보건학 박사학위논문

Unraveling ecology and zoonotic pathogens of wild animals in Korea by environmental fecal analysis using high-throughput sequencing

환경 분변의 high-throughput sequencing을 활용한 한국에 서식하는 야생동물의 생태와 인수공통감염 병원체의 규명

2023년 8월

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이 논문을 보건학 박사 학위논문으로 제출함
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우철운의 보건학 박사 학위논문을 인준함
2023년 8월

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Abstract

Unraveling ecology and zoonotic pathogens of wild carnivores in Korea by fecal analysis using high-throughput sequencing

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Keyword : Wild animals; Diet; Parasite; Virus; Zoonosis; One Health

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Habitat destruction of wild animals, accelerated by anthropogenic factors such as urbanization and deforestation, has reduced the physical distance between wild animals and humans. In line with this situation, zoonotic pathogens derived from wild animals can pose a serious public health threat. This study aimed to investigate the diet and zoonotic pathogens of wild animals using the feces of three wild animals inhabiting Korea: raccoon dog (*Nyctereutes procyonoides*), leopard cat (*Prionailurus bengalensis*), and Eurasian otters (*Lutra lutra*). Based on high-throughput sequencing, it was confirmed that raccoon dogs living in Seosan, Chungcheongnam-do mainly eat fish, amphibians, and insects, and leopard cats living in Chungcheongnam-do and Gyeongsangbuk-do eat small rodents or birds. The fecal parasitomes of the three wild animals detected by high-throughput sequencing had different compositions depending on the wild animals, which is thought to be influenced by the diet of each wild animal. In addition, 5 zoonotic parasites that can cause infections to humans were identified. The fecal viromes of the three wild animals detected by metagenomic sequencing had different

compositions depending on the wild animals, which is thought to be influenced by the gut microbiome or diet of each wild animal. In addition, five viral genera containing viruses known to cause zoonotic infections were identified. In the future, it is expected that zoonotic diseases will be increased due to the increased proximity between humans and wildlife due to urbanization. Vigilance may need to be continuously exercised, such as by monitoring zoonotic pathogens in the feces of wildlife, as was done in this study. In addition, from the perspective of One Health, it is regarded that continuous monitoring can be used as basic data to manage wild animals and the surrounding environment in a healthy way.

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Introduction

Globally, the interest in coexistence with wildlife that may harbor zoonotic pathogens is growing. Human activities such as changing land use, extracting of natural resources, animal production systems, modern transportation, and the use of antimicrobial drug not only alter the proximity between humans and wildlife but also increase the transmission of zoonotic diseases [1]. The reduced biodiversity caused by human activities has made humans more exposed to zoonotic pathogens [2]. The Korean peninsula is considered one of the 'hot spots' where emerging infectious diseases frequently occur [3]. Additionally, a high-density livestock industry in Korea could lead to the widespread occurrence of zoonotic diseases [4]. However, in Korea, studies on pathogens infecting humans and livestock are mainly conducted, while studies on pathogens inherent in wild animals are limited [4]. Therefore, identifying zoonotic pathogens inherent in wild animals can serve as a preemptive action to alleviate the burden of potential zoonotic diseases.

Wild animals can become infected with pathogens such as parasites and/or viruses due to ecological factors such as diet and season, biological factors such as age and body condition, and anthropogenic factors such as human-induced translocations and introductions [5]. Additionally, even if wild animals are not infected with pathogens, parasites and/or viruses can spread to the environment through their feeding behavior and/or defecation [5]. Therefore, the composition of parasites and/or viruses in wild animals may vary depending on the hosts and its diet profiles. Furthermore, the composition of zoonotic pathogens would differ for each wild animal and/or depending on the animal's diet.

The studies of wild animals, i.e., their ecology and the pathogens they carry, have historically been based primarily on morphological

observations. For example, morphological identification is conducted by observing the egg, cyst, or larval morphology unique to each parasite [6]. In addition, dietary analysis of wild animals has relied on morphological observations of undigested remains of fruits, seeds, hair, skin, feathers, bones and so on. Clearly, these traditional methods have advanced our understanding of wildlife. However, these methods have the disadvantage of requiring extensive morphological and anatomical knowledge. In addition, taxonomic analysis at the species or genus level is sometimes difficult.

Therefore, this study aimed to identify the diet of three wild carnivores and the composition of their parasites and viruses by introducing high-throughput sequencing. To conduct this study, environmental feces of three wild carnivores, raccoon dogs (*Nyctereutes procyonoides*), leopard cats (*Prionailurus bengalensis*), and Eurasian otters (*Lutra lutra*) were collected in Korea. In Chapter 1, the diet of raccoon dog was analyzed by high-throughput sequencing. The vertebrate and invertebrate diets of raccoon dog were analyzed by amplifying the 12S rRNA gene and 16S rRNA gene, respectively. In addition, a blocking primer was developed to suppress 12S rRNA gene of raccoon dog and its performance was evaluated. In Chapter 2, 12S rRNA gene sequencing and metagenomic sequencing were performed to evaluate leopard cat's diet. Metagenomic sequencing compared the abundance of vertebrates, invertebrates, and plants in leopard cat's diet. In addition, we compared the results of leopard cat's vertebrate diet obtained by metagenomic sequencing with those obtained by 12S rRNA gene sequencing. In Chapter 3, the composition of the parasites in the feces of raccoon dog, leopard cat, and Eurasian otter was identified by high-throughput sequencing. Furthermore, by linking the detected parasites with the diets of the three carnivores, we discussed whether the composition of the detected parasites was related to the diets of the carnivores. In Chapter 4, the composition of the viruses in the feces of raccoon

dog, leopard cat, and Eurasian otter was identified by high-throughput sequencing. Furthermore, by linking the detected viruses with the diets of the three carnivores, we discussed whether the composition of the detected viruses was related to the diets of the carnivores.

The results of this study provide information on the ecology of wild carnivores through diet analysis, as well as information on the parasite and virus composition of each animal. Since a study applying high-throughput sequencing to analyze the diet, parasites, and viruses of three wild carnivores have not been properly conducted in Korea, this study is expected to provide comprehensive knowledge on relevant research fields. The results of this study can be used as a preemptive measure to prevent potential zoonotic diseases by providing information on zoonotic pathogens inherent in each wild animal. Moreover, since the leopard cat and the Eurasian otter are classified as endangered species in Korea [7], the result of this study can be used as basic knowledge for their protection. Overall, the results of this study can provide basic information on how to manage each wild animal in a healthy way and can ultimately contribute to 'One Health'.

Chapter 1. Using DNA metabarcoding and a novel canid-specific blocking oligonucleotide to investigate the composition of animal diets of raccoon dogs (*Nyctereutes procyonoides*) inhabiting the waterside area in Korea

1.1. Introduction

The raccoon dog (*Nyctereutes procyonoides*) is a medium-size canid native to East Asia including Korea [8], and was introduced to European countries in the first half of the 20th century [9]. In Korea, the raccoon dog is among one of extant carnivoran species, including the leopard cat, Eurasian otter, yellow-throated marten, and least weasel [10]. While many of these carnivorans are endangered in Korea [10] and classified in a vulnerable category according to the Korean Red List of Threatened Species [7], the raccoon dog maintains a decent population [11] due to its high genetic diversity, adaptability, versatility in feeding habits, and reduction in the population of its predators and competitors [12-15]. Its high adaptability has also been reported in other countries. For example, in Japan, raccoon dogs are known to inhabit urban forests, such as the Imperial Palace [16], in the middle of Tokyo, a crowded megacity. In order to elucidate the origin of its high environmental adaptability, it is necessary to further investigate into the details of its ecology and food habits.

The raccoon dog is opportunistically feeding on plants, invertebrates, fishes, amphibians, reptiles, birds, and small mammals [16-23]. Through such predation and feeding, the raccoon dog is thought to directly and indirectly affect the ecosystem [24, 25], and by investigating their predation and feeding behavior, we can gain better understandings of their involvement in ecosystem functions. To investigate their predation

and feeding habits, the fecal contents are often surveyed. To date, many studies have been reported on the fecal analysis of raccoon dogs in countries such as Belarus [17], Denmark [18], Finland [19, 20], Germany [21, 22], and Japan [16, 23, 26–28]. However, all of these studies rely on morphological observations of undigested remains of fruits, seeds, hair, skin, feathers, bones, and so on. Furthermore, the morphological observation is laborious and requires expertise in morphology and osteology of a broad range of organisms eaten by raccoon dogs. In addition, the morphological observation can be difficult if species or genus level identification is warranted due to the limited taxonomic resolution.

Recently, studies on fecal analysis for dietary investigations of wildlife using DNA barcoding have been reported (e.g., [29–35]). The method using DNA barcoding has the advantage that identification is objective and does not require morphological and osteological expertise. The dietary analysis using DNA barcoding is usually performed by extracting DNA from the collected fecal samples and analyzing the sequences of the extracted DNA. For carnivores, DNA markers such as vertebrate 12S rRNA gene [36] and invertebrate 16S rRNA gene [32] are targeted and sequenced. In addition, high-throughput sequencing (HTS) technologies have also been introduced for diet analyses of wildlife, such as bats in Finland [31], brown bears in Italy [32], Eurasian otters in Korea [33], and leopard cats in China [34] and Pakistan [35]. Due to its high sensitivity and ability to generate large amounts of genetic information, HTS can provide taxonomically more detailed information on dietary profiles of wildlife.

A caveat to the DNA barcoding-based method is that if the predator under investigation is a vertebrate and universal vertebrate primers are used for PCR amplification (for preparation of DNA libraries), the predator's DNA will also be amplified. This is problematic because, with the limited sequencing resource, the inclusion of DNA reads of the predator reduces the number of DNA

reads of prey animals, resulting in the reduction in sensitivity in the detection of prey animals. Therefore, it is better to suppress the PCR amplification of the predator's DNA. To selectively prevent the amplification of the unwanted predator's DNA, the method using blocking oligonucleotides was invented [37]. The blocking oligonucleotide is designed to anneal at the template site between the forward and reverse primers, and it can block amplification by modification by a hydrocarbon at its 3'-end (called C3 spacer) [38, 39]. The method using blocking oligonucleotides has been widely applied to dietary surveys of wildlife, such as brown bear [32], leopard cat [34, 35], and Eurasian otter [33]. However, as far as we notice, the blocking oligonucleotide for the raccoon dog has not been reported yet.

In this study, we aimed to 1) develop the blocking oligonucleotide for the raccoon dog, and 2) investigate diet profiles of raccoon dogs in Korea using HTS-based DNA metabarcoding. For DNA metabarcoding, we sequenced vertebrate 12S rRNA gene and invertebrate 16S rRNA gene. For sequencing vertebrate 12S rRNA gene, the developed blocking oligonucleotide was used. The use of DNA barcoding is expected to facilitate dietary research of wildlife, elucidate their roles and functions in ecosystems, and help protect them.

1.2. Materials and methods

1.2.1. Fecal samples

This study analyzed fecal samples of raccoon dogs, which were analyzed for zoonotic pathogens in our previous study [40]. Briefly, the samples were collected in a waterside area with reclaimed paddy fields in Seosan city in Chungcheongnam-do in Korea on May 21, 2017. The sampling area is close to an artificial freshwater lake named Ganwol-ho and surrounded by agricultural landscape. A total of 15 samples presumed to be raccoon dog feces were collected (S1.1 Fig and S1.1 Table). Of them, 11 samples were

confirmed to be those of raccoon dogs by the raccoon dog-specific PCR assay [41] performed in our previous study [40]. The remaining four samples were excluded from subsequent analysis. The samples were stored at −80℃ until DNA extraction. The samples were collected in a public area. Therefore, it was not necessary to obtain permission to collect samples.

1.2.2. DNA extraction

DNA was extracted from collected fecal samples by a PowerMax® Soil DNA Isolation Kit (Mobio Laboratory, Inc., Carlsbad, CA, USA). Each fecal sample was preliminary homogenized in 5 ml of ultra-pure water using a sterile wooden spatula [33]. About 0.2 g of each homogenized sample was added into a 2 ml tube of the DNA isolation kit with additional 300 mg of 0.1 mm diameter glass beads and 100 mg of 0.5 mm diameter glass beads [42]. The samples were further homogenized by a bead beater (BioSpec Products, Inc., Bartlesville, OK, USA) for 3 min. After homogenization, DNA from each fecal sample was extracted and purified by the kit's protocol and finally eluted into 50 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0). The eluted DNA was kept at −80℃ until subsequent analysis.

1.2.3. Design of the blocking oligonucleotide RacBlk

To block unwanted amplification of the raccoon dog's DNA by universal vertebrate primers targeting 12S rRNA gene [36], we designed a canid-specific blocking oligonucleotide called RacBlk according to the method of designing blocking oligonucleotides reported by Vestheim and Jarman [37]. RacBlk has a 3-carbon spacer at its 3'-end and is designed to bind to the raccoon dog's 12S rRNA gene and block its amplification (Table 1.1). Specifically, RacBlk is comprised of 27 nucleotides and the first to sixth nucleotides of the RacBlk overlap with the 12SV5F, which is the forward primer for amplification of 12S rRNA gene. In addition, it was designed to avoid binding to the DNA of prey of raccoon dogs. However, since the target region of RacBlk is complementary to the

sequences of other Canidae species such as *Canis lupus familiaris* (domestic dog) (Table 1.1), RacBlk also blocks amplification of their DNA. This was unavoidable due to the high sequence similarity of the target region of the 12S rRNA gene among species of Canidae. In Korea, four wild Canidae species have been documented: *Nyctereutes procyonoides* (raccoon dog), *Vulpes vulpes* (red fox), *Cuon alpinus* (dhole), and *Canis lupus* (Eurasian wolf) [10]. However, with the exception of the raccoon dog, these animals are highly endangered or perhaps extinct in South Korea [10]. Therefore, the chance of interaction between raccoon dogs and these canids is low. The interaction between raccoon dogs and domestic dogs may not be impossible, but due to size relationships and lifestyle differences, it is highly unlikely that raccoon dogs prey on domestic dogs.

Table 1.1. Sequence of the blocking oligonucleotide RacBlk. The 12S rRNA gene sequences of raccoon dog (*Nyctereutes procyonoides*) and its related species and potential prey were aligned with RacBlk.

Accession number	Species (common name)	Starting position	Sequence (5′–3′) [a]																										End position
RacBlk [b]			C	T	C	T	A	G	A	G	G	G	A	T	A	T	A	A	A	G	C	A	C	C	G	C	C	A	A	
KF709435.1	*Nyctereutes procyonoides koreensis* (raccoon dog)	617	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	591
NC_002008.4	*Canis lupus familiaris* (domestic dog)	617	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	591
NC_008434.1	*Vulpes vulpes* (red fox)	617	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	591
NC_013445.1	*Cuon alpinus* (dhole)	617	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	591
NC_012374.1	*Rattus rattus* (black rat)	619	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	593
NC_027932.1	*Micromys minutus* (harvest mouse)	614	·	·	·	·	·	·	C	A	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	588
NC_016428.1	*Apodemus agrarius* (striped field mouse)	616	·	·	·	·	·	·	C	A	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	590
NC_007236.1	*Gallus gallus gallus* (chicken)	1868	·	·	·	·	·	·	C	A	·	·	C	·	A	·	C	C	C	·	·	·	·	·	·	·	·	·	·	1842
NC_006291.1	*Carassius carassius* (crucian carp)	1543	·	·	·	·	·	·	C	·	·	·	·	·	G	·	C	·	C	T	·	·	·	·	·	A	·	·	C	1517
NC_015806.1	*Silurus asotus*	616	·	·	·	·	·	·	C	A	·	·	C	·	G	·	C	·	C	T	·	·	·	·	·	·	·	·	·	590

	(Amur catfish)																													
KM590550.1	*Rana coreana* (Korean brown frog)	603	·	·	·	·	·	·	A	·	·	·	C	·	C	C	C	·	G	T	·	G	C	A	G	T	T	·	A	577
KT878718.1	*Pelophylax nigromaculatus* (dark-spotted frog)	3009	·	·	·	·	·	·	C	A	·	·	C	·	C	C	C	·	G	T	·	G	C	A	G	T	T	C	A	2983

[a] The dots represent the complementary base types of the corresponding RacBlk bases.

