019_1[4]	November 25, 2019	Oki Island, Shimane Prefecture	25	HapD	3869	24 446
Dor2_2019				11.7		43 240	147
Dor5_2020	Y2020_1[4]	August 25, 2020	Kiyosato, Hokuto City, Yamanashi Prefecture	3.4	HapC	31 374	79 441
Dor6_2020				9.2		56 336	58 989
Dor7_2020	Y2020_2	September 24, 2020		96.2	HapA	107 472	65 327
Dor8_2020				19.1		20 636	79 473
Dor9_2020				19.5	HapC	34 672	62 032
Dor10_2020				4.26	HapA	25 154	65 095
DorGA5-1_2020	YGA2020_1	October 29, 2020		91		59 308	111 244
DorGA5-2_2020				24.2		43 365	–
DorGA7-1_2020	YGA2020_2			18.8	HapA	92 666	76 843
DorGA7-2_2020				19		160 770	99 633
DorGA7-3_2020				25.2		190 097	–
DorGA7-4_2020				66.6		268 155	–
DorGA7-5_2020				71.2		64 667	–
Dor3_2021	Y-12[4]	June 10, 2021	Kiyosato, Hokuto City, Yamanashi Prefecture	36.4	HapB	–	780
Dor14_2021	SY7-12-1	July 28, 2021		19.9	HapB	29 559	–
Dor15_2021	SY7-12-2	July 28, 2021		80	HapB	607	–
Dor16_2021	SY7-2	August, 2021		98.8	HapB	15 400	476
Dor17_2021	SY6-17[4]	September 1, 2021		14.1	HapA	160 067	24 938
Dor18_2021				1.38		5330	25 907
Dor19_2021				27		–	21 411
Dor20_2021	SY6-26[4]	September 1, 2021		36	HapA	4957	–
Dor21_2021				25.4		20 830	–
Dor22_2021	SY7-11	September 24, 2021		104	HapC	3413	–
Dor24_2021	SY8-10	September 24, 2021		23.8	HapC	107 363	–
Dor25_2021	SY8-18	September 24, 2021		9.38	HapB	157 344	259 094
Dor26_2021	Y50	September 27, 2021		17.9	HapB	15 187	159 591
Dor28_2021	Y48[4]	September 27, 2021		0.542	HapB	–	130 294
Dor29_2021	Y49	September 27, 2021		4.48	HapB	26 910	83 856
Dor38_2021	SY3-24	October 18, 2021		2.26	HapC	107 396	199 348
Dor39_2021	SY4-25	October 18, 2021		7.76	HapC	50 007	132 469
Dor40_2021				15.4		6965	117 078
Dor41_2021	SY6-21	October 18, 2021		35.2	HapC	145 767	161 346
Dor42_2021				1.87		190 072	–
Dor43_2021				4.3		141 943	189 108
Dor45_2021	SY7-8	October 18, 2021		60.2	HapC	29 070	107 519
Dor46_2021	SY7-12	October 18, 2021		18.2	HapB	5396	–
Dor47_2021	SY7-21	October 18, 2021		78	HapB	471	–
Dor51_2021	GA26	October 19, 2021		6.94	HapA	13 812	–
Dor52_2021				50.6		23 746	35 878
Dor53_2021				37.8		545	43 607
Dor54_2021				40		173 790	–
Dor55_2021	Y37	October 19, 2021		89.6	HapC	91 807	53 352
Dor56_2021				40.6	HapB	66 648	137 260
Dor57_2021				23.6	HapC	116 936	–
Dor58_2021	Y10[4]	October 19, 2021		18.9	HaoC	8227	138 051
Dor59_2021	Y46[4]	October 19, 2021		7.68	HapB	8042	95 979
Dor60_2021				13.2		79 313	70 256

[1]: DNA concentration (ng/μl) calculated by Qubit fluorometer.
[2]: Cytochrome *b* gene haplotypes obtained in this study (see text for accession numbers in the DNA database).
[3]: Reads for animals and plants left after removing those for negative control, infrequent (less than 100), and bait reads for all samples.
[4]: The Japanese dormouse was present in the nest box.

Fig. 2. Photographs of the artificial nest box (A) and feces on bryophyte plants (B) in Yamanashi Prefecture.

at –20°C until DNA extraction. Because visits by dormice to the nest boxes were infrequent and fecal samples were obtained rarely, we could perform only sporadic sampling. Therefore, the numbers of samples obtained differed depending on month and location (Table 1).

DNA extraction

We used a commercial DNA extraction kit to extract genomic DNA from 3–5 pieces of feces so as not to exceed the maximum weight of samples (200 mg) as indicated in the kit instruction (QIAamp DNA Stool Mini Kit; Qiagen, Hilden, Germany). Before DNA extraction, we removed as much moss as possible from the feces and extensively cut feces using anatomical scissors. We followed the instructions of the kit, except for the duration of vortex mixing with inhibition buffer (from 1 min to 5 min) and the duration of DNA in the filter with elution buffer (from 1 min to 3 min). The DNA concentration was calculated using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA; Table 1).

Molecular method for species identification of the Japanese dormouse

As a target for PCR, we used the mitochondrial cytochrome *b* (*Cytb*) gene for species identification. The primers were designed previously (Yasuda et al. 2005, 2007, 2012): L14115 and Gjap-cyt-H323. The targets were 344 bp in length. PCR amplification was performed using an automated thermal cycler (Life Touch Thermal Cycler; Bioer Technology, Hangzhou, China). We used the KOD FX Neo kit (Toyobo, Osaka, Japan) for PCR. Aliquots of 20 ng of template DNA were added to 50 µL of PCR mixture containing 2× PCR buffer for KOD FX Neo, 0.4 mM dNTPs, 0.3 µM of each primer, and 1 µL of KOD FX Neo DNA polymerase. The PCR conditions were as follows: initial denaturation at 94°C for 1 min; followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and extension at 68°C for 30 s; and a final extension at 68°C for 5 min. A negative control was included in each PCR, and we confirmed no amplification in the negative control lane by agarose gel electrophoresis. Single bands were purified using the QIAquick PCR Purification Kit (Qiagen). For multiple bands, we used the QIAquick Gel Extraction Kit (Qiagen) to purify the target amplicon. PCR products were sequenced with the BigDye Terminator Cycle Sequencing kit v3.1 (Thermo Fisher Scientific) using the same primers as PCR in both directions, followed by DNA purification by ethanol precipitation, and evaluation using an ABI3130 genetic analyzer (Thermo Fisher Scientific). To identify species, we conducted BLAST searches (Altschul et al. 1990) of the DDBJ/ENA/GenBank International DNA Database on the NCBI website. We identified sequences with the highest identity. To assess the genetic relationships of the obtained and previously determined sequences, we constructed phylogenetic trees using the neighbor-joining method (Saitou and Nei 1987) in MEGA version 10.2 (Kumar et al. 2018). The obtained sequences were deposited in DNA databases under accession numbers LC762420–LC762423.

