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［投稿論文：研究論文］

DNA Metabarcoding Analysis of Long-Eared Owl *Asio otus* Pellets Reveals Small Animals as Its Prey

DNA メタバーコーディング解析が可能にしたトラフズク *Asio otus* のペリットにおける小型餌動物の検出

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Abstract: The feeding habits of apex predators such as raptors have a major influence on the local ecosystem, but their predation targets have been analyzed by visual observation that requires extremely proficient skills of taxonomic classification to identify small animals. Identifying prey animals at the species level reveals predator biology as well as important information for conserving predators, but it is difficult using conventional techniques. In this study, we aimed to clarify the diet of the long-eared owl *Asio otus* by DNA metabarcoding of its pellets in the central region of Kanagawa Prefecture in Japan, using three types of universal primer sets. The results indicate that the long-eared owl feeds on various types of warm-blooded animals including birds, rodents and bats in winter. Our findings also demonstrate the high efficiency and reliability of DNA metabarcoding in the study of predator–prey relationship.

猛禽類などの頂点捕食者の餌となる動物を種レベルで同定することは捕食者の生態を明らかにし、保全するために重要な情報となる。しかし、餌動物を特定するためには目視を主流とした高度な分類学的知識などが必要とされる。そこで、本研究では分子生物学的手法によって簡易的に餌動物を特定することにした。３種類のユニバーサルプライマーを用いて神奈川県中部に生息するトラフズク *Asio otus* のペリットを DNA メタバーコーディング手法によって解析した。その結果、トラフズクは冬季に小鳥や齧歯類、コウモリなど様々な小型の恒温動物を捕食していることが明らかとなった。捕食者と被食者の関係を研究するにあたって DNA メタバーコーディングが高効率で信頼性の高い手法であることが再確認されたと言える。

Keywords: diet, metabarcoding, universal primer
食性、メタバーコーディング、ユニバーサルプライマー

1 INTRODUCTION

Information regarding predators such as their niche, distribution, and community in the ecosystem is important for revealing their potential prey. In particular, Apex predators, also called as alpha predators or top predators, reign supreme in the food chain, and their top-down control of mesopredators and herbivores significantly influences the local predator–prey interactions (Estes et al., 2011).

Several studies have been performed on the prey species of raptors based on visual observations such as the pellet (the undigested material that raptor spit out is called pellet) analysis (Korpimäki, 1985), stomach content analysis (Tomback, 1975), direct behavior observations (Meyer et al., 2004), and video monitoring of nests

(McPherson et al., 2016). In the case of owl species, the pellet analysis has been commonly used because owls are nocturnal animals, occupying a wide range of forest areas (Glading et al., 1943). However, this method requires enough caution to avoid oversight and overestimation of prey species. Raptors usually tear their prey apart as they feed, and several parts and bones of the prey are digested by the gastric juice; hence, morphological identification of prey animals after digestion requires a high level of exclusive knowledge and experience. The identification of prey animals and the elucidation of prey selectivity strongly depend on the accuracy of experimental approach. The more accurate the identification of prey species, the better the elucidation of local predator–prey relationship (Hardy et al., 2010).

DNA metabarcoding (method for detecting organisms based on DNA information contained in a sample) can overcome the limitations associated with conventional methods and potentially be a powerful method to enable the identification of prey to the species level (Sullins et al., 2018). With the application of this method for diet analysis, significant information can be obtained to elucidate food web trophic structure (Pompanon et al., 2012). The application of DNA metabarcoding to diet identification improves the precision of analysis. Moreover, it does not require high level of knowledge and experience, leading to novel findings and reconfirmation of predator–prey relationships. In addition, this method can provide a useful framework for the study of prey species, especially for researchers who do not have enough experience as morphologists.

Pellets of the long-eared owl, *Asio otus*, also known as the northern long-eared owl, distributed in the Holarctic Region such as Europe and North America, have been thoroughly analyzed in some countries (Tome, 1994; Birrer, 2009). However, there are few prey studies about long-eared owls in Asian areas like Japan (Chiba et al., 2005) and China (Zhao et al., 2011). In addition, most of these studies employed visual observation, and therefore further in-depth analysis using new technologies such as DNA metabarcoding is worth conducting. In fact, there have been reports of pellet analysis showing a great variety of prey species, although the long-eared owl is thought to feed mainly on rodents (Gryz and Krauze-Gryz, 2015). In this study, we

aimed to clarify the diet of long-eared owl by DNA metabarcoding of its pellets. We also aimed to elucidate the reliability and future application potential of DNA metabarcoding.