[b] The 1st to 6th nucleotides (underlined) of RacBlk are designed to overlap the forward primer 12SV5F, which targets the 12S rRNA gene of vertebrates.

1.2.4. High-throughput DNA sequencing

For each sample, three libraries were constructed and meta-genetically analyzed. The three libraries are: (1) a library prepared with primers that target the vertebrate 12S rRNA gene with RacBlk, (2) a library prepared with primers that target the vertebrate 12S rRNA gene without RacBlk, and (3) a library prepared with primers that targets the invertebrate 16S rRNA gene. For vertebrates, 12S rRNA gene was amplified with primers 12SV5F and 12SV5R [36]. The vertebrate 12S rRNA libraries were constructed with and without the blocking oligonucleotide RacBlk. Technical duplicates were also prepared for six libraries constructed with RacBlk to test for reproducibility. For invertebrates, 16S rRNA gene was amplified with primers 16SMAV-F and 16SMAV-R [32]. Furthermore, MamMAVB1 was also included in PCR targeting the 16S rRNA gene for blocking amplification of mammal's DNA [32]. DNA sequencing was performed on an Illumina MiSeq system (Illumina, Inc., San Diego, CA, USA).

For vertebrate-specific PCR, 50 μl of each PCR reaction mixture consisted of 25 μl of Premix Taq™ (Takara Bio Inc., Otsu, Shiga, Japan), 0.1 μM of each of universal vertebrate primers attached to Illumina adapter sequences, 5 μM of RacBlk, 12 μl of molecular-graded water, and 1 μl of the DNA extract. PCR thermal condition was comprised of initial denaturation for 15 min at 95℃, followed by 45 cycles of 30 s at 95℃ and 30 s at 60℃. There was no elongation step [35]. Four concentrations of RacBlk at 2, 3, 4, and 5 μM were pretested and PCR amplification of raccoon DNA was inhibited at all of these tested concentrations (S1.2 Fig). To maximize the inhibition efficiency, 5 μM was chosen as a RacBlk concentration.

For invertebrate-specific PCR, 50 μl of PCR reaction mixture consisted of 25 μl of Premix Taq™ (Takara Bio), 0.2 μM of each of universal invertebrate primers attached to Illumina adapter sequences, 2 μM of MamMAVB1, 12 μl of molecular-graded

water, and 1 μl of the DNA extract. PCR thermal condition was comprised of initial denaturation for 15 min at 95℃, followed by 55 cycles of 30 s at 95℃ and 30 s at 55℃. There was no elongation step [32].

The resulting PCR products were purified by AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA). Next, index PCR was performed with Nextera XT Index kit (Illumina) to tag DNA libraries. The thermal cycle of index PCR comprised of 3 min at 95℃, followed by 8 cycles of 30 s at 95℃, 30 s at 55℃, and 30 s at 72℃, and final extension for 5 min at 72℃. The tagged libraries were purified again with AMPure XP beads (Beckman Coulter). The tagged and purified libraries were quantified and normalized by Quant-iT PicoGreen dsDNA reagent kit (Life Technologies, Carlsbad, CA, USA). The normalized libraries were loaded with 30% PhiX to a v3 600 cycle-kit reagent cartridge (Illumina) for 2 × 300 bp paired-end sequencing on an Illumina MiSeq system.

1.2.5. Sequencing processing and analysis

The adapter and tagging sequences for MiSeq were trimmed and the reads with quality scores below 20 were removed by MiSeq Reporter version 2.5 (Illumina). Then, ambiguous bases were trimmed by Trimmomatic v 0.33 [43]. Next, OBITools [44] was used for finding unique sequences and taxonomic assignment. The *illuminapairedend* command of OBITools was used to concatenate the paired-end forward and reverse reads. The *obiuniq* command was executed to group and dereplicate the resultant reads. The cut-off for read length was set to 80 bp for the 12S rRNA gene reads and 20 bp for the 16S rRNA gene reads by the *obigrep* command. The erroneous reads were further excluded by the *obiclean* command. After quality control of sequencing reads, the resultant sequencing reads were taxonomically assigned by the *ecotag* command against the custom 12S and 16S rRNA gene reference databases. Specifically, the taxonomic annotation was performed against 12S (vertebrate) and 16S (invertebrate) rRNA

genes databases generated from the latest snapshot (updated on March 13, 2022) of EMBL nucleotide sequences (http://ftp.ebi.ac.uk/pub/databases/ena/sequence/snapshot_latest/std/).

1.2.6. Statistical analysis

The statistical analysis was performed to evaluate the blocking efficacy of RacBlk on R version 4.1.0 with the phyloseq package [45] and vegan package [46]. First, we compared the number of sequence reads identified as the family Canidae (to which the raccoon dog belong) with and without RacBlk for each sample. Second, we evaluated about unintended inhibition by RacBlk. The use of blocking oligonucleotide may inhibit amplification of DNA of prey animals, resulting in change in dietary proportion measured. Therefore, we evaluated the change in dietary proportion due to the use of RacBlk. Specifically, the differences in composition of unique sequences from prey animals were compared with and without RacBlk. To analyze the composition of prey animals, the reads that were identified as Canidae were excluded. The remaining reads were rarefied into 120 reads per library, which was the smallest number of sequence reads from prey animals (excluding reads from Canidae) found in the library prepared without RacBlk. Rarefaction was necessary because the number of sequence reads from prey animals (excluding reads from Canidae) was much smaller in libraries prepared without RacBlk than those with RacBlk, and the comparison had to be done under the same condition, i.e., the same number of sequences. Note that rarefaction was performed only for the purpose to compare the sequencing results with and without RacBlk. No rarefaction was performed in other analyses. Based on the rarefied libraries, the difference in composition of unique sequences from prey animals were analyzed. The differences were characterized based on the Bray–Curtis dissimilarity (community structure) and Jaccard index (community membership). To compare the difference in composition of unique sequences from prey animals detected with and without RacBlk, the adonis2 function

in vegan package was used for performing permutational multivariate analysis of variance (PERMANOVA). The intra-sample and inter-sample variances of composition of unique sequences from prey animals were compared based on the beta dispersion calculated by the betadisper function in vegan package. The intra-sample variance was defined as the variance of the composition measured with and without RacBlk for the same sample, while the inter-sample variance was defined as the variance of composition between samples measured with (or without) RacBlk. Kruskal-Wallis rank sum tests and *post hoc* Wilcoxon rank-sum tests were used to compare the differences between the intra- and inter-sample variances.

1.3. Results

1.3.1. Sequencing statistics

From a total of 11 raccoon dog's fecal samples, 11 pairs of vertebrate libraries with and without RacBlk were obtained. Additionally, six vertebrate libraries prepared with RacBlk were technically duplicated. For invertebrates, one sample was not PCR amplified, resulting in a total of 10 libraries (S1.2 Table). From 38 libraries including duplicates, a total of 4,676,053 high-quality sequence reads were obtained, consisting of 1,367,071 reads of vertebrates without RacBlk, 2,513,355 reads of vertebrates with RacBlk, and 795,627 reads of invertebrates.

1.3.2. Performance of the blocking oligonucleotide RacBlk

Fig 1.1A shows relative abundance of vertebrates identified at the family level. Without RacBlk, the median relative abundance of Canidae was 99.0%, ranging from 51.6% to 99.9%, suggesting that most of the sequence reads were of DNA from the host animal (i.e., raccoon dog). With RacBlk, the median relative abundance of Canidae read was 55.8%, ranging from 5.3% to 72.9%, indicating that RacBlk inhibited amplification of DNA of the host animal. The reduction was statistically significant ($p < 0.001$; Wilcoxon rank-

sum tests; Fig 1.1B). It was also confirmed that there was no significant difference in the community structure of prey animals detected with and without RacBlk ($p > 0.05$; PERMANOVA; Fig 1.1C), and that the variance of the prey structure measured with and without RacBlk for the same sample (intra-sample variance) was significantly smaller than the variance of the structure between samples measured with (or without) RacBlk (inter-sample variance) ($p < 0.0001$; Wilcoxon rank-sum test; Fig 1.1D). The similar results were obtained for the community membership (Fig 1.1E and 1.1F). These suggest that the addition of RacBlk did not significantly change the composition of the detected prey animals. In addition, we compared the relative abundance of specific prey genera measured with and without RacBlk (Fig 1.1G). The differences measured with and without RacBlk were within an order of magnitude for all genera (Fig 1.1H). The result of reproducibility of sequencing with RacBlk based on technical duplicates is shown in S1.3 Fig. The result shows high reproducibility of sequencing since the intra-sample variability was significantly smaller than the inter-sample variability.

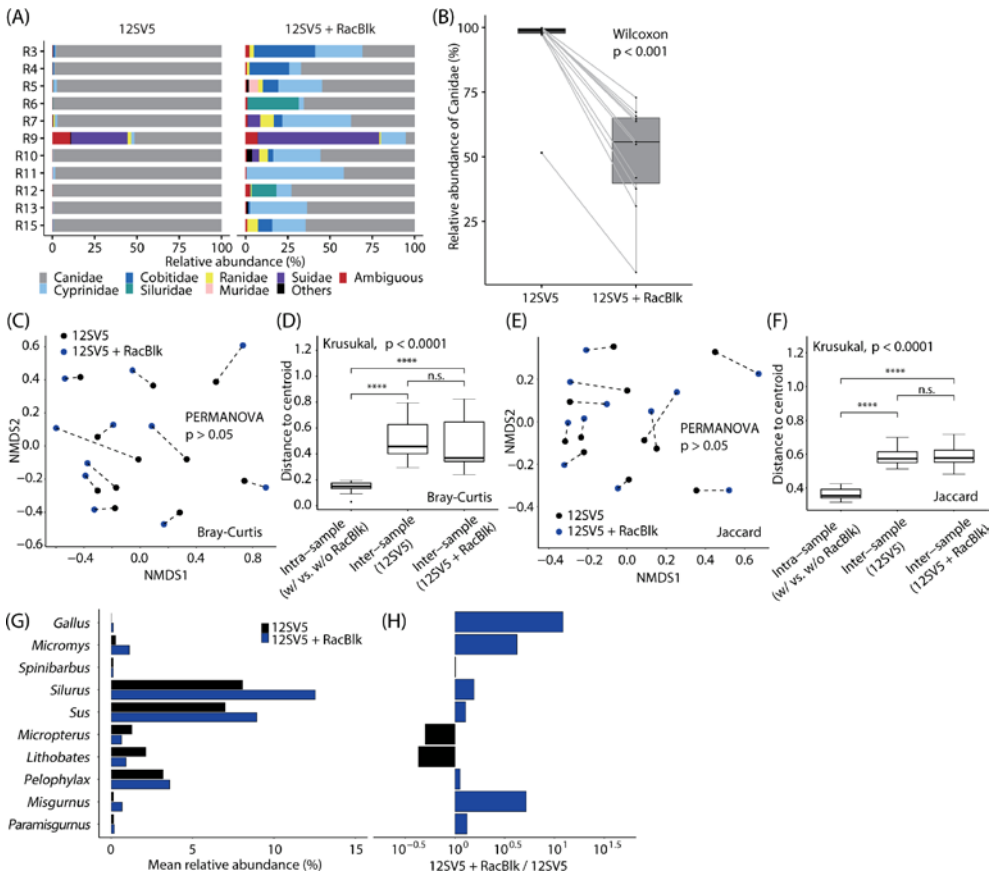


Fig 1.1. Performance of blocking oligonucleotide RacBlk. The 12S rRNA gene libraries prepared with the universal vertebrate primer pair 12SV5 are compared with and without RacBlk. (A) Relative abundance of vertebrates identified at the family level. (B) Relative abundance of Canidae to which the raccoon dog belongs. (C) Non-metric multidimensional scaling (NMDS) plot showing the Bray–Curtis dissimilarity of community structure of prey animals with and without RacBlk. (D) Boxplot showing the intra- and inter-sample variances of the Bray–Curtis dissimilarity of prey composition. (E) NMDS plot showing the Jaccard index of community membership of prey animals with and without RacBlk. (F) Boxplot showing the intra- and inter-sample variances of the Jaccard index of prey membership. (G) Mean relative abundances of the top 10 vertebrate genera detected with and without RacBlk. The reads identified as sequences of Canidae were excluded from the calculation of relative abundance. (H) Ratio of mean relative abundances measured with and without RacBlk. In the panels (C) and (E), the data from the same sample were connected by a line. In the panels (D) and (F), the four asterisks (****) represent $p < 0.0001$ by the *post hoc* Wilcoxon rank-sum test. The abbreviation “n.s.” represents that there is no significant difference.

1.3.3. Vertebrate composition

Fig 1.2A shows mean relative abundance of vertebrates at the family level as measured by 12S rRNA gene sequencing. The majority of vertebrate diets that were eaten by raccoon dogs in our sampling site was found to be freshwater fishes, with the families such as Cyprinidae (carp or minnows) (50.2%), Cobitidae (true loaches) (17.0%), and Siluridae (catfishes) (13.0%). Additionally, the families Ranidae (true frogs) (5.5%), Suidae (pigs or boars) (9.0%), and Muridae (rodents) (1.2%) were identified. Although most of the sequence reads identified as Cyprinidae and Cobitidae were ambiguous at the genus level, *Spinibarbus* (cyprinid fishes), *Misgurnus* (true loaches), and *Paramisgurnus* (large-scale loaches) were identified (Fig 1.2B). Additionally, *Silurus* (catfishes) and *Micropterus* (black bass) were identified from other fish families. Most of the sequence reads of Ranidae were classified into *Pelophylax* (green frogs) or *Lithobates* (true frogs). The mammalian genera such as *Sus* (domestic pig or wild boar) and *Micromys* (harvest mouse) were also identified.

1.3.4. Invertebrate composition

Fig 1.2C shows mean relative abundance of invertebrates at the family level as measured by 16S rRNA gene sequencing. More than half of the sequence reads were identified at the family level. Some of the detected families were aquatic, such as Cyprididae (freshwater ostracods) (12.6%), Plumatellidae (freshwater bryozoans) (9.5%), and Moinidae (water fleas) (3.2%). Others included terrestrial organisms such as Drosophilidae (flies) (15.5%), Gryllotalpidae (mole crickets) (10.2%) and Tenebrionidae (darkling beetles) (5.1%). The sequence reads identified as Tenebrionidae and Plumatellidae were ambiguous at the genus level. However, the sequence reads identified as other families were identified down to the genus level (Fig 1.2D). For instance, aquatic genera such as *Heterocypris* (freshwater ostracods) and *Moina* (water fleas) were identified. The genera of insects such as *Gryllotalpa* (mole crickets), *Orthetrum* (dragonflies), *Micronecta* (water boatmen), *Eurycotis*

(Florida woods cockroach), and *Labidura* (earwigs) were also identified. *Drosophila* (flies) was also abundantly detected in many samples.