DNA metabarcoding analysis

Considering the omnivorous nature of the Japanese dormouse, we focused on one marker in mitochondrial

DNA for invertebrate dietary profiling and one marker in nuclear DNA for plant dietary profiling. Diet of vertebrates suggested in Minato (2018; e.g., Japanese tit, *Parus minor*) was not considered in this study. We determined partial sequences of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*; Zeale et al. 2011) and the internal transcribed spacer between 5.8S rDNA and 28S rDNA (*ITS2*; Moorhouse-Gann et al. 2018).

Two-step tailed PCRs with first and second PCRs were conducted to prepare libraries for NGS analyses. The first PCR amplified the target region and the second PCR attached sequence adapters connected to the flow-cell in the Illumina MiSeq NGS platform (Illumina, San Diego, CA, USA) and each sample-specific index for sample identification. PCR was conducted in an automated thermal cycler (Life Touch thermal cycler; Bioer Technology). For the first PCR, KAPA HiFi HotStart ReadyMix (Kapa Biosystems Inc., Wilmington, DE, USA) was used in a 25-μL PCR mixture containing 2× KAPA HiFi HotStart ReadyMix, 0.3 μM of each universal primer as described below, and templates (20 ng DNA), adjusted with PCR-grade water. For *COI*, we used ZBJ-ArtF1c (5'-AGATATTGGAACWTTATATTTTATTTTTGG-3'; Zeale et al. 2011) and ZBJ-ArtR2c (5'-WACTAATCAAT TWCCAAATCCTCC-3'; Zeale et al. 2011) to assess invertebrate materials. For *ITS2*, we used a universal primer pair UniPlantF (5'-TGTGAATTGCARRATY CMG-3'; Moorhouse-Gann et al. 2018) and UniPlantR (5'-CCCGHYTGAYYTGRGGTCDC-3'; Moorhouse-Gann et al. 2018) to assess plant materials. Each primer has a sequence at the 5'-end for priming the second PCR and sequencing primers and has six N nucleotide bases for efficient sequencing by MiSeq. The first PCR primers were as follows: [forward] 5'-ACACTCTTTCCCTACA CGACGCTCTTCCGATCTNNNNNN***-3' (*** is each universal forward primer as described above) and [reverse] 5'-GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCTNNNNNN+++-3' (+++ is the universal reverse primer described above). The lengths of the PCR products were expected to be 289 bp for *COI* and 305–595 bp for *ITS2* in the first PCR (Zeale et al. 2011; Moorhouse-Gann et al. 2018). The first PCR conditions for *COI* were as follows: initial denaturation at 95°C for 15 min; followed by 35 cycles of denaturation at 98°C for 20 s, annealing at 57°C for 90 s, and extension at 72°C for 60 s; and a final extension at 72°C for 10 min. The first PCR conditions for *ITS2* were as follows: initial denaturation at 95°C for 10 min; followed by 35 cycles of denaturation at 98°C for 20 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 2 min. The first PCR products were purified using AMPure XP beads (Beckman Coulter Inc., Brea, CA, USA) and eluted with 35 μL of PCR-grade water.

For the second PCR, KAPA HiFi HotStart ReadyMix (Kapa Biosystems Inc.) was used in a 24-μL PCR mixture containing 2× KAPA HiFi HotStart ReadyMix, 0.29 μM of each index primer (described below), and template (2 μL of the purified first PCR product), adjusted with PCR-grade water. The index primers for the second PCR were shared by all the molecular markers: [forward] 5'-AATGATACGGCGACCACCGAGATCTACAC-[8-bp index]-ACACTCTTTCCCTACACGACGCTCTT CCGATCT-3' and [reverse] 5'-CAAGCAGAAGACGG CATACGAGAT-[8-bp index]-GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT-3'. The sequences of the 5'-end of the 8-bp index in the forward and reverse index primers were P5 and P7 adapters, respectively, which were attached to the MiSeq flow-cell and those of the 3'-side were primed to overhang regions of the first PCR product. With these primers, the second PCR added 69 bp to the first PCR products. Combinations of forward and reverse 8-bp indices were used for sample identification. The second PCR conditions were identical for all molecular markers: initial denaturation at 95°C for 3 min; followed by 12 cycles of denaturation at 98°C for 20 s, annealing at 55°C for 15 s, and extension at 72°C for 15 s; and a final extension at 72°C for 5 min. The same volumes of the second PCR products were mixed in a tube and purified using AMPure XP beads.

We used an E-gel electrophoresis system with E-Gel™ SizeSelect™ II Agarose Gels 2% to extract the target DNA fragments (Thermo Fisher Scientific). The extracted samples were subjected to library quantification and quality check using a Qubit Fluorometer and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively, and sequenced by several runs of Illumina MiSeq NGS and reagent kits with 300 pair-end cycles for *COI* and 500 cycles for *ITS2*. The PhiX control spike-in was set to 20–30%.

Data filtering and DNA database search

We used Claident software (Tanabe and Toju 2013) to filter the data and search DNA databases. First, we converted the bcl files generated by MiSeq into FASTQ files via *bcl2fastq*. DNA sequence data in the FASTQ file were assigned to the samples based on an 8-bp index and the primer regions and their flanking sequences were removed via *clsplitseq*. We next used *clconcatpair* to merge the

forward and reverse paired-end sequences generated at each cluster on the flow-cell. Low-quality and noisy data were removed using *clfilterseq* and *clcleanseqv*. Sequences of < 100 bp and < 150 bp were omitted for *COI* and *ITS2*, respectively. Clustering of the denoised sequences was conducted via *clclassseqv* with a 98% minimum identity to assess the operational taxonomic units (OTUs) of the dietary items. Sequence data omitted because of their noisiness but similar to those of clustered OTUs were recovered using *clrecoverseqv*. For the *COI* sequences, we checked chimera sequences using *clrunuchime* and excluded them (for *ITS2*, we did not check because the option was not available). Next, we BLAST searched the DNA database for dietary OTUs using *clrunuchime*. Finally, the lowest common ancestor (LCA) algorithm was performed in *classigntax* to identify conservative OTUs. OTUs with < 100 reads were removed as low-frequency OTUs. If we could not obtain the taxonomic names by LCA analysis, we repeated the BLAST search individually to identify similar sequences. We followed the scientific names based on those deposited in the DNA databases. The filtered read data for each sample are summarized in Supplementary Tables S1–S6. We converted the filtered dietary reads into relative read abundance (rra) data for each month, where the numbers of reads for each OTU in a month were summed, the sum was divided by the number of samples in each month, and each read number was converted to a proportion (0–1) to the total read in each month. This procedure provides the number of reads per sample per month for each OTU (Tables 2–5). Although the frequency-based occurrence data should be supplementarily examined in addition to the rra data since the rra data might provide biased estimation of the food items by difference in DNA extraction efficiency or PCR amplification bias, we could not examine the occurrence data because of small sample size.

