Diets of Rodents Revealed through DNA Metabarcoding

Author: Sato, Jun J.

Source: Mammal Study, 50(1)

Published By: Mammal Society of Japan

URL: https://doi.org/10.3106/ms2024-0018

Mammal Study 50 (2025) DOI: 10.3106/ms2024-0018

Invited Review

Series of Reviews for Mammal Ecology in Japan celebrating volume 50 of Mammal Study [Ecology and Genetics]

Diets of rodents revealed through DNA metabarcoding

Jun J. Sato[1,2]

[1] *Department of Biological Science, Fukuyama University, Higashimura-cho, Aza, Sanzo, 985, Fukuyama 729-0292, Japan*
[2] *Research Center for Green Science, Fukuyama University, Higashimura-cho, Aza, Sanzo, 985, Fukuyama 729-0292, Japan*

Abstract. Rodents are the most abundant mammals on Earth, prevalent on all continents except Antarctica, and they fulfill important ecological roles in various ecosystems. Recent progress in high-throughput DNA-sequencing technology has enabled identification of diet components in various environmental samples, such as feces or stomach contents, through DNA metabarcoding. Studies using this method have begun to clarify the diets of small, nocturnal, and elusive rodents. This review summarizes previous studies that used DNA metabarcoding to clarify the diets of native or exotic species in Rodentia to understand progress and unresolved problems in such analyses. Furthermore, the methodology of dietary DNA metabarcoding in rodents is discussed, together with issues that should be taken into consideration. Finally, future perspectives and directions to be considered in the use of DNA metabarcoding in dietary studies of rodents are discussed.

Key words: dietary DNA metabarcoding, ecosystem, food web, NGS, Rodentia.

We are now facing tremendous challenges related to loss of biodiversity, and the extinction rate is such that the planetary boundary related to preservation of biosphere integrity is considered to have been transgressed (Richardson et al. 2023). The recent and future extinction rates of vertebrate species have been calculated to be more than 100 times higher than in the past two million years (Ceballos et al. 2020). Therefore, there has been a great deal of discussion regarding the conservation of biodiversity worldwide, and the Convention on Biological Diversity accepted the Kunming-Montreal Global Biodiversity Framework in 2022 with a global vision to achieve "living in harmony with nature" by 2050 (see approved decision at https://www.cbd.int/gbf/, Accessed 19 March 2024). Despite this trend in biodiversity conservation worldwide, it is still challenging to live in harmony with nature. Although human–wildlife conflict and coexistence have been discussed (e.g., König et al. 2020 and papers in a special issue of *Conservation Biology* volume 34, issue 4), it is not easy to live in harmony with nature without a detailed understanding of ecosystems. To prevent the deterioration of biological diversity and achieve the vision of living in harmony with nature mentioned above by 2050, it is our responsibility to develop and improve methods to decipher the mechanisms that maintain ecosystems by assessing interactions among wildlife.

Food webs are a result of fundamental biological interactions formed through various relationships between predatory animals and consumed prey, and have long been examined by analyzing the diets of animals. For example, studies of the food habits of Japanese mammals determined by analysis of stomach contents have been reported in *Mammal Study* since the era of its predecessor, *The Journal of the Mammalogical Society of Japan* (e.g., Asahi and Watanabe 1967 for the exotic Formosan tree squirrel; Chiba 1968 for the Japanese serow). Food webs consist of simple relations of trophic interactions, but they are difficult to decipher because of the need to understand the diets of various animals. It is particularly challenging to trace the diets of small, nocturnal, and therefore elusive mammalian species, such as rodents (Rodentia; rats, mice, voles, squirrels, dormice, and so forth) because their elusive ecology makes it difficult to observe their dietary behaviors directly (Verde Arregoitia

E-mail: jsato@fukuyama-u.ac.jp

and D'Elía 2020) and to identify their prey species precisely from the tiny remains of dietary organisms in their feces or gastrointestinal tract. Nevertheless, without understanding the ecological role and status of wild rodents in their ecosystems through dietary analysis, it is not possible to clarify an important part of the food webs surrounding them that support the fundamental function of each ecosystem (see below). It is necessary to clarify the diets of rodents to develop measures to allow us to live in harmony with nature.

This review discusses why understanding the food chains around rodents is important, summarizes recent studies that have applied fecal or gastrointestinal DNA metabarcoding methodologies to rodents along with ways to avoid confounding factors that bias the results of DNA metabarcoding when examining rodents, and finally considers the future prospects and directions of research in this area.

Why should rodent diets be clarified?

The order Rodentia includes approx. 2500 species with a worldwide distribution, and accounts for approx. 40% of extant mammalian species; they play important ecological roles in various ecosystems including deserts, tropical rainforests, and urban environments on all continents of the world except Antarctica (Lacher et al. 2016). Rodents have various ecological characteristics, with terrestrial, arboreal, fossorial, and semiaquatic adaptations. Therefore, they have important ecological functions in various ecosystems through their consumption of a wide range of foods. Verde Arregoitia and D'Elía (2020) classified the diets of rodents into eight categories: carnivore, folivore, frugivore, granivore, omnivore, specialist herbivore, vermivore, and unknown. Their tooth and jaw morphologies have diversified through evolution to cope with various food resources in diverse environments (Lacher et al. 2016). As a result of their feeding behaviors, rodents are considered to have various ecological roles in ecosystems, including roles in the dispersal of seeds and fungal spores through scatter hoarding, pollination, and vegetation change (e.g., increases in plant production and diversity) (Lacher et al. 2016, 2019). Clarifying the dietary contents of rodent species in each ecosystem would therefore contribute to our understanding of various aspects of the mechanisms underlying the dynamics and functions of ecosystems.

As a consequence of their species richness, various rodents often coexist within the same community. Therefore, rodents are good models to understand the mechanisms of coexistence in each ecosystem. Particularly, in small and isolated islands such as in Japan with forests covering two thirds of the land area, rodents must have coexisted with niche partitioning in forest ecosystems (Sato J. J. et al. 2018). Rodents are also observed around human settlements and pose problems in terms of agriculture and public health (Lacher et al. 2016). However, human–wildlife conflicts have not been resolved in many cases because of a lack of understanding of the ecological requirements of rodents. Understanding their dietary needs may allow us to evaluate better whether rodents are beneficial or pest animals around agricultural fields (e.g., Sato et al. 2022; Murano et al. 2023). On the other hand, rodents are responsible for zoonotic diseases, including Lyme disease, hantaviruses, and many other diseases (Lacher et al. 2016). Rodents sometimes show cyclic population boosts and declines depending on resource dynamics (Saitoh et al. 2007) and cause public-health issues with regard to the spread of zoonotic diseases in periods of increasing population size (Han et al. 2015). As the Rodentia includes the greatest number of zoonotic host species among mammals (Han et al. 2016), it is necessary to monitor and control rodent population dynamics. To determine the mechanisms underlying expansion of rodent populations, their dietary needs must be clarified. Therefore, knowledge of rodent diets is required to understand the mechanisms of ecosystem dynamics and avoid adverse effects on humans.

Traditional dietary analysis and difficulties to overcome

Information regarding diet has long been acquired through traditional dietary analysis, including direct observation of dietary behaviors through tracing of animals or examination of fecal or stomach contents by microscopy (e.g., Ota 1968; Minato 2018). However, these methods have limitations in their ability to capture comprehensive food lists or achieve precise taxon identification of species from fluid (blood, nectar, or plant sap) or tissue such as parts of invertebrates remaining in such samples (Nielsen et al. 2018). Soft tissues of animals are often not visible in feces or stomach contents because of digestion. Even if such remnants could be visualized, precise taxon identification would still be difficult, thus allowing only broad taxonomic categorization of dietary components. Moreover, taxon identification from tiny remains requires analysis by experts. For example, Ota

(1968) performed dietary analyses of the stomach contents of rodents inhabiting Hokkaido (*Clethrionomix* [now *Myodes*], *Apodemus*, *Rattus*, and *Mus* species), and reported dietary components at a broad level, e.g., starch glue, plant fiber (leaf, stem, and root), seed, fruit, mushroom, lichen, and animal, without precise identification of the taxon for each dietary item. These traditional methods have provided valuable insights into rodent species. However, more comprehensive details are needed to gain a more precise understanding of rodent diets and the food webs. More sensitive methods that are unaffected by digestion and that can identify component taxa in greater detail from small dietary remains are required.

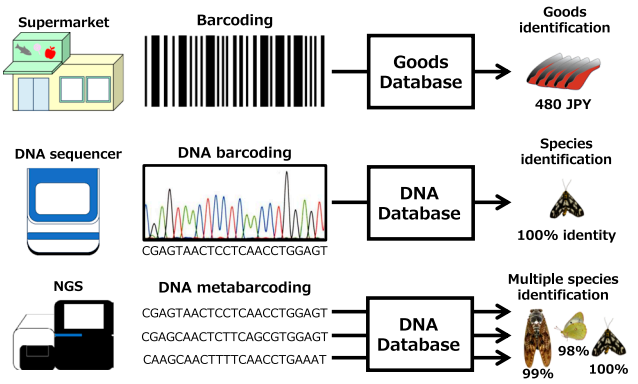

Fig. 1. Overview of the DNA metabarcoding method for simultaneous identification of multiple species with next-generation sequencing (NGS) in reference to the common barcoding method used in supermarkets for identification of goods and DNA barcoding for identification of a single organism with Sanger sequencing.

DNA metabarcoding

Recent progress in high-throughput DNA-sequencing technology has clarified previously unresolved issues in fields such as ecology and evolutionary biology. Next-generation sequencing (NGS) developed between 2005 and 2007 radically altered biodiversity research. It should be noted that NGS here means the second-generation sequencing technology and the wording of NGS is not appropriate at the time of publication of this review. DNA metabarcoding is a groundbreaking method that has been facilitated by the development of NGS and has been applied to studies of biodiversity and ecosystems.

DNA metabarcoding means simultaneous identifications of multiple taxonomic units (species, genus, family, and so on) using DNA sequences. More specifically, it can be used to clarify the presence of multiple organisms in environmental samples (e.g., water, soil, and feces) through interpretation of the obtained DNA sequences as barcodes that allow species identification by reference to DNA databases (Fig. 1). Basically, this method requires sampling of environmental samples, DNA extraction, PCR with universal primers, DNA purification, 2nd PCR with primers including index and adapter sequences, NGS sequencing, and bioinformatics (see the latter half of this review). NGS has enabled simultaneous sequencing of multiple species (Fig. 1). Such massively parallel sequencing methods together with the development of useful universal primers that allow PCR amplification of diverse species in a taxonomic group have facilitated environmental DNA studies examining DNA from environmental samples such as water, soil, feces, or others (e.g., invertebrates, Zeale et al. 2011; Jusino et al. 2019; plants, Taberlet et al. 2007; Moorhouse-Gann et al. 2018; fish, Miya et al. 2015; mammals, Ushio et al. 2017; amphibians, Sakata et al. 2022; birds, Ushio et al. 2018). Other universal primers were summarized by Taberlet et al. (2018), Ando et al. (2020), and Tournayre et al. (2020).

DNA metabarcoding analysis of dietary samples (feces or stomach contents) provides information to infer not only the basic dietary needs of wildlife and other biological interactions in ecosystems (Cuff et al. 2021) but also the conservation status of endangered or protected animals, niche overlap and partitioning among sympatric guilds, ecological roles of animals at the boundaries between humans and nature (e.g., agricultural land), and the influences of exotic species (see the next section for example). There have been several detailed reviews of dietary DNA metabarcoding, with discussion of the potential and pitfalls of this method (Alberdi et al. 2018, 2019; Ando et al. 2020; Cuff et al. 2021; Tercel et al. 2021; see Bohmann et al. 2022 and Tedersoo et al. 2022 for more general DNA metabarcoding methods). The advantages of this method include high sensitivity, high taxonomic coverage and resolution, and noninvasiveness (in the case of examining fecal specimens). However, some challenges remain, including PCR bias resulting from differences in primer efficiency, contamination, low taxonomic resolution due to insufficient global DNA databases, and secondary consumption (Cuff et al. 2021; Tercel et al. 2021). Therefore, care is required when designing DNA metabarcoding analyses to avoid these issues.

Rodent dietary studies that used DNA metabarcoding are discussed below. Research of this field is mainly progressing in North America (eleven studies), Europe

(five), and Japan (five), but only a few in Africa (one), South America (two), Oceania (one), and other Asian regions (one study in each of East, Southeast, and West Asian countries) (Table 1). Especially, no other research of the dietary DNA metabarcoding for rodents has been reported in Japan at the time of the submission except for our research group. Therefore, below, we focused on our previous research for the case study of rodents in Japan. Determination of the components of dietary samples (feces or stomach contents) of rodents using this method has provided important insights into their ecosystems and interactions with human society. For example, through clarifying the ecological roles of rodents, the DNA metabarcoding method has been applied to various studies assessing dietary trend of endangered or protected rodent species for conservation, the extent of niche overlap or partitioning among coexisting rodents, ecological roles of rodents in agricultural ecosystems, effects of exotic and invasive rodent species on the ecosystem, and rodents adapted to extreme environments, which I reviewed below.

What information can be obtained by dietary DNA metabarcoding of rodent species?

Endangered or protected species

The fecal DNA metabarcoding method is useful to gain an understanding of the dietary requirements of endangered or protected rodent species for their conservation because it is noninvasive, except for the requirement for capture in traps, handling, and sampling. Although they play important ecological roles and 358 species are listed as threatened in the IUCN Red List, rodents are usually not charismatic species and therefore do not have a global conservation focus (Lacher et al. 2016). In Japan, among the 22 rodent species, four species in the Ryukyu islands (three *Tokudaia* species and *Diplothrix legata*) are designated as Endangered by IUCN and one species is protected by the Japanese law (Japanese dormouse, *Glirulus japonicus*; see below). Clarifying the diets of such threatened or protected rodent species provides valuable information about how to preserve their habitats. Multiple DNA metabarcoding studies for this purpose have been reported (Table 1).