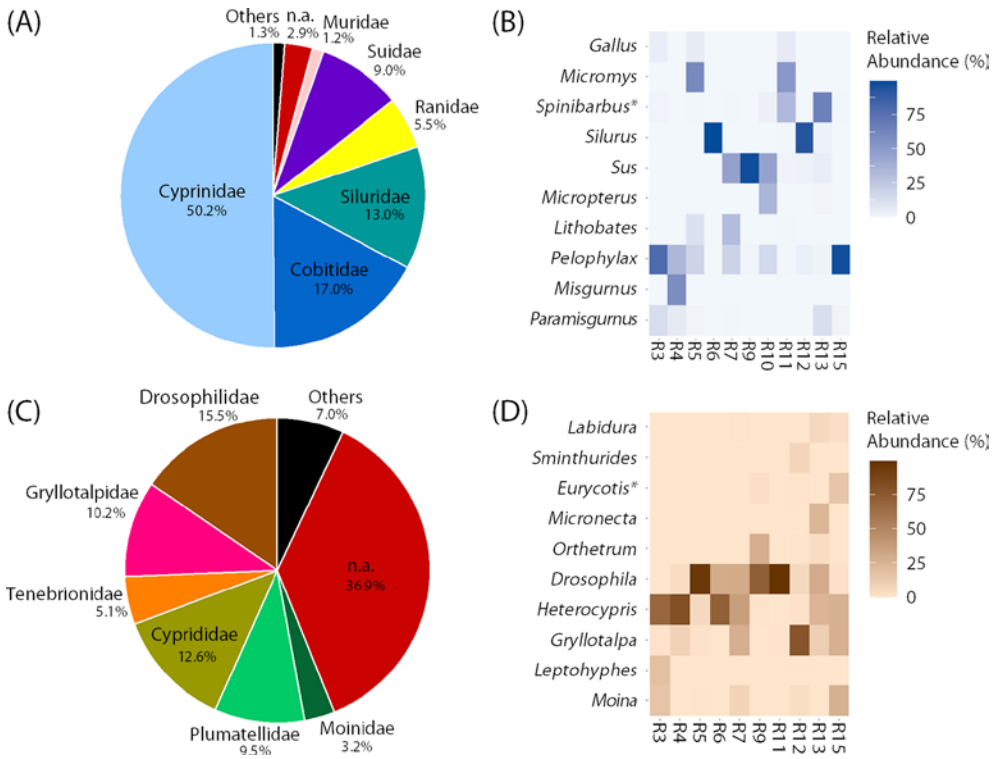


Fig 1.2. Dietary composition of raccoon dogs. (A) Mean relative abundance of vertebrates at the family level as measured by 12S rRNA gene sequencing. The data obtained using the blocking oligonucleotide RacBlk are shown. The sequence reads identified as the family Canidae were excluded from the calculation for relative abundance. (B) Relative abundance of the 10 most abundant vertebrate genera detected by 12S rRNA gene sequencing with RacBlk. The unidentified reads at the genus level and genera belong to the family Canidae were excluded from the calculation for relative abundance. (C) Mean relative abundance of invertebrates at the family level as measured by 16S rRNA gene sequencing. The sequence reads assigned to non-targeted organisms such as bacteria and vertebrates were excluded from the calculation for relative abundance. (D) Relative abundance of the 10 most abundant invertebrate genera detected by 16S rRNA gene sequencing. The unidentified reads at the genus level and reads assigned to non-targeted organisms such as bacteria and vertebrates were excluded from the calculation for relative abundance. The abbreviation “n.a.” represents the ambiguous reads were not classified into specific family. The detection of *Spinibarbus* and *Eurycotis* (with *) may be misidentification (see text for details).

1.4. Discussion

In this study, we used DNA metabarcoding of the invertebrate 16S rRNA gene and vertebrate 12S rRNA gene with the newly developed blocking oligonucleotide RacBlk to study dietary composition of raccoon dogs inhabiting a coastal area with paddy fields in Korea in late spring. We found that vertebrate diets of raccoon dogs mainly consisted of aquatic species such as freshwater fishes, frogs, and aquatic arthropods and fossorial and terrestrial insects in our study area. The results seem reasonable given the geographical characteristics of the study area. In addition, the results seem to be consistent with previous studies showing that raccoon dogs are generalists who eat a wide range of diets available in their habitat [17, 18, 21–23, 47].

1.4.1. Fishes are dominant prey for raccoon dogs inhabiting waterside areas

The organisms identified from fecal samples of raccoon dogs in this study were in congruence with the fauna reported in South Korea. For example, vertebrate 12S rRNA gene sequencing identified freshwater fishes, such as *Silurus*, *Micropterus*, *Misgurnus*, and *Paramisgurnus*, and frogs, such as *Pelophylax* and *Lithobates*, at the genus level (Fig 1.2B). Notably, fishes represented 80.2% of vertebrate DNA at the familly level if combining Cyprinidae (50.2%), Cobitidae (17.0%), and Siluridae (13.0%). In Korea, two species of *Silurus*, i.e., *Silurus asotus* (Amur catfish) and *Silurus microdorsalis* (slender catfish), are known to inhabit rivers and lakes [48–51]. Similarly, *Misgurnus* species, such as *Misgurnus anguillicaudatus* (pond loach) and *Misgurnus mizolepis* (mud loach), and *Paramisgurnus* species, such as *Paramisgurnus dabryanus*, a senior synonym of *M. mizolepis*, are common in Korea [52]. Additionally, *Micropterus salmoides* (largemouth bass) is known to be widely distributed in freshwater systems of South Korea [49, 51, 53]. Previous studies reported [17–22, 26] that fishes are regarded as one of important food sources for raccoon dogs especially in

waterside areas (i.e., coast and/or lake areas) although these studies did not identify fish taxa due to morphological observations. Our result obtained in the coastal area in Korea is in line with the tendency reported by the existing studies conducted in other countries. Species of frogs such as *Pelophylax nigromaculatus* (dark-spotted frog) and *Lithobates catesbeianus* (= *Rana catesbeiana*) (American bullfrog) are also commonly observed in Korea [54, 55]. For instance, the dark-spotted frog inhabits freshwater such as ponds around rivers, lakes, swamps and rice paddies [56]. Studies in Finland [19, 20] and Germany [22] reported Rana spp. as prey for raccoon dogs. Moreover, it has been recently reported that raccoon dogs are hunting the American bullfrog [57].

Mammals such as *Sus* (wild boar) and *Micromys* (harvest mouse) were also detected by vertebrate 12S rRNA gene sequencing (Fig 1.2B). These mammalian species are known to inhabit South Korea [58-60]. For instance, the population of wild boar (Sus scrofa) is known to be rapidly increased from the 2010s even threatening urban livings in Korea [58, 59]. Although the raccoon dog is thought to be unable to directly hunt animals larger than themselves, such as wild boars and deer, they have been reported to consume their carcasses [17, 21, 22]. The scavenging behavior of raccoon dogs is known to be especially noticeable in winter [17]. Meanwhile, this study was conducted in late spring. The smaller proportion of the family Suidae (9.0%) than those of fish families Cyprinidae (50.2%), Cobitidae (17.0%), and Siluridae (13.0%) (Fig 1.2A) may reflect this seasonal trend. The family Muridae represents 1.2% of vertebrate DNA (Fig 1.2A), and *Micromys* was identified at the genus level (Fig 1.2B). Some of murid species are reported as dietary items of raccoon dogs in Denmark [18] and Finland [19].

Invertebrate 16S rRNA gene sequencing identified *Gryllotalpa* as one of the most abundant invertebrate genera (Fig 1.2D). In Korea, *Gryllotalpa orientalis* (mole cricket) is known to inhabit [61]. The

mole cricket is known to inhabit wetlands and paddy fields by burrowing [62], and was reported to be predated by raccoon dogs by morphological observation of fecal contents in a Japanese study [28]. Furthermore, Tenebrionidae that belong to the order Coleoptera (beetles) was identified at the family level (Fig 1.2C). In line with the result of previous studies, the order Coleoptera were reported as one of main prey for raccoon dogs [17, 21, 22, 28, 63] and inhabited in diverse environments [64, 65]. Tenebrionidae is regarded as one of diverse groups in the order Coleoptera. In Korea, 129 species of Coleoptera are known to inhabit [66–68]. We also identified *Orthetrum*, *Micronecta*, and *Labidura* (Fig 1.2D). Several species belonging to those genera were reported to inhabit Korea [69–71]; however, knowledge about whether raccoon dogs feed on them is limited. Additionally, 16S rRNA gene sequencing detected small-size invertebrates such as *Heterocypris* and *Moina*. Due to their small size, these organisms are unlikely to be directly preyed on by raccoon dogs. Perhaps it was due to secondary predation, and they are thought to be accidentally swallowed or attached to larger aquatic organisms that were preyed on by raccoon dogs.

Overall, we found that the most dominant food for raccoon dogs is fishes at our sampling site located in the coastal area of Korea, representing 80.2% of vertebrate DNA. The family Ranidae was also abundant (5.5%) (Fig 1.2A). These results indicate that readily available freshwater fishes and frogs, rather than small mammals such as mice, are preferentially consumed by raccoon dogs in our study area and season. This seems to be in line with existing knowledge. For example, previous studies reported that raccoon dogs eat other animals, such as birds and amphibians, when small mammals are not readily available [19, 21]. Among the fishes detected, the family Cyprinidae was the most abundant and accounted for 50.2% of vertebrate DNA (Fig 1.2A), suggesting that they are main prey for raccoon dogs in our survey area. In Korea, Cyprinidae species, such as *Cyprinus carpio* (common carp) and *Carassius auratus* (crucian carp), abundantly exist [49–51]. We

speculate that the detection of *Spinibarbus* at the genus level (Fig 1.2B) may be misidentification of *Cyprinus* and *Carassius* because *Spinibarbus* is known to inhabit East Asia [72] but has not been reported in South Korea. Indeed, the sequence of target region of *Spinibarbus* is identical or similar to the sequences of other Cyprinidae species (S1.4 Fig), indicating the possibility of misidentification between species of Cyprinidae. Similarly, we identified *Eurycotis* by invertebrate 16S rRNA gene sequencing (Fig 1.2D). However, there have been no reports that *Eurycotis* inhabit Korea. Therefore, we surmise that sequence similarity caused a misidentification between *Eurycotis* and the indigenous cockroach *Periplaneta* (S1.5 Fig).

1.4.2. Blocking oligonucleotide RacBlk reduces PCR amplification of the raccoon dog's DNA

In wildlife dietary surveys using PCR, not only preys' DNA but also the predator's DNA can be possibly amplified, which is problematic because it can reduce the detection sensitivity of the preys' DNA. To alleviate this problem, the idea of using the blocking oligonucleotide, which selectively inhibits PCR amplification of a predator's DNA, was invented [37]. Blocking oligonucleotides have been widely used in dietary studies of wildlife such as the leopard cat [35], Eurasian otter [33], Antarctic krill [37], brown bear [32], penguin [73], and seal [74]. In this study, we designed the blocking oligonucleotide RacBlk for the raccoon dog. RacBlk has successfully reduced the amplification of the canid DNA in PCR that targeted the vertebrate 12S rRNA gene (Fig 1.1A and 1.1B). Furthermore, we could confirm that there was no significant change in dietary composition with and without RacBlk (Fig 1.1C and 1.1E), indicating that unintended inhibition of the amplification of preys' DNA was insignificant.

The blocking oligonucleotide RacBlk significantly reduced PCR amplification of DNA of Canidae, but the inhibition was not complete. In fact, 5.3%–72.9% sequence reads were those of Canidae even

with RacBlk (Fig 1.1A and 1.1B). The reduction seems to be lower than the reduction with blocking oligonucleotides reported by previous studies. For example, blocking oligonucleotides for the leopard cat and Eurasian otter developed by previous studies reported reducing the predators' DNA down to 0% and 0.21%, respectively [33, 35]. The lower reduction efficiency may be due to the shorter length of RacBlk (27 nt) than other blocking oligonucleotides (40–50 nt). In fact, the fact that longer blocking oligonucleotides are associated with higher blocking efficiencies has been reported by previous studies [75, 76]. We do not know the mechanism behind, but one possible explanation is that a short-length blocking oligonucleotide allows annealing and extension of the universal primer to the target region of the blocking oligonucleotide before the blocking oligonucleotide is annealed to it, resulting in amplification of the target organism (which should be blocked) by the universal primers. The short length of RacBlk was unavoidable due to the limited 12S rRNA gene site with sequence specific to the raccoon dog. In addition, due to the limited targeting site, the selection of the region whose sequence is identical or similar to those of other species of Canidae was unavoidable (Table 1.1). However, we don't expect this to be a problem. As mentioned above, three other species of Canidae have been reported in Korea, i.e., *Vulpes vulpes*, *Cuon alpinus*, and *Canis lupus* (Eurasian wolf) [10]. However, it is highly unlikely that they inhabit the study area because they are highly endangered or extinct in South Korea [10]. We also anticipate that the interaction between raccoon dogs and domestic dogs (*Canis lupus familiaris*) is unlikely because of difference in lifestyle. However, it may not be impossible. Future research needs to elucidate their relationships.