For identifying plant taxa, we made the following assumptions because of the presence of possibly unreliable and insufficient data in the global DNA databases. First, if a few minor data in the DNA database caused failure of identification, we did not consider such problematic sequences. For example, the obtained sequence was frequently similar to distantly related taxa, e.g., *Actinidia* sp. (> ten sequences in the database), *Asplenium scolopendrium* (one sequence), and *Aesculus chinensis* (one sequence) with the same sequence identity values. In this case, we assumed that our sequence corresponded to that of *Actinidia* sp. and recognized the other sequences (*A. scolopendrium* and *A. chinensis*) as error sequences in the database. This assumption is considered valid considering that almost all other OTUs were *Actinidia* sp. (Supplementary Tables S4–S6). Similarly, we adopted *Wisteria* (> ten sequences) and *Humulus* (> ten sequences) and therefore excluded *Millettia japonica* + *Lilium tsingtauense* (one sequence each) and *Keteleeria davidiana* + *Oryza meridionalis* (one sequence each), respectively, with the same sequence identity values. Second, the sequence of *Clematoclethra scandens* was typically a top hit, followed by *Actinidia* sp. (Supplementary Tables S4–S6). However, because the genus *Clematoclethra* is present in China, but not in Japan, we chose *Actinidia* sp. as the dietary taxon despite *A. scolopendrium* and *A. chinensis* having identical sequence identity values (see the reason above).

Results

Species identification using Cytb *sequences*

We determined nucleotide sequences from the start codon of the mitochondrial *Cytb* gene to the last base before the reverse primer Gjap-cyt-H323 (some sequences were not fully determined; 271–322 bp). With these sequences, we identified all the fecal samples to be from the Japanese dormouse. We detected four haplotypes (HapA–HapD; Fig. 3A), which were identical to sequences in the database and were consistent with the geographic variations (Fig. 3B). Specifically, sequences detected in Oki and Yamanashi were identical to those detected previously (Yasuda et al. 2007, 2012; Fig. 3B). Sequences from samples in the same nest box were typically identical, and samples Dor7-10_2020 and Dor55-57_2021 from a nest boxes Y2020-2 and Y37, respectively, showed multiple sequences. In the nest box Y2020-2, Dor7_2020, Dor8_2020, and Dor10_2020 were HapA, while Dor9_2020 was HapC. In the nest box Y37, Dor55_2021 and Dor57_2021 were HapC, while Dor57_2021 was HapB. These findings suggest that two maternal individuals (mate-pairs or father-offspring) were present in each nest box.

Sequences of dietary items by DNA metabarcoding analysis

We performed six independent analyses of Dor1–10 in 2019 and 2020, DorGA5 and GA7 in 2020, and Dor3–60 in 2021 for invertebrate and plant dietary analyses. The numbers of filtered reads for the dietary items are listed in Supplementary Tables S1–S6. We identified almost all dietary items at the genus level (82.1% and 99.5% for invertebrates and plants, respectively).

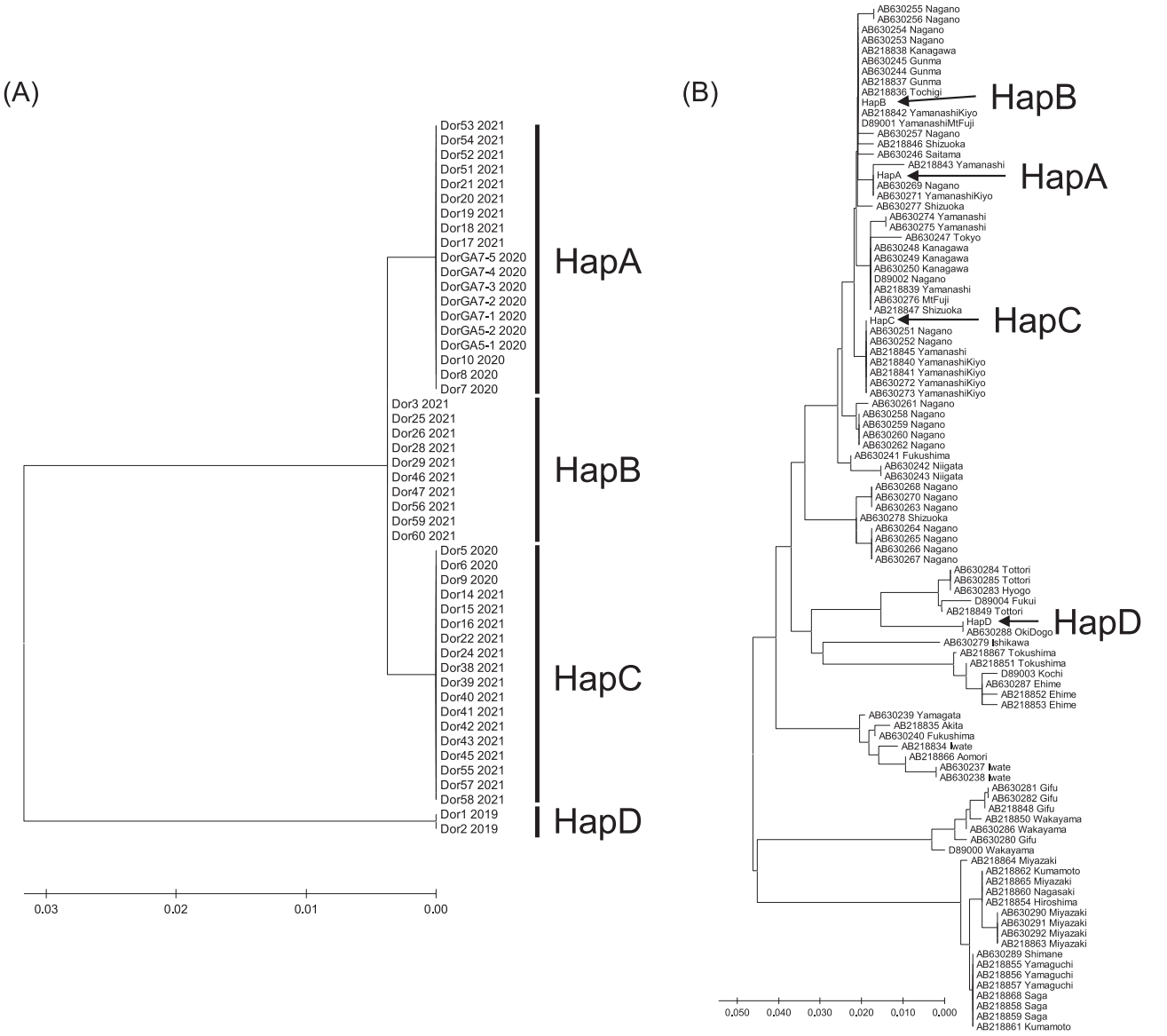

Fig. 3. Phylogenetic trees based on the neighbor-joining method constructed based on 268-bp sequences of the mitochondrial DNA cytochrome *b* gene for only samples examined in this study (A) and for haplotypes obtained in this study and those downloaded from the DNA database (data from Yasuda et al. 2007, 2012) (B). HapA, B, C, and D mean haplotypes obtained in this study.