2 MATREIALS AND METHODS

From the collected samples, randomly selecting ten pellets, bones of length 1 cm or more were removed, and the remaining residue was used for DNA extraction, using the NucleoSpin Plant II Maxi Kit (U0772A; TaKaRa, Shiga, Japan). In the first polymerase chain reaction (PCR), a portion of the mitochondrial COI (cytochrome oxidase subunit I) region of the COI primer set, mitochondrial 12S rRNA region of the MiMammal primer set, and mitochondrial 16S rRNA region of the gBird primer set were amplified using the following primers: 5 ′ -GGWACWGGWTGAACWGT WTAYCCYCC-3 ′ (IntF) and 5 ′ -TAHACTTCNGGGTGKCCRAARAATCA-3 ′ (HCOmR) for COI (Folmer et al., 1994); 5 ′ -NNNNNNGGGTTGGTAAATTTCGT GCCAGC-3 ′ (MiMammal-U-F) and 5 ′ -NNNNNNCATAGTGGGGTATCTAATC CCAGTTTG-3 ′ (MiMammal-U-R), 5 ′ -NNNNNNGGACTGGTCAATTTCGTG CCAGC-3 ′ (MiMammal-E-F) and 5 ′ -NNNNNNCATAGTGAGGTATCTAATCT CAGTTTG-3 ′ (MiMammal-E-R), 5 ′ -NNNNNNGGGTTGGTTAATTTCGTGCC AGC-3 ′ (MiMammal-B-F) and 5 ′ -NNNNNNCATAGTGGGGTATCTAATCCCA GTTTG-3 ′ (MiMammal-B-R) for MiMammal (Ushio et al., 2017); 5 ′ -NNNNNNCAAGTATTGAAGGTGATG-3 ′ (gBirdF) and 5 ′ -NNNNNNCCCTGGGGTAGCTTGG-3 ′ (gBirdR) for gBird (Seibutugiken Co., Ltd.). The forward primers (IntF, MiMammal-U-F, MiMammal-E-F, MiMammal-B-F and gBirdF) included the adapter sequence 5 ′ -ACACTCTTTCCCTACACGAC GCTCTTCCGATCT-3 ′ before the above region-specific sequences, and the reverse primers (HCOmR, MiMammal-U-R, MiMammal-E-R, MiMammal-B-R and gBirdR) included the adapter sequence 5 ′ -GTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCT-3 ′ before the above sequences. DNA library was prepared with each primer set using the two-step tailed PCR method (Miya et al., 2015). The target amplicons were subjected to MiSeq sequencing using the 600-cycle MiSeq Reagent Nano Kit

v3 (MS-102-3003; Illumina, San Diego, California). To determine the species identity based on the sequences obtained by MiSeq, the data were processed using FASTQ Barcode Splitter, which is a component of FASTX Toolkit. We only extracted sequences with the readings that exactly matched the primers used. The primer sequences were deleted from the read sequences obtained from MiSeq analysis, and then sequences with the quality value of less than 20 were removed. The sequences that had 40 bases or less were discarded with its pair sequence using the Sickle tool. High-quality pair reads were combined using FLASH tool, and OTU (operational taxonomic units) clustering was performed using USEARCH with the binding sequence. The sequence reads that had more than 97% homology were classified, the remaining OTUs were subjected to a BLAST search against the nr database, and lineage estimation was performed. Non-dietary items (e.g., Large mammalian, Bacteria, and Protists) and those with low taxonomic resolution (e.g., Eukaryota) were discarded.

3 RESULTS

The pellets for DNA metabarcoding analysis were collected almost every day from mid-February to mid-March, 2018. The monitoring point of the long-eared owls, that is, an overwintering tree, is located in the Central Kanagawa Prefecture in Japan and is surrounded by cropland, grassland, industrial and commercial installations, and urban residential areas (Fig 1). To prevent DNA contamination of the human body and surrounding organisms at the sampling site, we laid a vinyl sheet under the overwintering tree and collected the pellets of long-eared owls wearing gloves. Overall, 40 pellet samples were collected from the vinyl sheet laid under the overwintering tree. The recovered pellets were immediately placed in a plastic bag and frozen.