1.4.3. Limitations of DNA metabarcoding in dietary surveys for omnivore animals

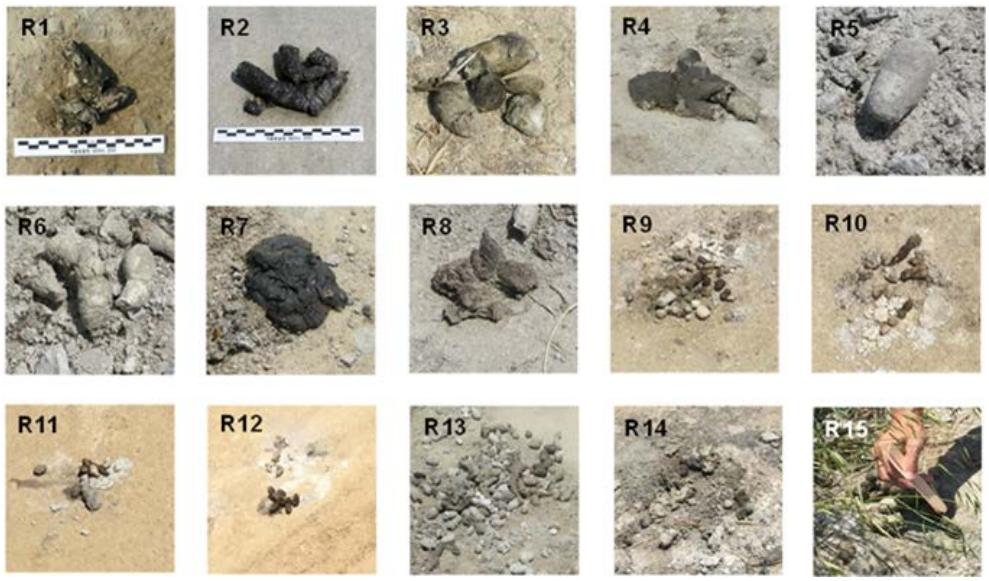
DNA barcoding is a useful tool for wildlife dietary research. However, it is not without problems, as pointed out elsewhere (e.g., [33, 77]). We recognize that this study also has such limitations. In

particular, we would like to acknowledge that there is ongoing argument over whether DNA-based methods can accurately quantify the proportion of dietary content [78-80]. This study reported relative abundance rather than detected or undetected since the information of relative abundance also includes the information of detected or undetected and may provide some insights such as detection specificity and sensitivity (or insensitivity) of the sequencing method used. In addition, there may be limitations specific to dietary analysis of omnivorous animals such as raccoon dogs. First, the raccoon dog is an omnivorous carnivore that also eats plants [17, 18, 21-23, 47]. However, this study focused on the animal diets and does not report the plant diets. In our preliminary analysis, large amounts of Pinaceae DNA (~80% of total reads) was detected in fecal samples (S1.6 Fig). We suspect that the detection of Pinaceae plants is due to the contamination by pollen since it is known that massive amounts of pine pollen are dispersed in spring in Korea [81]. Similarly, the detection of large amount of *Drosophila* might be due to their infestation on feces after defecation. Further validation is required for these issues of post-defecation contamination. Second, DNA metabarcoding does not allow inter-phylum comparison of abundance of vertebrates and invertebrates. Similarly, it does not allow inter-kingdom comparison of abundance of animals and plants. The comparison has to be limited to within the phylum or kingdom. For the comparison on the same scale between phyla or between kingdoms, methods such as metagenomic sequencing are required. Lastly, there may be the lack of taxonomic resolution of the selected DNA marker, primer pair, and/or reference database depending on the detected organisms. In our study, large amounts of fishes belonging to Cyprinidae and Cobitidae were detected (Fig 1.2A); however, many of them were ambiguous at the genus level. In this study, we used the primer pair 12SV5F and 12SV5R targeting 12S rRNA gene of vertebrates, which is widely used to identify common vertebrate species [36, 82]. For omnivorous opportunists such as the raccoon dog, it is difficult to predict the

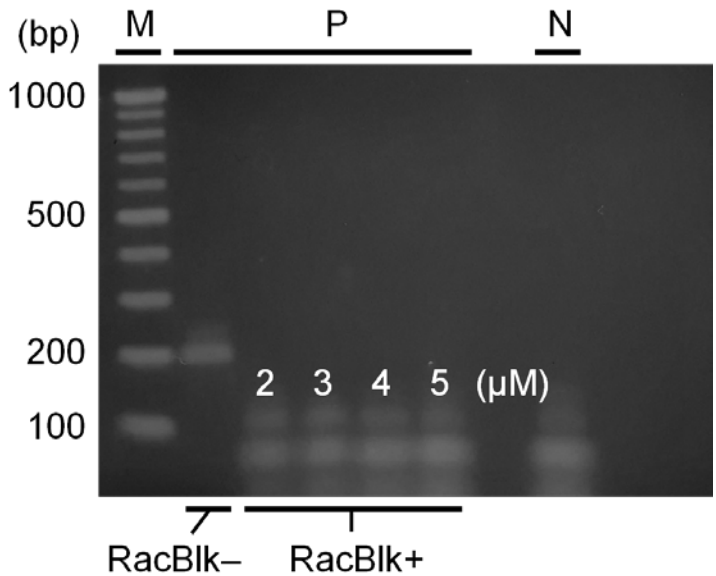
type of foods in advance, making it difficult to preselect the most appropriate sequencing strategy (e.g., target gene and primer pair). One way to alleviate this problem is to target multiple DNA markers and/or use multiple primer pairs for a single group of organisms. However, this should be decided according to the taxonomic resolution required and the sequencing resources available.

1.5. Conclusion

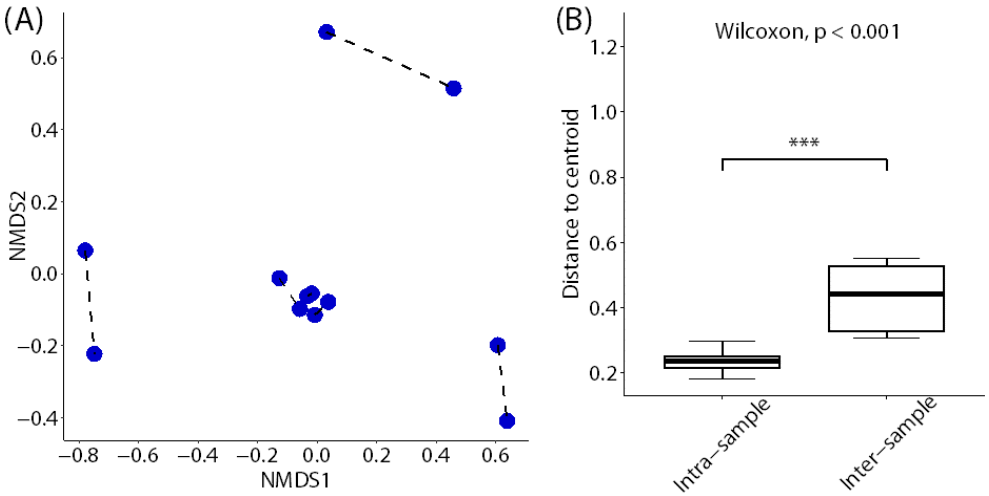
Using DNA metabarcoding and a canid-specific blocking oligonucleotide developed in this study, we identified that the main foods of raccoon dogs inhabiting the waterside of paddy fields in Korea were fishes such as Cyprinidae and insects such as mole crickets. The results seem reasonable in light of the Korean fauna and their well-known opportunistic feeding behaviors. The raccoon dog, which is relatively abundant in Korea, is known not only to play a role in the ecosystem, but also to be a reservoir of zoonotic pathogens [83]. Therefore, understanding their ecology is essential not only for conservation biology but also for public health. Understanding their feeding habits helps to understand their ecology. As a method to investigate their feeding habits, this study presented the baseline information on DNA metabarcoding, which does not require specialized knowledge such as osteology. By using convenient and objective DNA barcoding, the dietary habits and ecology of raccoon dogs are expected to be better understood by future research.



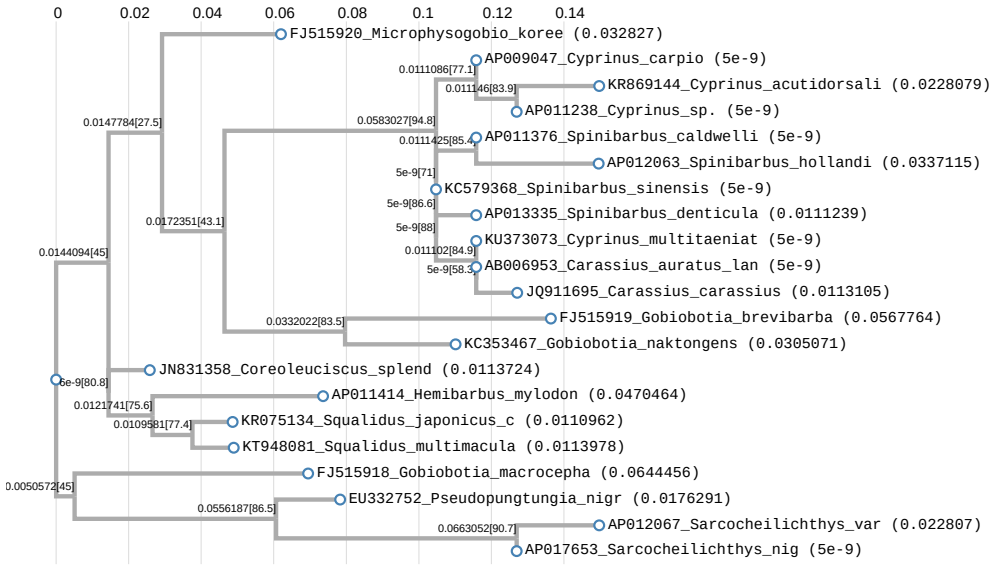
S1.1 Fig. Fecal samples of raccoon dogs. The samples were collected from the Seosan reclaimed paddy fields in Seosan city in Chungcheongnam-do, South Korea.



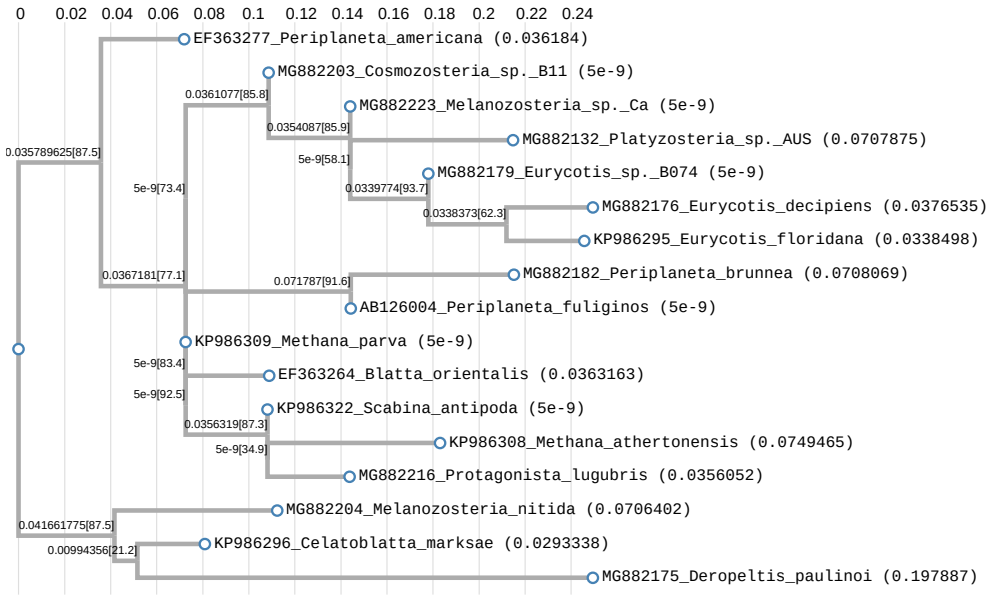
S1.2 Fig. Vertebrate-specific PCR assay with and without the blocking oligonucleotide RacBlk. Standard DNA extracted from a raccoon dog tissue sample was used as a template, and four concentrations of RacBlk of 2, 3, 4, and 5 μM were tested. Abbreviations: M, DNA marker; RacBlk–, without RacBlk, RacBlk+, with RacBlk; P, positive control; and N, negative control.



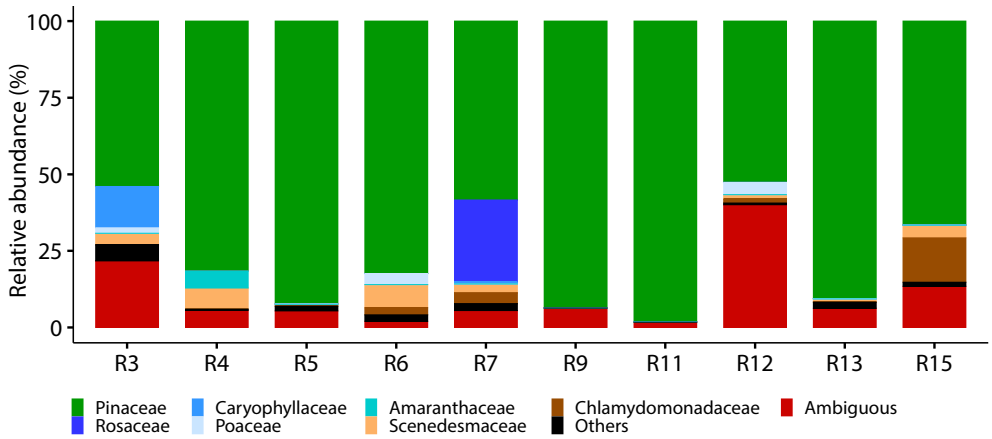
S1.3 Fig. Reproducibility of dietary composition characterized by technical duplicates. The data obtained by the vertebrate 12S rRNA sequencing with RacBlk are shown. (A) Non-metric multidimensional scaling (NMDS) plot showing the Bray–Curtis dissimilarity of composition of prey animals. The data from the same sample are connected by a line. (B) Boxplot showing the intra- and inter-sample variances of prey composition. The three asterisks (***) represent $p < 0.001$ by the Wilcoxon rank-sum test.



S1.4 Fig. Comparison of sequence similarity among species of the family Cyprinidae. All of sequences of Cyprinidae species belonging to the genera *Cyprinus*, *Carassius* and *Spinibarbus* and the genera that are known to be endemic in Korea were retrieved and aligned. For the alignment, the sequences were trimmed to the targeted amplified region of primers 12SV5F and 12SV5R. Multiple sequence alignment was performed by CLUSTALW (https://www.genome.jp/tools-bin/clustalw) with slow and accurate pairwise alignment method and default parameter settings. The FastTree method was used.



S1.5 Fig. Comparison of sequence similarity among species of the family Blattidae. All of sequences of species belonging to the familly Blattidae were retrieved from the reference database and aligned. For the alignment, the sequences were trimmed to the targeted amplified region of primers 16SMAV-F and 16SMAV-R. Multiple sequence alignment was performed by CLUSTALW (https://www.genome.jp/tools-bin/clustalw) with slow and accurate pairwise alignment method and default parameter settings. The FastTree method was used.



S1.6 Fig. Relative abundance of plants identified at the family level. The data were obtained by sequencing the internal transcribed spacer 2 (ITS2) region with universal plant-specific primers ITS-p3 and ITS-u4 [84]. The analyses were performed mainly with USEARCH v.11.0.667 [85] according to the previously reported method [86]. Briefly, taxonomic assignment was performed by SINTAX algorithm of USEARCH with 0.5 of a cutoff value [87] against the ITS2 database [88, 89]. Sequencing statistics and accession number of raw sequences are listed in S1.3 Table.

S1.1 Table. Metadata of fecal samples.

Sample ID	Sampling date	Sampling location		Origin [a]
	YYYY/MM/DD	Latitude	Longitude	
R3	2017/05/21	36°40'34.80"N	126°28'10.85"E	Raccoon dog
R4	2017/05/21	36°40'07.82"N	126°28'40.65"E	Raccoon dog
R5	2017/05/21	36°39'59.61"N	126°28'46.67"E	Raccoon dog
R6	2017/05/21	36°40'18.71"N	126°29'13.25"E	Raccoon dog
R7	2017/05/21	36°40'15.01"N	126°30'25.57"E	Raccoon dog
R9	2017/05/21	36°39'39.54"N	126°27'49.62"E	Raccoon dog
R10	2017/05/21	36°39'41.34"N	126°27'55.72"E	Raccoon dog
R11	2017/05/21	36°39'45.32"N	126°28'08.19"E	Raccoon dog
R12	2017/05/21	36°39'47.84"N	126°28'16.84"E	Raccoon dog
R13	2017/05/21	36°37'50.97"N	126°29'28.88"E	Raccoon dog
R15	2017/05/21	36°37'44.85"N	126°28'37.22"E	Raccoon dog
R1	2017/05/21	36°42'40.86"N	126°28'14.45"E	n.a.
R2	2017/05/21	36°40'48.54"N	126°27'48.91"E	n.a.
R8	2017/05/21	36°40'56.67"N	126°29'49.92"E	n.a.
R14	2017/05/21	36°37'48.87"N	126°28'48.62"E	n.a.

