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Original paper

Genetic analyses of Japanese field vole *Alexandromys* (*Microtus*) *montebelli* winter diet in apple orchards with deep snow cover

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Abstract. The Japanese field vole *Alexandromys* (*Microtus*) *montebelli* is prevalent in apple orchards and is the dominant cause of damage to fruit trees during winter. A recent study revealed that the Japanese field vole bred and increased its population during winter in regions with deep snow. Understanding what food resources support the voles during winter can assist in formulating a better understanding of the factors of the annual variations in orchard damage. In this study, we sampled faeces of the voles from November 2018 to May 2021 and performed the DNA metabarcoding analysis for plant dietary profiling with a molecular marker on the internal transcribed spacer region in the nuclear genome. We obtained results from 60 samples, and the food sources detected most frequently were the broadleaf docks *Rumex obtusifolius*, followed by the cultivated apples *Malus* spp. The detection frequency of Fabaceae sp. declined after March, and the one of apple rootstocks increased instead. During March and April, the various parts of fruit trees and *Rumex* spp. were the main diets for most of the voles. The biomass supplied by herbaceous plants, especially *Rumex* spp. could affect the extent of vole damage to fruit trees in winter.

Key words: DNA metabarcoding, faeces, rodent pest, *Rumex*, winter breeding.

The Japanese field vole *Alexandromys* (*Microtus*) *montebelli* is the primary cause of damage to fruit trees during winter (Udagawa 1965; Konuma and Kureha 1984). In areas with deep winter snowfalls, the vole is notorious for feeding on roots and bark of fruit trees; often, the damage is irreparable, killing the trees. The degree of damage varies widely from year to year and region to region. Although rare, they can destroy an entire orchard (Udagawa 1965; Aomori Prefectural Agricultural Pest Control Office 1985). The winter conditions which promote severe damage by voles have not been thoroughly investigated and are not well enough understood to be predictable. A better understanding of the conditions which can manifest into substantial damages by voles is necessary to mitigate the economic impact on farmers and the overall loss of production.

The basic ecology of the Japanese field vole has been very well studied (Watanabe 1962; Kaneko 1975). However, the knowledge of their winter ecology in areas with deep snow cover is primarily unknown (Kaneko 1975). A recent study on the Japanese field vole population dynamics in apple orchards in Aomori Prefecture (northern Japan) revealed that under the deep snow cover, voles reproduced and increased their populations (Murano et al. 2022). Although the *Microtus* and *Alexandromys* voles are widely distributed throughout the northern hemisphere, the records of winter breeding under snow in the field are rare, with no records of intensive winter breeding enough to cause the population increase (Hansson 1984; Tast 1984; Aars and Ims 2002). Therefore, reproduction during winter had been considered uncommon and not a significant factor in vole population dynamics. Huitu et al. (2003) demonstrated in their experimental setting in western Finland that when sufficient food was available and the predation pressure was eliminated, the field vole *M. agrestis* popu-

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lation under snow cover could increase. Based on these results, the recent finding of increased vole populations in the apple orchards in Aomori during winter raised a new question; what food resources support active breeding during the winter? Identifying the food resources will provide insight into the conditions that influence the winter population dynamics of the voles.

Voles prefer green plants, but they are also opportunistic herbivores (Watanabe 1962; Kaneko 1975); their diet is significantly affected by seasonal changes in the vegetation of their habitat. In regions like Aomori Prefecture, herbaceous vegetation can suddenly and dramatically change following seasons. While various plants grow until November, most will die or enter a wintering regime in December. During winter, the ground remains covered with snow for approximately three months, from late December until March. Following the snow melts in late March, the ground area shows drastic changes from almost no green vegetation to a rich cover, with herbaceous vegetation at the beginning of May. Understanding how voles respond to this seasonal vegetation change and what food resources they utilise during these dramatic months could provide insight into the winter biology of the vole and the effects of herbaceous vegetation on the population dynamics of the vole.

DNA metabarcoding analyses have been increasingly applied to a variety of fields, and the development of high-throughput next-generation sequencing (NGS) technology provides a method for simultaneously identifying multiple organisms in environmental samples such as water or faeces. Especially, recent studies of dietary DNA metabarcoding analyses have clarified greater detail of the dietary species contents in the faeces or gastrointestinal tract than traditional morphological and microhistological observations through microscopes (e.g., Soininen et al. 2009, 2013, 2015; Ando et al. 2013, 2020; Kartzinel et al. 2015; Lopes et al. 2015; de Sousa et al. 2019). This high detectability makes it possible to assess the interspecific, geographic, and seasonal variations in the diet from the faeces of small rodents (Sato et al. 2018, 2019, 2022). This technology could be utilized for identifying which parts of the fruit tree the voles feed on. Grafting is the predominant technique employed in orchard farming, where the fruit-bearing plant (scions) is grafted onto another plant which serves as the stem (rootstock). The scions and the rootstocks are closely related but different plant species, therefore we could distinguish one from the other by the metabarcoding analysis.