Several studies have been performed in the USA using fecal DNA metabarcoding analysis of endangered local rodent populations. Iwanowicz et al. (2016) used the nuclear *ITS2* marker to examine the plant diet of the Pacific pocket mouse, *Perognathus longimembris pacificus*, which is federally classified as an endangered rodent species in the USA, and suggested that this species consumes native and nonnative plants depending on their seasonal availability. Vandergast et al. (2023) recently extended this research using the same marker, and suggested that for conservation of the Pacific pocket mouse it may be necessary to maintain native forbs while reducing nonnative grasses. Castle et al. (2020) used both fecal microhistology and DNA metabarcoding of the *ITS2* region to study the diet of the critically endangered Amargosa vole, *Microtus californicus scirpensis*, which inhabits marshland dominated by bulrush (a potential dietary plant) in California, USA. They showed that in addition to protein-poor bulrush, the voles consumed a diverse range of other plant species to fulfill their nutritional requirements. The results imply that the monoculture of bulrush, which has been facilitated for conservation of the Amargosa vole, was inappropriate and that more diverse plant species should be preserved. Aylward et al. (2022) used DNA metabarcoding of the nuclear *ITS2* and chloroplast *trnL* regions to examine the plant diet of the endangered salt marsh harvest mouse, *Reithrodontomys raviventris*, in coastal areas of California, USA, and characterized the basic diet and niche breadth of this species. Their results imply the possibility of dietary competition with coexisting rodents such as the native western harvest mouse, *R. megalotis*, and the nonnative house mouse, *Mus musculus*. Goldberg et al. (2020) used three plant markers (*ITS1*, *ITS2*, and *trnL*) to evaluate the diet of the northern Idaho ground squirrel, *Urocitellus brunneus*, which is federally recognized as a threatened species in the USA, and demonstrated a dietary preference for specific plants, such as yampah (*Perideridia*), due to their importance for overwinter survival despite their low availability. All of these studies in the USA uncovered novel dietary characteristics of endangered local populations and prompted discussion of how these rodents and their habitats should be conserved based on dietary features or biological interactions with coexisting rodent species.

In Japan, Sato et al. (2023) performed noninvasive fecal DNA metabarcoding analyses of a protected animal in Japan, the Japanese dormouse (designated as a natural monument in 1975 by the Agency for Cultural Affairs, Japan). In that study, plant and invertebrate dietary components were assessed using the *ITS2* and mitochondrial *COI* gene regions, respectively; the results implied that the fruit of the hardy kiwi, *Actinidia arguta*, is an important resource in autumn to increase body weight before

Table 1. Previous fecal or stomach DNA metabarcoding studies for rodents

Aims of the study	Papers	Rodent species examined	Country	Markers		Samples	Baits	Traps
				Plant	Animal			
To clarify the diets of endangered or protected animals	Iwanowicz et al. (2016)	Pacific pocket mouse *Perognathus longimembris pacificus*	USA	*ITS2*		feces	millet seed (*Panicum milaceum*)	Sharman trap
	Castle et al. (2020)	Amargosa vole *Microtus californicus scirpensis*	USA	*ITS2*		feces	peanut butter (*Arachis*), oats (*Avena*)	Sharman trap
	Goldberg et al. (2020)	Northern Idaho ground squirrel *Urocitellus brunneus*	USA	*ITS1*, *ITS2*, *trnL*		feces	oats, peanut butter, imitation vanilla extract	Tomahawk live trap or focal trap
	Aylward et al. (2022)	Salt marsh harvest mouse *Reithrodontomys raviventris*	USA	*ITS2*, *trnL*		feces	oats (*Avena*), sunflower (*Helianthus*), walnut (*Juglans*), millet (*Panicum*), Canarygrass (*Phalaris*)	Sharman trap
	Vandergast et al. (2023)	Pacific pocket mouse *Perognathus longimembris pacificus*	USA	*ITS2*		feces	millet seed (*Panicum milaceum*)	Sharman trap
	Sato et al. (2023)	Japanese dormouse *Glirulus japonicus*	Japan	*ITS2*	*COI*	feces	none	Nest box
To clarify the niche overlap or partitioning	Soininen et al. (2014)	Grey-sided vole *Myodes rufocanus* Tundra vole *Microtus oeconomus* Norwegian lemming *Lemmus lemmus*	Norway	*trnL*		stomach	raisins and rolled oats	Snap trap
	Soininen et al. (2015)	Brown lemming *Lemmus trimucronatus* Collard lemming *Dicrostonyx groenlandicus*	Canada	*trnL*		feces	none	none
	Lopes et al. (2015)	Tiny tuco-tuco *Ctenomys minutus* Flamarion's tuco-tuco *Ctenomys flamarioni*	Brazil	*ITS1*, *trnL*	*16S*	feces	none	Snap trap with rubber
	Sato et al. (2018)	Large Japanese wood mouse *Apodemus speciosus* Small Japanese wood mouse *Apodemus argenteus*	Japan	*trnL*		feces	sunflower seeds (*Helianthus*)	Sharman trap
	Sato et al. (2019)	Large Japanese wood mouse *Apodemus speciosus*	Japan	*trnL*	*COI*	feces	oats (*Avena*)	Sharman trap
	Lopes et al. (2020)	Seven species of tuco-tucos *Ctenomys*	Brazil	*ITS1*, *trnL*		feces	none	Snap trap with rubber
	Petrosky et al. (2021)	Seven species of earthworm mice Chrotomyini	The Phillipines	*trnL*	*16S*	stomach	fresh earthworms or fried coconut (*Cocos*) coated in peanut butter (*Arachis*)	Snap trap
	Klure et al. (2023)	Bryant's woodrat *Neotoma bryanti* Desert woodrat *Neotoma lepida*	USA		*COI*	feces	not described	Sharman trap
To clarify the role in the agricultural ecosystem	Sato et al. (2022)	Large Japanese wood mouse *Apodemus speciosus*	Japan	*trnL*	*COI*, *16S*	feces	oats (*Avena*), grapes (*Vitis*)	Sharman trap
	Zhang et al. (2022)	Gansu zokor *Eospalax cansus* Smith's zokor *Eospalax smithii*	China	*ITS1*, *trnL*		stomach	none	Ground arrow trap
	Murano et al. (2023)	Japanese field vole *Alexandromys* (*Microtus*) *montebelli*	Japan	*ITS2*		feces	sunflower seeds (*Helianthus*)	Sharman trap

Table 1. (continued)

Aims of the study	Papers	Rodent species examined	Country	Markers		Samples	Baits	Traps
				Plant	Animal			
To clarify the dietary requirement of exotic species	Pinho et al. (2022)	House mouse *Mus musculus*	Cabo Verde	*trnL*	*16S*	stomach, intestines	oats, tuna, peanut butter	Sharman trap
	Holthuijzen et al. (2023)	House mouse *Mus musculus*	Midway Atoll, USA	*ITS2*	*COI*	colon-feces	peanut butter (Arachis), oats (*Avena*)	Trapper 24/7
	Gabrielson et al. (2024)	House mouse *Mus musculus* Black rat *Rattus rattus* Pacific rat *Rattus exulans*	Hawaii, USA	*rbcL*	*COI*	feces	peanut butter (*Arachis*), coconuts chunks (*Cocos*)	House mouse: Sharman trap Black rat and Pacific rat: Tomahawk single door live trap
To clarify the basic dietary needs of rodents								
: in high-altitude alpine ecosystem	Valentini et al. (2009)	Golden marmot *Marmota caudata*	Pakistan	*trnL*		feces	not described	not described
: in arctic tundra ecosystem	Soininen et al. (2009)	Tundra vole *Microtus oeconomus* Grey-sided vole *Myodes rufocanus*	Norway	*trnL*		stomach	none	snap trap
: in arctic tundra ecosystem	Soininen et al. (2013)	Norwegian lemming *Lemmus lemmus*	Norway	*ITS1*, *trnL*		stomach	none	Snap trap
: in a transitional zone between subarctic and continental climates	Neby et al. (2024)[1]	Bank vole *Myodes glareolus* Tundra vole *Microtus oeconomus*	Norway	*trnL*	*COI*	feces	freshly cut carrots, apples, oat seeds, peanuts, and peeled sunflower seeds	Ugglan live trap
: in limestone karst ecosystem	Latinne et al. (2014)	Neill's long-tailed giant rat *Leopoldamys neilli* Herbert's long-tailed giant rat *Leopoldamys herberti* Long-tailed giant rat *Leopoldamys sabanus*	Thailand	*rbcL*		feces	ripe banana (*Musa*)	trap (no detail)
: in flammable ecosystem experiencing frequent fire	Wanniarachchi et al. (2022)	Heath mouse *Pseudomys shortridgei* Bush rat *Rattus fuscipes*	Australia	*trnL*	*COI*	feces	mixture of oats, peanut butter, golden syrup, and pistachio essence	Elliott trap (= Sharman trap)
To improve the DNA metabarcoding method								
: on the quantitative potential	Neby et al. (2021)	Tundra vole *Microtus oeconomus*	Norway	*trnL*		feces	captive	captive
: on the quantitative potential	Stapleton et al. (2022)	Desert woodrat *Neotoma lepida*	USA	*trnL*		feces	oats (but after being captured, the rats were fed cactus, juniper, creosote in captive state)	Sharman trap
: on the non-specific amplification of the host species in PCR	Klure et al. (2022)	Bryant's woodrat *Neotoma bryanti* Desert woodrat *Neotoma lepida* White-throated woodrat *Neotoma albigula*	USA		*COI*	feces	not described	Sharman trap

[1]: This study examined five dietary DNA metabarcoding markers including *trnL* and *COI* because the primer targets also include eukaryotes and fungi (not listed here).

hibernation in winter. Such knowledge is useful for planning efforts to conserve their habitat and thus protect this dormouse species. Similar application of this technique is possible to the other endangered rodent species in Japan (species in *Tokudaia* and *Diplothrix*). DNA metabarcoding will continue to be useful in studies of the fundamental dietary requirements of endangered and protected rodents for their conservation.

Niche overlap or partitioning

DNA metabarcoding can be used to determine the basic dietary requirements of sympatric animals, and therefore provide information on how habitat resources are shared or partitioned by these animals, known as niche overlap and niche partitioning, respectively (e.g., Kartzinel et al. 2015; Sato J. J. et al. 2018; Lu et al. 2023). It is important to assess such basic biological interactions to understand ecosystem dynamics. Niche overlap and partitioning also define how the current species distribution has been configured during the process of evolution (see Sato 2017 for examples of mammals in Japan). Several studies performed to clarify the niches of animals with sympatric distributions have been reported (Table 1).

Using the *trnL* and *ITS1* markers, Lopes et al. (2015) suggested that two herbivorous and subterranean tuco-tucos belonging to the genus *Ctenomys* in Brazil, i.e., Tiny tuco-tuco, *C. minutus*, and Flamarion's tuco-tuco, *C. flamarioni*, which are present in a narrow sympatric zone, have different food compositions, possibly to avoid interspecific competition and enable coexistence of the two species. They also showed a mechanism of coexistence in which one species consumed a limited resource (*C. flamarioni*), while the diet of the other species became more heterogeneous (*C. minutus*). On the other hand, Lopes et al. (2020) further investigated the diets of seven *Ctenomys* species in Brazil and examined dietary overlap among the closely related species with different distributions. Their results showed that the plant dietary niches overlapped among different species, implying that interspecific competition may be avoided by allopatric distributions among *Ctenomys* species.

In Japan, Sato J. J. et al. (2018) used fecal DNA metabarcoding of the *trnL* gene to examine two coexisting wood mouse species, the large Japanese wood mouse, *Apodemus speciosus*, and the small Japanese wood mouse, *A. argenteus*. As observed in the study of two *Ctenomys* species in Brazil (Lopes et al. 2015), the diet of the large Japanese wood mouse seemed to be restricted to Fagaceae plants (a limited resource), while the small Japanese wood mouse consumed a variety of plants, especially tall trees (heterogeneous resources), possibly to avoid interspecific competition and enable coexistence of the two species. On the other hand, Sato et al. (2019) showed dietary overlap with little differentiation of the large Japanese wood mouse inhabiting different islands in the Seto Inland Sea, Japan. Among the eight islands (Shikoku Island was represented by Imabari), Fagaceae, Lardizabalaceae, and Rosaceae plants and Noctuidae moths were frequently detected as components of the diet of these wood mice from six, six, seven, and seven islands, respectively. Although species-level identification of the dietary items was not necessarily achieved with precise taxonomic resolution, the results imply that the large Japanese wood mouse has a specific dietary niche that is not altered by island-specific environments.

Other studies showing niche partitioning have also been reported. Two species of woodrat, Bryant's woodrat, *Neotoma bryanti* and Desert woodrat, *N. lepida*, which inhabit xeric environments in the USA and had been assumed to be herbivorous, were demonstrated to show niche segregation in invertebrate diets by DNA metabarcoding of the *COI* gene, despite overlap in their plant diet (mostly C3 plants as estimated by stable isotope analyses) (Klure et al. 2023). Petrosky et al. (2021) tested how dietary niche partitioning allows coexistence of closely related rodents in the tribe Chrotomyini in the Philippines (Luzon Island), which consume earthworms extensively. Analysis of stomach contents using both animal (16S rRNA) and plant (*trnL*) markers showed different dietary requirements of these rodent species in terms of the dietary composition of animals (mammal, amphibian, insect, earthworm, and centipede) and plants (17 families and one order of angiosperms), which could be explained by adaptive radiation.