[a] The origin of fecal samples were identified by the raccoon dog specific PCR assay.

Abbreviation: n.a., not amplified by the raccoon dog-specific PCR assay.

S1.2 Table. Sequencing statistics. The statistics of vertebrate 12S rRNA gene sequencing and invertebrate 16S rRNA gene sequencing is shown.

Sample ID	12S rRNA gene of vertebrates without RacBlk		12S rRNA gene of vertebrates with RacBlk		16S rRNA gene of invertebrates	
	Accession number	No. sequence reads	Accession number	No. sequence reads	Accession number	No. sequence reads
R3	SAMN26117575	119,583	SAMN26117564	161,761	SAMN26117586	76,834
R3_dup	-	-	SAMN26117606	169,293	-	-
R4	SAMN26117576	121,867	SAMN26117565	164,162	SAMN26117587	81,379
R4_dup	-	-	SAMN26117607	178,742	-	-
R5	SAMN26117577	132,174	SAMN26117566	158,509	SAMN26117588	80,272
R5_dup	-	-	SAMN26117608	146,458	-	-
R6	SAMN26117578	90,684	SAMN26117567	159,838	SAMN26117589	84,111
R7	SAMN26117579	118,744	SAMN26117568	128,580	SAMN26117590	73,268
R7_dup	-	-	SAMN26117609	133,462	-	-
R9	SAMN26117580	140,180	SAMN26117569	179,233	SAMN26117591	92,981
R10	SAMN26117581	97,605	SAMN26117570	134,897	n.a.	n.a.
R11	SAMN26117582	141,697	SAMN26117571	147,294	SAMN26117592	81,507
R12	SAMN26117583	124,751	SAMN26117572	127,689	SAMN26117593	138,308
R12_dup	-	-	SAMN26117610	116,491	-	-
R13	SAMN26117584	144,171	SAMN26117573	144,589	SAMN26117594	5,338
R15	SAMN26117585	135,615	SAMN26117574	143,413	SAMN26117595	81,629
R15_dup	-	-	SAMN26117611	118,944	-	-
Total		1,367,071		2,513,355		795,627

Abbreviation: RacBlk, blocking oligonucleotide for the raccoon dog; n.a., not amplified by PCR.
Symbol: -, duplicate unavailable.

S1.3 Table. Sequencing statistics of plant internal transcribed spacer (ITS) sequencing.

Sample ID	Accession number	No. sequence reads
R3	SAMN26117596	40,673
R4	SAMN26117597	34,750
R5	SAMN26117598	21,023
R6	SAMN26117599	12,230
R7	SAMN26117600	24,467
R9	SAMN26117601	23,048
R10	n.a.	n.a.
R11	SAMN26117602	19,482
R12	SAMN26117603	19,099
R13	SAMN26117604	19,097
R15	SAMN26117605	20,678
Total		234,547

Abbreviation: n.a., not amplified by PCR.

Chapter 2. Combining vertebrate mitochondrial 12S rRNA gene sequencing and shotgun metagenomic sequencing to investigate the diet of the leopard cat (*Prionailurus bengalensis*) in Korea

2.1. Introduction

The leopard cat (*Prionailurus bengalensis*) is a small feline weighing 1.7–7.1 kg [90]. Due to its adaptability to deforestation and habitat changes, it is distributed in various regions in Asia, such as tropical rainforests, temperate broadleaf forests, coniferous forests, shrub forests, and grasslands [91]. However, in South Korea, its population has declined due to habitat loss, illegal hunting, and road killing, so the Korean Ministry of Environment classified it as a class II endangered species [7, 60]. In addition, it has been pointed out that in South Korea, the risk of regional extinction is high due to their reduced genetic diversity [92]. The leopard cat is the only surviving wild feline species in South Korea and is considered one of the top predators [10]. Therefore, further research on their ecological roles such as food habits is needed to preserve them and ensure their genetic diversity.

The leopard cat is considered to be a hypercarnivore that mainly feeds on rodents, especially murids [93–103]. It was also reported that leopard cats feed on other mammals such as ungulates, lagomorphs, and shrews, as well as birds, reptiles, amphibians, insects, and fishes [93–104]. There is also some literature on the diet of relative species of the leopard cat. For example, the flat-headed cat (*Prionailurus planiceps*) is known to eat fish, but also shrimp, birds, small rodents, and domestic poultry [105]. The fishing cat (*Prionailurus viverrinus*) is a species regarded as a generalist and known to feed on a variety of prey including rodents,

birds, and fish [106]. The rusty-spotted cat (*Prionailurus rubiginosus*) is known to eat rodents [107].

Studies on predation and feeding behavior of leopard cats have been conducted in countries, such as Singapore [93], Malaysia [94], Thailand [95, 96], Russia [97], Indonesia [98], Pakistan [99], India [100], Cambodia [101], Laos [102], Korea [103], and Japan [104, 108]. In these studies, prey items were identified by direct observation of hunting [98], observation of stomach contents [104], and microscopic observation of hair, teeth, feathers, toenails, and bones contained in collected fecal samples [93-97, 99-103]. However, these methods require not only labor and time, but also expertise in morphology and osteology. Furthermore, the identification of closely related prey items is often difficult by morphological observation.

The recent introduction of DNA barcoding in wildlife dietary analysis [31-33, 73, 74, 109] has made it possible to overcome the difficulties described above. In carnivore and omnivore dietary studies [31-33, 73, 74, 109], DNA markers such as the vertebrate mitochondrial 12S rRNA gene [36] have been targeted to analyze DNA derived from diets in collected fecal samples. DNA barcoding has also been applied to dietary analysis of leopard cats in Pakistan [35], China [29, 34], and Korea [110]. In the Korean study [110], bone or tissue fragments were isolated from fecal samples, DNA was extracted from each of the isolates, and each DNA extract was analyzed individually by Sanger sequencing. In the Chinese study [29], DNA was extracted collectively from fecal samples, the DNA markers were amplified and cloned, and each clone was individually analyzed by Sanger sequencing. In the remaining studies [34, 35], high-throughput sequencing (HTS) was used to analyze DNA collectively extracted from feces without cloning nor isolating individual tissue or bone fragments. The HTS-based DNA barcoding is particularly useful for analyzing dietary diversity and detecting rare species due to its sensitivity. For the leopard cat

dietary survey, the blocking oligonucleotide has also been developed to suppress the amplification of DNA derived from the leopard cat when performing the vertebrate-specific PCR targeting the mitochondrial 12S rRNA gene [35]. The use of blocking oligonucleotides helps increase the detection sensitivity of prey DNA by suppressing the amplification of the predator DNA.

In this study, we aimed to apply HTS technologies to the dietary analysis of leopard cats in Korea. Using HTS, we analyzed the vertebrate mitochondrial 12S rRNA gene in feces of leopard cats collected in winter in inland areas of Korea. The previously reported blocking oligonucleotide [35] was used to suppress the amplification of leopard cat DNA. In addition to vertebrate mitochondrial 12S rRNA gene sequencing, we also applied shotgun metagenomic sequencing. The use of shotgun metagenomic sequencing allows for detection of not only target organisms (i.e., vertebrates) but also non-target organisms such as invertebrates and plants. We compared the results of vertebrate mitochondrial 12S rRNA gene sequencing and shotgun metagenomic sequencing, and discussed whether the identified genera were reasonable in light of the Korean fauna.

2.2. Materials and methods

2.2.1. Fecal samples

Previously collected fecal samples of leopard cats for the investigation of zoonotic pathogens [40] and antimicrobial resistance genes [111] were used for dietary analysis in this study. Briefly, a total of twenty-two samples were collected in inland areas of Sejong and Gongju cities in Chungcheongnam-do province, and Daegu and Sangju cities and Goryeong gun in Gyeongsangbuk-do province during February 2019 in Korea (S2.1 Fig and S2.1 Table). Six samples (L1 and L8-L12) were collected in an area in Gongju city, Chungcheongnam-do, where a small stream originating from a tractional pond is flowing around and a paddy field is spread

out. This area is surrounded by mountains and is adjacent to a reservoir. The sample L2 was collected from a location right next to the aforementioned reservoir. Here too, rice fields are spread out, and it is surrounded by mountains. Five samples (L3–L6 and L13) were collected from a basin of a river flowing through Gongju city. This basin is covered with plants and grass and is surrounded by mountains. Two samples (L7 and L14) were collected around a confluence of the aforementioned river and a small stream in Sejong city, Chungcheongnam-do. There is a wetland right next to this sampling area, which is surrounded by mountains. Eight samples (L15–L22) were collected in Gyeongsangbuk-do province, and one of them (L15) was collected in Sangju city. In the area where the sample L15 was collected, a small stream originating from a tractional pond is flowing around, rice fields are spread out, and it is surrounded by mountains. The remaining seven samples (L16–L22) were collected from a basin of a river flowing through Gyeongsangbuk-do province. As with other sampling sites, there are paddy fields around this place and are surrounded by mountains. No permission was required to collect the fecal samples since they were collected in public places. About 10 g of each sample was collected with a wooden spatula and placed in a 50 ml tube. The samples taken were transported to the laboratory on ice and stored at −80℃ for analysis. Our previous study [40] has confirmed that the collected fecal samples are from leopard cats by the leopard cat-specific PCR assay using a primer pair PrioF/PrioR [112].

2.2.2. DNA extraction

DNA extraction from the collected scat samples was performed by a PowerMax® Soil DNA Isolation Kit (Mobio Laboratory, Inc., Carlsbad, CA, USA). First, 5 ml of ultra-pure water was added to each of the 50 ml tubes, and the inner part of the fecal sample was used whenever possible to minimize potential contamination from the environment and homogenized using a wooden spatula [33]. Second, 0.2 g of each homogenized sample, 300 mg of 0.1 mm diameter glass beads, and 100 mg of 0.5 mm diameter glass beads

were added into a 2 ml tube of the DNA isolation kit. Third, the samples were additionally homogenized by a bead beater (BioSpec Products, Inc., Bartlesville, OK, USA) for 3 min. Finally, from each sample, DNA was extracted and purified using the kit's protocol and eluted in 50 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0). The eluted DNA extracts were stored at −80℃ until analysis.

2.2.3. Shotgun metagenomic sequencing

In this study, we used shotgun metagenomic data previously obtained for the purpose of investigating antimicrobial resistance genes [111]. In the present study, we specifically analyzed dietary content. Briefly, eleven randomly selected from a total of twenty-two samples were subject to shotgun metagenomic sequencing. Shotgun metagenomic sequencing was performed on an Illumina NextSeq system (Illumina, San Diego, CA, USA) with 150 bp paired-end chemistry. For details, see our previous study [111].

From the resulting shotgun metagenomic sequencing reads, low-quality reads were eliminated and adapter sequences were trimmed using Trimmomatic version 0.39 [43] with default settings. Then, paired-end reads were merged by FLASH version 1.2.11 [113]. Finally, the paired shotgun metagenomic reads were taxonomically annotated against the RefSeq database using BLASTX with default parameters (e-value cutoff = 10^{-5}, % identity cutoff = 60, and alignment length cutoff = 15 bp) in the Metagenomics Rapid Annotation (MG-RAST) server version 4.0.3 [114]. As of July 2022, the genome sequences of 2,255 vertebrate species have been reported as RefSeq, and these are considered to be contained in the MG-RAST database. Shotgun metagenomic sequence reads included those that are not related to the diet, such as bacteria and fungi. We extracted only sequences assigned to the phyla Chordata, Streptophyta, and Arthropoda, which are thought to be related to the diet of the leopard cat. Also, the reads assigned to the family Felidae to which the leopard cat belongs were excluded because they are likely to come from the leopard cat itself rather than the

diet.

2.2.4. Vertebrate mitochondrial 12S rRNA gene sequencing

All twenty-two samples collected were subjected to vertebrate mitochondrial 12S rRNA gene amplicon sequencing. Of them, seven samples were technically duplicated. The libraries were prepared with a primer pair of 12SV5F and 12SV5R that target the vertebrate mitochondrial 12S rRNA gene and a blocking oligonucleotide *PrioB* that inhibits the amplification of leopard cat's DNA [35, 36]. The primers were added with adapter sequences for tagged sequencing of Illumina MiSeq. Each PCR mixture with a volume of 50 μl contained 25 μl of Premix Taq™ (Takara Bio Inc., Otsu, Shiga, Japan), 0.1 μM of each primer, 2 μM of *PrioB*, 13 μl of molecular grade water, and 1 μl of DNA extract. PCR started with initial denaturation at 95℃ for 15 min, followed by 45 cycles of 30 s at 95℃ and 30 s at 60℃. There was no elongation step [35]. The resulting amplicons were purified with AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA). The purified amplicons were tagged with a Nextera XT Index kit (Illumina), with PCR starting with initial denaturation at 95℃ for 3 min, followed by 8 cycles of 30 s at 95℃, 30 s at 55℃, and 30 s at 72℃, and completing by final extension at 72℃ for 5 min. The tagged libraries were purified with AMPure XP beads (Beckman Coulter). The purified tagged libraries were quantified by a Quant-iT PicoGreen dsDNA reagent kit (Life Technologies, Carlsbad, CA, USA). The quantified libraries were normalized, pooled, and introduced to a v3 600 cycle-kit reagent cartridge (Illumina) with 30% PhiX control. The loaded libraries were sequenced on an Illumina MiSeq system with 300 bp paired-end chemistry.

From the resulting vertebrate mitochondrial 12S rRNA gene sequence reads, low quality reads with a score of less than 20 were removed and the adapter sequences were trimmed using MiSeq Reporter version 2.5 (Illumina). In addition, ambiguous base callings were trimmed by Trimmomatic v 0.33 [43]. Then, OBITools [44]

was used for subsequent processing and analyses. The paired-end reads were concatenated by the *illuminapairedend* command. The concatenated reads were merged and dereplicated by the *obiuniq* command and filtered with a cut-off length of 80 bp by the *obigrep* command. The *obiclean* command was used to exclude erroneous reads. Using the *ecotag* command, the high-quality reads obtained were taxonomically assigned against our custom reference database [109] consisting of vertebrate mitochondrial 12S rRNA gene sequences downloaded from the EMBL nucleotide database. The database contains reference mitochondrial 12S rRNA gene sequences of a total of 12,574 vertebrate species.

2.2.5. Statistical analysis

Statistical analysis was performed to compare the results of shotgun metagenomic sequencing and vertebrate mitochondrial 12S rRNA gene sequencing. The differences in diversity, structure, and membership of prey animals identified by both methods were compared at the class level. The phyloseq [45] and vegan [46] packages in R version 4.1.0 were used. Prior to the comparison, from both sequencing results, only reads classified as the phylum Chordata were included and the reads identified as the family Felidae were excluded. The *tax_glom* function of phyloseq was used to merge taxa of the same group at a given taxonomic rank. Then, using *rarefy_even_depth* function of phyloseq, each library was rarefied to 2,700 reads, which is the round-down number of the minimum number of the Chordata reads (i.e., 2,744 reads per library) obtained by shotgun metagenomic sequencing (S2.2 Table). The *estimate_richness* function of phyloseq was used to compute α diversity indices of the rarefied libraries, and the Wilcoxon rank-sum test was used to compare of the results obtained by the two sequencing methods. In addition, we compared the differences in prey community structure and membership obtained by the two different sequencing methods. The Bray-Curtis dissimilarity and Jaccard index were used to represent the community structure and membership, respectively. The vegan package was used to perform

permutational multivariate analysis of variance (PERMANOVA) to compare the differences. Additionally, the Mantel test was performed based on the Bray-Curtis dissimilarity with 10,000 permutations, in order to evaluate the Spearman's rank correlation between the distance matrices of shotgun metagenomic sequencing and vertebrate mitochondrial 12S rRNA gene sequencing.