For *COI* data of Dor1–10 in 2019 and 2020, we obtained 322 753 total reads (average = 40 344, min = 3869, max = 107 472) excluding infrequent sequences (< 100 reads; Supplementary Table S1). In total, 51 OTUs were obtained with 96.7% average identity to the database sequence (Supplementary Table S1), which were assigned to 22 genera (six unidentified), 19 families (two unidentified), and six orders (Table 2). For *COI* data of DorGA5 and GA7 in 2020, we obtained 879 028 total reads (average = 125 575, min = 43 365, max = 268 155) excluding infrequent sequences (< 100 reads; Supplementary Table S2). In total, 36 OTUs were obtained with 96.5% average identity to the database sequence (Supplementary Table S2), which were assigned to 27 genera (five unidentified), 21 families (two unidentified), and nine orders (Table 2). For *COI* data of Dor3–60 in 2021, we obtained 1 806 920 total reads (average = 58 288, min = 471, max = 190 072) excluding infrequent sequences (< 100 reads; Supplementary Table S3). In total, 165 OTUs were obtained with 97.2% average identity to the database sequence (Supplementary Table S3), which were assigned to 88 genera (ten unidentified), 52 families (five unidentified), and ten orders (one unidentified; Table 3). For *ITS2* data of Dor1–10 in 2019 and 2020, we obtained 434 950

Table 2. Relative read abundance (rra) of the dietary invertebrate OTUs detected in samples collected in 2019 and 2020

Class	Order	Family	Genus	Oki November $n = 2$	Yamanashi August $n = 2$	Yamanashi September $n = 4$	Yamanashi October $n = 7$
Arachnida	Araneae	Amaurobiidae	*Tegecoelotes*	0.00	0.00	0.00	
Arachnida	Araneae	Pimoidae	*Weintrauboa*				0.01
Arachnida	Araneae	Tarsonemidae	unidentified				0.00
Chromadorea	Rhabditida	Rhabditidae	*Oscheius*				0.00
Collembola	Entomobryomorpha	Tomoceridae	*Tomocerus*	0.03	0.00	0.00	0.01
Insecta	Blattodea	Ectobiidae	*Blattella*	0.00	0.00	0.00	
Insecta	Coleoptera	unidentified	unidentified				0.00
Insecta	Diptera	Syrphidae	*Sphecomyia*	0.00	0.00	0.01	
Insecta	Diptera	Campichoetidae	*Campichoeta*	0.00	0.00	0.00	
Insecta	Diptera	Cecidomyiidae	unidentified				0.00
Insecta	Diptera	Ceratopogonidae	*Atrichopogon*				0.00
Insecta	Diptera	Chironomidae	*Rheocricotopus*	0.00	0.06	0.00	
Insecta	Diptera	Culicidae	*Lutzia*				0.01
Insecta	Diptera	Dolichopodidae	*Chrysotimus*	0.00	0.33	0.00	0.01
Insecta	Diptera	Drosophilidae	*Drosophila*	0.00	0.00	0.54	0.01
Insecta	Diptera	Drosophilidae	*Stegana*	0.00	0.52	0.01	0.83
Insecta	Diptera	Fanniidae	*Fannia*	0.00	0.00	0.42	0.02
Insecta	Diptera	Muscidae	*Helina*	0.00	0.00	0.00	
Insecta	Diptera	Muscidae	*Limnophora*				0.00
Insecta	Diptera	Muscidae	*Muscina*				0.01
Insecta	Diptera	Sciaridae	*Camptochaeta*				0.00
Insecta	Diptera	Syrphidae	*Argentinomyia*	0.00	0.00	0.00	
Insecta	Diptera	Tachinidae	*Ceromasia*				0.01
Insecta	Diptera	Tachinidae	*Phytomyptera*	0.00	0.00	0.00	
Insecta	Diptera	Tachinidae	*Smidtia*				0.00
Insecta	Diptera	Tachinidae	unidentified	0.00	0.00	0.00	
Insecta	Diptera	unidentified	unidentified	0.00	0.00	0.00	0.00
Insecta	Hemiptera	Aphididae	*Phyllaphis*				0.00
Insecta	Hemiptera	Lachnidae	*Cinara*				0.01
Insecta	Hymenoptera	Pamphiliidae	*Cephalcia*				0.00
Insecta	Lepidoptera	Erebidae	*Amata*	0.79	0.00	0.00	0.01
Insecta	Lepidoptera	Erebidae	*Coenipeta*				0.00
Insecta	Lepidoptera	Erebidae	*Eilema*				0.00
Insecta	Lepidoptera	Erebidae	*Cyana*	0.01	0.00	0.00	
Insecta	Lepidoptera	Erebidae	*Sypnoides*	0.01	0.00	0.00	
Insecta	Lepidoptera	Geometridae	*Cabera*				0.00
Insecta	Lepidoptera	Geometridae	*Macaria*	0.00	0.00	0.00	
Insecta	Lepidoptera	Geometridae	unidentified	0.02	0.00	0.00	
Insecta	Lepidoptera	Limacodidae	*Phrixolepia*				0.00
Insecta	Lepidoptera	Lymantriidae	*Lymantria*	0.01	0.00	0.00	
Insecta	Lepidoptera	Noctuidae	*Acronicta*				0.00
Insecta	Lepidoptera	Noctuidae	*Cosmia*				0.01
Insecta	Lepidoptera	Noctuidae	*Feralia*				0.02
Insecta	Lepidoptera	Noctuidae	*Lithophane*	0.00	0.00	0.00	
Insecta	Lepidoptera	Noctuidae	*Orthosia*	0.01	0.00	0.00	
Insecta	Lepidoptera	Noctuidae	unidentified				0.00
Insecta	Lepidoptera	Saturniidae	*Citheronia*	0.00	0.00	0.01	
Insecta	Lepidoptera	Tortricidae	*Ancylis*	0.00	0.00	0.00	
Insecta	Lepidoptera	Tortricoidea	unidentified	0.00	0.00	0.00	
Insecta	Lepidoptera	unidentified	unidentified	0.11	0.07	0.00	
Insecta	Orthoptera	Rhaphidophoridae	unidentified	0.00	0.01	0.00	
Insecta	Orthoptera	Tettigoniidae	*Cosmetura*				0.00
Insecta	Psocoptera	Psocidae	*Metylophorus*				0.00

Background color:	0	$0 < \text{rra} < 0.005$	$0.005 \leq \text{rra} < 0.1$
	$0.1 \leq \text{rra} < 0.4$	$0.4 \leq \text{rra} < 0.8$	$0.8 \leq \text{rra} < 1.0$

Table 3. Relative read abundance of the dietary invertebrate OTUs detected in samples collected in 2021