In this study, we analysed the diet of the Japanese field vole using DNA metabarcoding analyses. Then we reviewed the seasonal change of the diet variations and considered the winter food resources supporting the Japanese field vole in the apple orchards in Aomori Prefecture.

Materials and methods

Samples and study area

We conducted the field survey from November 2018 to May 2021 at eight apple orchards in Aomori Prefecture, Japan. The winter field surveys from January to March were conducted primarily at three of the eight orchards (Fig. 1, Table 1). All orchards had apple trees *Malus domestica*, and orchards A and C had a few cherry *Prunus avium* and peach *P. persica* trees as well. There were vegetable and strawberry gardens beside orchards A, C, and H. Orchard B had decorative flowering trees along the edge of the orchard, while orchard F had a mimosa *Albizia julibrissin* tree at its edge. The white clover *Trifolium repens* is dominant in many orchards (Table 1). There were no crop fields within 100 m of any orchards. Fifteen compost boxes with lids were installed over the vole burrows at the three orchards before snow accumulated allowing us to trap voles on the ground level irrespective of snow depth. During the snow-free periods, five traps were set at eight orchards. Traps were set at 9:00 and checked at 15:00. We caught

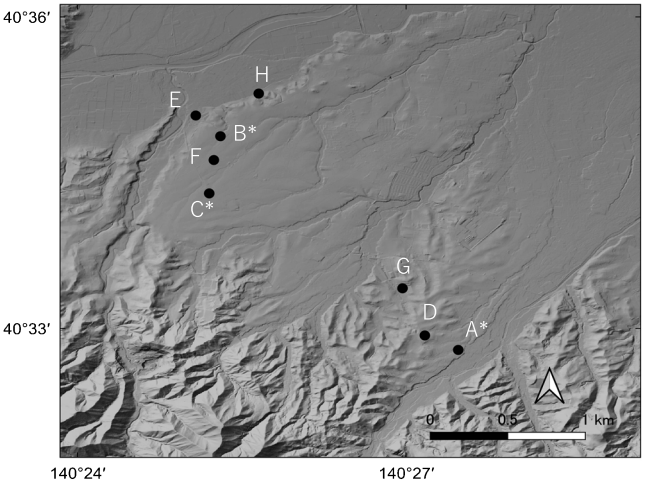

Fig. 1. The location of trapping sites. The alphabets are consistent with Table 1. Star symbols (*) indicate the orchards where composts were installed for the winter samplings.

Table 1. List of orchard fields surveyed and the number of samples successfully analysed

Orchard ID	Nov.	Jan.	Feb.	Mar.	Apr.	May	Dominant undergrowth
A*	2	6	5	2	3	1	*Trifolium repens*
B*	2	4	3	6	4	2	*T. repens* Poaceae spp.
C*	1	2	3	2	1		*T. repens*
D	2			2	1		Poaceae spp.
E					1		*T. repens*
F	1				1		*T. repens*
G	1					1	Poaceae spp.
H	1						Poaceae spp.
Total	10	12	11	12	11	4	

* indicates the orchards where the winter trappings were conducted.

79 individuals of the Japanese field vole using live traps (27 × 7 × 9 cm; Hokkaido Forest Management Corporation, Japan) with sunflower seeds (*Helianthus annuus*) as bait. Upon capture, we recorded the sex and the weight of the trapped voles and released them on the site. Traps were carried to the laboratory to collect the remaining faeces in the traps. The collected faeces were preserved at –20°C in 1.5-ml plastic tubes until DNA extraction. The mean temperature of the study site is 10.6°C with the lowest –1.5°C in January and the highest 23.5°C in August. During the study period, a stable snow cover formed during December and melted in the middle of March each year (Japan Meteorological Agency, https://www.jma.go.jp/jma/menu/arcdata.html, Accessed 13 February 2023), without significant differences in snow cover duration over the three years. We obtained permission from Aomori Prefecture to live-trap the voles (approval codes: 4153, 4110, 4094) and conducted protocols approved by the Animal Care and Use Committee of Hirosaki University. 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).

We also collected samples of four plant species assumed to be involved in the Japanese field vole diet as DNA sequence references. The samples were foliage of two major types (Fuji and Orin) of apple tree *M. domestica*, Chinese apple *M. pumila* (Marubakaido in Japanese) commonly used as rootstocks for the cultivation of apple varieties, and the broadleaf dock *Rumex obtusifolius*.

