DNA metabarcoding dietary analyses of the wood mouse *Apodemus speciosus* on Innoshima Island, Japan, and implications for primer choice

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Abstract

We used DNA metabarcoding to assess the seasonal diets of the large Japanese wood mouse, *Apodemus speciosus* (Muridae, Rodentia), in forest edges adjacent to citrus orchards on Innoshima Island, Japan. We used one chloroplast and three mitochondrial DNA barcoding markers to determine mouse diets. Among the various plant and invertebrate diets, *A. speciosus* typically consumed Chinese cork oak (*Quercus variabilis*) in early spring (likely acorns preserved during winter) and gypsy moths (*Lymantria dispar*, a forest pest) in late spring and summer. In addition, we found that *A. speciosus* also preyed on orchard pests, including the gutta stink bug and other potentially harmful invertebrates. The season during which *A. speciosus* preyed on stink bugs corresponded with the harvest of orchard products. This study revealed several of the ecological roles of *A. speciosus* within the boundary zone between forest and human ecosystems. Furthermore, based on the performance of various mitochondrial markers in dietary profiling of invertebrate food items, we recommend the multi-locus DNA metabarcoding method to comprehensively assess the diet of *A. speciosus*.

Keywords eDNA · Food web · Pest control · Primer choice · Seasonal dietary variations · Wood mice

Introduction

Assessing the ecological roles of organisms in forest ecosystems is crucial for understanding how forest-dwelling species promote forest sustainability and provide essential ecosystem services for humans (Brockerhoff et al. 2017). Such endeavors are especially important given accelerating global deforestation (Hansen et al. 2013) as well as the urgency of meeting the United Nations Sustainable Development Goals over the next

decade, particularly target 15 (Life on Land; <https://sdgs.un.org/goals/goal15>, accessed 1 June 2021). In Japan, two-thirds of the land base is forested. These forests are fundamental to the country's biodiversity (Hill et al. 2019) but are unique in experiencing the extremely high-intensity anthropogenic pressures exerted over a small land area. Therefore, identifying the ecological roles of forest-dwelling species is fundamental for building a society that exists in harmony with nature and preserving forest sustainability.

The large Japanese wood mouse, *Apodemus speciosus* (Muridae, Rodentia), is endemic to Japan. This species is one of the most ancient mammals in Japan (Sato 2016) and is widely distributed in forests as well as at the boundary between forests and nearby human settlements from northern Hokkaido to southern Honshu, Shikoku, Kyushu, and other adjacent small islands (Ohdachi et al. 2015). Previous studies have documented conflicts due to the interactions between human infrastructure (roads, buildings, or city urbanization) and the forest ecosystem based on wood mouse genetics (Hirota et al. 2004; Sato et al. 2014, 2020). Thus, *A. speciosus* represents a wildlife species that can be studied to elucidate specific aspects of the interplay between forest and anthropogenic ecosystems.

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One strategy for assessing interrelationships between forest and anthropogenic ecosystems is food-web analysis involving the assessment of wildlife diets. Previous morphological analyses of *A. speciosus* feces and stomach contents were unable to adequately identify fragments of prey species, even with the aid of a microscope (Abe and Oya 1974; Mizushima and Yamada 1974; Tatsukawa and Murakami 1976; Tachibana et al. 1988). More recently, a DNA metabarcoding approach, based on high-throughput sequencing, was used to determine the diets of wood mice (*A. speciosus* and/or *A. argenteus*) on Hokkaido and islands in the Seto Inland Sea; this allowed for dietary comparisons under interspecific competition and across geographic space (Sato et al. 2018, 2019). Sato et al. (2019) assessed *A. speciosus* diets on several islands in the Seto Inland Sea, but this study was temporally limited and did not characterize seasonal variation. Documenting seasonal variation in diets would provide more insight into the ecological role of *A. speciosus* within forest edges adjacent to human settlements.

Applications of DNA metabarcoding in diet studies have been increasing, and several promises and pitfalls are associated with this approach (de Sousa et al. 2019; Ando et al. 2020). A key issue is the lack of consensus concerning the choice of primers to use in analyses. The mitochondrial cytochrome c oxidase subunit I (*COI*) and 16S ribosomal RNA (*16S*) genes are the most frequently targeted genomic regions for DNA metabarcoding of invertebrates. Accordingly, several universal primers have been developed for these regions (Tournayre et al. 2020). Despite their name, universal primers are not always strictly universal due to differences in the polymerase chain reaction (PCR) efficiency of each primer based on the target species. This issue leads to variation in the detection of dietary components among primers (Tournayre et al. 2020; Browett et al. 2021). To date, researchers do not agree on whether to use multiple primers for a single locus or to combine primers for multiple loci (Alberdi et al. 2018; Corse et al. 2019; Browett et al. 2021) or to use a single, well-designed primer (Elbrecht et al., 2019; Tournayre et al. 2020). Therefore, the performance of each barcoding primer should be assessed to judge which is appropriate for each case study. However, such a comparison of marker performances has not yet been conducted for DNA metabarcoding analyses involving rodents. In fact, no studies have examined how primer choice affects the representation of seasonality for any organism.