Class	Order	Family	Genus	Yamanashi July $n = 2$	Yamanashi August $n = 1$	Yamanashi September $n = 10$	Yamanashi October $n = 19$
Arachnida	Araneae	Clubionidae	*Clubiona*	0.00	0.00	0.00	0.00
Arachnida	Araneae	Pimoidae	*Weintrauboa*	0.00	0.00	0.00	0.00
Arachnida	Araneae	Salticidae	*Hyllus*	0.00	0.00	0.00	0.00
Arachnida	Araneae	Tetragnathidae	*Menosira*	0.00	0.00	0.00	0.00
Arachnida	Araneae	Theridiidae	*Takayus*	0.00	0.00	0.01	0.00
Arachnida	Scorpiones	unidentified	unidentified	0.00	0.50	0.00	0.00
Collembola	Entomobryomorpha	Tomoceridae	*Tomocerus*	0.00	0.00	0.00	0.00
Insecta	Coleoptera	Carabidae	unidentified	0.01	0.00	0.00	0.00
Insecta	Coleoptera	Cerambycidae	*Grammoptera*	0.00	0.00	0.00	0.01
Insecta	Coleoptera	Coccinellidae	*Harmonia*	0.00	0.00	0.00	0.00
Insecta	Coleoptera	Curculionidae	*Curculio*	0.00	0.00	0.00	0.00
Insecta	Coleoptera	Endomychidae	*Endomychus*	0.00	0.00	0.00	0.00
Insecta	Coleoptera	Staphylinidae	*Quedius*	0.00	0.00	0.00	0.00
Insecta	Coleoptera	unidentified	unidentified	0.00	0.00	0.00	0.00
Insecta	Diptera	Bibionidae	*Bibio*	0.00	0.00	0.00	0.03
Insecta	Diptera	Carnidae	*Meoneura*	0.00	0.00	0.00	0.00
Insecta	Diptera	Chironomidae	*Cricotopus*	0.00	0.00	0.00	0.00
Insecta	Diptera	Chironomidae	*Tanytarsus*	0.00	0.00	0.00	0.00
Insecta	Diptera	Chloropidae	*Tricimba*	0.00	0.00	0.01	0.00
Insecta	Diptera	Drosophilidae	*Amiota*	0.00	0.00	0.00	0.00
Insecta	Diptera	Drosophilidae	*Apenthecia*	0.00	0.00	0.00	0.00
Insecta	Diptera	Drosophilidae	*Drosophila*	0.01	0.00	0.21	0.14
Insecta	Diptera	Drosophilidae	*Stegana*	0.00	0.00	0.00	0.34
Insecta	Diptera	Limoniidae	*Libnotes*	0.00	0.00	0.00	0.00
Insecta	Diptera	Limoniidae	unidentified	0.00	0.00	0.00	0.04
Insecta	Diptera	Muscidae	*Helina*	0.00	0.00	0.00	0.00
Insecta	Diptera	Muscidae	*Neomyia*	0.00	0.00	0.00	0.00
Insecta	Diptera	Muscidae	*Phaonia*	0.00	0.00	0.00	0.00
Insecta	Diptera	Mycetophilidae	*Exechia*	0.00	0.00	0.00	0.00
Insecta	Diptera	Phoridae	*Megaselia*	0.00	0.00	0.01	0.00
Insecta	Diptera	Pipunculidae	*Eudorylas*	0.00	0.03	0.00	0.00
Insecta	Diptera	Psychodidae	*Psychoda*	0.00	0.00	0.00	0.00
Insecta	Diptera	Syrphidae	*Dasysyrphus*	0.02	0.00	0.00	0.00
Insecta	Diptera	Syrphidae	*Epistrophe*	0.00	0.00	0.00	0.03
Insecta	Diptera	Syrphidae	*Meliscaeva*	0.00	0.00	0.00	0.00
Insecta	Diptera	Syrphidae	*Syrphus*	0.00	0.00	0.00	0.02
Insecta	Diptera	Tachinidae	*Ceromasia*	0.00	0.00	0.00	0.00
Insecta	Diptera	Tachinidae	*Oswaldia*	0.00	0.00	0.00	0.00
Insecta	Diptera	Tachinidae	*Triarthria*	0.00	0.00	0.00	0.00
Insecta	Diptera	unidentified	unidentified	0.00	0.00	0.00	0.00
Insecta	Hemiptera	Aphididae	*Euceraphis*	0.00	0.00	0.00	0.03
Insecta	Hemiptera	Aphididae	*Longistigma*	0.00	0.00	0.05	0.02
Insecta	Hemiptera	Lachnidae	*Cinara*	0.02	0.00	0.00	0.01
Insecta	Hemiptera	Lachnidae	*Tuberolachnus*	0.00	0.00	0.00	0.00
Insecta	Hemiptera	Pentatomidae	*Dinorhynchus*	0.00	0.00	0.00	0.00
Insecta	Hymenoptera	Braconidae	*Pauesia*	0.00	0.00	0.00	0.00
Insecta	Hymenoptera	Pamphiliidae	*Cephalcia*	0.00	0.00	0.01	0.00
Insecta	Hymenoptera	Tenthredinidae	*Pristiphora*	0.00	0.00	0.00	0.00
Insecta	Hymenoptera	Vespidae	*Vespula*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Crambidae	*Palpita*	0.00	0.04	0.00	0.00

Table 3. (Continued)

Class	Order	Family	Genus	Yamanashi July $n = 2$	Yamanashi August $n = 1$	Yamanashi September $n = 10$	Yamanashi October $n = 19$
Insecta	Lepidoptera	Depressariidae	*Psilocorsis*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Epicopeiidae	*Psychostrophia*	0.00	0.02	0.00	0.00
Insecta	Lepidoptera	Erebidae	*Coenipeta*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Erebidae	*Cratoplastis*	0.00	0.00	0.00	0.08
Insecta	Lepidoptera	Erebidae	*Eilema*	0.00	0.10	0.00	0.00
Insecta	Lepidoptera	Erebidae	*Epitausa*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Erebidae	*Ghoria*	0.00	0.09	0.00	0.03
Insecta	Lepidoptera	Erebidae	*Lithosia*	0.00	0.03	0.00	0.00
Insecta	Lepidoptera	Erebidae	*Oxidercia*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Erebidae	*Zale*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Gelechiidae	unidentified	0.00	0.00	0.01	0.00
Insecta	Lepidoptera	Geometridae	*Cabera*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Geometridae	*Cystidia*	0.00	0.00	0.00	0.01
Insecta	Lepidoptera	Geometridae	*Deileptenia*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Geometridae	*Dioptrochasma*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Geometridae	*Ennomos*	0.02	0.00	0.00	0.00
Insecta	Lepidoptera	Geometridae	*Macaria*	0.00	0.00	0.04	0.00
Insecta	Lepidoptera	Geometridae	*Melanthia*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Geometridae	unidentified	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Limacodidae	*Microleon*	0.00	0.00	0.00	0.02
Insecta	Lepidoptera	Nepticulidae	*Stigmella*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Noctuidae	*Feralia*	0.04	0.00	0.00	0.02
Insecta	Lepidoptera	Noctuidae	*Heliocheilus*	0.00	0.00	0.02	0.00
Insecta	Lepidoptera	Noctuidae	*Neumichtis*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Noctuidae	unidentified	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Nolidae	*Pseudoips*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Notodontidae	*Drymonia*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Pyralidae	*Ceroprepes*	0.00	0.00	0.00	0.10
Insecta	Lepidoptera	Saturniidae	*Saturnia*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Sphingidae	*Marumba*	0.01	0.00	0.00	0.00
Insecta	Lepidoptera	Sphingidae	*Neogene*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Tortricidae	*Acleris*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Tortricidae	*Argyroploce*	0.00	0.00	0.02	0.02
Insecta	Lepidoptera	Tortricidae	*Eccopsis*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Tortricidae	*Isochorista*	0.00	0.00	0.00	0.01
Insecta	Lepidoptera	Tortricidae	*Lobesia*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Tortricidae	*Pseudogalleria*	0.00	0.01	0.00	0.00
Insecta	Lepidoptera	Tortricidae	*Strophedra*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Tortricidae	*Syndemis*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	Tortricidae	*Tortricodes*	0.00	0.00	0.00	0.00
Insecta	Lepidoptera	unidentified	unidentified	0.86	0.15	0.23	0.00
Insecta	Orthoptera	Acrididae	*Nomadacris*	0.00	0.00	0.00	0.01
Insecta	Orthoptera	Mogoplistidae	*Ornebius*	0.00	0.00	0.03	0.00
Insecta	Orthoptera	Tettigoniidae	*Cosmetura*	0.00	0.00	0.00	0.00
Insecta	Orthoptera	Tettigoniidae	*Metrioptera*	0.00	0.00	0.00	0.00
Insecta	Plecoptera	Nemouridae	*Nemoura*	0.00	0.00	0.00	0.00
Insecta	Psocoptera	Stenopsocidae	*Stenopsocus*	0.00	0.00	0.00	0.00
Insecta	unidentified	unidentified	unidentified	0.00	0.03	0.31	0.00