DNA extraction

We used a commercial DNA extraction kit to extract the genomic DNA from three pieces of faeces from each vole (QIAamp DNA Stool Mini Kit; Qiagen, Hilden, Germany). Before the DNA extraction, we extensively cut the three pieces of faeces with anatomical scissors. We followed the instruction of the DNA extraction kit except for the length of vortex time with inhibit buffer (5 min) and the time for DNA maintained in the filter with elution buffer (3 min). For leaf tissues of the four plant samples described above, we used DNeasy Plant Mini Kit (Qiagen) to extract DNA following instructions. The concentration of the eluted DNA was calculated by Qubit Fluorometer (ThermoFisher Scientific, Waltham, USA) and referred to in the polymerase chain reactions (PCRs) described below.

DNA metabarcoding analyses with the next-generation sequencer

Two-step tailed PCRs were conducted to prepare libraries for the NGS analyses. The first PCR amplified the target region with forward and reverse universal primers. We applied the pair of primers targeted on the internal transcribed spacer region between 5.8S rDNA and 28S rDNA in the nuclear genome (hereafter *ITS2*). The second PCR attached sequence adapters used for sequencing on the Illumina MiSeq NGS platform (Illumina, San Diego, USA) and each sample-specific index used for the sample identification after the NGS run. All the PCR were conducted in an automated thermal cycler (Life Touch thermal cycler; Bioer Technology, Hangzhou, China). For the first PCR, KAPA HiFi Hotstart Ready

Mix (Kapa Biosystems Inc., Wilmington, USA) was used. The PCR reaction mixture in 25 μL contains 2× KAPA HiFi HotStart ReadyMix, 0.3 μM of each universal primer as described below, and templates (20 ng DNA), adjusted by PCR-grade water. To amplify the *ITS2* region of plants, we used universal primers UniPlantF (5'-TGTGAATTGCARRATYCMG-3'; Moorhouse-Gann et al. 2018) and UniplantR (5'-CCCGHYTGAYYTGRGG TCDC-3'; Moorhouse-Gann et al. 2018). Each primer has tailed sequences at their 5'-end to be primed by the second PCR and sequencing primers and also has six N bases for efficient sequencing by MiSeq. The structures of the first PCR primers are therefore as follows: [forward primer] 5'-ACACTCTTTCCCTACACGACGCTCTTCC GATCTNNNNNNTGTGAATTGCARRATYCMG-3' and [reverse primer] 5'-GTGACTGGAGTTCAGACGTGTG CTCTTCCGATCTNNNNNNCCCGHYTGAYYTGRG GTCDC. The target length of the first PCR product is approximately 380 bp. The first PCR condition was 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, extension at 72°C for 30 s, and a final extension at 72°C for 2 min. After PCR, we checked the amplification of the target-length fragment by agarose gel electrophoresis. A negative control (with PCR-grade water instead of the sample) was also included in each PCR and no amplification on the negative control lane in the electrophoresis was confirmed. The negative control PCR products were also examined in the following steps including DNA sequencing. The sequence data obtained in the negative control samples were used in the data filtering step (see below). The first PCR products were purified via AMpure XP beads (Backman Coulter Inc., Brea, USA) and eluted with 35 μL PCR-grade water.

For the second PCR, KAPA HiFi Hotstart Ready Mix (Kapa Biosystems Inc., Wilmington, USA) was also used. The PCR reaction mixture (24 μL) contains 2× KAPA HiFi HotStart ReadyMix, 0.29 μM of each index primer as described below, and an aliquot of templates (2 μL of the purified first PCR product), adjusted by PCR-grade water. The index primers for the second PCR were as follows: [forward primer] 5'-AATGATACGGCGACCA CCGAGATCTACAC-[8-bp index]-ACACTCTTTCCCT ACACGACGCTCTTCCGATCT-3' and [reverse primer] 5'-CAAGCAGAAGACGGCATACGAGAT-[8-bp index]-GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CT-3'. Sequences of the 5'-side of the 8-bp index in forward and reverse index primers are P5 and P7 adapters, respectively, which are attached to the MiSeq flow-cell. Sequences of the 3'-side of the 8-bp index are primed to overhang regions of the first PCR fragments. With the index primers, the second PCR adds 69-bp sequence to the first PCR products. Combinations of forward and reverse 8-bp indices were used for sample identification. The second PCR conditions 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, extension at 72°C for 15 s, and a final extension at 72°C for 5 min. Each second PCR product that was adjusted to equi-molar in DNA concentration was mixed and then purified by the AMpure XP beads.

We used E-gel electrophoresis with E-Gel™ SizeSelect™ II Agarose Gels 2% to extract the targeted DNA fragment (ThermoFisher Scientific, Waltham, USA). The extracted samples were subjected to library quantification and quality check by Qubit Fluorometer and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA), respectively, and then sequencing by several runs of Illumina MiSeq reagent v2 kit and MiSeq reagent Nano kit V2 with 500 cycles. We added the PhiX control (20%) as a spike-in.