In this study, we used a DNA metabarcoding approach for fecal samples by employing several short barcoding markers for the target regions (shorter markers are better for degraded fecal samples; Vamos et al. 2017; Tournayre et al. 2020). Our objectives were to identify seasonal variation in the plant and invertebrate diet of *A. speciosus* on Innoshima Island, Japan, and to elucidate its roles in forest and agro-ecosystems. We also discuss primer selection

based on comparisons of three mitochondrial DNA primers for invertebrates.

Materials and methods

Study site and obtained samples

Innoshima Island was not included in previous studies of the *A. speciosus* diet on islands in the Seto Inland Sea (Sato et al. 2019). The present study took place at the base of Mt. Okuyama, near the town of Mukunoura, Innoshima Island, Hiroshima Prefecture, Japan (Fig. 1). The island is located within a typical temperate zone climate. Approximately one-third of the island area (1251.96 of 3976 ha) is dominated by forest, and 40% of the forested area is composed of broad-leaved trees. The forest near Mukunoura is dominated by deciduous tree species, with high prevalence of Chinese cork oak (*Quercus variabilis*). Citrus orchards, typically *Citrus hassaku*, occur near the forest study site in Mukunoura (Fig. 1; citrus fruit agriculture is a major industry on the island). This island therefore provides a suitable location for assessing the interplay between forest and agro-ecosystems.

We captured 19 individuals of *A. speciosus* using 40–60 Sherman live traps set in a 50×50-m area from March to August 2019. We sampled wood mice every month to assess seasonal differences in diet. The trapping period included the rainy season (called Tsuyu in Japan; Table 1, late June to late July). At the study site, the Tsuyu season occurred from 26 June to 25 July in 2019. The capture rate of wood mice declines dramatically in September of every year in our study area (personal observation of the first author); therefore, we stopped sampling in September. Traps baited with oats (*Avena sativa*) were set out for one night. Upon capture, we collected several tissue samples using an ear punch for genetic species identification (see below) and then released the individuals. Each mouse sample was collected from different individuals, because ear-punched individuals were never re-sampled. We transported traps to the laboratory within 30 min of release and collected feces in the trap using sterilized tweezers. Feces were collected into 1.5-ml plastic tubes. Fecal samples were then preserved at −80 °C until DNA extraction. We obtained permission from Hiroshima Prefecture to collect the wood mice samples in the Innoshima island (no approval code from the prefecture) and conducted protocols approved by the Animal Care and Use Committee of Fukuyama University (approval code: H30-Animal-8). We followed the guidelines of the Procedure of Obtaining Mammal Specimens established by the Mammal Society of Japan (<https://www.mammalogy.jp/en/guideline.pdf>; accessed 1 June 2021).

Fig. 1 **A** Map of the study area including the area surrounding Mukunoura, Innoshima Islands, Japan, **B** the study area in spring, and **C** the study area in summer. Both photos were taken from the study area looking toward Mukunoura

Avoidance of contamination

For all molecular experiments described below, we used filtered tips and experimental globes. Before performing each experiment, we wiped the laboratory bench with DNA Away (Thermo Scientific, Waltham, MA, USA) to

remove any remaining DNA. DNA extraction and PCR for both tissue and fecal samples were performed in the same laboratory (but at different benches), and sequencing occurred in a different building. We conducted negative control experiments for PCR and sequencing as described below to control for contamination.

Table 1 Sample information

Sample ID	Capture date	“Tsuyu” ¹	Reads before rarefaction ²				Accession no. ⁴
			<i>COI</i>	<i>16S-epp</i>	<i>16S-kp</i>	<i>trnL</i>	
Inn2019-3	2019.03.12	before	107,005	75,939	45,677	148,302	LC600288
Inn2019-4	2019.03.12	before	114,958	35,958	59,732	312,864	LC600289
Inn2019-6	2019.04.27	before	188,384	134,672	20,913	na ³	LC600290
Inn2019-7	2019.04.27	before	55,418	37,600	31,058	162,353	LC600291
Inn2019-8	2019.04.27	before	104,490	100,559	68,815	214,189	LC600292
Inn2019-9	2019.04.27	before	53,340	61,957	24,571	183	LC600293
Inn2019-10	2019.04.27	before	46,788	177,717	75,310	158,434	LC600294
Inn2019-11	2019.05.11	before	3941	150,712	na ³	1533	LC600295
Inn2019-12	2019.05.11	before	29,949	14,514	39,302	966	LC600296
Inn2019-13	2019.6.18	before	48,406	106,369	44,691	31,342	LC600297
Inn2019-14	2019.6.18	before	117,437	315,060	76,346	7173	LC600298
Inn2019-15	2019.6.18	before	8702	10,513	20,872	670	LC600299
Inn2019-16	2019.7.27	after	7981	112,315	na ³	41,050	LC600300
Inn2019-17	2019.7.27	after	79,958	198,650	32,751	30,304	LC600301
Inn2019-18	2019.7.27	after	14,395	58,881	40,583	6193	LC600302
Inn2019-19	2019.7.27	after	18,135	136,463	na ³	na ³	LC600303
Inn2019-20	2019.8.20	after	25,290	98,160	46,672	105,853	LC600304
Inn2019-21	2019.8.20	after	448,714	100,612	81,712	na ³	LC600305
Inn2019-22	2019.8.20	after	253,314	29,179	na ³	6589	LC600306