2.3. Results

2.3.1. Sequencing statistics

From twenty-two fecal samples, twenty-two vertebrate mitochondrial 12S rRNA gene sequence and eleven shotgun metagenomic sequence libraries were obtained. For vertebrate mitochondrial 12S rRNA gene sequencing, seven additional libraries were obtained as technical replicates. A total of 2,816,069 high-quality reads were obtained by vertebrate mitochondrial 12S rRNA gene sequencing (S2.3 Table). A total of 122,474,517 high-quality shotgun metagenomic sequencing reads were obtained. Among them, a total of 352,045 reads (0.29%) were assigned to the phyla Arthropoda, Chordata, or Streptophyta, which are thought to be derived from the diet of leopard cats (S2.2 Table).

2.3.2. Shotgun metagenomic sequencing

Among the reads assigned to the phyla related to the diet of leopard cats, their relative abundances were 41.7% for Chordata, 47.1% for Streptophyta, and 11.2% for Arthropoda. The relative abundance of diet-related phyla detected in each sample is shown in Fig 2.1A. At the class level, abundant vertebrates and their relative abundance within the phylum were Mammalia (62.7%), Amphibia (19.3%), Actinopterygii (ray-finned fishes) (7.3%), and Aves (birds) (3.8%) (Fig 2.1B). Among the Streptophyta, the abundant classes were Magnoliopsida (dicotyledons) (76.3%), Bryopsida (mosses) (12.9%), Liliopsida (monocotyledons) (7.6%), and Isoetopsida (quillworts) (1.2%) (Fig 2.1C). Among the Arthropoda, the abundant classes were Insecta (94.0%) and Arachnida (spiders,

ticks, and mites) (5.5%) (Fig 2.1D).

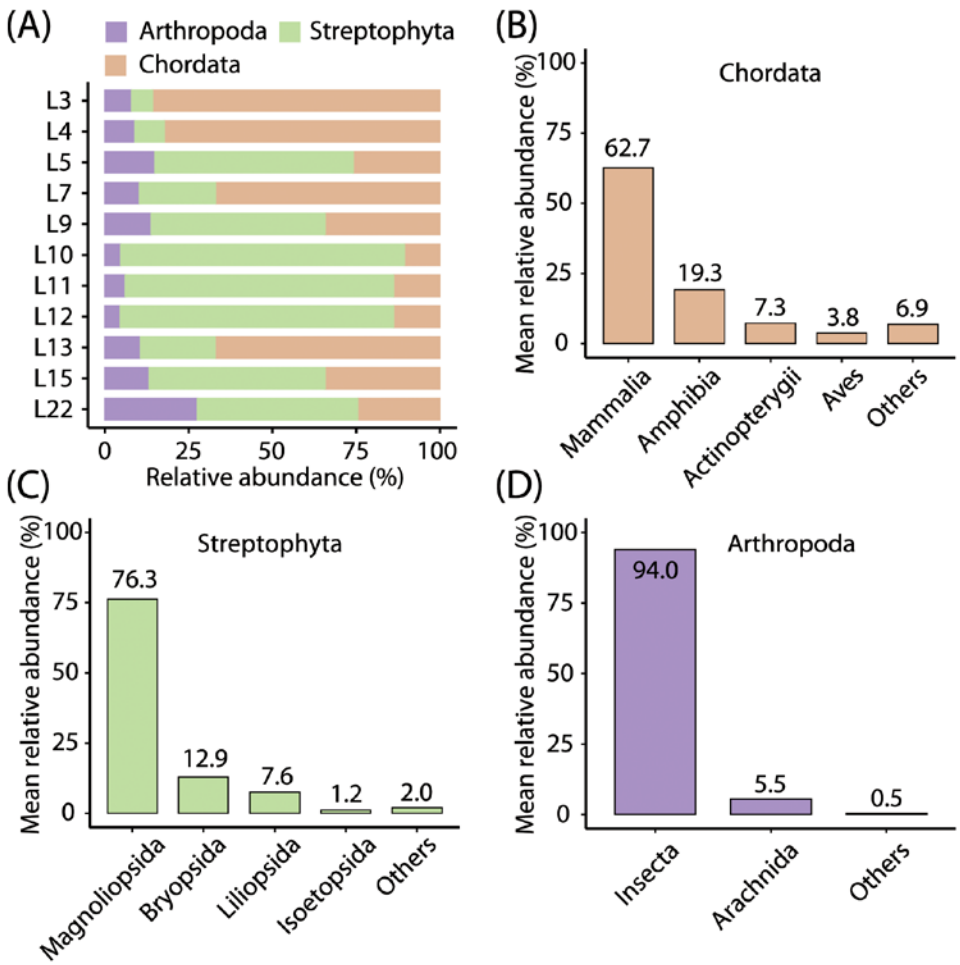


Fig 2.1. Shotgun metagenomic sequencing results. Mean relative abundance of the leopard cat diet-related phyla detected from their fecal samples are shown (n = 11). The reads classified into the family Felidae were excluded because they may be derived from DNA of the leopard cat itself. (A) Relative abundance of the three leopard cat diet-related phyla in each sample. Mean relative abundance of each class within each phylum of (B) Chordata, (C) Streptophyta, and (D) Arthropoda.

2.3.3. Vertebrate mitochondrial 12S rRNA gene sequencing

Fig 2.2 shows the relative abundance of the 20 most abundant genera detected by vertebrate mitochondrial 12S rRNA gene sequencing. Murids such as *Micromys* (harvest mouse), *Apodemus* (field mouse), and *Rattus* (rat) were abundantly detected. They belong to a group of rodents. Other small-size rodents, such as *Sciurus* (red squirrel) and *Myodes* (vole), were also detected. Relatively large herbivores such as *Hydropotes* (water deer) were also detected. Moreover, carnivores such as *Mustela* (weasel) and *Canis* (domestic dog) were also detected in some samples. Birds, such as *Streptopelia* (dove) and *Phalacrocorax* (cormorant), fishes, such as *Squaliobarbus* (barbel chub) and *Lepomis* (bluegill sunfish), and amphibians, such as *Pelophylax* (true frog), were also detected. Technical duplications show reproducible results (S2.2 Fig). There was no spatial difference in the prey composition between Chungcheongnam-do and Gyeongsangbuk-do, where sampling was carried out (S2.3 Fig).

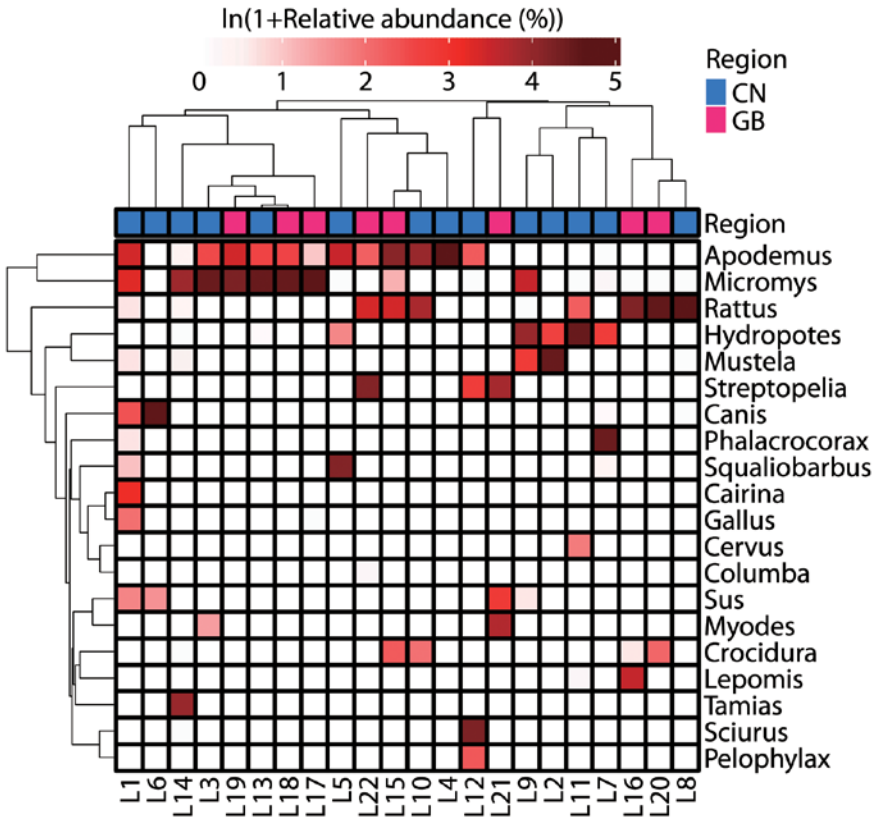


Fig 2.2. Vertebrate mitochondrial 12S rRNA gene sequencing results. Relative abundance of the top 20 genera identified from fecal samples (n = 22) by vertebrate mitochondrial 12S rRNA gene sequencing is shown. The ambiguous reads that were not identified at the genus level and the reads that were assigned to genera belonging to the family Felidae were excluded from the calculation. The dendrograms represent the similarity (dissimilarity) of log-transformed relative abundance results between samples (x-axis) or between prey animals (y-axis). The dissimilarity is represented by branch lengths based on Euclidean distance. Abbreviation: CN, Chungcheongnam-do; GB, Gyeongsanbuk-do.

2.3.4. Comparison between shotgun metagenomic and vertebrate mitochondrial 12S rRNA gene sequencing

The results obtained by shotgun metagenomic sequencing and vertebrate mitochondrial 12S rRNA gene sequencing were compared at two taxonomic levels, i.e., class and genus levels. The class-level comparisons are shown in Fig 2.3. The proportion of the class Mammalia was highest by both shotgun metagenomic and vertebrate mitochondrial 12S rRNA gene sequencing (Fig 2.3A). However, the proportion of mammals is highly distinct by vertebrate mitochondrial 12S rRNA gene sequencing, while more diverse classes such as Amphibia and Actinopterygii were detected more evenly by shotgun metagenomic sequencing. These tendencies were also confirmed by α diversity analysis (Fig 2.3B). We found that the Chao1 estimator (community richness) and the Shannon index (community diversity) were statistically significantly higher by shotgun metagenomic sequencing than by vertebrate mitochondrial 12S rRNA gene sequencing ($p < 0.01$; Wilcoxon rank-sum test). Moreover, β diversity analysis found that both Bray-Curtis dissimilarity (community structure) and Jaccard index (community membership) differed statistically significantly between shotgun metagenomic and vertebrate mitochondrial 12S rRNA gene sequencing ($p < 0.01$ and 0.001, respectively; PERMANOVA) (Fig 2.3C). No correlation was found between the distance matrices of shotgun metagenomic sequencing and vertebrate mitochondrial 12S rRNA gene sequencing at the class level ($r = -0.05, p > 0.05$; Mantel test).

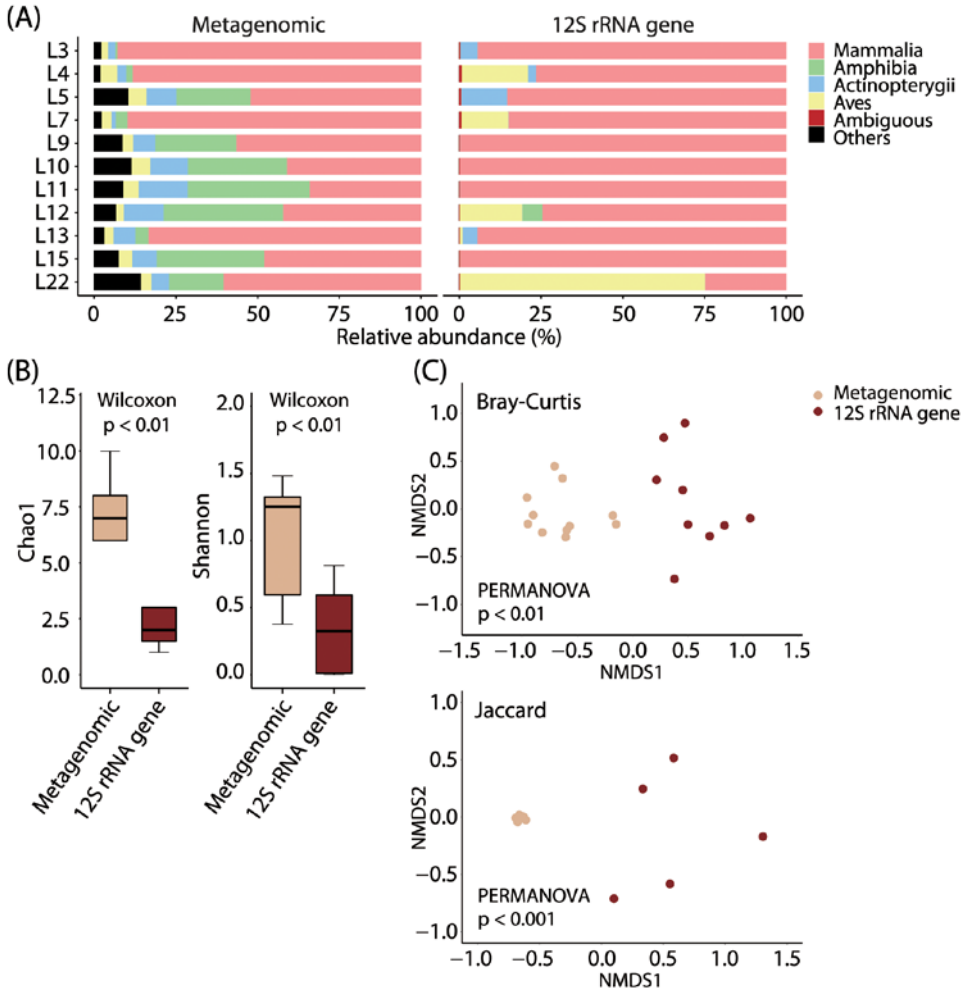


Fig 2.3. Comparisons between shotgun metagenomic and vertebrate mitochondrial 12S rRNA gene sequencing. The class-level results are shown. The reads assigned to the family Felidae were excluded from the comparisons. (A) Relative abundance of classes within the phylum Chordata. (B) Alpha diversity. The Chao1 estimator (community richness) and Shannon index (community diversity) based on the class-level results are shown. (C) Beta diversity. Non-metric multidimensional scaling (NMDS) plots based on the Bray–Curtis dissimilarity (community structure) and Jaccard index (community membership) based on the class-level results are shown.