Data filtering and DNA database search

We used the program Claident (Tanabe and Toju 2013) to perform data filtering and dietary profiling with DNA database searches. Firstly, we converted bcl file generated by MiSeq into FASTQ file via *bcl2fastq*. DNA sequence data in the FASTQ file were classified into each sample based on 8-bp index by *clsplitseq*. The primer regions and their flanking sequences were also removed via *clsplitseq*. We then used *clconcatpair* to merge the forward and reverse paired-end sequences. Low quality and noisy data were removed by using *clfilterseq* and *clcleanseqv*. At the same time, sequences with less than 150 bp were omitted. Clustering of the denoised sequences was then conducted via *clclassseqv* with 98% minimum identity to obtain Operational Taxonomic Unit (OTU) of the dietary items. Sequence data omitted because of their noisiness but found to be similar to that of clustered OTU were recovered through *clrecoverseqv*. Then, we searched sequences on the DNA database to identify the obtained OTU by the BLAST (Altschul et al. 1990) search using *clrunuchime*. Finally, lowest common ancestor (LCA) algorithm was performed via *classigntax* and therefore conservative OTUs were identified. OTUs that have less than ten reads were removed as low frequency OTUs.

The number of reads assigned to the negative controls

was removed from that of examined samples. Furthermore, we did not consider reads assigned to the sunflower *H. annuus*, assuming that these reads were from the bait used in sampling. For the three plant species (Fuji and Orin of *M. domestica*, *M. pumila*, and *R. obtusifolius*) examined, we could find exact sequences in the DNA database searches for each plant species. On the other hand, minor possible contaminant reads were also obtained. For example, when examining the sample of *M. pumila*, all the obtained sequences are expected to match with those of *M. pumila* in the DNA database. As we expected, we found that the large number of reads obtained from the sample of *M. pumila* was assigned to *M. pumila* in the DNA database. However, a minor number of reads from the sample of *M. pumila* were erroneously assigned to *R. obtusifolius* in the DNA database, suggesting that the sample of *R. obtusifolius* that we also examined with that of *M. pumila* was contaminated into the sample of *M. pumila* during the process of the experiments. In such a case, we subtracted the number of reads obtained for contaminants from those of samples, assuming that such numbers of reads could be obtained from those for contaminants. Infrequent reads with less than 0.1% of the total number of reads per sample were also removed.

To correct for differences in the number of reads per sample, rarefaction analysis was conducted with the package vegan (Oksanen et al. 2019) in R (R Core Team 2020), in which we fixed the number (10 000 reads) based on the minimum reads obtained among samples (minimum 11 085, maximum 354 031, average 168 830). We confirmed that the rarefaction curves were saturated at the 10 000 reads, indicating that we could discuss the diet with the rarefied reads.

We then examined the seasonal dietary changes based on the relative read abundance data. To summarize the seasonal variation of key plant species, the identified sequences were classified into five groups; fruits (*Malus* and *Prunus*), *Rumex* sp., Fabaceae sp., other plants and unclear.

To analyse seasonal changes in the detection rate of *Malus* spp., we calculated the monthly occurrence percentage, by dividing the number of samples in which rootstocks and apple varieties were detected by the total number of samples in each month. Generalized linear model (GLM) analyses were also conducted to test whether the presence or absence of snow cover affected the frequencies of fruit trees and rootstock occurrence. The number of samples in which apple varieties (or rootstock) sequences were detected and the number of samples in which they were not detected were used as response variables. The presence or absence of snow cover was used as the explanatory variable. We used "binomial" for the error structure and "logit" for the link function.

Results

During the data filtering step, we excluded low-quality and chimeric sequences. We also removed sequences with an *E*-value > 10^{-10} (low homology in the BLAST search) and with < 10 reads (infrequent sequences). After the data filtering step, we obtained 10 846 821 reads for 3656 OTUs from 60 faecal samples of 60 different individuals out of 79 voles and from four plant samples. Among 60 samples, 35 were from the snow season (January to March), while 25 were from the snow-free periods (November, April, and May). The sequence lengths of the detected OTUs were 150–385 bp (271.6 bp on average). We removed reads for baits and contaminants from the rarefied read data (10 000 reads in each sample) and examined 592 956 rarefied reads in total for the dietary analyses.

Characteristics of winter dietary items of the Japanese field vole

We identified 275 sequences with 519 984 reads at the family or the taxa below the family level (Table 2), while 237 sequences with 26 576 reads could not be identified at the family level (Table 2). The number of samples containing a specific sequence for each taxonomic unit was also given in Table 2. Various taxonomic units of plants, extending to 16 families were found. The most frequently detected herbaceous species was the broadleaf dock. Forty-nine out of 60 samples contained some sequences of them, and the number of reads assigned to the broadleaf dock was also the largest. The second most frequently observed taxonomic unit was *Malus* spp., which should originate from cultivated apple trees, including rootstock and apple varieties. The total number of reads of *Malus* spp. was also the second highest. Although species were unidentified, Fabaceae and Caryophyllaceae were also listed frequently. For Fabaceae, we re-submitted the UIS (unidentified sequence in Table 2), which was not identified in our LCA analysis, to the BLAST search and found that it was almost identical to sequences of *T. repens*: the highest hit was 100% sequence identity in the database.