¹Rainy season called Tsuyu (from June 26 to July 25 in 2019 around the sampling location)

²Reads left after the negative control treatment and removal of infrequent reads (less than 10) for all samples

³Not applicable due to the PCR failure

⁴The mitochondrial Dloop sequences deposited to the DNA database

DNA extraction

We used two commercial DNA extraction kits, the DNeasy Blood & Tissue Kit and QIAamp DNA Stool Mini Kit (Qia-gen, Hilden, Germany), to extract DNA from ear tissue and fecal samples, respectively, following the manufacturer’s instructions. Prior to extraction, three pieces of feces were chopped as finely as possible using sterilized surgical scissors within each tube. Following DNA extraction from both tissue and fecal samples, the sample concentration of DNA was calculated using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and a Qubit fluorometer (Thermo Fisher Scientific), respectively, to adjust the DNA concentration to 100 ng/μL and 5 ng/μL in tissue and fecal samples, respectively, for PCR.

Species identification

We followed methods in Sato et al. (2014) to determine a partial nucleotide sequence of the *Dloop* region in the mitochondrial DNA using the Sanger DNA sequencing method for the collected samples. We then compared the obtained sequences with previously determined sequences

through a BLASTn search (Altschul et al. 1990) for species identification.

DNA metabarcoding

We performed two-step PCRs to prepare libraries for subsequent next-generation sequencing (NGS) analyses. All PCR amplifications described below were performed using an automated thermal cycler (LifeTouch thermal cycler; Bioer Technology, Hangzhou, China). In each PCR, we targeted three molecular markers in the mitochondrial DNA, one of *COI* and two of *16S* (*16S-epp* and *16S-kp*), for invertebrate dietary profiling, and one molecular marker in the chloroplast DNA, the P6 loop of the *trnL* (UAA) intron (*trnL*), for plant dietary profiling (Table 2). We used these primers based on their performance in previous studies on wood mice (Sato et al. 2018, 2019) and published reviews (Alberdi et al. 2017; Ando et al. 2020). In the first PCR, we amplified each target region using KAPA HiFi Hot-start Ready Mix (Kapa Biosystems Inc., Wilmington, MA, USA). The first PCR reaction mixture (25 μL) contained 2× KAPA HiFi HotStart ReadyMix, 0.3 μM of each universal primer (Table 2), and template DNA (20–45 ng), and was adjusted using PCR-grade water as necessary. We used

Table 2 Universal primers used in this study

Primer ID ¹	direction	Primer names and sequences ²	Target length ³	References
<i>COI</i>	Fw	ZBJ-ArtF1c (5'-AGATATTGGAACWTTATATTTTATTTTGG-3')	157 bp	Zeale et al. (2011)
	Rv	ZBJ-ArtR2c (5'-WACTAATCAATTWCCAAATCCTCC-3')		
<i>16S-epp</i>	Fw	Coleop_16Sc (5'-TGCAAAGGTAGCATAATMATTAG-3')	106 bp	Epp et al. (2012)
	Rv	Coleop_16Sd (5'-TCCATAGGGTCTTCTCGTC-3')		
<i>16S-kp</i>	Fw	IN16STK-1F-mod (5'-TAACTCAGATCATGTAA-3')	~ 110 bp	Kartzinel and Pringle (2015)
	Rv	IN16STK-1R-mod (5'-TTAGGGATAACAGCGTWA-3')		
<i>trnL</i>	Fw	trnL-g (5'-GGGCAATCCTGAGCCAA-3')	10–143 bp	Taberlet et al. (2007)
	Rv	trnL-h (5'-CCATTGAGTCTCTGCACCTATC-3')		

¹Names of the target regions used in this study

²These primer sequences were combined to adapter sequences as described in the text

³Information based on Ando et al. (2020)

specific primers targeted to the *COI*, *16S-epp*, *16S-kp*, and *trnL* genomic regions (Table 2). The 3'-end of each primer used in the first PCR had an adapter sequence targeted by the second PCR and sequencing primers. Between the specific and adapter primer sequences, there were six base-pair (bp) sequences (6 N) for efficient sequencing on the Illumina MiSeq NGS platform (Illumina, San Diego, CA, USA). The sequences of the first PCR primers were 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNN***-3' (***) represents the first PCR forward primers; Table 2) and 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNN+++-3' (+++ represents the first PCR reverse primers; Table 2). Therefore, the total target length included the length of each specific primer, a 79-bp adapter, and 6-bp sequences (Table 2).

The first PCR conditions for *COI* and *16S-epp* 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 *16S-kp* were as follows: initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 98 °C for 20 s, annealing at 50 °C for 60 s, and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Finally, the first PCR conditions for *trnL* were as follows: initial denaturation at 95 °C for 15 min, followed by 40 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 5 min. We confirmed that there was no amplification of the negative control samples (one per 4–8 samples) using agarose gel electrophoresis analyses. The products of the first PCR were purified using AMPure XP beads (Beckman Coulter Inc., Brea, CA, USA).