Another study showed dietary overlap between two lemming species on Bylot Island in Canada, albeit with a sympatric distribution, which was explained by abundant quantities of plants belonging to the genus *Salix* (Salicaceae) on which they feed, enabling coexistence of two species with similar diets at the same site (Soininen et al. 2015).

Soininen et al. (2014) also examined the effects of population density on the plant dietary niches of three rodent species in the arctic region of Norway—the gray red-backed vole, *Myodes rufocanus*, the tundra vole, *M. oeconomus*, and the Norwegian lemming, *Lemmus lemmus*—using DNA metabarcoding of the *trnL* gene in stomach contents. They detected no influence of population density on the dietary composition of any of the three species, and showed instead that the presence of habitat with available food plants was an important factor determining their diets.

As outlined above, DNA metabarcoding has illuminated the mechanisms of coexistence or competition observed in ecosystems worldwide, and the factors influencing niche overlap or partitioning, leading to a greater understanding of how the ecosystems work.

Ecological roles in agricultural ecosystems

Human–wildlife conflicts occur in areas representing the boundaries between human and animal populations, e.g., on farms (König et al. 2020). Rodents are often considered agricultural pest species. In fact, extensive rodent-caused crop losses have been reported worldwide, and they have been estimated to correspond to the loss of food for 200 million people (Lacher et al. 2016). Voles have often been implicated as pest species with deleterious effects on ecosystems (e.g., crop damage) rather than beneficial effects (e.g., weed removal) (Fischer et al. 2018). However, organisms generally do not show only deleterious or beneficial effects (Shapiro and Báldi 2014); rodents also provide ecological benefits through their dietary behavior (e.g., pollination, forest sustainability, or biological control of invertebrate pests). Whether rodents have beneficial and/or deleterious effects on ecosystems depends on the situation in the local environment, and cannot be determined easily without understanding their diets. To elucidate the ecological roles of rodents in agricultural ecosystems, several DNA metabarcoding studies have been conducted in cultivated spaces and the surrounding areas (Table 1).

Sato et al. (2022) examined the seasonal diet of the large Japanese wood mouse on Innoshima Island in the Seto Inland Sea, Japan, using the *COI* and 16S rRNA markers to detect invertebrate species and the *trnL* marker to detect plant species. They showed that the mice consume various moths, stinkbugs, and cicadas, which can cause feeding damage in nearby orchards where oranges are cultivated (local specialty of Innoshima Island). In addition, they consume the gypsy moth, *Lymantria dispar*, which is a forest pest. Therefore, the wood mouse may have beneficial roles for agriculture and forest sustainability by controlling insect pests.

Zhang et al. (2022) applied the *ITS1* and *trnL* markers to clarify the diets of two species of herbivorous and subterranean zokors in China that have been considered to have detrimental effects on newly cultivated forests through feeding on tree roots, the Gansu zokor, *Myospalax cansus*, and Smith's zokor, *M. smithii*. In contrast to previous assumptions, their observations implied that these zokors consume a diverse range of plants and therefore have roles in preventing any one plant species from dominating the ecosystem, maintaining plant species diversity in the cultivated forests. The authors recommended that the presence of zokors be accepted to a certain extent in forest management, despite their feeding damage to the forest trees.

Using the *ITS2* marker, Murano et al. (2023) examined the diet of the Japanese field vole, *Alexandromys* (*Microtus*) *montebelli*, in apple farms in Aomori Prefecture, the largest apple producer in Japan. They showed that the Japanese field vole consumes cultivated apples but also feeds on weeds (*Rumex*) that are usually removed from the farm to allow nutrition from the soil to be utilized only by the apple trees. The results imply that if the weeds were not removed from the orchards, they would be consumed by the vole, leading to a reduction in feeding damage to the apple trees. On the other hand, if the weeds are removed, the rodents would consume apples, and the feeding damage may be much more severe. Therefore, such tradeoffs must be taken into account when growing apple trees in areas where the field vole is present.

In summary, the ecological roles of rodents should be clarified to determine whether they are beneficial or pest species in agricultural and other cultivated areas, and to elucidate novel beneficial and deleterious effects of wild rodents on ecosystems. Studies using DNA metabarcoding have great potential to achieve these goals.

Exotic and invasive rodent species

Invasive species are a cause for concern in that they can exacerbate biodiversity loss through the extinction of native species (Bellard et al. 2016). It was estimated that invasive species threaten 14% of critically endangered terrestrial vertebrate species in the IUCN Red List (Dueñas et al. 2021). Exotic rodents are prevalent all over the world, and their invasiveness means that they pose a potential risk to native ecosystems, particularly on small islands (Bellard et al. 2016, 2017; Dueñas et al. 2021). There have been some DNA metabarcoding studies to elucidate the effects of feeding by exotic rodents on native ecosystems (Table 1).

To investigate the diet of the invasive house mouse introduced onto the Cabo Verde islands (where there are no native rodents), Pinho et al. (2022) used the *trnL* and 16S rRNA genes as markers to perform DNA metabarcoding analyses for plants and animals, respectively, in gastrointestinal tract samples. They showed that more than 50% of the dietary items of the mouse were from indigenous, endemic, and agricultural crops species, while 19% were from exotic species. More specifically for the latter, the mouse consumed invasive plants and pest species, such as cockroaches and aphids, indicating that simply eradicating invasive rodents is not always beneficial for the current ecosystem, necessitating careful manage-

ment based on the predicted effect of eradication.

Gabrielson et al. (2024) also examined the plant and invertebrate items in the diets of three introduced rodents on O'ahu Island in Hawaii (where there are no native rodents) that are commensal to human movement and are globally invasive, i.e., the house mouse, the black rat, *Rattus rattus*, and the Pacific rat, *R. exulans*, using the *rbcL* (for plants) and *COI* (for animals) markers. Nearly half of the detected plant taxa (45%) and invertebrate taxa (43%) were introduced species. Similarly, the majority of seeds in feces were from introduced plant species. These observations imply that rodent removal could lead to increases in the populations of nonnative plant and invertebrate species on the island. The issue is also not simple because the result may imply that the seed dispersal of native plants might also be reduced by the removal of exotic rodents. On the other hand, it was also shown that these invasive rodents were associated with deterioration of the native ecosystem through their consumption of native Hawaiian plant or invertebrate species that are the subject of conservation efforts. Based on these results, the authors stressed that rodent-control programs should be considered very carefully.

Similarly, using both arthropod (*COI*) and plant (*ITS2*) markers, Holthuijzen et al. (2023) demonstrated that arthropods and plants consumed by the invasive house mice on Sand Island in Midway Atoll were mostly non-native, and showed a concern for the effect of eradication of the mice on the ecosystem.

Removal of invasive species should be performed while understanding the predicted effects of their removal on the current ecosystem. These complex food chains among native and introduced species cannot be clarified without using the DNA metabarcoding method.

Rodents adapted to various ecosystems

In addition to studies to examine the universal effects of niche overlap or partitioning, ecological roles in agroecosystems, or impacts of invasive species as described above, dietary DNA metabarcoding has been widely applied to various elusive and ecologically specialized rodents worldwide to understand their basic dietary requirements (Table 1): the plant diets of the golden marmot, *Marmota caudata*, adapted to the high-altitude alpine ecosystem in Pakistan (Valentini et al. 2009); the plant diets of the tundra vole, the gray red-backed vole, and the Norwegian lemming adapted to the arctic tundra ecosystem in the northernmost peninsula of Norway (Soininen et al. 2009, 2013); the diets of the bank vole, *M. glareolus*, and tundra vole in an ecosystem at transitional zone between subarctic and continental climates in southern Norway where the cyclic population dynamics are observed for both rodents (Neby et al. 2024); the plant diets of three *Leopoldamys* species adapted to the limestone karst ecosystem in Thailand (mainly Neill's long-tailed giant rat *L. neilli*; Latinne et al. 2014), and the dietary shift of rodents in an environment changed by fire in a flammable ecosystem in Australia (heath mouse, *Pseudomys shortridgei*, and bush rat, *R. fuscipes*; Wanniarachchi et al. 2022). DNA metabarcoding is an efficient method for such studies, even in these extreme ecosystems.

Rodents as model animals to improve the dietary DNA metabarcoding method

As rodents are small animals, they have long been used as models in laboratory experiments (Lacher et al. 2016). The ease of handling can also be applied to wild rodents. Wild rodents can be used as model organisms to test the efficiency of dietary DNA metabarcoding because they are easy to capture, breed, and feed in the laboratory, and therefore experiments can be performed to examine factors influencing the results of this method using captured and artificially fed wild rodents (Table 1). Neby et al. (2021) fed captive tundra voles from northern Norway with three plant species in different proportions and evaluated the quantitative capability of DNA metabarcoding. Stapleton et al. (2022) similarly examined artificially fed *Neotoma* species in the USA to evaluate the quantitative potential of DNA metabarcoding. Klure et al. (2022) evaluated the effectiveness of an invertebrate primer, ANML (targeting a portion of the mitochondrial *COI* gene), by examining the extent of nontarget PCR amplification of the host *Neotoma* species fed an artificially controlled diet. Experiments using such controlled model species can be used to understand and improve the DNA metabarcoding method. Rodents are suitable targets for developing the methodology in future studies, as the house mouse and Norway rat, *R. norvegicus*, are major model animals used in experimental biology.

Methodological flow and potential bias of the DNA metabarcoding method

To understand the dietary characteristics of rodents better, continued technical improvement of the DNA metabarcoding method is required together with sharing of potential caveats. The DNA metabarcoding method

includes many steps, and various combinations of these steps are possible. Because I cannot introduce all the combinations of steps in this review, here I focused on explanations of my own methodological flow from field work to laboratory experiments and data processing involving dietary DNA metabarcoding analyses in rodents based on previous studies (Sato J. J. et al. 2018, 2019, 2022, 2023; Murano et al. 2023). The flow that I explained seems to be frequently used by other end users. In addition, caveats regarding the potential biases introduced during the workflow are discussed. Before starting a DNA metabarcoding study, careful design is necessary at each step because many factors affect the ecological conclusions. Figures 2, 3, and 4 present the methodological flow as well as the caveats at each step, with reference to experiences in the authors' previous studies (Sato J. J. et al. 2018, 2019, 2022, 2023; Murano et al. 2023). For additional background information on the DNA metabarcoding method, the reader should refer to Alberdi et al. (2018, 2019), Piper et al. (2019), Zinger et al. (2019), Ando et al. (2020), Cuff et al. (2021), Tercel et al. (2021), Bohmann et al. (2022), Liu et al. (2020), and Tedersoo et al. (2022).

Trapping

As the first step in the sampling procedure, we elaborate on the selection of suitable bait to capture wild rodents (Fig. 2). Of course, it would be preferable to use no bait, to avoid its detection by DNA metabarcoding. However, the sampling efficiency might probably be unacceptable without the use of suitable bait. Oats as bait and a piece of grape as a water source are often used to capture the large Japanese wood mouse which necessitates ignoring *Avena* (oats) and *Vitis* (grape) detected in dietary samples (e.g., Sato et al. 2022) (Table 1). Seeds of the sunflower (*Helianthus annuus*) are also often used as a bait, in which case it is necessary to ignore the presence of sunflower in dietary analysis (e.g., Sato J. J. et al. 2018; Murano et al. 2023) (Table 1). The bait and water source in traps should be chosen carefully, depending on what is to be identified in the diet. Cotton (*Gossypium*) used for insulation in traps should also be taken into account as a possible source of contamination in plant diet analyses of rodents (e.g., Wanniarachchi et al. 2022). When using nest boxes without bait, as in a previous study of the Japanese dormouse (Sato et al. 2023) (Table 1), it is not necessary to remove data for bait or water supply from the results. However, care is required to exclude any organisms brought into the nest box (e.g., moss) or traces

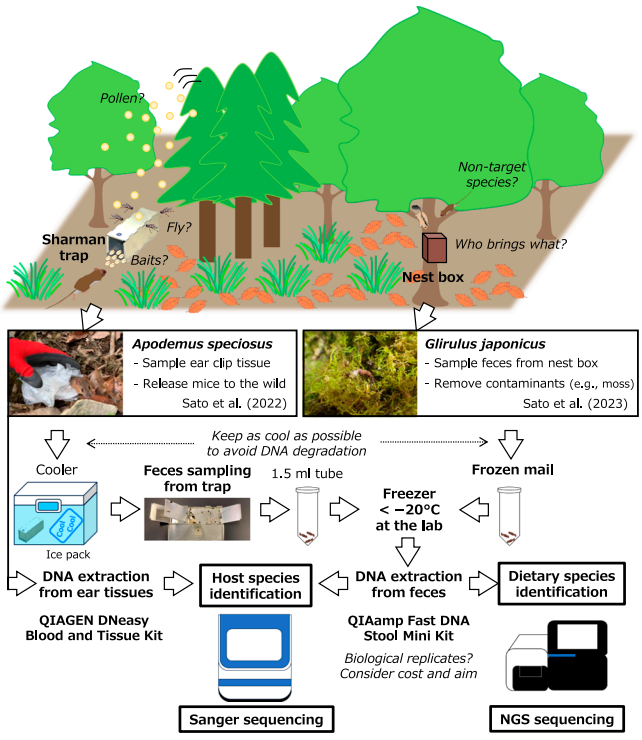

Fig. 2. Flow of the dietary DNA metabarcoding method from fieldwork to DNA extraction from rodents based on previous studies of the large Japanese wood mouse (*Apodemus speciosus*; Sato et al. 2022) and the Japanese dormouse (*Glirulus japonicus*; Sato et al. 2023). Caveats to be considered are shown in italics. Sizes of animals, plants, and traps are adjusted for ease of understanding and are not to scale. See text for detailed explanations. Permission to reuse the photograph of dormouse feces with moss in Sato et al. (2023) was obtained from the Mammal Society of Japan.

of other rodent species (e.g., the small Japanese wood mouse) (Fig. 2).