Table 2. List of plant taxa detected in the faeces of the Japanese field vole (*Alexandromys montebelli*), with the detection frequencies and the total number of reads

Family	Genus*	Species	Number of samples contained the species		Number of reads	Remarks
			Nov., Apr., and May (n = 25)	Jan., Feb., and Mar. (n = 35)		
Cultivated plants						
Convolvulaceae	*Ipomoea*	*trifida*	1	0	3670	
	Ipomoea		1	0	1586	
Cucurbitaceae	*Cucurbita*	*pepo*	0	1	9	
Rosaceae	*Fragaria*		1	0	12	
	Malus		21	25	92 005	Including apple varieties and rootstocks
	Prunus		3	8	21 561	
Other plants						
Araliaceae	*Hydrocotyle*		0	1	10	
Asteraceae	*Erigeron*		1	0	222	
	Taraxacum		1	0	35	
	UIS		0	1	13	
Caryophyllaceae	UIS		5	7	13 054	
Fabaceae	*Albizia*		1	1	9812	
	UIS		7	16	6458	
Geraniaceae	*Geranium*		0	2	743	
Lamiaceae	*Lamium*	*purpureum*	2	0	222	
	Lamium		1	0	177	
Oleaceae	*Ligustrum*	*japonicum*	1	0	12	
Oxalidaceae	*Oxalis*		0	4	1441	
Plantaginaceae	*Plantago*	*asiatica*	0	6	383	
Poaceae	*Agrostis*	*stolonifera*	1	1	87	
	Agrostis		5	3	978	
	Avena		1	0	10	
	Poa	*infirma*	1	0	333	
	Poa		5	4	5608	
	Triticum	*aestivum*	0	1	19	
	UIS		3	3	540	
Polygonaceae	*Fallopia*	*sachalinensis*	0	1	6607	
	Fallopia		0	1	530	
	Persicaria	*thunbergii*	2	0	195	
	Persicaria		1	0	262	
	Rumex	*obtusifolius*	21	28	283 040	
	Rumex		12	25	79 676	
	UIS		2	0	33	
Primulaceae	UIS		1	0	1	
Ranunculales	*Akebia*		0	1	17	
UIS			1	4	385	Rosales spp.
Unclear						
UIS			15	17	26 576	Including the sequences classified as "Streptophyta"

* UIS stands for "unidentified sequence". The sequences described as UIS may contain multiple species.