In the second PCR, we amplified each target region using KAPA HiFi Hotstart Ready Mix and a pair of primers that contained adapters (P5 and P7) and indices as sample identifiers. The PCR mixture (24 µL) contained 2 × KAPA HiFi

HotStart ReadyMix, 0.29 µM of each index primer below, and 2 µL of a purified first PCR product, and was adjusted using PCR-grade water as necessary. The primers for the second PCR were designed to be attached to the 5' end of the first PCR product, and they contain the index sequence in the middle part and the P5 or P7 adapter at the 5' end: [forward primer] 5'-AATGATACGGCGACCACCGAGATCTACAC-[8-bp index]-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3' and [reverse primer] 5'-CAAGCAGAAGACGGCATACGAGAT-[8-bp index]-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'. A 69-bp sequence was therefore added to the first PCR products after the second PCR. The conditions of the second PCR were as follows: 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. We confirmed that there was no amplification of the negative control samples (one per 4–8 samples) using agarose gel electrophoresis. The second PCR products were mixed on an equi-volume basis, wherein negative control samples were also included, and purified using AMPure XP beads.

Following purification, gel electrophoresis was performed using E-Gel™ SizeSelect™ II Agarose Gels 2% to extract target DNA fragments (Thermo Fisher Scientific). We then assessed the concentration and quality of the extracted samples using a Qubit fluorometer and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Finally, we conducted several runs of sequencing using Illumina MiSeq with Nano or Micro 300-cycle reagent kits with a 30% PhiX control spike-in in each run.

Data filtering and homology search

We used Claident (Tanabe and Toju 2013) for data filtering and conducted a DNA database search using BLASTn for dietary profiling. The analysis codes for data processing are

provided in Supplementary Information [S11](#). First, the bcl file obtained from MiSeq was converted to a FASTQ file using the *bcl2fastq* command. For the FASTQ file, DNA sequence data were demultiplexed based on an 8-bp index and the primer regions and their flanking sequences were removed using *clsplitseq*. Subsequently, forward and reverse paired-end sequences were merged using *clconcatpair*. Low-quality data, noisy data, too-short sequences, and chimeras were filtered using *clfilterseq* and *clcleanseqv*. Filtered sequences were clustered using *clclassseqv* with a 98% minimum identity to obtain operational taxonomic units (OTUs). Sequences that were noisy but found to be similar to an OTU cluster were recovered using *clrecoverseqv*. Chimeras were searched for again using a specific database and were removed using *clrunuchime* (except for *trnL*). For these filtered OTU sequences, a BLASTn search for dietary OTUs in the DNA database (the animal *COI* and animal mtDNA databases for *COI* and *16S*, respectively, as available in Claident by default and in the cpDNA database for *trnL* constructed in this study through a search with the term “chloroplast” in the NCBI nucleotide database) was performed using *clidentseq*, and finally, lowest common ancestor (LCA) analyses were performed using *classigntax*. OTUs that had fewer than 10 reads were classified as low-frequency OTUs and removed (Sato et al. 2018, 2019). When we could not identify a certain level of taxonomic resolution in the LCA analyses, we repeated the BLAST search individually to confirm top hit sequences. Raw data, in the form of FASTQ files that had been subjected to the *clsplitseq* analyses, are available from DRA (DDBJ Sequence Read Archive) under the accession number DRA011398.

Rarefaction and statistical analyses

Although there was no evidence of amplification in the negative controls, some minor reads were assigned to them. Therefore, the number of reads detected in the negative controls was removed from those of other samples. For example, if a sample and a negative control had 500 and 10 reads for OTU1, respectively, we considered the read number obtained from the sample to be 490 (500 – 10). We also removed infrequent reads, i.e., those accounting for <0.1% of the total number of reads per sample. We then used rarefaction to account for differences in the number of reads obtained per sample (Table 1) in each marker individually with the package *vegan* (Oksanen et al. 2019) in R (R Core Team 2020). We determined saturation on the rarefaction curves at a selected number of reads, where the selected number was based on either the minimum (3900, 10,000, and 20,000 for *COI*, *16S-epp*, and *16S-kp*, respectively) or a smaller arbitrary (6000 for *trnL*) number. We confirmed that the rarefaction curves were saturated at the points of the pre-determined threshold values reported above (Supplementary Information [S12](#)). We

calculated pairwise Bray–Curtis and Jaccard indices, which represent differences in diet between sampled individuals, based on relative read abundance data and binary (0/1) occurrence data, respectively. The obtained matrices were used to construct a non-metric multidimensional scaling (NMDS) plot, again using the package *vegan* in R. We divided samples into two seasons, spring (March, April, May, and June) and summer (July and August), based on the timing of the rainy (Tsuyu) season, and assessed differences in diet between seasons using PerMANOVAs with 10,000 permutations.

Results

Species identification

We confirmed that all sequences of the mitochondrial *Dloop* region from each of the 19 individuals matched those of *A. speciosus* using a BLASTn search. For samples 6, 7, 10, 15, 19, and 21, a novel haplotype (Inn4) was obtained. For all other samples, the haplotypes matched those from previous findings (Inn1 and Inn2; Sato et al. 2017). The obtained sequences were deposited in the DDBJ/ENA/GenBank international DNA database under accession numbers LC600288–LC600306 (Table 1).