The genus-level comparisons are shown in Table 2.1, which shows major genera detected by vertebrate mitochondrial 12S rRNA gene and/or shotgun metagenomic sequencing and their inhabitation status in Korea. Most of the major genera detected by vertebrate mitochondrial 12S rRNA gene sequencing, such as *Apodemus* (field mouse), *Micromys* (harvest mouse), and *Hydropotes* (water deer), are known to inhabit Korea. However, many of the major genera identified by shotgun metagenomic sequencing are not known to naturally inhabit Korea. For instance, *Xenopus* (clawed frog) and *Danio* (danio) were abundantly identified. Mammals such as *Pan* (chimpanzee), *Pongo* (orangutan), *Macaca* (macaque), *Ailuropoda* (giant panda), *Monodelphis* (short-tailed opossum), and *Ornithorhynchus* (platypus) were also identified. However, they are not known to live naturally in Korea. Marine chordates such as *Branchiostoma* (lancelet) and *Ciona* (sea squirt) were identified, but the detection of these organisms is likely erroneous given that fecal sampling was done inland.

Table 2.1. Major genera detected and their inhabitation status in Korea. Twenty most abundant vertebrate and chordate genera detected by vertebrate mitochondrial 12S rRNA gene (n = 11) and/or shotgun metagenomic (n = 11) sequencing are shown.

Genus	Common name	12S rRNA (%) [a]	Metagenomic (%) [a,b]	Inhabitation in Korea [ref.]
Apodemus	Field mouse	25.8	n.d.	Confirmed [10]
Micromys	Harvest mouse	18.9	n.d.	Confirmed [10]
Hydropotes	Water deer	14.3	n.d.	Confirmed [10]
Rattus	Rat	9.7	9.1	Confirmed [10]
Phalacrocorax	Cormorant	7.7	0.001	Confirmed [115]
Streptopelia	Dove	7.2	n.d.	Confirmed [116]
Sciurus	Red squirrel	6.2	0.003	Confirmed [10]
Squaliobarbus	Barbel chub	5.8	n.d.	Confirmed [117]
Mustela	Weasel	1.4	n.d.	Confirmed [10]
Crocidura	Shrew	1.3	n.d.	Confirmed [10]
Pelophylax	True frog	0.8	n.d.	Confirmed [118]
Cervus	Deer	0.5	0.003	Nearly extinct, but raised on farms and very few individuals live in the wild [10, 119-122]
Myodes	Vole	0.3	n.d.	Confirmed [10]
Sus	Wild boar	0.1	1.1	Confirmed [10]
Columba	Pigeon	0.02	n.d.	Confirmed [116]
Micropterus	Largemouth bass	0.02	n.d.	Confirmed [49]
Lepomis	Bluegill sunfish	0.02	n.d.	Confirmed [49]
Euryoryzomys	Rice rat	0.02	n.d.	Not confirmed
Canis	Dog	0.01	17.1	Pet animal
Xenopus	Clawed frog	n.d.	19.3	Not confirmed
Homo	Human	n.d.	8.0	Confirmed
Danio	Danio	n.d.	7.2	Pet animal
Bos	Cattle	n.d.	6.6	Domestic

				animal
Mus	House mouse	n.d.	5.8	Confirmed [10]
Branchiostoma	Lancelet	n.d.	4.4	Marine chordate
Ailuropoda	Giant panda	n.d.	2.7	Not confirmed
Ciona	Sea squirt	n.d.	2.5	Marine chordate
Gallus	Fowl	n.d.	2.4	Domestic animal
Pan	Chimpanzee	n.d.	2.2	Not confirmed
Macaca	Macaque	n.d.	2.0	Not confirmed
Pongo	Orangutan	n.d.	1.7	Not confirmed
Monodelphis	Short-tailed opossum	n.d.	1.7	Not confirmed
Taeniopygia	Finch	n.d.	1.4	Pet animal
Equus	Horse	n.d.	1.3	Domestic animal
Ornithorhynchus	Platypus	n.d.	1.2	Not confirmed
Oryctolagus	Rabbit	n.d.	0.5	Pet animal

[a] The reads assigned to the family Felidae and ambiguous reads at the genus level were excluded from calculation of mean relative abundance.

[b] The relative abundance of each genus was calculated for the total number of reads of identified genera belonging to the phylum Chordata.

2.4. Discussion

In this study, we applied vertebrate mitochondrial 12S rRNA gene sequencing and shotgun metagenomic sequencing to investigate the dietary content of leopard cats inhabiting inland areas of Korea in winter. We found that the leopard cats fed mainly on mammals, especially murids, and confirmed that the detected prey items were plausible in light of the Korean fauna. In addition, shotgun metagenomic sequencing revealed that the amount of plant DNA in the feces of leopard cats is comparable to that of animal DNA. Furthermore, some inconsistencies between vertebrate mitochondrial 12S rRNA gene sequencing and shotgun metagenomic sequencing, and misidentification of prey at the genus level by shotgun metagenomic sequencing were identified. Below, we discuss these points.

2.4.1. Mammals as main prey for leopard cats in Korea

Both vertebrate mitochondrial 12S rRNA gene sequencing and shotgun metagenomic sequencing revealed that mammals are the main prey of leopard cats in our study areas (Fig 2.3A). In particular, vertebrate mitochondrial 12S rRNA gene sequencing revealed murids, such as *Micromys*, *Apodemus*, and *Rattus*, and other small-size rodents, such as *Sciurus* and *Myodes*, as the predominant prey (Fig 2.2 and Table 2.1). Our observation was the same as that by previous Korean studies [103, 110] that reported these three murid genera and rodents as the main prey of leopard cats. However, this tendency is universal not only for leopard cats in Korea but also for those in other countries [29, 34, 35, 93–103, 108, 110].

Large mammals, such as *Sus* and *Hydropotes*, were also detected by vertebrate mitochondrial 12S rRNA gene sequencing. *Hydropotes inermis* (water deer) and *Sus scrofa* (wild boar) grow up to 15 kg [123] and 300 kg [59], respectively, while the leopard cat grows only up to 7.1 kg [90]. Due to the size difference, it can be thought

that hunting is not easy, if not impossible. Wild boar and deer, such as *Cervus nippon* (sika deer) and *Capreolus pygargus* (roe deer), were reported to be detected from the feces of leopard cats in the Russian Far East [97] and in Pakistan [99], too. These studies suggest that leopard cats may have scavenged the carcasses of these large animals, although there is no direct evidence presented. In addition to these studies, consumption of deer by hunting or scavenging by leopard cats has been reported, e.g., *Tragulus javanicus* (lesser mouse deer) in Thailand [95] and *Muntiacus* spp. (muntjac) in Cambodia [101] and Laos [102].

Carnivorous animals, such as *Canis* and *Mustela*, were also detected by vertebrate mitochondrial 12S rRNA gene sequencing. In Korea, the wolf is thought to be extinct [10]. Therefore, *Canis* detected is likely domestic dogs. In Pakistan, it has been reported that leopard cats prey on domestic dogs [99]. *Mustela nivalis* (least weasel) and *Mustela sibirica* (Siberian weasel) are known to inhabit Korea [10], and *M. sibirica* was reported to be detected from a feces of leopard cat in Korea [103]. Although it is unclear whether it is predation or scavenging, it has been reported that carnivorous animals were detected in the feces of leopard cats, e.g., *Nyctereutes procyonoides* (raccoon dog) in the Russian Far East [97], *Urva auropunctata* (small Indian mongoose) and *Herpestes edwardsii* (grey mongoose) in Pakistan [99], and civet in Cambodia [101].

We detected an OTU with a sequence highly similar to that of *Cervus* (Fig2.2 and Table 2.1), which is believed to be extinct in the wild in South Korea [10]. The detected sequence is phylogenetically distant from those of *Hydropotes*, an extant deer species in Korea, so the misidentification of *Hydropotes* is unlikely (S2.4 and S2.5 Figs). We consider that the detected sequence is from *Cervus*, as they may still exist in South Korea. In fact, species such as *Cervus nippon* (sika deer), *Cervus elaphus* (red deer), and *Cervus canadensis* (elk) are imported from abroad and farmed

for their antlers and blood [119, 120]. It has been also reported that the sika deer has been released into the wild by religious events, and about 50 to 100 individuals are known to inhabit Mt. Songni near Chungcheongbuk-do and Gyeongsangbuk-do [10, 121]. There have also been reports of some deer escaping farmland or being illegally released by farmers in Korea [121]. Therefore, albeit in very low numbers, there is a possibility that deer belonging to *Cervus* inhabit the wild environment of Korea, and it is possible that they were hunted by leopard cats.

The genus *Euryoryzomys* detected (Table 2.1) is not known to inhabit Korea. We consider that the detection of *Euryoryzomys* was a misidentification of *Myodes* because these two rodents have very similar vertebrate mitochondrial 12S rRNA gene sequences (S2.6 Fig). One hypothesis is that the intra-genus variability in the vertebrate mitochondrial 16S gene sequences within the genus *Myodes* may be comparably large to the inter-genus variability between *Myodes* and *Euryoryzomys*. However, this needs to be clarified in future research.

2.4.2. Non-mammalian vertebrates and invertebrates as secondary prey

This study found that mammals are the main prey for the leopard cats in Korea, which may be a universal tendency worldwide. However, exceptions have been also reported, such as leopard cats inhabiting the subtropical Iriomotejima Island in Japan, whose main prey is frogs [104]. Therefore, there may be the regional differences, but their adaptability may allow them to flexibly change their target prey depending on the type of prey locally available. In addition to regional effects, there may also be seasonal effects [99, 101, 103]. In Korea, which is located in the temperate zone, poikilotherms, such as amphibians, hibernate in the cold winter, so they may be inevitably excluded from the predation of leopard cats. However, we found that amphibians, including *Pelophylax* (Fig 2.2 and Table 2.1), were present as prey for leopard cats in a relatively

high proportion (Fig 2.1B), even though our samples were collected in the cold winter. A previous study has also reported that the proportion of amphibians in feces of leopard cats in Korea is higher in winter than in other seasons [103]. We do not know the reason. One possible explanation is that the activity of poikilotherms declined in the cold winter, making them easy targets for leopard cats. However, future seasonal investigations need to confirm the tendency and reveal the cause behind.

In addition to Amphibia, Actinopterygii and Aves were relatively abundantly detected as non-mammalian vertebrates by shotgun metagenomic sequencing (Figs 2.1B and 2.3A). Vertebrate mitochondrial 12S rRNA gene sequencing also identified genera belonging to Actinopterygii, such as *Squaliobarbus* and *Lepomis*, and Aves, such as *Streptopelia* and *Phalacrocorax* (Fig 2.2 and Table 2.1). The detection of fishes makes sense because fecal samples were collected near rivers and reservoirs. Moreover, the detection of the abovementioned fish genera is reasonable as species belonging to these genera have been reported to inhabit Korea, such as *Squaliobarbus curriculus* (barbel chub) [117], *Lepomis macrochirus* (bluegill sunfish) [49], *Streptopelia orientalis* (oriental turtle dove) [116], and *Phalacrocorax carbo* (great cormorant) [115]. In countries other than Korea, several studies have reported that leopard cats eat fish [34, 35, 97, 104, 108] and birds [29, 34, 35, 93-102, 108]. In Korea, studies reported that leopard cats eat fish, such as *Monopterus albus* (Asian swamp eel) [110], and birds, such as *Phasianus colchicus* (common pheasant) [110], *Turdus pallidus* (pale thrush) [110], and *Garrulus glandarius* (Eurasian jay) [103]. In this study, we also confirmed that leopard cats eat fish and birds, although they are less common than mammals.

Arthropods were also detected in relatively large numbers by shotgun metagenomic sequencing, most of which were found to be insects (Fig 2.1D). Many studies have reported that leopard cats feed on arthropods such as insects [93-95, 97, 99, 101, 103, 108]

and arachnids [94]. In Korea, cicadas and grasshopper are reported to be detected from feces of leopard cats [103]. It was remarkable that relatively abundant insects were detected from the leopard cat feces collected in the winter in this study, as insects are inactive in the cold winter in Korea. The detection of insects in winter has also been reported in the previous Korean study [103]. The reason is unknown, but we speculate that leopard cats may feed on insect larvae and/or nymphs in winter. In addition, in this study using shotgun metagenomic sequencing, the types of insects detected are unknown. In the future, target-specific sequencing, such as invertebrate 16S rRNA gene sequencing [32], will need to reveal the species of insects that leopard cats specifically prey on.

2.4.3. Large amount of plant DNA in feces of leopard cats

Shotgun metagenomic sequencing revealed that the amount of plant DNA in the feces of leopard cats is comparable to the amount of animal DNA (Fig 2.1A). Although the amount of DNA is not necessarily proportional to the amount of biomass, it is remarkable that based on the amount of DNA, the amount of plants is comparable to the amount of animals in the feces of leopard cats. The leopard cat is known to eat grass. They are thought to eat grass for intestinal regulation [108] and parasite removal [99], and/or ingestion of minerals and vitamins [103]. In fact, it has been reported that graminoids (grass-like plants, mostly monocot plants) were detected in the feces of leopard cats [103, 108]. However, the majority of plants detected in this study were Magnoliopsida (Fig 2.1C), which are dicot plants. Therefore, the plants detected in this study do not appear to be consistent with the general dietary habit of leopard cats. One explanation is that graminoids might have become scarce at the time of our fecal sampling done in winter. Meanwhile, the plants belonging to the class Magnoliopsida such as *Bellis perennis* (daisy) were also reported to have been detected abundantly from feces of leopard cats, and the possibility that they were ingested secondarily by eating herbivorous prey was suggested [99]. Another study also

reported the detection of dicotyledonous plants such as the genus *Solanum* and subfamily Rosoideae in the feces of leopard cats [29]. These suggest that leopard cats may flexibly adapt to seasonally and locally available plants. Note that similar to the vertebrate results, the plants identified at the genus level by shotgun metagenomic sequencing are likely biased toward species whose genomes have already been sequenced (S2.4 Table). Therefore, to precisely identify plant taxa that were consumed or secondarily ingested by leopard cats, future research should perform sequencing of plant DNA markers (e.g., ITS) for which a broader range of plant taxa are covered in reference databases.

2.4.4. Inaccurate genus-level prey identification by shotgun metagenomic sequencing

In this study, we explored the possibility for shotgun metagenomic sequencing to identify the diet of leopard cats at the genus level (Table 2.1). In the light of the Korean fauna, we conclude that many of the genera identified by shotgun metagenomic sequencing are erroneous. While many genera, except *Euryoryzomys*, identified by vertebrate mitochondrial 12S rRNA gene sequencing are known to inhabit Korea and reasonable in light of the known eating habits of leopard cats, most of the genera identified by shotgun metagenomic sequencing are not reasonable. For example, the inhabitation of *Xenopus*, *Danio*, *Ailuropoda*, *Pan*, *Macaca*, *Pongo*, *Monodelphis*, and *Ornithorhynchus* in Korea is unknown. Moreover, the detection of marine organisms such as *Branchiostoma* and *Ciona* is not reasonable because the samples were collected inland far from the sea.