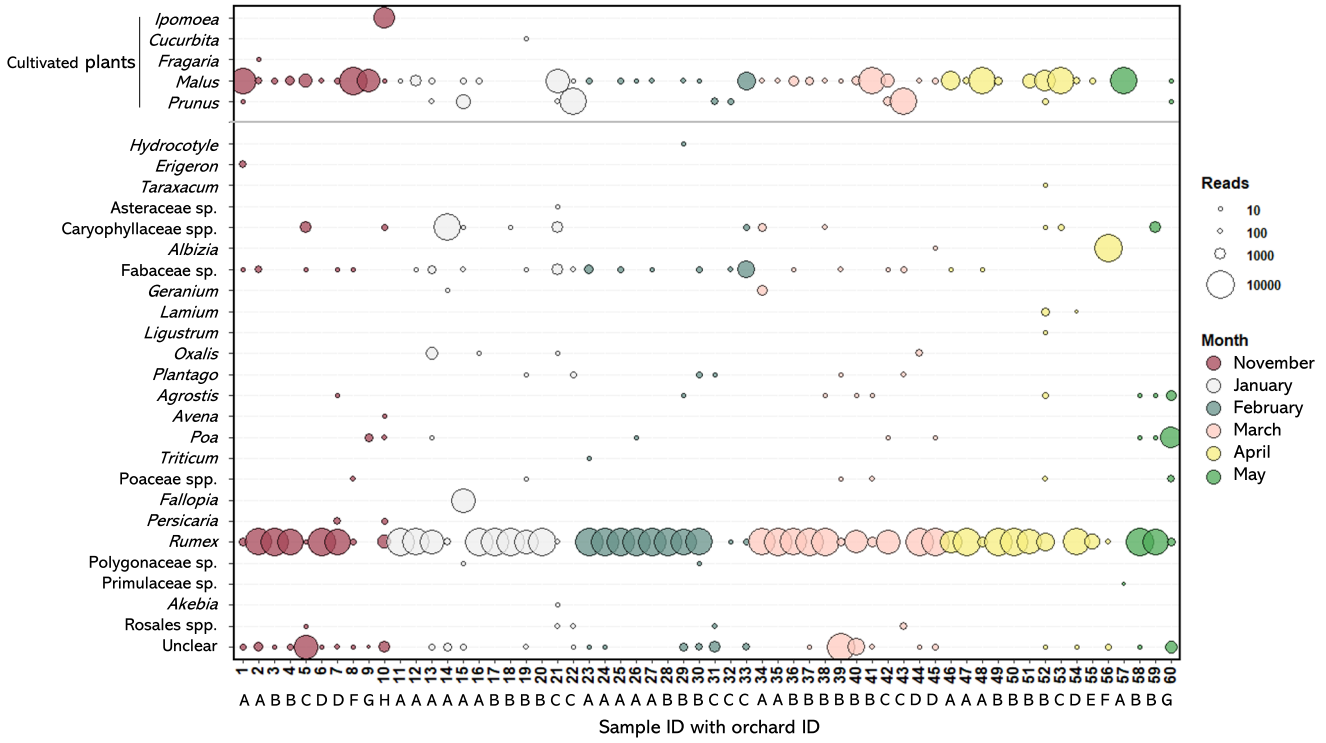

Fig. 2. The list of plant taxa identified by DNA metabarcoding analysis and the number of reads detected from each sample. The numbers on the horizontal axis represent sample IDs and alphabets represent orchard IDs in Table 1. The size of circles indicates the number of reads.

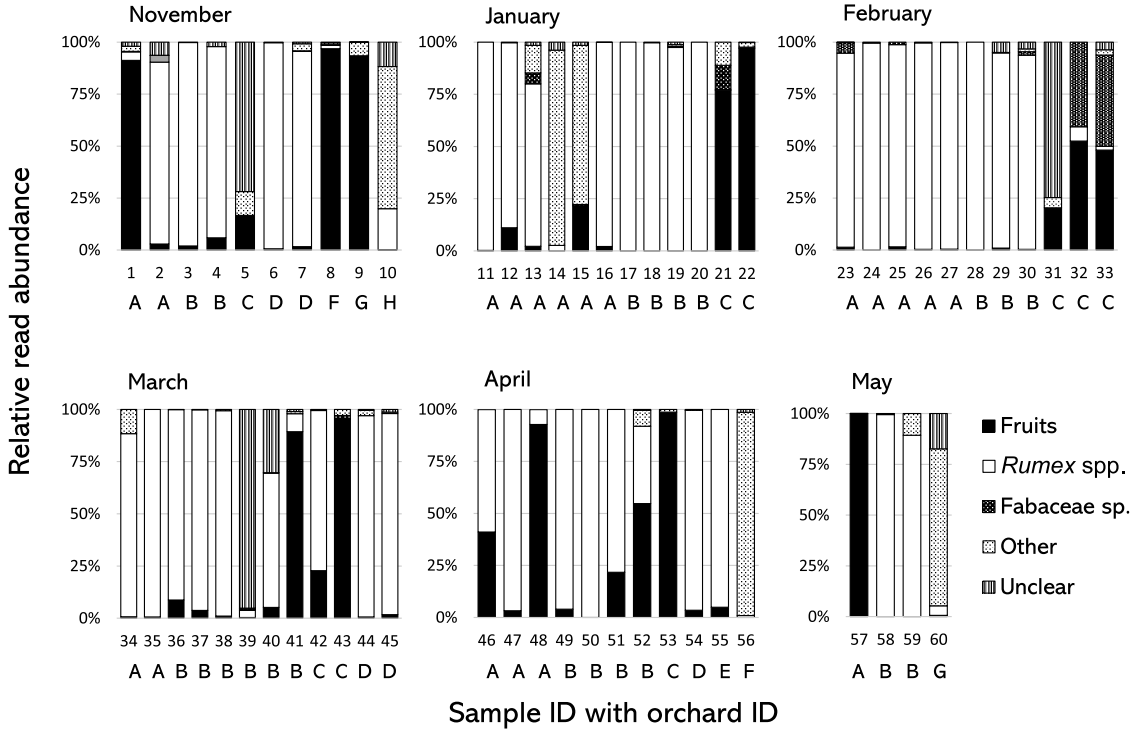

Fig. 3. The relative read abundance (RRA) of voles' diet. The detected plant species were classified into five groups; fruits (*Malus* and *Prunus*), *Rumex* spp., Fabaceae sp., other (all other plant species), and unclear. The numbers on the horizontal axis represent sample IDs and alphabets represent orchard IDs in Table 1.

We, therefore, assumed that the UIS for Fabaceae was from *T. repens*. Other taxonomic units found in three or more samples under snow cover were *Agrostis* sp., *Poa* sp., Poaceae UIS, *Plantago asiatica*, and *Oxalis* sp. We have observed sweet potato *Ipomoea trifida*, summer squash *Cucurbita pepo*, and strawberry *Fragaria* sp. Because they were planted in vegetable gardens adjacent to orchards, they were classified as "cultivated plants". On average, the number of plant taxa contained in a sample (three pieces of faeces) was 4.0 (min. 1, max. 10; Fig. 2).