Background color:	0	$0 < rra < 0.005$	$0.005 \leq rra < 0.1$
	$0.1 \leq rra < 0.4$	$0.4 \leq rra < 0.8$	$0.8 \leq rra < 1.0$

Table 4. Relative read abundance of the dietary plant OTUs detected in samples collected in 2020

Order	Family	Genus	Oki November $n = 2$	Yamanashi August $n = 2$	Yamanashi September $n = 4$	Yamanashi October $n = 3$
Ericales	Actinidiaceae	*Actinidia*	0.05	0.98	1.00	1.00
Fagales	Betulaceae	*Alnus*	0.00	0.01	0.00	
Fagales	Fagaceae	*Castanopsis*	0.95	0.00	0.00	
Fagales	Fagaceae	*Quercus*	0.00	0.00	0.00	
Poales	Poaceae	*Triticum*	0.00	0.00	0.00	
Rosales	Rosaceae	*Prunus*	0.00	0.00	0.00	

Background color:	0	0 < rra < 0.005	0.005 ≤ rra < 0.1
	0.1 ≤ rra < 0.4	0.4 ≤ rra < 0.8	0.8 ≤ rra < 1.0

total reads (average = 54 369, min = 147, max = 79 473) excluding bacteria, fungi, contaminants, infrequent sequences (< 100 reads), and other unknown organisms (Supplementary Table S4). In total, 33 OTUs were obtained with 98.1% average identity to the database sequence (Supplementary Table S4), which were assigned to six genera, five families, and four orders (Table 4). For *ITS2* data of DorGA5 and GA7 in 2020, we obtained 287 720 total reads (average = 95 907, min = 76 843, max = 111 244) excluding bacteria, fungi, contaminants, infrequent sequences (< 100 reads), and other unknown organisms (Supplementary Table S5). In total, 21 OTUs were obtained with 96.7% average identity to the database sequence (Supplementary Table S5), which were assigned to one genus, one family, and one order (Table 4). For *ITS2* data of Dor3–60 in 2021, we obtained 2 187 598 total reads (average = 99 436, min = 476, max = 259 094) excluding bacteria, fungi, contaminants, apples (*Malus* sp.) and oranges (*Citrus* sp.) used to keep dormice temporarily in the nest box (Dor17–21 in 2021), infrequent sequences (< 100 reads), and other unknown organisms (Supplementary Table S6). In total, 146 OTUs were obtained with 98.3% average identity to the database sequence (Supplementary Table S6), which were assigned to 25 genera (one unidentified), 23 families, and 11 orders (Table 5).

Based on the read data in Supplementary Tables S1–S6, rra ratios were calculated (Tables 2–5). For invertebrate dietary components in 2019 to 2020, the genus *Amata* in Erebidae (Lepidoptera; approximately 80%) was the main component, followed by unidentified Lepidoptera sp. (approximately 10%) in the fecal sample from Oki in November 2019, whereas Diptera dominated in fecal samples from Yamanashi in August to October of 2020 (approximately 90%; *Lutzia* in Culicidae, *Drosophila* and *Stegana* in Drosophilidae, *Fannia* in Fanniidae, *Muscina* in Muscidae, and *Ceromasia* in Tachinidae) (Table 2). Lepidoptera were detected in Yamanashi in August (7%, unidentified Lepidoptera sp.), September (1%, *Citheronia* in Saturniidae), and October (3%, *Cosmia* and *Feralia* in Noctuidae) albeit at a lower rate than Diptera (Table 2). In 2021, Diptera was the main item in October (60%) fecal samples (*Drosophila* and *Stegana* in Drosophilidae, unidentified species in Limoniidae, and *Dasysyrphus* and *Syrphus* in Syrphidae), and Lepidoptera were detected less frequently in October (29%; Table 3). By contrast, in 2021 samples, Lepidoptera was the main item in July (93%), August (44%), and September (32%), and was higher than Diptera (3%, 3%, and 23%, respectively; Table 3). Also, 2020 and 2021 samples had higher proportions of Hemiptera in autumn, including *Cinara* in Lachinidae (1% in October 2020, 2% in July 2021, and 1% in October 2021), *Longistigma* in Aphididae (5% in September 2021 and 2% in October 2021), and *Euceraphis* in Aphididae (3% in October 2021; Table 3). Scorpiones sp. (Arachnida) dominated 50% of reads detected for one sample from August 2021 in Yamanashi. Although species of this taxon are not distributed in the study area, the sequence identity to the database sequence was high (96.8%; Supplementary Table S3).

Various plant dietary components were detected in Oki and Yamanashi in 2019 to 2020. *Castanopsis* (Fagaceae) was the main item in Oki (95%, November), and *Actinidia* (Actinidiaceae; mostly *Actinidia arguta*) dominated fecal samples from Yamanashi (98–100%, August to October) (Table 4). The latter taxon was observed in Oki, albeit at a lower rate (5%; Table 4). In 2021, *Actinidia*

Table 5. Relative read abundance of the dietary plant OTUs detected in samples collected in 2021

Order	Family	Genus	Yamanashi June $n = 1$	Yamanashi August $n = 1$	Yamanashi September $n = 7$	Yamanashi October $n = 13$
Caryophyllales	Polygonaceae	*Rumex*	0.00	0.00	0.00	0.00
Cornales	Cornaceae	*Cornus*	0.00	0.00	0.00	0.00
Cornales	Hydrangeaceae	*Deutzia*	0.00	0.00	0.02	0.00
Ericales	Actinidiaceae	*Actinidia*	0.00	0.00	0.89	0.99
Ericales	Clethraceae	*Clethra*	0.00	0.34	0.00	0.00
Ericales	Pentaphylacaceae	*Eurya*	0.00	0.00	0.01	0.00
Ericales	Pentaphylacaceae	unidentified	0.00	0.00	0.00	0.00
Ericales	Primulaceae	*Myrsine*	0.00	0.00	0.00	0.00
Fabales	Fabaceae	*Wisteria*	0.27	0.00	0.00	0.00
Fagales	Betulaceae	*Carpinus*	0.00	0.00	0.00	0.00
Fagales	Fagaceae	*Castanea*	0.00	0.45	0.00	0.00
Fagales	Fagaceae	*Quercus*	0.00	0.00	0.00	0.00
Fagales	Juglandaceae	*Pterocarya*	0.37	0.00	0.00	0.00
Gentianales	Rubiaceae	*Lasianthus*	0.00	0.00	0.00	0.00
Laurales	Lauraceae	*Lindera*	0.00	0.00	0.00	0.00
Porellales	Frullaniaceae	*Frullania*	0.19	0.22	0.00	0.00
Porellales	Porellaceae	*Porella*	0.00	0.00	0.00	0.00
Ranunculales	Eupteleaceae	Euptelea	0.00	0.00	0.00	0.00
Rosales	Cannabaceae	*Celtis*	0.00	0.00	0.07	0.00
Rosales	Cannabaceae	*Humulus*	0.00	0.00	0.00	0.00
Rosales	Rosaceae	*Prunus*	0.00	0.00	0.00	0.00
Rosales	Ulmaceae	*Ulmus*	0.17	0.00	0.00	0.00
Sapindales	Aceraceae	*Acer*	0.00	0.00	0.00	0.00
Sapindales	Anacardiaceae	*Toxicodendron*	0.00	0.00	0.00	0.00
Sapindales	Rutaceae	*Zanthoxylum*	0.00	0.00	0.00	0.00
Vitales	Vitaceae	*Parthenocissus*	0.00	0.00	0.00	0.00