Dietary profiling

We obtained read data for the *COI*, *16S-epp*, *16S-kp*, and *trnL* barcoding markers from 19, 19, 15, and 16 wood mice, respectively, after next-generation sequencing analyses (Table 1). For invertebrates, rarefied *COI*, *16S-epp*, and *16S-kp* data indicated the presence of 230, 20, and 21 OTUs, respectively. Among these, *16S-epp* and *16S-kp* data included host reads, in which 98% and 94% of the total number of reads, respectively, were indicative of Murinae species and were assumed to belong to the host (*A. speciosus*). We then excluded these host reads from further analyses. We also removed reads for a nematode from both *COI* and *16S-epp* data, on the assumption that nematodes are not a component of wood mouse diets, as they are sometimes observed on the skin of the wood mouse (personal observation of the first author). Finally, we removed samples 3, 8, 9, 15, 16, 17, 20, and 21 from *16S-epp* analyses and sample 15 from *16S-kp* analyses, as the number of reads became zero for all OTUs after the rarefaction analyses. The OTUs were reduced to zero because the relative read abundance for the host wood mouse (removed later) was predominant for these *16S* markers, and the reads for prey taxa were not sampled in the rarefaction analyses. For plants, we first removed samples 9, 11, 12, and 15 from rarefaction analyses of *trnL* due to a low number of reads relative to other samples (<6000 reads/sample; Table 1). The remaining rarefied *trnL* data included 63 OTUs. Among these,

38.8% of the total rarefied reads were assigned to *Avena* sp., which was assumed to be the bait used in this study, *A. sativa*, and removed from further analyses.

The NMDS plot of *COI* rarefied relative read abundance data (stress value [sv]=0.13) indicated a seasonal difference between spring and summer (Fig. 2A), which was supported by the results of the PerMANOVA ($P < 0.01$, $F = 3.41$, $R^2 = 0.17$). The occurrence data also supported the presence of a seasonal difference in the NMDS plot (Fig. 2B; sv=0.15) and PerMANOVA ($P < 0.01$, $F = 2.17$, $R^2 = 0.11$). The majority of the rarefied reads (77.9%) were assigned to Lepidoptera (Supplementary Information S13). The major lepidopteran families included Erebiidae (6.4% of the Lepidoptera reads), Lymantriidae (7.7%), and Noctuidae (5.9%) (Fig. 3A). Although we used the family name Lymantriidae based on the registered name in the DDBJ/ENA/Genbank DNA database, we note that this family is currently considered a subfamily (Lymantriinae) in Erebiidae (Zahiri et al., 2010). Moths that could be identified at the species

level and also occur in Japan included *Pidorus glaucopis* (Zygaenidae); *Trachea atriplicis*, *Mythimna separata*, and *Melanchra persicariae* (Noctuidae); and *Lymantria monacha* and *L. dispar* (Lymantriidae). In particular, *L. dispar*, the gypsy moth, was commonly observed in samples from June and July, representing 78.0% of the total reads for the family (Supplementary Information S14). Although we did not achieve species-level identification for Noctuidae, we note that the genus *Lithophane* accounted for 74.9% of the total reads for the family. We were unable to identify genera or species belonging to Erebiidae, but this family was a major component of moth prey in spring (Fig. 3). The second most frequently encountered OTU, at the order level, was Diptera, accounting for 18.1% of the total number of reads. Of these, Chironomidae accounted for 65.0% and Tachinidae accounted for 25.4% (Supplementary Information S13). Nearly all of the reads belonging to Chironomidae were from the species *Tanytarsus formosanus* (98.4%), but we were unable to obtain genus- or species-level identification

Fig. 2 Non-metric multidimensional scaling (NMDS) plot based on Bray–Curtis (A, C, E) and Jaccard (B, D, F) similarity indices for rarefied read data obtained from *COI* (A, B), *16S-epp* (C, D), and *16S-*

kp (E, F) analyses. Open circles and open diamonds indicate samples collected in spring and summer, respectively

Fig. 3 Read numbers assigned to each taxonomic category (orders and families) for *COI* (A), *16S-epp* (B), and *16S-kp* (C). Before plotting, the read numbers were rarefied and therefore normalized among samples to a pre-determined value for each marker as explained in the text. Chironomidae (Diptera) and unassigned Lepidoptera with very large read numbers (7778 primarily in August and 40,057 dis-

tributed throughout the sampling period, respectively) for *COI* were removed to show the other read data clearly; see Supplementary Information S13 for those additional data. Reads associated with an unassigned order, Murinae (presumably *A. speciosus*), and a nematode were excluded. We performed rarefaction on spring observations to account for differences in sample sizes between spring and summer

for Tachinidae. Finally, beetles (Carabidae) were most frequently detected in July (Fig. 3; Supplementary Information S14), for which the majority of reads belonged to *Carabus* (72.2%). We also detected a species of *Vespula* (Vespidae),

but only during the summer season (July and August; Supplementary Information S14).