It is also important to understand the characteristics of the trap and sampling season to consider possible contaminants and to interpret the results of the dietary DNA metabarcoding method correctly (Fig. 2). Sherman traps can be used to capture the large Japanese wood mouse. After collection, ear-clip samples are obtained for DNA analysis and the mice are released to the capture site. Then the traps, including feces, are transferred to the laboratory and fecal samples are collected into 1.5-mL plastic tubes using sterilized forceps (Fig. 2). There are various points at which contamination can occur in the process of trapping, transfer, and sampling. Sato et al. (2019) detected greater numbers of sequence reads of flies in the family Phoridae in the feces of wood mice from only three of eight islands surveyed (Ohsakishimojima, Kamikamagarijima, and Shimokamagarijima, where sampling was conducted in one day). They concluded

that the flies were not dietary components but were detected because of their small body size (approx. 2–3 mm), which allowed them to enter the traps through small gaps. Many flies in the Phoridae would be attracted to the rodent feces. The time elapsed after the deposition of feces by rodents in the traps would affect the abundance of such small coprophagous flies in the samples. Therefore, earlier sampling would be better, and closing the gaps in the traps may be efficient for exclusion of these insect contaminants. Small pollen grains may also be contaminants that should not be considered dietary components (Fig. 2). Sato et al. (2019) also detected greater numbers of sequence reads of the Japanese cedar, *Cryptomeria japonica*, in most fecal samples of wood mice collected in February from one locality (Imabari on Shikoku Island). This was in sharp contrast to the lack of detection of this plant in samples from the other islands in the Seto Inland Sea. They suspected that this was due to contamination during the trapping procedure, because the pollen of this plant is most abundant in February. Therefore, it is necessary to account for this type of contamination even in the wild, taking the characteristics of the traps and sampling season into consideration.

Furthermore, for successful DNA metabarcoding analyses, degradation of the DNA in a sample in the field should be considered. Fecal samples should be preserved by freezing (e.g., at –20°C) as soon as they are collected to avoid DNA degradation (Ando et al. 2020) (Fig. 2). However, it may be difficult to freeze samples immediately in field surveys. Fecal samples kept at ambient temperature for several hours before freezing are still acceptable for DNA metabarcoding analyses (e.g., Sato et al. 2019, 2022). However, it should be noted that this depends on the climatic conditions at the study site. In the western part of Japan (Hiroshima Prefecture), there is little problem with sample preservation in late autumn, winter, and early spring, but the humid and hot climate in summer is expected to exacerbate DNA degradation in fecal samples maintained at ambient temperature. A cooler (called cooler box in Japan) containing ice packs is usually used to keep samples as cool as possible in the field, and they are transferred to a freezer as soon as possible (usually less than one hour) (Fig. 2). The use of appropriate buffer for DNA preservation (e.g., RNA later or DNA/RNA shield) might be another procedure to prevent the DNA in the feces from degradation. The cautions on the trapping procedures described above could also be applied to any world rodent species captured by similar methods.

DNA extraction

In our recent studies, DNA was extracted from fecal samples using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) (Fig. 2). For the other kits, Qiagen DNeasy Blood and Tissue Kit, Qiagen DNeasy plant Kit, Qiagen Biosprint 96 DNA Blood Kit, Qiagen QIAamp PowerFecal Pro DNA Kit, Qiagen DNeasy PowerSoil Pro Kit, E.Z.N.A.1 soil DNA kit (Omega Bio-Tek, Georgia, USA), Stool DNA Isolation Kit (Norgen Biotek, Ontario, Canada), and the conventional phenol–chloroform method have been used for DNA extraction in studies listed in Table 1 depending on the target dietary species. The maximum weight of fecal samples for use in the QIAamp Fast DNA Stool Mini Kit is 200 mg, corresponding to 5–10 pieces of feces depending on size in the case of the large Japanese wood mouse. Based on the results of our previous studies, three to five pieces of feces (~50–150 mg) are sufficient to obtain DNA for subsequent analysis. However, whether the amount of feces is sufficient must be decided based on the research objectives. The diversity of detected dietary species is increased with the number of biological replicates (e.g., different individuals or different sets of fecal samples from the same individual; Mata et al. 2019). Three to five pieces of feces from the large Japanese wood mouse would likely be insufficient for complete characterization of their diet. For example, to examine the complete diet of the large Japanese wood mouse in a given season, several rounds of DNA extraction from different sets of fecal samples (< 200 mg) must be performed from a sufficient number of individuals (> 10 individuals if possible) collected in the season; of course, completeness may never be achieved. However, as repeating the procedure with multiple biological replicates is costly, the decision to examine biological replicates depends on a tradeoff between cost and the research objectives. It should be noted that pooling too many fecal samples in a single DNA extraction can lead to underestimation of the dietary species, probably due to saturation in the spin column of the DNA extraction kit (Mata et al. 2019). The filter in the spin column has a certain pore size. If the size of the fragments in the feces is larger than the pore size, it is trapped in the filter, leading to clogging. This situation would lead to blocking the DNA to pass through the filter. To avoid the saturation, the conventional phenol–chloroform method may be used for the DNA extraction. If the research does not intend to clarify the individual's diet, mixing the individual samples from a particular locality or season might

be a cost-effective way of understanding comprehensive characteristics of the species diet.

PCR

There are various library preparation methods through PCR. However, here I explained the most frequently used methods using two rounds of PCR and the Illumina MiSeq sequencing platform (Illumina, San Diego, CA, USA) (Fig. 3). The first PCR amplifies the target DNA regions with a universal primer set, and the second adds indices to demultiplex samples after sequencing and attaches adapters (P5 and P7) to both ends of the second PCR products that are necessary for connection to the flow cell used in the MiSeq platform (Fig. 3). General PCR settings and primer design should be determined with reference to other reports (e.g., Miya et al. 2015; Bohmann et al. 2022; Tedersoo et al. 2022). A KAPA HiFi Hot Start Ready Mix PCR kit (Kapa Biosystems, Woburn, MA, USA) has been used for both PCRs in previous studies (e.g., Sato et al. 2022, 2023) because of its high fidelity for proofreading activity; other polymerases such as KOD FX Neo (Toyobo, Osaka) could also be used. Several factors should be taken into consideration in the PCR.

The first issue that should be considered is avoidance of contamination. Although Alberdi et al. (2019) and McKnight et al. (2019) suggested that negative controls for contamination should be placed in each step of the DNA metabarcoding procedure, the most critical step for excluding contaminants is the PCR step. Therefore, special care should be taken to include negative controls in PCR. It should be noted here that including the negative control in the DNA extraction step has also often been adopted. To avoid contamination, the PCR solution should be prepared in a separate room from that used for adding DNA and performing PCR (Fig. 3). That is, the thermal cycler should not be placed in the same room in which the PCR solution is prepared. Different pipettes and tips are used for preparing PCR solutions and treating DNA. Before preparing the PCR solution, the bench should be decontaminated by wiping it with DNA AWAY (Thermo Fisher Scientific, Waltham, MA, USA) or other decontamination reagents. A lack of amplification in the negative control samples after PCR is checked by agarose gel electrophoresis, and the negative control PCR products are sequenced regardless of no band in the agarose gel electrophoresis and used to interpret the effects of contamination on the number of sequence reads for the samples (Alberdi et al. 2019). In previous studies (e.g., Sato et al. 2022, 2023), the sequence reads obtained with the negative control samples were subtracted from the relevant reads obtained for the samples. The other program-based removals of the negative control sequence reads are also known (Davis et al. 2018; McKnight et al. 2019).

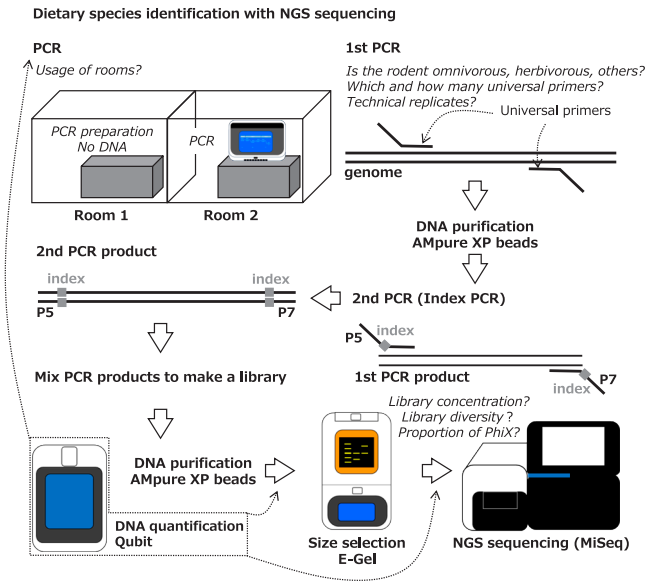

Fig. 3. Flow of the dietary DNA metabarcoding method from PCR to next-generation sequencing (NGS). Caveats to be considered are shown in italics. See text for detailed explanations.

If time and cost constraints permit, it is better to use multiple universal primers to reduce taxonomic bias and increase the taxonomic coverage of the obtained dietary species (Alberdi et al. 2018; Corse et al. 2019; Browett et al. 2021) (Fig. 3). One reason for using multiple primers is that it is difficult to predict the performance of primers in silico for degraded DNA, such as that in fecal samples (Corse et al. 2019). Corse et al. (2019) recommended the use of several primers for one locus (e.g., *COI*). It should, however, be noted that there is debate regarding the use of multiple or single well-designed primer sets in DNA metabarcoding analysis. Elbrecht et al. (2019) and Tournayre et al. (2020) recommended the use of a single well-designed primer set, partly because the use of multiple primers is costly and comparison of the relative read abundances among results obtained with these independent markers is difficult (Elbrecht et al. 2019). Sato et al. (2022) used three primer pairs (one for *COI* [ZBJ] and two for 16S rRNA) to assess invertebrates in the diet of the large Japanese wood mouse, and the results identified various species of Hemiptera only from 16S rRNA analyses. PCR using the *COI* (ZBJ) primer is

often biased toward amplifying Diptera and Lepidoptera, and therefore other complementary markers are required (Sato et al. 2019, 2022; Tournayre et al. 2020; Browett et al. 2021). Thus, the use of multiple primers is beneficial in terms of detecting more dietary items (Sato et al. 2022). Multiple plant markers are also useful to avoid missing even some common dietary plant components (Goldberg et al. 2020). Therefore, until universal primers that could amplify a large proportion of species within a clade are designed, it is recommended to use multiple primers. However, this would be dependent on budget constraints. The many omnivorous species of Rodentia with various organisms as dietary items present challenges in deciphering the total dietary components in light of both cost (time and/or money) and taxonomy (Tercel et al. 2021). Although the use of multiple primers may reveal the overall dietary habits of omnivorous species, this inevitably requires multiple PCR and NGS runs. Efforts to develop well-designed universal primers should therefore be continued. Several universal primers for invertebrate mitochondrial DNA have been extensively evaluated (Tournayre et al. 2020). Recently, Jusino et al. (2019) developed an efficient primer set for detecting dietary invertebrates at the species level (ANML) with less bias than the ZBJ primers, although ANML also amplifies the host mammal species (Klure et al. 2022). The analyses with host-amplifying primers might require blocking primers that prevent the host DNA amplification (Vestheim and Jarman 2008). In summary, it is necessary to decide how many and which universal primers are appropriate for the research objectives, taking into consideration budget and the effectiveness of candidate universal primers.

Host species identification is necessary for some fecal DNA metabarcoding analyses when samples are obtained without observing the host species (Fig. 2). Although Tournayre et al. (2020) recommended the use of DNA metabarcoding markers that capture both the host and prey species' DNA simultaneously in the case of bat guano samples, in which multiple bat feces may be overlaid, host species' identification should be separated from prey species' identification because it is difficult to design universal primers that are suitable for amplification of both host (e.g., rodent) and prey (e.g., insect) DNA. Sato et al. (2022) identified species of the large Japanese wood mouse by Sanger sequencing part of the mitochondrial DNA (*Dloop*) extracted using a QIAGEN DNeasy Blood and Tissue Kit (Qiagen) and amplified via PCR from ear-clip tissue samples (Fig. 2). Of course, the species can be identified by directly observing the external morphology of the individual of the large Japanese wood mouse before releasing it back into the wild, but genetic methods would support the validity of the identification. In that study, the *COI* (ZBJ) primers for invertebrate prey identification did not amplify the host rodent DNA, allowing efficient analyses of the prey species without the confounding effect of the host DNA (amplification of the host DNA would reduce the amount of data for target dietary species). On the other hand, for feces sampled from the nest box of the Japanese dormouse, species identification using genetic methods must be done before DNA metabarcoding analyses because other rodents, such as the small Japanese wood mouse, utilize the nest boxes of the Japanese dormouse (Fig. 2; Sato et al. 2023). In that study, the host species was genetically identified from fecal samples because invasive sampling cannot be performed for the Japanese dormouse due to its protected status in Japan. It is recommended that DNA metabarcoding primers be selected for amplifying only the prey species DNA efficiently, and the host rodent species should be identified with another round of Sanger sequencing experiments.