Based on the results of DNA metabarcoding of pellets of the long-eared owl, we obtained 127763 sequencing reads by MiSeq analysis, which contained 42834, 60419, and 24510 reads, using the sequences of the COI, Mimammal and gBird primer sets, respectively. Further classification of these reads to the species level

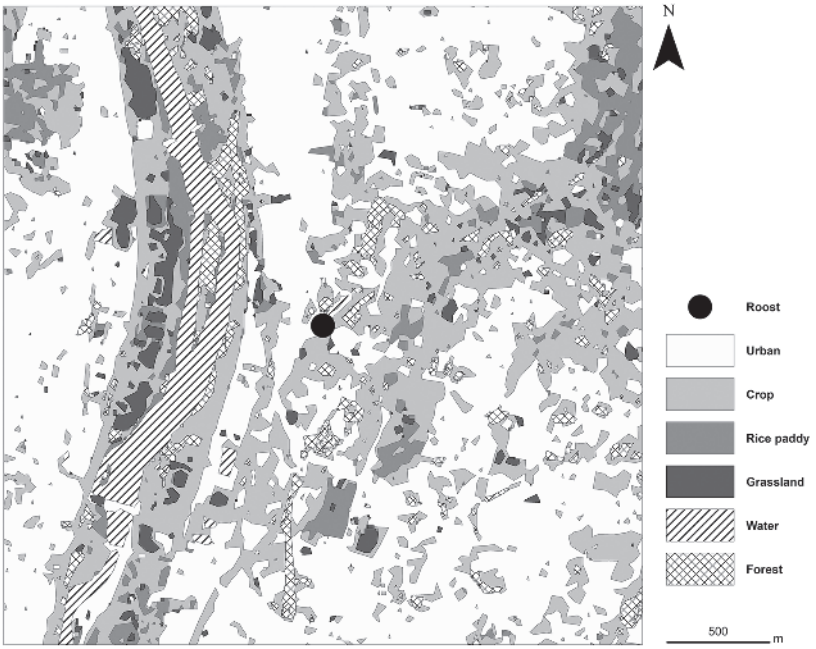

Figure 1 Map of the study area. Roost of the long-eared owl *Asio otus* is marked as a black point. Land use around the roost is illustrated. The Sagami River is in the vicinity. (High-Resolution Land Use and Land Cover Map of Japan. ver.18.03)

showed seven birds and two mammals (Table 1).

The species obtained for each primer were as follows. Six birds (Common Kingfisher *Alcedo atthis,* long-eared owl, Meadow Bunting *Emberiza cioides,* Brown-eared Bulbul *Hypsipetes amaurotis,* Eurasian Tree Sparrow *Passer montanus,* and Dusky Thrush *Turdus eunomus*) and two mammals (Japanese House Bat *Pipistrellus abramus* and House Mouse *Mus musculus*) were identified using the COI primer sets. Three birds (Eurasian Tree Sparrow, long-eared owl and Meadow Bunting) and two mammals (Japanese House Bat and House Mouse) were identified using the Mimammal primer sets. Five small birds (Eurasian Tree Sparrow, Dusky Thrush, Common Kingfisher, Meadow Bunting and Malagasy Bulbuls *H. madagascariensis*) were identified using the gBird primer sets.

Table 1 List of species detected from the pellets of long-eared owl *Asio otus* by DNA metabarcoding using each universal primer. Sequence reads and identity of the detected species are described.

Primer name		Reads	Species	Identity
CO1				
	Aves			
		9 206	*Alcedo atthis*	100%
		7 195	*Asio otus*	100%
		2 836	*Passer montanus*	100%
		1 010	*Turdus eunomus*	99.4%
		365	*Emberiza cioides*	100%
		160	*Hypsipetes amaurotis*	100%
	Mammalia			
		4 037	*Pipistrellus abramus*	100%
		2 251	*Mus musculus*	100%
Mimammal				
	Aves			
		2 857	*Passer montanus*	100%
		321	*Asio otus*	99.5%
		21	*Emberiza cioides*	100%
	Mammalia			
		1 541	*Pipistrellus abramus*	100%
		1 278	*Mus musculus*	100%
gBird				
	Aves			
		16 687	*Passer montanus*	100%
		7 421	*Turdus eunomus*	99.7%
		128	*Alcedo atthis*	99.7%
		108	*Emberiza cioides*	100%
		89	*Hypsipetes madagascariensis*	98%