For the *16S-epp* rarefied relative read abundance data, the NMDS plot, based on Bray–Curtis distances, did not

indicate seasonal differences in diet (Fig. 2C; $sv = 0.13$), nor did the results of the PerMANOVA ($P > 0.05$, $F = 0.83$, $R^2 = 0.084$). Similarly, the occurrence data did not indicate a seasonal difference in either the NMDS plot (Fig. 2D; $sv = 0$) or PerMANOVA ($P > 0.05$, $F = 1.01$, $R^2 = 0.08$). Broadly, from *16S-epc* data, dietary components different from those from *COI* were detected, specifically Hemiptera (stink bugs) and Orthoptera (grasshoppers) (Fig. 3B). Within Hemiptera, we detected a Kempfer cicada (*Platypleura* sp.), which we assumed to be *Platypleura kaempferi*, given that only one species of this genus occurs in the study area, and the gutta stink bug *Physopelta gutta* (Supplementary Information S15). Within Orthoptera, we detected a bush-cricket, *Tettigonia* sp. (Supplementary Information S15). In contrast to the *COI* reads, Geometridae was the most frequently encountered moth family according to the rarefied *16S-epc* data (Fig. 3B). Among the Geometridae OTUs, we detected the genus *Biston* (Supplementary Information S15). A re-analysis of the BLASTn search uncovered identifications for a *Biston* sp., *Inurois fletcheri*, and *Wilemania nitobei*, none of which were assigned in the LCA analysis. The most frequently detected Geometridae OTU was not identified at the genus level. Similar to the results using *COI*, Lymantriidae (*Lymantria*) was observed in June (Supplementary Information S15).

The NMDS plot using *16S-kp* rarefied data indicated that samples 13 and 14 were outliers for both relative read abundance and occurrence data (Supplementary Information S16). These two samples did not share any OTUs with the other samples (only *Lymantria dispar* and two unassigned Lepidoptera taxa in samples 13 and 14, respectively, were detected; Supplementary Information S17); therefore, these two samples were removed from the analyses. After removing these samples, the NMDS plot based on the relative read abundance data ($sv = 0.0001$) suggested a seasonal difference in diet (Fig. 2E), which was confirmed using a PerMANOVA ($P < 0.05$, $F = 2.0$, $R^2 = 0.17$). The occurrence data supported the presence of a seasonal difference in the NMDS plot (Fig. 2F; $sv = 0.06$), although the PerMANOVA outcome was not significant ($P > 0.05$, $F = 1.20$, $R^2 = 0.11$). Dietary components in the spring samples were dominated by unassigned Lepidoptera (Fig. 3C). However, the summer samples were dominated by Cicadidae (Hemiptera; Fig. 3C; Supplementary Information S17). The LCA analysis also detected cicadas in the summer months, but that analysis assigned cicada sequences to Polyneurini, and the BLASTn re-analysis generally identified *Graptopsaltria nigrofuscata*. Thus, it appears that different species of cicada were detected using the two *16S* markers. As in the *16S-epc* analysis, the BLASTn re-analysis identified Largidae OTUs that had not been assigned to species in the LCA analysis as *P. gutta*. Within Lepidoptera, *L. dispar* was again identified

in June, and no genus or species resolution was available for Erebidae (Supplementary Information S17).

The NMDS plot of *trnL* rarefied relative read abundance data ($sv = 0.16$) indicated a seasonal difference in diet between spring and summer (Fig. 4A), which was supported by the results of the PerMANOVA ($P < 0.01$, $F = 3.22$, $R^2 = 0.24$). The occurrence data also supported the presence of a seasonal difference in the NMDS plot (Fig. 4B; $sv = 0.13$) and PerMANOVA ($P < 0.05$, $F = 1.64$, $R^2 = 0.14$). In spring, wood mouse diets were dominated by Fagaceae (Fig. 4C). Although the LCA analysis did not yield genus-level identification, nearly all observations with 100% identity in the DNA database belonged to *Quercus*, and thus we assumed all Fagaceae observations were of *Quercus*. *Quercus variabilis* dominates at the study site, with a minor proportion of *Q. serrata* (Nobukazu Nakagoshi, personal communication), and thus we assumed *Q. variabilis* to be the detected Fagaceae species. Further examination of the number of reads for this species showed that most of the reads for Fagaceae were derived from samples from early spring (93.6%; 76.2% in March and 17.4% in April; Supplementary Information S18). In summer, mouse diets were dominated by unassigned Laurales, which we were unable to identify at a greater taxonomic resolution, and Euphorbiaceae (Malpighiales) (Fig. 4C). Given that the majority of hits in the DNA database for the latter belonged to *Mallotus*, we assumed that these reads belonged to *Mallotus japonicus*, as the genus is monotypic in Japan. Other OTUs detected at the genus level and frequently observed in summer included *Lonicera* (Caprifoliaceae), *Rhododendron* (Ericaceae), and *Toxicodendron* (Anacardiaceae) (Supplementary Information S18). OTUs that were assigned to Ericaceae using LCA analysis were later confirmed as *Rhododendron* based on the BLASTn search re-analysis. Finally, Rutaceae OTUs were detected from three individuals in April, June, and August, albeit with a small number of reads (Supplementary Information S18). Nearly all Rutaceae sequences detected with 100% identity in the DNA database belonged to a *Citrus* species.