Considering PCR errors, some studies have examined the same samples multiple times by PCR (technical replicates; Alberdi et al. 2018, 2019; Ando et al. 2020) (Fig. 3). Examining multiple replicates is the best strategy to capture the diet of an individual animal precisely because such a strategy is less likely to miss a minor dietary component. However, whether this strategy should be adopted depends on the extent to which it is necessary to clarify rare dietary species. The balance between workload/cost and what could be obtained from replicate analyses should be considered (Alberdi et al. 2019; Elbrecht et al. 2019). This is because most rare dietary species are not the main topic of discussion. Such rare species are usually removed during the process of data filtering with a minimum sequence read copy number threshold (e.g., 10 or 100 absolute reads or 0–5% of total reads; see reviews and individual papers cited in this review), which reduces the variation in the obtained dietary items among PCR replicates (Alberdi et al. 2018). In fecal DNA metabarcoding analysis of the European free-tailed bat, *Tadarida teniotis*, much less variation was observed in dietary items among technical (PCR) replicates than among biological replicates (Mata et al. 2019). In the case of the authors' group, only one PCR is performed per individual (three to five pieces of feces < 200 mg) and dietary items with small numbers of sequence reads (i.e., below the cut-

off determined based on the number of reads obtained in the negative control samples) that would not be stably amplified in multiple PCR are removed. Therefore, missing some rare species does not pose a major problem in interpretation in terms of understanding general dietary trends. The decision should, however, depend on the aim of the study.

DNA purification

AMPure XP beads (Beckman Coulter, Brea, CA, USA) were used for our previous studies to purify PCR products (> 100 bp) after the first PCR using universal primers and the second PCR using primers with index sequences and adapters (Fig. 3). Other DNA purification methods using such as spin column (QIAquick PCR Purification Kit, QIAGEN) or enzyme (ExoSAP-IT Express PCR Product Cleanup, Thermo Fisher Scientific) can also be used.

Size selection

It should be taken into consideration that fecal or other dietary samples will include various genomes from a diverse range of species, which often results in nonspecific amplification via PCR. Therefore, it is necessary to check for the presence of nonspecific PCR products via agarose gel electrophoresis. Size selection can be performed to excise the PCR amplicons of the target size using E-Gel SizeSelect II (Thermo Fisher Scientific) (Fig. 3). Without this step, sequence-read data would also be assigned to irrelevant and nontarget items, thereby reducing data derived from dietary items.

DNA sequencing

Below I focused on the Illumina MiSeq that is the most widely used NGS platform for DNA metabarcoding (Braukmann et al. 2019; Liu et al. 2020) (Fig. 3), although Illumina iSeq has recently become another reasonable platform for this method. An appropriate reagent kit should be selected according to the length of the target sequences. In a previous study, the authors' group used a 300-cycle kit (150 cycles paired end) for *COI* (ZBJ), 16S rRNA, and *trnL* (Sato et al. 2022) because the target regions of these markers are short (< 200 bp) and can be covered by sequencing 150 bp in both forward and reverse directions. In the case of *ITS2* (Murano et al. 2023; Sato et al. 2023), a 500-cycle kit (250 cycles paired end) was used in a previous study because of the longer target region (Moorhouse-Gann et al. 2018). Care should be taken with regard to the final DNA concentration of the prepared library to avoid over-clustering in the MiSeq run, which would prevent discrimination of the position of the cluster in the flow cell, resulting in no data. Final library concentration, the diversity of the library, and the amount of PhiX spike-in also require careful consideration, particularly in the case of amplicon sequencing (e.g., DNA metabarcoding). This is because, at each cycle of amplicon sequencing, almost all clusters should have the same color fluorescence (i.e., the same base), whereby MiSeq tends to misrecognize the position of nearby clusters. Therefore, several preliminary runs should be performed with small-scale kits (Micro or Nano kits in the case of using MiSeq) to assess which final library concentration (4 pM, 6 pM, or 8 pM) and proportion of PhiX spike-in provide the best performance to obtain the appropriate cluster density and sufficient amount of data. These caveats should be noted when combining DNA libraries of different lengths because this may affect the cluster density.

DNA concentration

Quantification of the DNA concentration is crucial in several steps in DNA metabarcoding analysis. A Qubit 4 Fluorometer (Thermo Fisher Scientific) can be used to calculate DNA concentration before the first PCR (to prepare the same DNA concentration for each sample), for E-Gel size selection (to adjust DNA to < 500 ng in a final volume of 22.5 μL according to the instrument), and for MiSeq sequencing (to prepare 4 nM library solution according to the manufacturer's instructions) (Fig. 3). Although the running cost is a concern, 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) or other fragment size analyzer can also be used for DNA quantification in addition to DNA qualification. The calculation should be as accurate as possible, because miscalculation can lead to failure of sequencing on MiSeq.

Data filtering, treatment, and interpretation

After sequencing, a bioinformatics pipeline should be selected to demultiplex samples, remove primers, merge paired-end reads, filter data, and to search databases to identify dietary species (Fig. 4A). Briefly, Fig. 4A shows major steps in the data filtering steps: unnecessary primers regions are removed from each of forward (Fw) and reverse (Rv) sequences; both Fw and Rv sequences are merged into one sequence; low quality (usually < Q30, meaning < 99.9% accuracy), infrequent (a user defined fixed value), and chimera sequences (detected by comparison with the DNA database or comparison of obtained sequences with each other) are removed; OTUs (Opera-

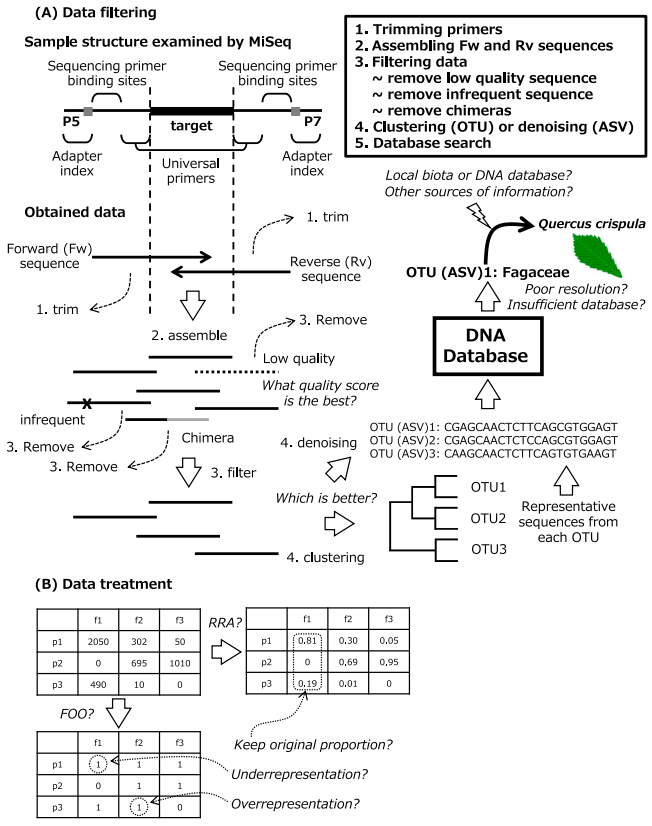

Fig. 4. Flow of the dietary DNA metabarcoding method from data filtering to DNA database search (A) and data treatment after obtaining the data matrix composed of sequence read numbers for each fecal sample and prey (B). Major steps in the data filtering are shown with numbers in the upper-right box (the same numbers are also used in the figure). OTU and ASV mean Operational Taxonomic Unit and Amplicon Sequence Variant, respectively. A symbol x on the merged sequence means that this sequence is infrequent (rare). In the three tables in (B), "f" indicates feces and "p" indicates prey. FOO and RRA indicate frequency of occurrence and relative read abundance, respectively. Upper-left table, right table, and lower-left table show the original number of sequence reads, relative reads, and occurrence data, respectively. Caveats to be considered are shown in italics. See text for detailed explanations.

tional Taxonomic Units generated by clustering method) or ASVs (Amplicon Sequence Variants generated by denoising method) should be chosen for the following database searches (in the clustering method, only representative OTUs defined by usually >97% sequence similarity are examined [Alberdi et al. 2018] and the other OTUs are ignored, leading to removing error sequences but loosing possibly correct sequences, while in the denoising method, the statistically defined confident sequences are determined by sequence frequencies and all the ASV sequences are used thereafter); and international DNA databases combined with local biota or DNA databases are used for species identification. There are many steps where the researchers should decide what options to use for their aims. These bioinformatics procedures are not the main topic of this review and the reader is directed to other technical reviews and reports for details (e.g., Creedy et al. 2022; Tedersoo et al. 2022; Hakimzadeh et al. 2023). Programs that can process these steps are described in Hakimzadeh et al. (2023) who summarized 32 various pipelines and showed their own specific characteristics, making the choices of a pipeline difficult because of numerous options. In previous studies by the authors' group, Claident (Tanabe and Toju 2013), which was not listed in Hakimzadeh et al. (2023), was used as the bioinformatics pipeline (detailed settings are described in the Supplementary Information of Sato et al. 2022). For other pipelines, Hakimzadeh et al. (2023) should be consulted. Several critical considerations as explained below should be performed prior to data treatment after bioinformatics processing and before interpretation.

Frequency of occurrence (FOO) and relative read abundance (RRA) should be taken into consideration when treating and interpreting sequence-read data from NGS analysis (Fig. 4B). Occurrence data are based on the presence or absence of dietary items. Even if only a small number of sequence reads are obtained for a given dietary item, the item must be interpreted as present. That is, we interpret both 10 and 2050 sequence reads (just example values) as indicating presence (Fig. 4B). Overrepresentation of rare species (sometimes contaminants) and underrepresentation of frequent species are therefore both concerns in the use of occurrence data. In particular, "rare-item inflation" has been observed in various empirical studies (see Deagle et al. 2019 and references therein). A simulation study showed that RRA was more accurate than FOO to capture the true dietary composition (Deagle et al. 2019). If the study aim is to identify rare dietary items (e.g., detection of invasive rare insects for biosecurity surveillance; Piper et al. 2019), care is required regarding the use of FOO or RRA in the analyses due to the risk of overrepresentation of the species of interest. On the other hand, RRA data are severely affected by unusually high numbers of sequence reads detected from a small number of samples (outliers; Sato et al. 2019), and therefore do not always provide accurate dietary profiles. There is still ongoing debate regarding the use of FOO and RRA, and which should be used in any particular case depends on the study objectives. Until such debate has been resolved, presenting results from both methods represents a conservative way to

understand the dietary characteristics of animals (e.g., Sato J. J. et al. 2018).

Quantitative clarification of diet is of fundamental importance to understand how much of each dietary item a species requires. As the results generated by DNA metabarcoding through high-throughput DNA sequencing are composed of the abundance of sequence reads, the method may be viewed as a means of assessing the diet quantitatively, allowing inference of biomass. However, many biological and technical factors introduce biases to distort the proportion of species originally included in the dietary samples (feces or stomach contents). Biological factors include certain characteristics of the prey species that lead to distortion. For example, larger invertebrate prey may lead to greater numbers of reads and a reduction in the detection of rare prey species (Elbrecht et al. 2017). The rate of degradation of DNA or digestion of tissues in the gastrointestinal tract would also differ among prey species and tissue types. Furthermore, mitochondrial DNA copy numbers differ according to the tissues consumed (for the case in humans, see D'Erchia et al. 2015), and therefore greater sequence abundance cannot be interpreted only as indicating greater numbers of prey tissues but also as greater DNA copy numbers in a small number of tissues. It should also be noted that predominant numbers of reads are sometimes detected from a small number of samples, swamping the quantitative trends of sequence reads in other samples (Sato J. J. et al. 2018). Technical biases include those introduced during the experimental procedure, such as DNA extraction or PCR (Braukmann et al. 2019). DNA extraction efficiency differs between hard insect tissues and soft tissues, and between plant seeds and fruits (i.e., DNA extraction bias). PCR bias may result in preferential amplification of some taxa in PCR, while others may not be amplified. Primer mismatch is the greatest influencing factor causing unequal amplification in PCR (Piñol et al. 2015). A simulation study suggested that the results of DNA metabarcoding would not be quantitative unless PCR efficiency (e.g., mainly due to primer–template mismatch) were the same for all species in a sample (Piñol et al. 2019). Meta-analyses of DNA metabarcoding studies have suggested that the relationship between biomass and the number of sequence reads is weak with large variance (Lamb et al. 2019). Therefore, DNA extraction efficiency and PCR technical bias significantly distort the quantitative features of dietary samples together with biological bias.

It should however be noted that a weakly positive relationship between sequence reads and amounts of prey has been observed in meta-analysis (Lamb et al. 2019). As a simple rule, larger or smaller amounts of prey lead to larger or smaller numbers of sequence reads, respectively. Controlling biological and technical biases may lessen the variance. Piñol et al. (2019) suggested that analyses could be quantitative depending on the primer choice. In an experiment with mock arthropod community samples, Krehenwinkel et al. (2017) showed that the bias in PCR amplification could be alleviated using degenerate primers or primers targeting conserved regions, and supported the possibility of quantitative inferences with DNA metabarcoding. Stapleton et al. (2022) used DNA metabarcoding of the *trnL* region to examine the diets of wild-caught and artificially fed desert woodrats, *N. lepida*, and demonstrated a generally positive quantitative correlation with the amounts of plant food materials, although they also showed that the quantitative estimates had large errors. As outlined above, whether DNA metabarcoding can provide realistic quantitative results is still a matter of some debate. Therefore, researchers should consider the potential for error when interpreting results quantitatively. Further characterization of primers and other factors that potentially affect quantitative analysis are necessary in future studies. Simultaneous examination of internal standards as controls may be effective for quantification (Thomas et al. 2016; Harrison et al. 2021). In addition, because the number of total sequence-reads assigned to each sample is quite variable, the rarefaction analysis, adjusting the total sequence reads to a unified value (e.g., the minimum total sequence read among samples; but see Chao and Jost [2012] for discussions about which to use equal size or equal completeness [coverage] of detected items for characterizing diversity), has often been used to compare quantities among samples. The rarefaction analysis assigns the number of sequence-reads for each dietary species according to the proportion of sequence reads obtained for the dietary species in the reduced total sequence reads for the sample.