Background color:	0	$0 < \text{rra} < 0.005$	$0.005 \leq \text{rra} < 0.1$
	$0.1 \leq \text{rra} < 0.4$	$0.4 \leq \text{rra} < 0.8$	$0.8 \leq \text{rra} < 1.0$

predominated in autumn fecal samples (89% in September, 99% in October; Table 5). In September 2021, *Celtis* (Cannabaceae, 7%) and *Eurya* (Pentaphylacaceae, 1%; identified as *Eurya japonica* with 100% sequence identity) were also detected. In June and August, plant taxon diversity was elevated (Table 5): *Pterocarya* (Juglandaceae, 37%), *Wisteria* (Fabaceae, 27%), and *Ulmus* (Ulmaceae, 17%) in June and *Castanea* (Fagaceae, 45%; identified as *Castanea crenata* with 99.7% sequence identity) and *Clethra* (Clethraceae, 34%; identified as *Clethra barbinervis* with 99.7% sequence identity) in August. We detected the bryophyte genus *Frullania* (Frullaniaceae) in June (19%) and August (22%) samples (Table 5), but we considered them to have been carried by dormice into the nest box and did not regard them as dietary components.

Discussion

Noninvasive method for species identification

We report a method for species identification and assessment of the geographic variation of the Japanese dormouse using fecal samples and primers developed by Yasuda et al. (2005, 2012). Such a method is beneficial

because the direct capture or handling of the Japanese dormouse is problematic as a result of it being a protected natural monument in Japan. For dietary profiling, species identification using feces is mandatory because other small mammals, such as the small Japanese wood mouse *Apodemus argenteus*, are likely to share an artificial nest box and produce feces (Minato 2018; Takatsuki et al. 2022). It is therefore critical to identify species before dietary analysis. Furthermore, the procedure in this study can be used to clarify geographic variations of the Japanese dormouse. The Japanese dormouse was demonstrated to be genetically distinct locally, maintaining nine phylogroups that were inferred to have differentiated in the late Miocene to Pliocene (Yasuda et al. 2012). Our noninvasive procedure may facilitate phylogeographic studies and contribute to the circumscription of the conservation unit for this species.

Dietary profiling of the Japanese dormouse

Previous studies of dormouse diet were based on direct observation (Minato et al. 1997; Iwabuchi 2008; Aoki and Moriya 2009; Ochiai et al. 2015; Minato 2018; Takatsuki and Suzuki 2022). These studies suggested this species to be omnivorous and to have seasonal variation in dietary components, consistent with our findings. However, these previous studies were based on broad taxonomic units. This is also the case for the other dormouse species in Europe (e.g., *Glis glis*, Nowakowski and Godlewska 2006; *Eliomys quercinus* in Spain, Gil-Delgado et al. 2010; *Dryomys nitedula*, Nowakowski and Godlewska 2006, Juškaitis and Baltrūnaitė 2013a; *Muscardinus avellanarius*, Juškaitis and Baltrūnaitė 2013b). We provided taxonomic resolution at the genus level or sometimes at the species level for the dietary items. In this study, sufficient sampling to fully discuss the diet of dormice was difficult because of their elusiveness and domestic regulation. We therefore focused on their main dietary characteristics and discussed the potential and pitfalls of our methodology. In future, we should collect more samples with more efficient strategy such as finding highly utilized nest boxes or higher density regions to assess the seasonal variation of diets more fully. Placing more nest boxes would also be efficient.

Direct observations in previous feeding experiments and field surveys demonstrated that dormice eat insects such as beetles, butterflies, centipedes, dragonflies, earwigs, flies, grasshoppers, ladybirds, mantises, moths, spiders, wasps, and aquatic insects (Aoki and Moriya 2009; Minato 2018). In this study, Lepidoptera was predominantly detected in Oki samples, whereas Lepidoptera and Diptera were two main items in Yamanashi samples. In Yamanashi, the proportions of these two taxa differed seasonally although it should be noted that the sample size in summer was so small. Nevertheless, Lepidoptera decreased and Diptera increased in fecal samples toward autumn. Also, Hemiptera (specifically, aphids) increased toward autumn. In fact, Minato (2018) observed that the Japanese dormice in Yamanashi consumed aphids in gall of the Koyama's Spruce (*Picea koyamae*) in September. Consumption of insects is high in summer and decreases toward autumn, and fruits are often consumed instead in autumn (Ochiai et al. 2015; Takatsuki and Suzuki 2022). Lepidoptera showed a similar tendency in this study, but Diptera and Hemiptera did not. Thus, insects might still be important resources in autumn by the Japanese dormouse. It is also likely that some fruit-related flies were consumed simultaneously when dormice searched for fruits such as *Actinidia* sp. in autumn (see below). Such accidental consumption may introduce bias in the dietary components, as suggested in a recent DNA metabarcoding study (Tercel et al. 2021).

Regarding dietary plant components, *Actinidia* sp. (mostly the hardy kiwi, *A. arguta*) was detected in almost all samples obtained in autumn (September and October 2020 and 2021) in Yamanashi, consistent with a prior report (Minato 2018). By contrast, dormice tended to have a more diverse plant diet in summer (June to August) although small sample size should provide only preliminary dietary trend. Nevertheless, dormice appeared not to consume *Actinidia* sp. exclusively in summer, while probably preferentially selecting this plant species in autumn. Autumn is important for accumulating fat for hibernation (Shibata 2000). Dormice typically weigh 14–20 g in summer and in spring after hibernation, but > 20 g is needed to prepare for hibernation (Minato 2018). Dormice therefore increase their weight in autumn by eating nutritious, high-carbohydrate foods to prepare for hibernation. In this study, *A. arguta* was detected at a high rate in autumn. This plant has been reported as an important food resource for various mammalian species, which disperse its seeds (e.g., black bears, macaques, racoon dogs, and martens; Yasumoto and Takatsuki 2015; Naoe et al. 2019). This is consistent with a direct observation that a portion of the September diet of the Japanese dormouse in Yamanashi included the fruit of *A. arguta*, which ripens from September to October (Iwabuchi et al. 2008; Minato 2018). In addition, other plants that produce ripened fruits in autumn (*Celtis* sp.

and *E. japonica*) were detected in September 2021. The consumption of fruits in autumn is consistent with a recent microscopy study (Takatsuki and Suzuki 2022). Fructose, a carbohydrate present in fruits and honey, can be converted into fat via several metabolic pathways; therefore, many animals use fruits for energy storage (Johnson et al. 2020). As such, the fruit of *A. arguta* may be an important dietary resource enabling Japanese dormice to survive hibernation.