Discussion

Dietary characteristics of *A. speciosus* on Innoshima Island and its ecological roles

The dietary components of *A. speciosus*, including both invertebrates and plants, shifted between spring (March–June) and summer (July–August) in our study area. Thus, the rainy season (Tsuyu), which runs from late June to late July, is likely a period of change in wood mouse diets. The rainy season is followed by a hot, humid summer in the study area, wherein various invertebrates become more

Fig. 4 Non-metric multidimensional scaling (NMDS) plot based on Bray–Curtis (A) and Jaccard (B) similarity indices for rarefied relative read data and occurrence data, respectively, obtained from *trnL* analysis. Open circles and open diamonds indicate samples obtained in spring and summer, respectively. (C) Read numbers assigned to each taxonomic category (orders and families) for *trnL*. Before plotting, the read numbers were rarefied and therefore normalized among samples to a pre-determined value for each marker as explained in the text. Reads associated with unassigned orders and *Avena* sp. were removed

active (e.g., cicadas; Hayashi and Saisho 2011). We found that wood mice consumed more *Carabus* beetles in July than any other surveyed month during the study (Fig. 5A), and they consumed large amounts of *Platypleura* (assumed to be *P. kaempferi*) and an unassigned cicada (assumed to be *G. nigrofusca*) in July and August (Fig. 5F, H). Although only a minor diet component, wood mice consumed *Vespula* sp. in both July and August (Fig. 5B). *Carabus* has previously been reported as a diet item for *A. speciosus* in the Seto Inland Sea (Sato et al. 2019), but *P. kaempferi*, *G. nigrofusca*, and *Vespula* sp. are novel dietary species. Given that *A. speciosus* is a terrestrial species, we suspect that larvae, exuviae, or dead individuals were the prey item, rather than live, mature adults of those flying species.

For plants, broadly, consumption of a Fagaceae species (assumed to be *Quercus variabilis*) was high in early spring and then decreased toward the rainy season (Fig. 5I; Supplementary Information S18). This observation suggests that wood mice may eat acorns preserved during the winter season. Although a direct observation of feeding on acorns is necessary to verify this hypothesis, the wood mouse may aid in the seed dispersal of *Q. variabilis* within its home range of ca. 500–2000 m² each year (Oka 1992; Oishi et al. 2018). On the other hand, in summer, consumption of *Rhododendron*

sp. increased in July, and an unassigned Laurales species was frequently detected in July and August (Fig. 5J, K). *Mallotus* sp. (assumed to be *Mallotus japonicus*) was more frequently consumed in August (Fig. 5L). In terms of the plant diet, the rainy season also appeared to be a turning point for changes in dietary habits.

Our findings suggest that *A. speciosus* may play an ecological role in pest control. We found that the wood mice ate *Lymantria dispar*, a cyclical-outbreak forest pest (Inoue et al. 2019), in June and July (Fig. 5C), suggesting that *A. speciosus* may suppress this forest pest by feeding on it, similar to the white-footed mouse *Peromyscus leucopus* in the USA (Elkinton et al. 1996). We also found that *A. speciosus* preyed on the gutta stink bug (*P. gutta*) and an unassigned Largidae species (assumed to be *P. gutta*) in March (Fig. 5E, G). Gutta stink bugs adversely affect citrus crops (Umeya and Okada 2003), and *C. hassaku* fruits are typically ripe in February and March on Innoshima Island. Predation by *A. speciosus* in March may therefore indicate a role of this mouse in reducing an orchard pest in the forest edge. The two cicada species we detected in the diet of *A. speciosus*, *P. kaempferi* and *G. nigrofusca*, pierce fruit and consume tree sap at both the larval and adult stages (Umeya and Okada 2003). We also found that *A. speciosus* consumed

Fig. 5 Monthly seasonal changes in dietary operational taxonomic units (OTUs). The vertical line indicates the number of rarefied reads per sample in each month. The most likely identity of each OTU (order, family, genus, or species) is shown above each panel along with the barcoding marker used: (A) *Carabus* - COI, (B) *Vespula*

- COI, (C) *Lymantria dispar* - COI, (D) Sciaridae - COI, (E) *Physopelta gutta* - 16S-epp, (F) *Platyleura* - 16S-epp, (G) Largidae - 16S-kp, (H) Cicadidae - 16S-kp, (I) *Quercus* - trnL, (J) *Rhododendron* - trnL, (K) Laurales - trnL, and (L) *Mallotus* - trnL

Noctuoidea moth species in the families Erebiidae, Lymantriidae, and Noctuidae, including many phytophagous moth species that could be harmful to orchard production (Umeya and Okada 2003), although we did not detect the notorious fruit-piercing moth species in Japan (Hattori 1969). In terms of the other moth species discussed, it is possible that *Biston* sp., which was abundant in *A. speciosus* feces according to the 16S-epp analysis, could have an adverse effect on citrus fruit production (Umeya and Okada 2003). Although the number of detected reads was low, we detected *M. separata*, which damages crops such as corn, and *M. persicariae*, which is a pest of soybean and Brassicaceae crops (Umeya and Okada 2003). We additionally detected a fly in the family Sciaridae in spring (Fig. 5D), which was assumed to be *Bradysia* sp. based on the BLASTn re-analysis. This genus is a pest of agricultural crops (Umeya and Okada 2003). We speculate that *A. speciosus* may perform an ecological role of suppressing the abundance of these pest species, similar to the common bent-wing bat (*Miniopterus schreibersii*) in

the Mediterranean (Aizpurua et al. 2018). This potential role of *A. speciosus* in pest control should be tested in future studies that incorporate more intense sampling of wood mice.