In addition to contamination introduced during the sampling steps as described above (e.g., pollen detected in the feces of the large Japanese wood mouse in February in Imabari or coprophagous flies attached after defecation of feces of the large Japanese wood mouse in the western islands of the Seto Inland Sea, Sato et al. 2019; mosses in the nest boxes of the Japanese dormouse, Sato et al. 2023), secondary predation and passive consumption provide other types of contamination. Thus, the die-

tary species detected in fecal samples may not always represent what the host species positively attempted to consume but instead may reflect what a prey animal itself had consumed or the contents of a plant on which the host had fed. As an example of the latter case, let us consider the detection of fruit flies in the autumn diet of the Japanese dormouse (Sato et al. 2023). In that study, the dormouse was found to have intensively consumed the hardy kiwi in the autumn season, probably the fruits of the plant according to the phenology. Therefore, it is likely that some fruit fly tissues remaining within the fruits of the kiwi had been passively delivered to the dormice. It is not easy to interpret the significance of the contribution of these flies to the diet of the dormouse. Such inadvertent consumption should always be taken into consideration in interpretation of the data based on an understanding of the ecology of prey species.

Insufficient data in a database adversely affects species identification of dietary items (Fig. 4A). Clarification of the identity of prey species often requires information on local fauna and flora. Sato J. J. et al. (2018) identified the Fagaceae *trnL* sequence in the feces of two *Apodemus* species as from *Quercus crispula* because it was the only Fagaceae species known to be present in the surveyed area (Uryu Experimental Forest Station, Hokkaido, Japan) (Fig. 4B). It is therefore important to collect lists of local faunal and floral species. Construction of a local reference DNA database is also informative and effective if the DNA data in the global DNA database are insufficient for precise taxon identification (Ando et al. 2020). Another weak point of the DNA metabarcoding method is that it is not possible to know which parts of tissues are preyed upon because all of the cells basically yield the same DNA information. Nevertheless, it is sometimes critical to understand the tissue types when interpreting the ecological roles of rodents. Sato J. J. et al. (2018) interpreted the Fagaceae species (*Q. crispula*) detected in fecal samples of the large Japanese wood mouse and the small Japanese wood mouse as being from acorns, as individuals consuming the Fagaceae species were abundant in autumn season (September and October in Hokkaido, Japan). Based on this interpretation, these two wood mouse species were suggested to have competed for acorns as a winter survival resource and to have eventually achieved niche partitioning. Therefore, it is also important to understand the phenology of the dietary species across seasons in addition to the list of local faunal and floral species and their DNA database. One should always test assumptions made based on DNA metabarcoding analysis using other experiments or observations. A study of the vervet monkey, *Chlorocebus pygerythrus*, in South Africa showed that observational data complemented data obtained by DNA metabarcoding, although the latter showed greater coverage and better resolution than the former (Brun et al. 2022). Although not focused on dietary analysis, another study found that traditional methods such as direct observations (camera, pitfall, and mist net traps) provided complementary data for environmental DNA analyses of mammals in the southwest Amazon basin in South America (Mena et al. 2021). It should be recognized that dietary DNA metabarcoding has inherent disadvantages that cannot be resolved and require other sources of information (see Nielsen et al. 2018 for comparisons among DNA metabarcoding, visual, stable isotope, and fatty acid analyses).

Future perspectives and directions for further study

Clarifying the diets of rodents around the world will increase our understanding of the basic mechanisms underlying various ecosystems and how the rodents interact with human society, which will change the way we live. Over the last quarter century, there have been marked advances in the methods used for dietary analysis, as represented by the development of NGS technology. To achieve the Convention on Biological Diversity's vision of "living in harmony with nature by 2050," we can further improve the methodology of dietary analysis by filling the gaps in the current DNA metabarcoding method outlined above and continuing to apply novel techniques to this issue. Three points for future perspectives and directions to achieve this vision are discussed below.

First, a method is required to identify as diverse a range of species as possible as accurately as possible. Let us consider the possibility of designing universal primers for groups including diverse species that could amplify a large proportion of species within a clade. Current design of "universal primers" has been restricted for several biological and technical reasons. The high evolutionary rate of mitochondrial DNA and degradation of DNA in fecal samples are two major biological reasons for this limitation. Mitochondrial DNA for which DNA metabarcoding primers (e.g., *COI*) are usually designed has notoriously high mutation and evolutionary rates, which make it difficult or impossible to design universal primers for diverse taxa. Accepting this problem, it is necessary to

search for other candidate primer regions in the nuclear genome. However, as nuclear DNA has less variation than mitochondrial DNA, longer sequences may be necessary to identify species with sufficient variable sites. A previous study suggested that nuclear ribosomal markers are less effective for DNA metabarcoding than the mitochondrial *COI* gene due to their high degree of conservation (Krehenwinkel et al. 2017). At this point, a technical limitation appears because the maximum DNA length that can be examined using a second-generation DNA sequencer (e.g., Illumina platform) is about 400 bp. In this sense, long-read sequencing technology has the potential to improve the dietary DNA metabarcoding method. Commercial PCR kits have already been adapted to this demand for long-read sequencing. For example, the KAPA HiFi Hot Start Ready Mix PCR kit that is usually applied to dietary analysis can amplify targets up to 15 kb from genomic DNA, which is sufficient for species identification in terms of genetic variation. The third-generation DNA sequencing technologies developed by Oxford Nanopore Technologies (ONT; Deamer et al. 2016) and Pacific Biosciences (PacBio; Rhoads and Au 2015) have recently been used for DNA metabarcoding analyses with long reads of several thousands of base pairs to clarify the spatiotemporal dynamics of the fungal community in soil (Furneaux et al. 2021), assessing the composition of microbial eukaryotes in soil (Jamy et al. 2020) or ocean (Latz et al. 2022), and detecting apicomplexan parasites in blood samples from dogs for public health (Huggins et al. 2024). These studies imply the potential utility of long-read sequencing technology with high taxonomic resolution, albeit with some technical concerns such as high error and chimera rates and low sequencing depth. However, this technology has not been applied to dietary analysis examining fecal or stomach content samples. For arthropods, Gajski et al. (2024) developed a DNA metabarcoding method for species identification of diverse spiders using MinION (Oxford Nanopore Technologies, Oxford, UK), an ONT platform, with approx. 5-kb sequences from both mitochondrial (*COI* and *cytb*) and nuclear (18S-28S rRNA) genes. This method could be useful for fecal dietary studies of animals. However, there is a tradeoff between longer DNA sequences and DNA degradation. In that study, long PCR for nuclear genes with a target of 4.5 kb failed in 25% of the samples despite preparation of these DNA samples from whole spider bodies, which would contain less degraded DNA than feces (Gajski et al. 2024). Less abundant nuclear DNA may be undetectable in PCR due to DNA degradation in fecal samples. Therefore, fresh samples in sufficient amounts would be needed for long-read DNA metabarcoding of fecal samples. In addition, reference databases for species identification should also be established for long-read sequences, which would require costly and laborious work. Further studies are required to explore potential barcoding regions in the nuclear genome of minimal length to circumvent the problem of DNA degradation in feces and to construct a DNA database for these regions.

Second, bioinformatics procedures should be made more user-friendly. The most challenging task for most users of DNA metabarcoding, particularly beginners, lies in the bioinformatics (Liu et al. 2020; Hakimzadeh et al. 2023). Several processes, such as data filtering (denoising or clustering), selection of data for database searches (OTU or ASV), database design, and organism classification, affect the outcomes and interpretation of the results. The lack of consensus on methods among researchers does not allow proper comparisons among studies (O'Rourke et al. 2020). For example, the difficulty of data filtering includes the requirement to remove rare read sequences (10 or 100 reads, 0–5% of total reads, and so forth) in the denoising steps or the arbitrary setting of some threshold, such as sequence similarity (97%, 98%, and so forth), to define the molecular operational taxonomic unit (MOTU) in the clustering step. Procedures of MOTU clustering or removal of PCR errors and cross-sample contamination are crucial steps that affect ecological conclusions based on the results of DNA metabarcoding (Calderón-Sanou et al. 2020). However, a simulation study suggested that this can lead to misinterpretation of the true diversity of dietary items, depending on the percentage of removed reads, thereby making the ecological interpretations unclear (Littleford-Colquhoun et al. 2022). Therefore, how rare sequence reads should be treated is a difficult question depending on whether the focus is on abundant or rare food items. It is also problematic that the bioinformatics pipelines and tasks adopted by researchers to date are becoming increasingly variable. Nevertheless, detailed data treatments are often not reported, which makes comparisons among studies difficult (reviewed in the *COI* metabarcoding study of metazoan species; Creedy et al. 2022).

A user-friendly MiFish Pipeline has been developed for use in the field of environmental DNA analysis of fish from water samples (Sato Y. et al. 2018; Zhu et al. 2023), in which a matrix of detected fish species by sequence read numbers for each sample is generated by uploading

an FASTQ file (created from the sequencing platform) and quickly setting some easy options. The ecology, IUCN Red List status, or other biological characteristics of the detected fish species can also be provided in the matrix, which would be useful for discussions of prey species if these traits are provided for every species detected in the dietary samples. Such a standardized and easy-to-use pipeline should be developed and maintained for dietary DNA metabarcoding analyses. Other user-friendly GUI programs have been introduced (Liu et al. 2020; Hakimzadeh et al. 2023). Such progress in the development of bioinformatics tools would make it easier to compare outcomes between studies and facilitate fruitful discussion among groups. However, it should be noted that the detailed settings should be clarified even if researchers adopt default settings in such a user-friendly pipeline (Creedy et al. 2022).

Finally, it is necessary to consider how to clarify the whole ecosystem via accumulation of dietary data of animals at different trophic levels using DNA metabarcoding. Although a great deal remains to be determined to clarify even the diets of rodents, success in this area would still be insufficient to understand the ecosystem. Temporal and spatial dynamics of predatory carnivorous species, primary consumers such as invertebrates, and producers (i.e., plants) should be examined to understand how rodent populations increase or decrease depending on the dynamics of the ecosystem. Connecting species by qualitative and quantitative DNA metabarcoding must be accomplished to comprehend the complicated networks within an ecosystem. Without understanding the mechanisms underlying the complicated networks, it is not possible to understand the mechanisms of nature and how we can live in harmony with it. Therefore, it is necessary to continue to improve the DNA metabarcoding method to clarify all animal food items qualitatively and quantitatively at all time points and locations to elucidate the complicated ecosystems. This is the only way that we can live in harmony with nature.

Acknowledgments: I would like to thank Haruka Aiba, Nobuyuki Azuma, Satoe Kasahara, Shusaku Minato, Chie Murano, Takashi Saitoh, Takuya Shimada, and Shigeru Uemura for collaborative works of the dietary studies of rodents. I am also grateful to Daisuke Kyogoku, Taketo Komura, Yuji Isagi, Tomoyasu Shirako for their kind instructions of the DNA metabarcoding method (experiments and data analyses). I also would like to express my appreciation to students in Fukuyama University, Chiaki Inamori, Kouhei Maeda, Yuya Ohtsuki, Naoki Nishiura, Kumi Mouri, Haruna Matsuda, Honoka Fujita, Kouki Yasuda, Takashi Wada for their enthusiastic research from field works, experiments, and to data analyses. This work was supported by JSPS KAKENHI (Grant No. 22K05699).

References

Alberdi, A., Aizpurua, O., Bohmann, K., Gopalakrishnan, S., Lynggaard, C., Nielsen, M. and Gilbert, M. T. P. 2019. Promises and pitfalls of using high-throughput sequencing for diet analysis. Molecular Ecology Resources 19: 327–348.

Alberdi, A., Aizpurua, O., Gilbert, M. T. P. and Bohmann, K. 2018. Scrutinizing key steps for reliable metabarcoding of environmental samples. Methods in Ecology and Evolution 9: 134–147.

Ando, H., Mukai, H., Komura, T., Dewi, T., Ando, M. and Isagi, Y. 2020. Methodological trends and perspectives of animal dietary studies by noninvasive fecal DNA metabarcoding. Environmental DNA 2: 391–406.

Asahi, M. and Watanabe, S. 1967. On the Formosan tree squirrel, *Callosciurus caniceps thaiwanensis* Bonhote, introduced into Tomogashima, IV Stomach contents. Journal of Mammalogical Society of Japan 3: 152–157 (in Japanese).

Aylward, C. M., Statham, M. J., Barthman-Thompson, L., Kelt, D. A. and Sacks, B. N. 2022. Dietary characterization of the endangered salt marsh harvest mouse and sympatric rodents using DNA metabarcoding. Ecology and Evolution 12: e9121. DOI: 10.1002/ece3.9121.

Bellard, C., Cassey, P. and Blackburn, T. M. 2016. Alien species as a driver of recent extinctions. Biology Letters 12: 20150623. DOI: 10.1098/rsbl.2015.0623.

Bellard, C., Rysman, J. F., Leroy, B., Claud, C. and Mace, G. M. 2017. A global picture of biological invasion threat on islands. Nature Ecology and Evolution 1: 1862–1869.

Bohmann, K., Elbrecht, V., Carøe, C., Bista, I., Leese, F., Bunce, M., Yu, D. W., Seymour, M., Dumbrell, A. J. and Creer, S. 2022. Strategies for sample labelling and library preparation in DNA metabarcoding studies. Molecular Ecology Resources 22: 1231–1246.

Braukmann, T. W. A., Ivanova, N. V., Prosser, S. W. J., Elbrecht, V., Steinke, D., Ratnasingham, S., de Waard, J. R., Sones, J. E., Zakharov, E. V. and Hebert, P. D. N. 2019. Metabarcoding a diverse arthropod mock community. Molecular Ecology Resources 19: 711–727.

Browett, S. S., Curran, T. G., O'Meara, D. B., Harrington, A. P., Sales, N. G., Antwis, R. E., O'Neill, D. and McDevitt, A. D. 2021. Primer biases in the molecular assessment of diet in multiple insectivorous mammals. Mammalian Biology 101: 293–304.