One or two predominant plant species were detected in the majority of samples (Fig. 3). *Rumex* spp. were predominant in the samples taken during February at orchards A and B, and the samples from orchard C mainly contained fruits, Fabaceae sp., and unclear (Fig. 3). During March and April, *Rumex* spp., fruits, and unclear were the majority. The predominant sequence detected in the sample 56 from orchard F was *Albizia* sp.

Separation of apples and rootstock

Using the reference data obtained from the plant samples of rootstock and apple varieties, we subdivided the 30 241 sequences which were classified as *Malus* spp. into those from the rootstock or apple variety part of the tree (Table 3). The *Malus* sequences were detected in 46 of 60 samples, of which 16 samples contained the sequences of apple varieties only, 13 samples did rootstocks only, 11 samples included both, and six were indistinguishable due to insufficient informative sites in the *ITS2* marker. The sequences originating from apple varieties were frequently found in November, but the percentage of occurrence decreased during the snow season, and then again increased in April (Table 3). When the six survey months were divided by seasons, the occurrence percentage of apple varieties ranged from 27.2% to 33.3% in the snow season and from 50.0% to 80.0% in the snow-free season, respectively (Table 3). A GLM analysis indicated that the occurrence of apple varieties was significantly fewer in the snow season than in the snow-free season ($\chi^2 = 6.332$, $P = 0.012$). As for rootstock, four out of ten samples contained rootstocks in November, but the percentage became low in January and February. The percentage rose in March and remained high throughout April (Table 3). The occurrence percentage of rootstocks ranged from 8.3% to 66.7% in the snow season and from 0% to 72.7% in the snow-free season, respectively. A GLM analysis did not support the effects of snow cover on the occurrence of rootstocks, although the observed data showed overdispersion ($\chi^2 = 1.140$, $P = 0.286$).

Discussion

This is the first report on the winter diet of the Japanese field vole under deep snow cover, which is comparable to the diet in the snow-free season. It has been reported that the vole utilises a wide variety of green plants opportunistically (Watanabe 1962; Kaneko 1975); our study revealed a strong dependence on some specific plants (e.g., the broadleaf dock; Table 2, Fig. 3) in both the snow and snow-free seasons.

The broadleaf dock is a perennial herb that prefers a sunny environment and is common in meadows and orchards (Yonekura 2006; Toyoshima and Takanashi 2007). It grows aggressively throughout the growing season, forms thick rhizomes for overwintering (Kobayashi et al. 1989) and has strong mowing resistance by regenerating from rhizomes when the above-ground portion is removed (Nemoto et al. 1983). Among the *Rumex* species observed in Japan, the broadleaf dock has the highest content of oxalic acid (Miyagi et al. 2010b), which is a plant defence substance and not favoured by herbivores. However, this defence substance accumulates only in its leaves; the stems are rich in its precursor, citric acid, which is used to produce of oxalic acid during leaf formation (Miyagi et al. 2010b). Additionally, the broadleaf dock plant contains high

Table 3. The number of samples where the sequences of apple varieties or rootstock were detected

	Nov. ($n = 10$)	Jan. ($n = 12$)	Feb. ($n = 11$)	Mar. ($n = 12$)	Apr. ($n = 11$)	May ($n = 4$)	Total number of reads
Apple varieties	8 (80.0%)	4 (33.3%)	3 (27.3%)	4 (33.3%)	6 (54.5%)	2 (50.0%)	18 474
Rootstocks	4 (40.0%)	1 (8.3%)	3 (27.3%)	8 (66.7%)	8 (72.7%)	0 (0.0%)	11 767

levels of organic nutrients such as glutamine, glutamic acid, asparagine, and serine (Miyagi et al. 2010b). The rhizomes of broadleaf docks are nutrient storage organs for overwintering. Their aggregated biomass of rhizomes with rich nutrients may have made this species very suitable as a winter food resource for voles. Even during the snow-free season, the broadleaf docks were frequently detected (Fig. 2). The broadleaf docks have strong cold tolerance (Miyagi et al. 2010a) and are one of few green plants available in orchards for some time after thaw. In addition to that, the rhizomes growing underground are the food resource that voles can access without taking the risk of going out of their burrows. Considering these factors, broadleaf docks are presumed to be a good year-round food resource for voles.