The wood mice likely consumed some citrus fruit in April, June, and August, although at low abundance. Although this could be viewed negatively by orchard growers, we suggest that *A. speciosus* may play an even more important role in pest control as suggested above, highlighting the value of having forest ecosystems in proximity to agricultural operations.

Performance of three different barcoding markers for animal dietary profiling

In the present study, we performed multi-locus DNA metabarcoding analyses for animal diet profiling and recovered 230, 20, and 21 OTUs using the COI, 16S-epp, and 16S-kp barcoding markers, respectively. Our findings suggest that

the *COI* primers developed by Zeale et al. (2011) were the most efficient markers for detecting invertebrate species represented in the diet of wood mice. The amplification of sequences from host wood mice (> 90% of the total reads) in the *16S-epp* and *16S-kp* analyses adversely affected the efficiency of these *16S* markers. *COI* also outperformed the *16S* genes in terms of the number of detected OTUs and taxonomic resolution in a study of bat diets (Tournayre et al. 2020), in which *COI* primers developed by Zeale et al. (2011) and *16S-epp* primers developed by Epp et al. (2012) gave rise the highest and lowest numbers of invertebrate OTUs, respectively, among the 10 *COI* and two *16S* barcoding markers. Nevertheless, even given this substantial difference in efficiency, we conclude that multi-locus DNA metabarcoding should be performed, because the three barcoding markers were complementary to one another in terms of detecting OTUs. In particular, Hemiptera was not detected using *COI*, but was found using *16S*. Cicadidae (the two species of cicadas mentioned above) was characteristically found in the wood mouse diet in summer, whereas Largidae (stink bugs, a pest species) was observed in early spring. Therefore, the *16S* markers proved to be useful for assessing seasonal dietary variation and the possible agricultural pest control ability of wood mice. Even the two *16S* markers used in the present study varied in performance, as different cicada species were detected from different samples (*Platypleura kaempferi* from a sample in July with *16S-epp* and *Graptosaltria nigrofuscata* from samples in August with *16S-kp*). We thus agree with the recommendation of previous studies to use multiple meta-barcoding markers (Alberdi et al. 2018; Browett et al. 2021), in contrast to Elbrecht et al. (2019). Because the detection of organisms is highly dependent on the sample contents (feces in this study), further case studies should be performed to determine which strategy with single or combined barcoding markers would be most effective for comprehensively assessing the diet of the wood mouse. Seasonal dietary analyses similar to those conducted in this study may be necessary because the efficiency of the primers used would change depending on the season and concomitant differences in dietary components.

Conclusions

Using the DNA metabarcoding approach, we demonstrated that a clear seasonal transition in diet occurred during the rainy season in the large Japanese wood mouse, *A. speciosus*. In addition, our results suggest the potential ecological role of this species in forest and agricultural pest management. Furthermore, we recommend multi-locus DNA metabarcoding analyses to achieve a comprehensive understanding of wood mouse diets. In future studies, we suggest the development of more efficient markers, the

design of blocking primers targeting host DNA (especially for *16S* markers), and the construction of a local DNA database (sequencing of the same markers used in this study for potential prey species collected in the field). These efforts would contribute to more precise clarification of the dietary components at the species and genus level and to improved understanding of the dynamics of diet shifts across seasons. In particular, with a local DNA database, one can potentially identify 23.8% of the reads for Noctuidae species and 77.9% of the reads for Lepidoptera that were not assigned to a lower taxonomic level and may include forest or agricultural pests.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13364-021-00601-7>.

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Author contribution JJS planned and financially supported the research project. JJS, YO, and NN were involved in the sampling of *Apodemus speciosus* from the Innoshima Island in the Seto Inland Sea, Japan. YO, NN, and KM undertook the laboratory work. JJS conducted the analyses. JJS drafted the manuscript. All authors reviewed the manuscript and joined the discussion.

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Availability of data and material (data transparency) We deposited genetic data obtained in this study to DDBJ (DNA Data Bank of Japan). The accession numbers for the sanger sequences are LC600288–LC600306. The accession number for the sequences produced by the next-generation sequencing is DRA011398. We also submit following supplementary data to support our findings.

Code availability We followed the instruction of Tanabe and Toju (2013) and R Core Team (2020) for the bioinformatics analyses using the program Claident and R, respectively. The analyses codes were described in Supplementary Information S11.

Declarations

Ethics approval (include appropriate approvals or waivers) We obtained permission from Hiroshima Prefecture to collect the wood mice samples in the Innoshima island (no approval code from the prefecture) and conducted protocols approved by the Animal Care and Use Committee of Fukuyama University (approval code: H30-Animal-8). We followed the guidelines of the Procedure of Obtaining Mammal Specimens established by the Mammal Society of Japan (<https://www.mammalogy.jp/en/guideline.pdf>; accessed 1 June 2021).

Consent to participate (include appropriate statements) All authors contributed to the study as described in the author's contribution above, read the manuscript, and discussed the contents.

Consent for publication (include appropriate statements) All authors agreed with the submission and publication of this manuscript in Mammal Research.

Competing interests (include appropriate disclosures).

The authors declare no competing interests.

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