Brun, L., Schneider, J., Carrió, E. M., Dongre, P., Taberlet, P., van de Waal, E. and Fumagalli, L. 2022. Focal vs. fecal: seasonal variation in the diet of wild vervet monkeys from observational and DNA metabarcoding data. Ecology and Evolution 12: e9358. DOI: 10.1002/ece3.9358.

Calderón-Sanou, I., Münkemüller, T., Boyer, F., Zinger, L. and Thuiller, W. 2020. From environmental DNA sequences to ecological conclusions: how strong is the influence of methodological choices? Journal of Biogeography 47: 193–206.

Castle, S. T., Allan, N., Clifford, D., Aylward, C. M., Ramsey, J., Fascetti, A. J., Pesapane, R., Roy, A., Statham, M., Sacks, B., et al.

2020. Diet composition analysis provides new management insights for a highly specialized endangered small mammal. PLOS ONE 15: e0240136. DOI: 10.1371/journal.pone.0240136.

Ceballos, G., Ehrlich, P. R. and Raven, P. H. 2020. Vertebrates on the brink as indicators of biological annihilation and the sixth mass extinction. Proceedings of the National Academy of Sciences of the United States of America 117: 13596–13602.

Chao, A. and Jost, L. 2012. Coverage-based rarefaction and extrapolation: standardizing samples by completeness rather than size. Ecology 93: 2533–2547.

Chiba, H. 1968. Some notes on the food habit of the Japanese serow, *Capricornis crispus crispus* (Temminck). Journal of Mammalogical Society of Japan 4: 20–25 (in Japanese).

Corse, E., Tougard, C., Archambaud-Suard, G., Agnèse, J.-F., Mandeng, F. D. M., Bilong, C. F. B., Duneau, D., Zinger, L., Chappaz, R., Xu, C. C. Y., et al. 2019. One-locus-several-primers: a strategy to improve the taxonomic and haplotypic coverage in diet metabarcoding studies. Ecology and Evolution 9: 4603–4620.

Creedy, T. J., Andújar, C., Meramveliotakis, E., Noguerales, V., Overcast, I., Papadopoulou, A., Morlon, H., Vogler, A. P., Emerson, B. C. and Arribas, P. 2022. Coming of age for COI metabarcoding of whole organism community DNA: towards bioinformatic harmonisation. Molecular Ecology Resources 22: 847–861.

Cuff, J. P., Windsor, F. M., Tercel, M. P. T. G., Kitson, J. J. N. and Evans, D. M. 2021. Overcoming the pitfalls of merging dietary metabarcoding into ecological networks. Methods in Ecology and Evolution 13: 545–559.

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A. and Callahan, B. J. 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6: 226. DOI: 10.1186/s40168-018-0605-2.

Deagle, B. E., Thomas, A. C., McInnes, J. C., Clarke, L. J., Vesterinen, E. J., Clare, E. L., Kartzinel, T. R. and Eveson, J. P. 2019. Counting with DNA in metabarcoding studies: how should we convert sequence reads to dietary data? Molecular Ecology 28: 391–406.

Deamer, D., Akeson, M. and Branton, D. 2016. Three decades of nanopore sequencing. Nature Biotechnology 34: 518–524.

D'Erchia, A. M., Atlante, A., Gadaleta, G., Pavesi, G., Chiara, M., de Virgilio, C., Manzari, C., Mastropasqua, F., Prazzoli, G. M., Picardi, E., et al. 2015. Tissue-specific mtDNA abundance from exome data and its correlation with mitochondrial transcription, mass and respiratory activity. Mitochondrion 20: 13–21.

Dueñas, M.-A., Hemming, D. J., Roberts, A. and Diaz-Soltero, H. 2021. The threat of invasive species to IUCN-listed critically endangered species: a systematic review. Global Ecology and Conservation 26: e01476. DOI: 10.1016/j.gecco.2021.e01476.

Elbrecht, V., Braukmann, T. W. A., Ivanova, N. V., Prosser, S. W. J, Hajibabaei, M., Wright, M., Zakharov, E. V., Hebert, P. D. N. and Steinke, D. 2019. Validation of COI metabarcoding primers for terrestrial arthropods. PeerJ 7: e7745. DOI: 10.7717/peerj.7745.

Elbrecht, V., Peinert, B. and Leese, F. 2017. Sorting things out: assessing effects of unequal specimen biomass on DNA metabarcoding. Ecology and Evolution 7: 6918–6926.

Fischer, C., Gayer, C., Kurucz, K., Riesch, F., Tscharntke, T. and Batáry, P. 2018. Ecosystem services and disservices provided by small rodents in arable fields: effects of local and landscape management. Journal of Applied Ecology 55: 548–558.

Furneaux, B., Bahram, M., Rosling, A., Yorou, N. S. and Ryberg, M. 2021. Long- and short-read metabarcoding technologies reveal similar spatiotemporal structures in fungal communities. Molecular Ecology Resources 21: 1833–1849.

Gabrielson, S. M. E., Mau, R. L., Dittmar, E., Kelley, J. P., Tarwater, C. E., Drake, D. R., Sperry, J. H. and Foster, J. T. 2024. DNA metabarcoding reveals diet composition of invasive rats and mice in Hawaiian forests. Biological Invasions 26: 79–105.

Gajski, D., Wolff, J. O., Melcher, A., Weber, S., Prost, S., Krehenwinkel, H. and Kennedy, S. R. 2024. Facilitating taxonomy and phylogenetics: an informative and cost-effective protocol integrating long amplicon PCRs and third-generation sequencing. Molecular Phylogenetics and Evolution 192: 107988. DOI: 10.1016/j.ympev.2023.107988.

Goldberg, A. R., Conway, C. J., Tank, D. C., Andrews, K. R., Gour, D. S. and Waits, L. P. 2020. Diet of a rare herbivore based on DNA metabarcoding of feces: selection, seasonality, and survival. Ecology and Evolution 10: 7627–7643.

Hakimzadeh, A., Abdala Asbun, A., Albanese, D., Bernard, M., Buchner, D., Callahan, B., Caporaso, J. G., Curd, E., Djemiel, C., Brandström Durling, M., et al. 2023. A pile of pipelines: an overview of the bioinformatics software for metabarcoding data analyses. Molecular Ecology Resources 24: e13847. DOI: 10.1111/1755-0998.13847.

Han, B. A., Kramer, A. M. and Drake, J. M. 2016. Global patterns of zoonotic disease in mammals. Trends in Parasitology 32: 565–577.

Han, B. A., Schmidt, J. P., Bowden, S. E. and Drake, J. M. 2015. Rodent reservoirs of future zoonotic diseases. Proceedings of the National Academy of Sciences of the United States of America 112: 7039–7044.

Harrison, J. G., John Calder, W., Shuman, B. and Alex Buerkle, C. 2021. The quest for absolute abundance: the use of internal standards for DNA-based community ecology. Molecular Ecology Resources 21: 30–43.

Holthuijzen, W. A., Flint, E. N., Green, S. J., Plissner, J. H., Simberloff, D., Sweeney, D., Wolf, C. A. and Jones, H. P. 2023. An invasive appetite: combining molecular and stable isotope analyses to reveal the diet of introduced house mice (*Mus musculus*) on a small, subtropical island. PLOS ONE 18: e0293092. DOI: 10.1371/journal.pone.0293092.

Huggins, L. G., Colella, V., Young, N. D. and Traub, R. J. 2024. Metabarcoding using nanopore long-read sequencing for the unbiased characterization of apicomplexan haemoparasites. Molecular Ecology Resources 24: e13878. DOI: 10.1111/1755-0998.13878.

Iwanowicz, D. D., Vandergast, A. G., Cornman, R. S., Adams, C. R., Kohn, J. R., Fisher, R. N. and Brehme, C. S. 2016. Metabarcoding of fecal samples to determine herbivore diets: a case study of the endangered pacific pocket mouse. PLOS ONE 11: e0165366. DOI: 10.1371/journal.pone.0165366.

Jamy, M., Foster, R., Barbera, P., Czech, L., Kozlov, A., Stamatakis, A., Bending, G., Hilton, S., Bass, D. and Burki, F. 2020. Long-read metabarcoding of the eukaryotic rDNA operon to phylogenetically and taxonomically resolve environmental diversity. Molecular Ecology Resources 20: 429–5443.

Jusino, M. A., Banik, M. T., Palmer, J. M., Wray, A. K., Xiao, L., Pelton, E., Barber, J. R., Kawahara, A. Y., Gratton, C., Peery, M. Z. and Lindner, D. L. 2019. An improved method for utilizing high-throughput amplicon sequencing to determine the diets of insectivorous animals. Molecular Ecology Resources 19: 176–190.

Kartzinel, T. R., Chen, P. A., Coverdale, T. C., Erickson, D. L., Kress, W. J., Kuzmina, M. L., Rubenstein, D. I., Wang, W. and Pingle, R. M. 2015. DNA metabarcoding illuminates dietary niche partitioning by African large herbivores. Proceedings of the National Academy of Sciences of the United States of America 112: 8019–8024.

Klure, D. M., Cragun, B. J. and Dearing, M. D. 2023. Sympatric rodents in a desert shrubland differ in arthropod consumption.

Journal of Arid Environments 214: 104999. DOI: 10.1016/j.jaridenv.2023.104999.

Klure, D. M., Greenhalgh, R. and Dearing, M. D. 2022. Addressing nontarget amplification in DNA metabarcoding studies of arthropod-feeding rodents. Mammal Research 67: 499–509.

König, H. J., Kiffner, C., Kramer-Schadt, S., Fürst, C., Keuling, O. and Ford, A. T. 2020. Human-wildlife coexistence in a changing world. Conservation Biology 34: 786–794.

Krehenwinkel, H., Wolf, M., Lim, J. Y., Rominger, A. J., Simison, W. B., Gillespie, R. G. 2017. Estimating and mitigating amplification bias in qualitative and quantitative arthropod metabarcoding. Scientific Reports 7: 17668. DOI: 10.1038/s41598-017-17333-x.

Lacher, T. E. Jr., Davidson, A. D., Fleming, T. H., Gómez-Ruiz, E. P., McCracken, G. F., Owen-Smith, N., Peres, C. A. and Vander Wall, S. B. 2019. The functional roles of mammals in ecosystems. Journal of Mammalogy 100: 942–964.

Lacher, T. E. Jr., Murphy, W. J., Rogan, J., Smith, A. T., Upham, N. S. 2016. Evolution, phylogeny, ecology, and conservation of the clade Glires: Lagomorpha and Rodentia. 2016. In (Wilson, D. E., Lacher, T. E. Jr. and Mittermeier, R. A., eds.) Handbook of the Mammals of the World, Vol. 6, Lagomorphs and Rodents I, pp. 15–26. Lynx Edicions, Barcelona.

Lamb, P. D., Hunter, E., Pinnegar, J. K., Creer, S., Davies, R. G. and Taylor, M. I. 2019. How quantitative is metabarcoding: a meta-analytical approach. Molecular Ecology 28: 420–430.

Latinne, A., Galan, M., Waengso-thorn, S., Rojandilok, P., Eiamampai, K., Sribuarod, K. and Michaux, J. R. 2014. Diet analysis of *Leopoldamys neilli*, a cave-dwelling rodent in Southeast Asia, using next-generation sequencing from feces. Journal of Cave and Karst Studies 76: 139–145.

Latz, M. A. C., Grujcic, V., Brugel, S., Lycken, J., John, U., Karlson, B., Andersson, A. and Andersson, A. F. 2022. Short- and long-read metabarcoding of the eukaryotic rRNA operon: evaluation of primers and comparison to shotgun metagenomics sequencing. Molecular Ecology Resources 22: 2304–2318.

Littleford-Colquhoun, B. L., Freeman, P. T., Sackett, V. I., Tulloss, C. V., McGarvey, L. M., Geremia, C. and Kartzinel, T. R. 2022. The precautionary principle and dietary DNA metabarcoding: commonly used abundance thresholds change ecological interpretation. Molecular Ecology 31: 1615–1626.

Liu, M., Clarke, L. J., Baker, S. C., Jordan, G. J. and Burridge, C. P. 2020. A practical guide to DNA metabarcoding for entomological ecologists. Ecological Entomology 45: 373–385.

Lopes, C. M., De Barba, M., Boyer, F., Mercier, C., da Silva Filho, P. J. S., Heidtmann, L. M., Galiano, D., Kubiak, B. B., Langone, P., Gielly, L., et al. 2015. DNA metabarcoding diet analysis for species with parapatric vs sympatric distribution: a case study on subterranean rodents. Heredity 114: 525–536.

Lopes, C. M., De Barba, M., Boyer, F., Mercier, C., Galiano, D., Kubiak, B. B., Maestri, R., da Silva Filho, P. J. S., Gielly, L., Coissac, E., et al. 2020. Ecological specialization and niche overlap of subterranean rodents inferred from DNA metabarcoding diet analysis. Molecular Ecology 29: 3144–3154.

Lu, Q., Cheng, C., Xiao, L., Li, J., Li, X., Zhao, X., Lu, Z., Zhao, J. and Yao, M. 2023. Food webs reveal coexistence mechanisms and community organization in carnivores. Current Biology 33: 647–659.

Mata, V. A., Rebelo, H., Amorim, F., McCracken, G. F., Jarman, S. and Beja, P. 2019 How much is enough? Effects of technical and biological replication on metabarcoding dietary analysis. Molecular Ecology 28: 165–175.

McKnight, D. T., Huerlimann, R., Bower, D. S., Schwarzkopf, L., Alford, R. A. and Zenger, K. R. 2019. microDecon: a highly accurate read-subtraction tool for the post-sequencing removal of contamination in metabarcoding studies. Environmental DNA 1: 14–25.