Clethra barbinervis (detected in August 2021) was also observed by Iwabuchi et al. (2008) and Minato (2018). August is the flowering season for this species. Iwabuchi et al. (2008) and Minato (2018) reported that dormice consumed the flower of this species, as well as Lepidoptera around the flower. Lepidoptera was detected frequently in August 2021, albeit in a single sample. The flowering season for *Pterocarya* sp. and *Wisteria* sp., detected in a fecal sample in June 2021, is in spring (April to June), suggesting that flowers are the consumed tissue. Dormice pollinate various plant species (Minato 2018). *Castanea crenata* in August might be at a post-flowering stage and we could not determine which tissues were consumed by dormice. Hard chestnuts are difficult for dormice to eat because of their poor jaw power. In feeding experiments, dormice did not consume hard nuts such as acorns and walnuts (Minato 2018). Iwabuchi et al. (2008) noted that, in summer after the flowering season, dormice might mainly eat tree bark and insects, possibly including the bark of *Castanea* species. We could not assess the *Ulmus* lifecycle; species of this genus were not identified because of 100% sequence identities among *Ulmus* species.

Potential and pitfalls of noninvasive species identification and dietary profiling

This is the first study of noninvasive species identification and dietary profiling using genetic methods for dormouse fecal samples. These methods provided insight into the evolution and ecology of this elusive and protected species. However, the methods are imperfect and should be improved by solving the following problems and incorporating several recommendations.

First, although the global DNA database provided highly similar sequences for the *COI* and *ITS2* markers (> 96.5% and > 96.7% on average, respectively), they were not 100%, limiting identifications to the genus level. A local reference DNA database would facilitate species-level identification (Ando et al. 2020), enabling clarification of the ecological requirements of this protected species. *ITS2* is an efficient marker for plant metabarcoding because of its discriminatory power among species and its recently established database (Cheng et al. 2016; Moorhouse-Gann et al. 2018; Banchi et al. 2020). This is consistent with our study in terms of the higher resolution for *ITS2* (high sequence identity with database sequences). However, the number of detected OTUs was less than that for other plant markers, such as the chloroplast *trnL* P6 loop region (e.g., Sato et al. 2019, 2022). Thus, further characterization of the *ITS2* marker is needed.

Second, the DNA databases contain some erroneous sequences. An OTU that accounted for half of reads from a fecal sample obtained in August 2021 in Yamanashi was identified as a species of Scorpiones (Supplementary Table S3). The sequence identity to the database sequence (accession no. KM838535) was high (96.8%). However, species of Scorpiones are observed only in the Ryukyu Islands in Japan (two species in Ischnuridae and Buthidae), not in the study area (Yamanashi). We have never used the nest boxes to hold species of Scorpiones. A possible explanation is erroneous registration of database sequences. The second hit sequence was a Tarsonemidae mite species (sequence identity was 90.2%). If this is accurate, these data would not provide dietary information for the Japanese dormouse. Another explanation is that dormice consumed exotic Scorpiones sp. artificially released into the wild. However, no such artificial release has been reported and the dormouse, with its poor mastication power, is unlikely to be able to consume armed Scorpiones spp. Researchers at our institute have not studied Scorpiones spp., making contamination unlikely. We also used special care when interpreting plant dietary taxa. We frequently detected *Actinidia* sp. (kingdom Viridiplantae; phylum Streptophyta; class Magnoliopsida; order Ericales; family Actinidiaceae), *A. scolopendrium* (Viridiplantae; Streptophyta; Polypodiopsida; Polypodiales; Aspleniaceae), and *A. chinensis* (Viridiplantae; Streptophyta; Magnoliopsida; Sapindales; Sapindaceae) with the same sequence identities, meaning that different classes or orders have identical sequences. In this study, we assumed *Actinidia* sp. to be correct based on the abundance of its data in the database.

Third, the sampling procedure was an issue. Although the noninvasive analysis provides insight into the ecology of protected animals, fecal samples must be collected with caution to prevent contamination (Sato et al. 2019). The Japanese dormouse collects plant materials, such as birch (*Betula* spp.) or bryophytes (Fig. 2B), to construct

nests for reproduction (Minato 2018). In fact, we detected *Frullania* bryophytes in samples obtained in June and August 2021. Moreover, Japanese dormice share artificial nest boxes with other animals like the small Japanese wood mouse *A. argenteus* or the Japanese tit *P. minor* (Minato 2018). These animals also bring plant materials into nest boxes (Minato 2018). Because DNA metabarcoding has higher sensitivity than traditional microscopic analyses for detecting dietary species in feces, care should be taken not to collect such materials brought by dormice or other animals. Of final note, flies gathering around fruits may be accidentally consumed (Tercel et al. 2021).

Fourth, our procedure using the mitochondrial *Cytb* gene marker cannot discriminate dormouse individuals. This precludes investigation of whether an obtained diet is from an individual. Because multiple dormice likely share a nest box (Minato 2018); we detected two *Cytb* haplotypes from the same nest box (Y2020_2 and Y37). Therefore, we recommend that a camera be set near the nest box or a genetic method capable of individual identification from feces be applied simultaneously.

Conclusions

We developed a NIG method to assess the diet of the protected Japanese dormouse from its feces in artificial nest boxes. To test the dietary trends of this species, more sampling of feces and more information on local fauna and flora are required. Our noninvasive DNA metabarcoding method provided insight into the diet of elusive, endangered, and protected animals. It may apparently provide more superior data to the traditional direct observation based on fecal analysis (Ochiai et al. 2015; Takatsuki and Suzuki 2022). However, the latter enables determination of the relative abundances of invertebrates and plants in fecal samples or the developmental stages of detected species (e.g., larvae or adults), which are difficult to determine via DNA metabarcoding because no universal primers target invertebrate and plant species simultaneously and all tissues have basically the same DNA contents. Combining these methods would enable clarification of the ecological role of the Japanese dormouse in forest ecosystems.

Supplementary data

Supplementary data are available at *Mammal Study* online.
Supplementary Table S1. Numbers of reads for each invertebrate OTU detected from feces of dormice Dor1–10 in 2020.
Supplementary Table S2. Numbers of reads for each invertebrate OTU detected from feces of dormice DorGA5 and DorGA7 in 2020.
Supplementary Table S3. Numbers of reads for each invertebrate OTU detected from feces of dormice in 2021.
Supplementary Table S4. Numbers of reads for each plant OTU detected from feces of dormice Dor1–10 in 2020.
Supplementary Table S5. Numbers of reads for each plant OTU detected from feces of dormice DorGA5 and DorGA7 in 2020.
Supplementary Table S6. Numbers of reads for each plant OTU detected from feces of dormice in 2021.

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