The food resources of small mammals under snow cover have been gradually revealed in recent studies in various regions. In North American forests, the abundance of berry shrubs plays an important role in the overwinter survival of red-backed vole *Myodes* (*Clethrionomys*) *rutilus* (Dyke 1971; Boonstra and Krebs 2006). In north-eastern Norway, the tundra vole *A. oeconomus* exhibits a strong preference for Polygonaceae forbs and Salicaceae deciduous shrubs (Soininen et al. 2013). These studies indicate that voles feed primarily on perennial plants, maintaining a large biomass in winter. Especially the tundra vole is phylogenetically closely related to the Japanese field vole (Martínková and Moravec 2012), and its frequent use of Polygonaceae family (mainly *Rumex* sp.) (Soininen et al. 2013) is consistent with the result of our study. In the apple orchards in Aomori Prefecture, perennial shrubs which could maintain large biomass during winter are rarely present as a result of regular undergrowth clearing. As there are no shrubs with large biomass above the ground, the broadleaf dock with relatively large underground biomass is thought to have become an important winter food resource for Japanese field voles.

Malus domestica utilised by voles in November mainly was apple varieties (Table 3). Voles probably consumed fallen fruits, as Japanese field vole does not climb trees. The frequency of apple varieties decreased as the fruits on the ground were removed or consumed before snowfall. The subsequent increase from March to May should be due to feeding on tree branches that have been dropped onto the ground by pruning. The significant seasonal differences in apple variety detection would reflect seasonal changes in human-induced availability. On the other hand, the rootstock was barely used in January, and the frequency of use gradually increased, with about 70% of the samples containing it in March and April (Table 3). This is consistent with the observation of apple farmers that tree damage usually occurs from February onward. The dependence on the broadleaf dock and Fabaceae sp. (assumed to be the white clover *T. repens*) until February appears to shift gradually to the broadleaf dock and cultivated trees from March onwards.

Further research is needed to determine if this increased detection of rootstocks in early spring is due to the depletion of Fabaceae sp., or other factors; such as seasonal changes in nutrient levels that made the rootstocks more attractive. It is of note that four of the ten individuals were feeding on the rootstocks in November, when there were still enough herbaceous plants on the ground. Since voles often excavate tunnels at the base of fruit trees (personal observation), there is a possibility of unintentional consumption during the excavation of these tunnels. The consumption of fruit tree by voles before winter has not been recorded to date. Fruit tree damage outside the winter season would also need to be investigated.

Our results also indicated that the voles whose diet consisted of less amount of broadleaf docks tended to feed on cultivated trees such as apples and plums (Fig. 3). In general, the removal of broadleaf docks is recommended to reduce the competition for nutrients with fruit trees (Yokota et al. 1989). From the viewpoint of vole damage prevention, however, the complete removal of the broadleaf dock may reduce winter herbaceous food resources for voles and induce damage to cultivated fruit trees, although the sampling error caused by the small samples (three pieces of faeces) should be considered.

Apple orchards in northern Japan are often dominated by white clover *T. repens* and Poaceae spp., as pasture grasses had been recommended as the undergrowth (Toyoshima and Takanashi 2007). Among the plants other than the broadleaf dock, the Fabaceae sp. (assumed to be *T. repens*) was the most frequently consumed, but its utilisation declined through March (Fig. 2). The possible reason for the decline could be that the species had been eaten up, or that a more favourable food resource had become available. Poaceae spp. (including *Agrostis* spp., *Avena* sp., *Poa* spp., *Triticum aestivum*, and UIS) were consumed primarily in May, during its budding season, but they were not a food resource during winter (Table 2, Fig. 2). Having perennial shrubs in orchards

would not be practical as it would provide summer habitat for voles, but letting a certain amount of perennial herbaceous plants which have a sizeable underground biomass grow in addition to pasture grasses, might reduce the vole's dependency on fruit trees during winter. Other frequently detected plants during winter (e.g., *P. asiatica* and Caryophyllaceae sp.) could also be the candidate plants that could be food resources during the winter months.

At the same time, we should carefully discuss the species' diet based on relative read abundances estimated from DNA metabarcoding data. Although recent meta-analysis has shown that relative read abundance and diet biomass correlate positively in general (Lamb et al. 2019), there are still many uncertainties. For example, Neby et al. (2021) pointed out that the utilisation of some specific plant species (*Trifolium* sp. in their experiment) by voles could be significantly underestimated in the faecal DNA metabarcoding data compared to the actual amount of food consumed, while other plant species showed relatively high positive correlations. In other words, the relative read abundance is affected by the extent of the detection rate for other species in the faeces. The number of reads of Fabaceae sp. was generally low in our study as well. The utilisation of Fabaceae sp. needs further investigation, considering the detection characteristics.

This study revealed that the broadleaf docks were an important food source for voles in apple orchards and the damage to fruit tree rootstock was concentrated in the latter half of winter and early spring, along with decreasing use of Fabaceae sp. (assumed to be *T. repens*). This study also suggests that the biomass of *Rumex* sp. in orchard may affect the consumption of fruit trees during winter. Future studies are necessary to identify the factors that influence the changes in diet during winter, and such information could be used to formulate more appropriate undergrowth management practices to reduce the vole's damage to fruit trees.

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