Mena, J. L., Yagui, H., Tejeda, V., Bonifaz, E., Bellemain, E., Valentini, A., Tobler, M. W., Sánchez-Vendizú, P. and Lyet, A. 2021. Environmental DNA metabarcoding as a useful tool for evaluating terrestrial mammal diversity in tropical forests. Ecological Applications 31: e02335. DOI: 10.1002/eap.2335.

Minato, S. 2018. The Japanese Dormouse–Conservation of Wild Animals and Environmental Education. Tokyo University Press, Tokyo, 272 pp.

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., Minamoto, T., Yamamoto, S., Yamanaka, H., Araki, H., Kondoh, M. and Iwasaki, W. 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. Royal Society Open Science 2: 150088. DOI: 10.1098/rsos.150088.

Moorhouse-Gann, R. J., Dunn, J. C., de Vere, N., Goder, M., Cole, N., Hipperson, H. and Symondson, W. O. C. 2018. New universal ITS2 primers for high-resolution herbivory analyses using DNA metabarcoding in both tropical and temperate zones. Scientific Reports 8: 8542. DOI: 10.1038/s41598-018-26648-2.

Murano, C., Sato, J. J., Wada, T., Kasahara, S. and Azuma, N. 2023. Genetic analyses of Japanese field vole *Alexandromys* (*Microtus*) *montebelli* winter diet in apple orchards with deep snow cover. Mammal Study 48: 219–229.

Neby, M., Kamenova, S., Devineau, O., Ims, R. A. and Soininen, E. M. 2021. Issues of under-representation in quantitative DNA metabarcoding weaken the inference about diet of the tundra vole *Microtus oeconomus*. PeerJ 9: e11936. DOI: 10.7717/peerj.11936.

Neby, M., Ims, R. A., Kamenova, S., Devineau, O. and Soininen, E. M. 2024. Is the diet cyclic phase-dependent in boreal vole populations? Ecology and Evolultion 14: e11227. DOI: 10.1002/ece3.11227.

Nielsen, J. M., Clare, E. L., Hayden, B., Brett, M. T. and Kratina P. 2018. Diet tracing in ecology: method comparison and selection. Methods in Ecology and Evolution 9: 278–291.

O'Rourke, D. R., Bokulich, N. A., Jusino, M. A., MacManes, M. D. and Foster, J. T. 2020. A total crapshoot? Evaluating bioinformatic decisions in animal diet metabarcoding analyses. Ecology and Evolution 10: 9721–9739.

Ota, K. 1968. Studies on the ecological distribution of the murid rodents in Hokkaido. Research Bulletin of the Hokkaido University Forests 26: 223–295 (in Japanese with English abstract).

Petrosky, A. L., Rowsey, D. M. and Heaney, L. R. 2021. Molecular assessment of dietary niche partitioning in an endemic island radiation of tropical mammals. Molecular Ecology 30: 5858–5873.

Pinho, C. J., Lopes, E. P., Paupério, J., Gomes, I., Romeiras, M. M. and Vasconcelos, R. 2022. Trust your guts? The effect of gut section on diet composition and impact of *Mus musculus* on islands using metabarcoding. Ecology and Evolution 12: e8638. DOI: 10.1002/ece3.8638.

Piñol, J., Mir, G., Gomez-Polo, P. and Agustí, N. 2015. Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. Molecular Ecology Resources 15: 819–830.

Piñol, J., Senar, M. A. and Symondson, W. O. C. 2019. The choice of universal primers and the characteristics of the species mixture determine when DNA metabarcoding can be quantitative. Molecular Ecology 28: 407–419.

Piper, A. M., Batovska, J., Cogan, N. O. I., Weiss, J., Cunningham, J. P., Rodoni, B. C. and Blacket, M. J. 2019. Prospects and challenges

of implementing DNA metabarcoding for high-throughput insect surveillance. Gigascience 8: giz092. DOI: 10.1093/gigascience/giz092.

Rhoads, A. and Au, K. F. 2015. PacBio Sequencing and Its Applications. Genomics Proteomics Bioinformatics 13: 278–289.

Richardson, K., Steffen, W., Lucht, W., Bendtsen, J., Cornell, S. E., Donges, J. F., Drüke, M., Fetzer, I., Bala, G., von Bloh, W., et al. 2023. Earth beyond six of nine planetary boundaries. Science Advances 9: eadh2458. DOI: 10.1126/sciadv.adh2458.

Saitoh, T., Osawa, J., Takanishi, T., Hayakashi, S., Ohmori, M., Morita, T., Uemura, S., Vik, J., Stenseth, N. C. and Maekawa, K. 2007. Effects of acorn masting on population dynamics of three forest-dwelling rodent species in Hokkaido, Japan. Population Ecology 49: 249–256.

Sakata, M. K., Kawata, M. U., Kurabayashi, A., Kurita, T., Nakamura, M., Shirako, T., Kakehashi, R., Nishikawa, K., Hossman, M. Y., Nishijima, T., et al. 2022. Development and evaluation of PCR primers for environmental DNA (eDNA) metabarcoding of Amphibia. Metabarcoding and Metagenomics 6: e76534. DOI: 10.3897/mbmg.6.76534.

Sato, J. J. 2017. A review of the process of mammalian faunal assembly in Japan: insight from the molecular phylogenetics. In (Motokawa, M. and Kajihara, H., eds.) Species Diversity of Animals in Japan. pp. 49–116. Springer Japan, Tokyo.

Sato, J. J., Kyogoku, D., Komura, T., Maeda, K., Inamori, C., Yamaguchi, Y. and Isagi, Y. 2019. Potentials and pitfalls of the DNA metabarcoding analyses for the dietary study of the large Japanese wood mouse *Apodemus speciosus* on Seto Inland Sea islands. Mammal Study 44: 221–231.

Sato, J. J., Matsuda, H., Fujita, H., Yasuda, K., Aiba, H. and Minato, S. 2023. Noninvasive genetic methods for species identification and dietary profiling of the Japanese dormouse *Glirulus japonicus* from fecal samples. Mammal Study 48: 245–261.

Sato, J. J., Ohtsuki, Y., Nishiura, N. and Mouri, K. 2022. DNA metabarcoding dietary analyses of the wood mouse *Apodemus speciosus* on Innoshima Island, Japan, and implications for primer choice. Mammal Research 67: 109–122.

Sato, J. J., Shimada, T., Kyogoku, D., Komura, T., Uemura, S., Saitoh, T. and Isagi, Y. 2018. Dietary niche partitioning between sympatric wood mouse species (Muridae: *Apodemus*) revealed by DNA meta-barcoding analysis. Journal of Mammalogy 99: 952–964.

Sato, Y., Miya, M., Fukunaga, T., Sado, T. and Iwasaki, W. 2018. MitoFish and MiFish pipeline: a mitochondrial genome database of fish with an analysis pipeline for environmental DNA metabarcoding. Molecular Biology and Evolution 35: 1553–1555.

Shapiro, J. T. and Báldi, A. 2014. Accurate accounting: how to balance ecosystem services and disservices. Ecosystem Services 7: 201–202.

Soininen, E. M., Ehrich, D., Lecomte, N., Yoccoz, N. G., Tarroux, A., Berteaux, D., Gauthier, G., Gielly, L., Brochmann, C., Gussarova, G., et al. 2014. Sources of variation in small rodent trophic niche: new insights from DNA metabarcoding and stable isotope analysis. Isotopes in Environmental and Health Studies 50: 361–381.

Soininen, E. M., Gauthier, G., Bilodeau, F., Berteaux, D., Gielly, L., Taberlet, P., Gussarova, G., Bellemain, E., Hassel, K., Stenøien, H. K., et al. 2015. Highly overlapping winter diet in two sympatric lemming species revealed by DNA metabarcoding. PLOS ONE 10: e0115335. DOI: 10.1371/journal.pone.0115335.

Soininen, E. M., Valentini, A., Coissac, E., Miquel, C., Gielly, L., Brochmann, C., Brysting, A. K., Sønstebø, J. H., Ims, R. A., Yoccoz, N. G. and Taberlet, P. 2009. Analysing diet of small herbivores: the efficiency of DNA barcoding coupled with high-throughput pyrosequencing for deciphering the composition of complex plant mixtures. Frontiers in Zoology 6: 16. DOI: 10.1186/1742-9994-6-16.

Soininen, E. M., Zinger, L., Gielly, L., Bellemain, E., Bråthen, K. A., Brochmann, C., Epp, L. S., Gussarova, G., Hassel, K., Henden, J.-A., et al. 2013. Shedding new light on the diet of Norwegian lemmings: DNA metabarcoding of stomach content. Polar Biology 36: 1069–1076.

Stapleton, T. E., Weinstein, S. B., Greenhalgh, R. and Dearing, M. D. 2022. Successes and limitations of quantitative diet metabarcoding in a small, herbivorous mammal. Molecular Ecology Resources 22: 2573–2586.

Taberlet, P., Bonin, A., Zinger, L. and Coissac, E. 2018. Environmental DNA: For Biodiversity Research and Monitoring. Oxford University Press, Oxford, 253 pp.

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T., Corthier, G., Brochmann, C. and Willerslev, E. 2007. Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. Nucleic Acids Research 35: e14. DOI: 10.1093/nar/gkl938.

Tanabe, A. S. and Toju, H. 2013. Two new computational methods for universal DNA barcoding: a benchmark using barcode sequences of bacteria, archaea, animals, fungi, and land plants. PLOS ONE 8: e76910. DOI: 10.1371/journal.pone.0076910.

Tedersoo, L., Bahram, M., Zinger, L., Nilsson, R. H., Kennedy, P. G., Yang, T., Anslan, S. and Mikryukov, V. 2022. Best practices in metabarcoding of fungi: from experimental design to results. Molecular Ecology 31: 2769–2795.

Tercel, M. P. T. G., Symondson, W. O. C. and Cuff, J. P. 2021. The problem of omnivory: a synthesis on omnivory and DNA metabarcoding. Molecular Ecology 30: 2199–2206.

Thomas, A. C., Deagle, B. E., Eveson, J. P., Harsch, C. H. and Trites, A. W. 2016. Quantitative DNA metabarcoding: improved estimates of species proportional biomass using correction factors derived from control material. Molecular Ecology Resources. 16: 714–726.

Tournayre, O., Leuchtmann, M., Filippi-Codaccioni, O., Trillat, M., Piry, S., Pontier, D., Charbonnel, N. and Galan, M. 2020. In silico and empirical evaluation of twelve metabarcoding primer sets for insectivorous diet analyses. Ecology and Evolution 10: 6310–6332.

Ushio, M., Fukuda, H., Inoue, T., Makoto, K., Kishida, O., Sato, K., Murata, K., Nikaido, M., Sado, T., Sato, Y., et al. 2017. Environmental DNA enables detection of terrestrial mammals from forest pond water. Molecular Ecology Resources 17: e63–e75.

Ushio, M., Murata, K., Sado, T., Nishiumi, I., Takeshita, M., Iwasaki, W. and Miya, M. 2018. Demonstration of the potential of environmental DNA as a tool for the detection of avian species. Scientific Reports 8: 4493. DOI: 10.1038/s41598-018-22817-5.

Valentini, A., Miquel, C., Nawaz, M. A., Bellemain, E., Coissac, E., Pompanon, F., Gielly, L., Cruaud, C., Nascetti, G., Wincker, P., Swenson, J. E. and Taberlet, P. 2009. New perspectives in diet analysis based on DNA barcoding and parallel pyrosequencing: the *trnL* approach. Molecular Ecology Resources 9: 51–60.

Vandergast, A. G., Brehme, C. S., Iwanowicz, D., Cornman, R. S., Adsit-Morris, D. and Fisher, R. N. 2023. Fecal metabarcoding of the endangered Pacific pocket mouse (*Perognathus longimembris pacificus*) reveals a diverse and forb rich diet that reflects local habitat availability. Ecology and Evolution 13: e10460. DOI: 10.1002/ece3.10460.

Verde Arregoitia, L. D. and D'Elía, G. 2020. Classifying rodent diets for comparative research. Mammal Review 51: 51–65.

Vestheim, H. and Jarman, S. N. 2008. Blocking primers to enhance PCR amplification of rare sequences in mixed samples—a case

study on prey DNA in Antarctic krill stomachs. Frontiers in Zoology 5: 12. DOI: 10.1186/1742-9994-5-12.

Wanniarachchi, S., Swan, M., Nevil, P. and York, A. 2022. Using eDNA metabarcoding to understand the effect of fire on the diet of small mammals in a woodland ecosystem. Ecology and Evolution 12: e9457. DOI: 10.1002/ece3.9457.

Zeale, M. R., Butlin, R. K., Barker, G. L., Lees, D. C. and Jones, G. 2011. Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. Molecular Ecology Resources 11: 236–244.

Zhang, X., Zou, Y., Zou, X., Xu, Z., Nan, X. and Han, C. 2022. DNA metabarcoding uncovers the diet of subterranean rodents in China. PLOS ONE 17: e0258078. DOI: 10.1371/journal.pone.0258078.

Zhu, T., Sato, Y., Sado, T., Miya, M. and Iwasaki, W. 2023. MitoFish, MitoAnnotator, and MiFish Pipeline: updates in ten years. Molecular Biology and Evolution 40: msad035. DOI: 10.1093/molbev/msad035.

Zinger, L., Bonin, A., Alsos, I. G., Bálint, M., Bik, H., Boyer, F., Chariton, A. A., Creer, S., Coissac, E., Deagle, B. E., et al. 2019. DNA metabarcoding-Need for robust experimental designs to draw sound ecological conclusions. Molecular Ecology 28: 1857–1862.

Received 30 March 2024. Accepted 22 August 2024.
Editor was Junco Nagata.