4 DISCUSSION

DNA metabarcoding is commonly used for environmental DNA analysis to detect amphibians and fish (Miya et al., 2015; Valentini et al., 2016; Bálint et al., 2018). In addition, diet analysis by DNA metabarcoding has been used for assessing

feeding preferences and population structure of vampire bats using blood meal and faucal samples (Bohmann et al., 2018) and for illustrating the dietary compositions of shorebird as well as generalist grasshopper, which revealed their diet demands for their survival and possible conservation (Huang et al., 2022; Yamamoto and Uchida, 2018). In this study, we conducted a diet analysis of the long-eared owl during the wintering period in Japan by DNA metabarcoding of its pellets. The results helped us identify a small bat (Japanese House Bat) and birds (Common Kingfisher, Meadow Bunting, Brown-eared Bulbul, Eurasian Tree Sparrow and Dusky Thrush), which are usually difficult to identify by morphological classification. These results suggest that, compared with those by conventional methods, this method can greatly reduce total effort and expense of the analysis.

Based on the results, we identified that the Malagasy Bulbuls, a close relative species of Brown-eared Bulbul, as the Brown-eared Bulbul is not registered in the sequence amplified by the gBird primer set. A species closely related to Malagasy Bulbuls might have been detected. We thought that the remaining seven species identified were reliable, namely, five small birds (Common Kingfisher, Meadow Bunting, Brown-eared Bulbul, Eurasian Tree Sparrow and Dusky Thrush) and two mammals (Japanese House Bat and House Mouse). All of them have been reported to inhabit the area of the monitoring point of this study (Survey of Samukawa town Animal and Plants 2016). The present study results directly indicated that relationship existed between these seven animals hunted by the long-eared owls. The long-eared owl is thought to fly around the range of approximately 969 ha near overwintering trees (Emin et al., 2018), and their winter food composition in Northern Turkey mainly comprises small rodents such as voles (Ahmet et al., 2017). As voles are not reported to inhabit the area around the monitoring point, the owls may instead catch house mouse and other small animals, although the predominant species among them are still unknown because we did not perform quantitative analysis in this study.

In the previous research in China and Japan, it is reported that the long-eared owls are preying on Japanese House Bat in urban areas, and therefore the results of this research reconfirm it (Chiba et al., 2005; Tian et al., 2015). There are few studies

in Asia that identified small birds at the species level, which the long-eared owls prey on, so the results of this study indicating that the long-eared owls comprehensive prey on small birds may be very significant.

In DNA metabarcoding, the quality of DNA contained in pellets is one of the most important factors to obtain good results. Several factors such as bacteria and fungi in pellets and exposure to ultraviolet rays or rain might significantly damage the DNA (Oehm et al., 2011). In this study, five fungal species were detected as contaminants, although we collected pellets almost every day to maintain fresh condition. One possibility for these contaminations is that fungal DNA is originally included in pellets. Frequently, raptors prey on small mammals and birds, and hence, there is a possibility that the fungi on these prey animals can be detected in pellets (Ciesielska et al., 2017). Another possibility is that fungi, which originally exist on vinyl sheet, simply adhere to pellets. In studies targeting animals living under wild conditions, it is difficult to avoid these contaminations, but we recommend the use of well-sterilized vinyl sheets in future studies.

In this study, all seven potential prey animals of the long-eared owl were detected using the COI primer set. The Mimammal primer set detected both prey mammals and the gBird primer set detected all five prey avians (assuming *H. madagascariensis* as *H. amaurotis*). In the future, prey animals can be efficiently detected using the COI primer set with the most comprehensive database (Andujar et al., 2018) in the first step and running analysis using suitable universal primers in the second step. On the other hand, the high sensitivity of DNA metabarcoding methods may produce abundant false positives as a consequence of, for example, secondary predation or contamination from any source (Sheppard et al., 2005). However, in food habitat analysis of large carnivores such as raptors, these restrictions can be dealt with by considering the biota of the survey site and the ecology of the target species.

5 CONCLUSION

This study demonstrates that DNA metabarcoding is a suitable method to obtain

fundamental information about small prey animals using pellets without requiring expert knowledge and complicated technique. In the future, the combination of DNA metabarcoding and observational data will allow researchers to obtain a great amount of reliable and accurate information on predator–prey relationships. The method described in this study can be applied for the prey analysis of other species, and it is tremendously effective for listing prey animals. DNA metabarcoding using multiple universal primers is highly likely to be the mainstream technique of prey animal analysis in the future.

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