The plausible explanations for why taxonomic assignment of plants and animals by shotgun metagenomic sequencing is inaccurate are given by Pearman et al. [124]. As one reason, they pointed out that plant and animal genome databases are far more incomplete than those of bacteria and fungi. We also consider that this is the reason why taxonomic assignment by shotgun metagenomic sequencing is

less accurate than by vertebrate mitochondrial 12S rRNA gene sequencing. In fact, the reference database for shotgun metagenomic sequencing used in this study contained genomic sequences of only 2,255 chordate species, which is much less than sequences of 12,574 vertebrate species contained in the reference database used for vertebrate mitochondrial 12S rRNA gene sequencing. As a result, many reads are presumed to have been biasedly misassigned to organisms whose whole genome sequences have been elucidated, such as *Xenopus tropicalis* [125], *Danio rerio* [126], *Bos taurus* [127], *Branchiostoma floridae* [128], *Ciona intestinalis* [129], *Macaca mulatta* [130], *Monodelphis domestica* [131], and *Taeniopygia guttata* [132]. However, we expect this problem will be alleviated in the future by the ongoing efforts made to expand genomic databases, such as the Earth BioGenome Project [133] and the Genome 10K [134].

2.4.5. Sequencing method biases

We used shotgun metagenomic and vertebrate mitochondrial 12S rRNA gene sequencing, and found the differences in taxonomic richness, and community structure and membership of prey between these two sequencing methods (Fig 2.3). Specifically, we found that more diverse taxa were detected by shotgun metagenomic sequencing than by vertebrate mitochondrial 12S rRNA gene amplicon sequencing (Fig 2.3B), and the similar tendency has been reported by previous studies [135–137]. This may be due to the difference in two sequencing approaches, i.e., the presence or absence of targeted amplification process. Bias that can occur during PCR process are caused by various factors such as temperature ramp rates [138], amplicon length, and PCR primer [139, 140]. As an example of bias, rare taxa are known to be underestimated during PCR process [141]. Therefore, the smaller number of taxa detected by vertebrate mitochondrial 12S rRNA gene sequencing in this study is plausible and may have been associated with the inclusion of PCR amplification in the process of library preparation. Additionally, we found that there are

differences in community structure and membership between these two sequencing methods (Fig 2.3C). In addition to the abovementioned amplification bias, the bias due to the difference in the number of copies of the target DNA marker to be amplified [142] may contribute to the observed differences.

In contrast, other previous study [143] reported that the results of amplicon-based sequencing show more diverse taxa than those of shotgun metagenomic sequencing. One reason could be related to inadequate sequencing depth [144]. In our study, only about 0.29% of all shotgun metagenomic reads were assigned to taxa related to prey of leopard cats. This underscores the importance of sequencing depth in wildlife dietary analysis by the shotgun metagenomic approach.

2.4.6. Caveats

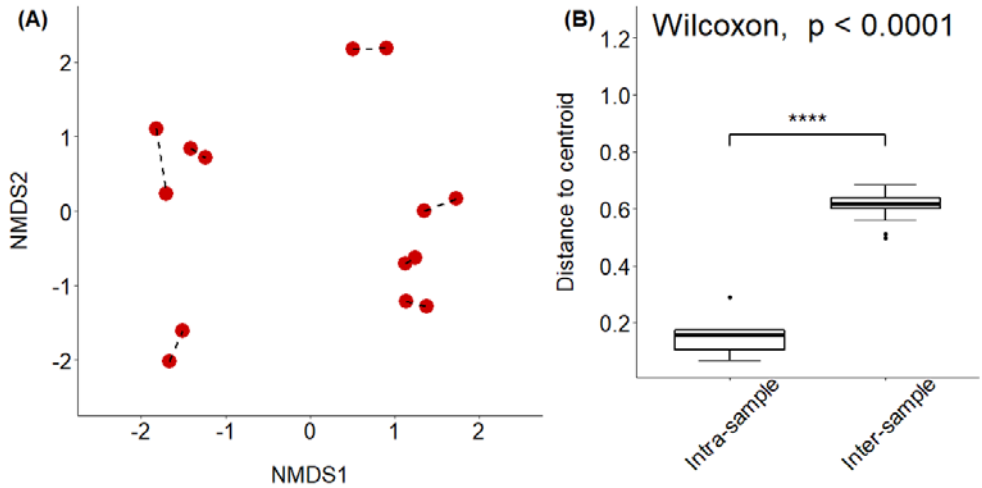
In this study, we used DNA sequencing to conduct a dietary survey of leopard cats. We recognize that quantified DNA does not necessarily represent the accurate proportion of biomass consumed, as correctly pointed out by previous studies [78-80]. As discussed above, DNA sequencing can be affected by a variety of factors, such as genome size, copy number variation, PCR bias, primer bias, and so on. Additionally, the difference in digestibility of dietary items may also affect the relative abundance of DNA measured. However, DNA sequencing can objectively identify dietary items without morphological and osteological expertise and detect rare dietary items. We believe that DNA sequencing helps complement existing methods due to its convenience, objectivity, and detection sensitivity.

2.5. Conclusion

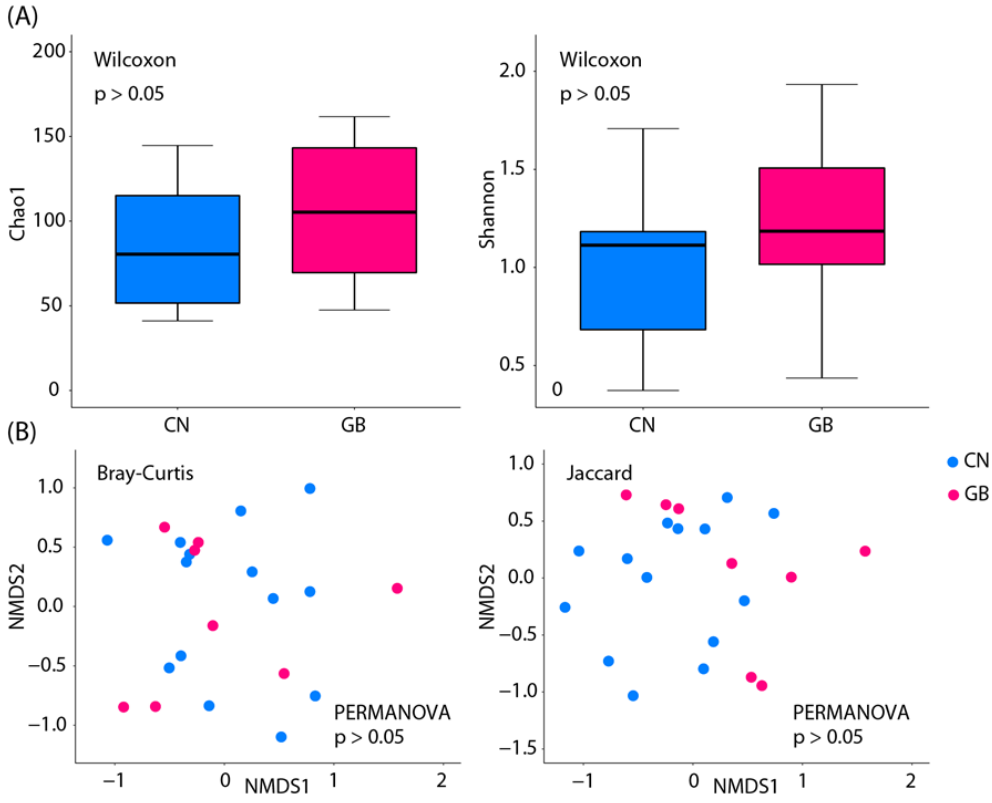
The combination of vertebrate mitochondrial 12S rRNA gene amplicon sequencing and shotgun metagenomic sequencing provided a comprehensive understanding of the dietary composition of the

leopard cat, an endangered species in Korea. By using shotgun metagenomic sequencing, we were able to grasp an overall picture of the dietary composition of leopard cats, including the presence of plants in a relatively large proportion. Both shotgun metagenomic sequencing and vertebrate mitochondrial 12S rRNA gene sequencing confirmed that mammals are the main prey. It was also confirmed that the genera of prey identified by vertebrate mitochondrial 12S rRNA gene sequencing were reasonable in light of the Korean fauna. However, the genera identified by shotgun metagenomic sequencing were inaccurate, which is likely due to the inadequate genomic database, and therefore care must be taken when using shotgun metagenomic sequencing to identify prey at lower taxonomic levels, such as species and genus levels. Meanwhile, the genome database is expanding, and it is expected that this problem will be alleviated in the future. We expect that the use of shotgun metagenomic sequencing will provide a novel opportunity to comprehensively understand the inter-phylum and inter-class dietary composition of wildlife in the near future.

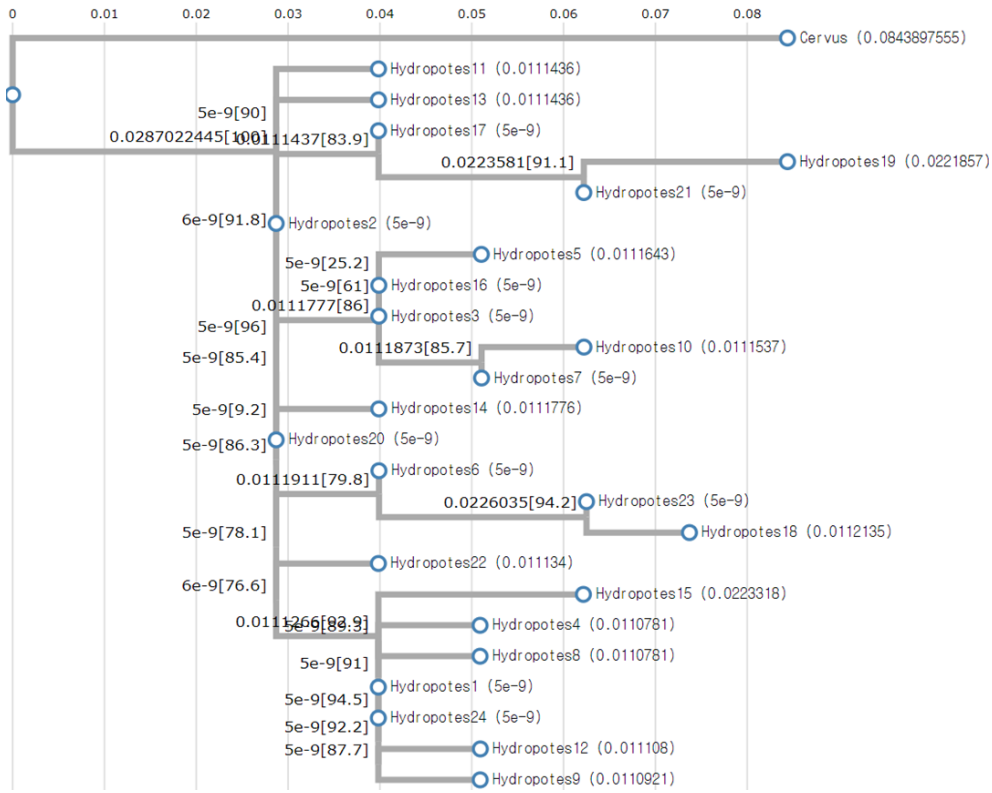
S2.1 Fig. Fecal samples of leopard cats.



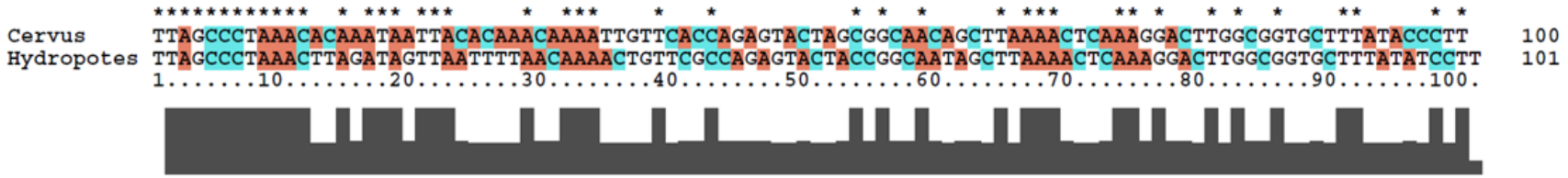
S2.2 Fig. Reproducibility of vertebrate mitochondrial 12S rRNA gene sequencing. (A) Non-metric multidimensional scaling (NMDS) plot representing the composition of vertebrate prey based on the Bray–Curtis dissimilarity. The data duplicated from the same sample is connected by a line. (B) The comparison between intra- and inter-sample variances of vertebrate prey compositions. The four asterisks (****) represent $p < 0.0001$ by the Wilcoxon rank-sum test.



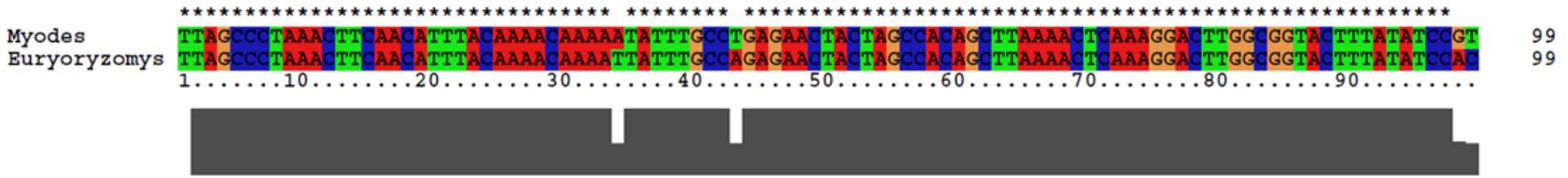
S2.3 Fig. Spatial comparisons of α and β diversities by vertebrate mitochondrial 12S rRNA gene sequencing. The reads assigned to the family Felidae were excluded from the comparisons, and each library was rarefied to 15,800 reads. (A) Alpha diversity. The Chao1 estimator (community richness) and Shannon index (community diversity) based on OTU-level are shown. (B) Beta diversity. Non-metric multidimensional scaling (NMDS) plots based on the Bray–Curtis dissimilarity (community structure) and Jaccard index (community membership) based on OTU-level are shown. Abbreviation: CN, Chungcheongnam-do (n = 14); and GB, Gyeongsanbuk-do (n = 8).



S2.4 Fig. Phylogenetic tree of vertebrate mitochondrial 12S rRNA gene sequences of OTUs assigned to Cervus (n = 1) and Hydropotes (n = 24). Clustal W version 2.1 [145] was used with default setting of slow and accurate pairwise alignment method.



S2.5 Fig. Alignment of vertebrate mitochondrial 12S rRNA gene sequences of OTUs taxonomically assigned to Cervus (n = 1) and Hydropotes (n = 24). The centroid sequence was determined against 24 OTUs of Hydropotes based on sequence similarity of 0.9, and the determined centroid sequence was used for alignment. Clustal X version 2.1 [145] was used. The gray peak increases as the sequences are similar. A gray peak appears for each nucleotide, and the peak is lowered in case of mismatch.



S2.6 Fig. Alignment of vertebrate mitochondrial 12S rRNA gene sequences of OTUs taxonomically assigned to Myodes (n = 1) and Euryoryzomys (n = 1). Clustal X version 2.1 [145] was used. The gray peak increases as the sequences are similar. A gray peak appears for each nucleotide, and the peak is lowered in case of mismatch.

S2.1 Table. Sample metadata.

Sample ID	Sampling date (YYYY/MM/DD)	Latitude	Longitude	City or Gun
L1	2019/02/20	36°36'1.83"N	127°8'2.71"E	Gongju, Chungcheongnam-do
L2	2019/02/20	36°34'32.91"N	127°9'43.39"E	Gongju, Chungcheongnam-do
L3	2019/02/21	36°27'19.5"N	127°9'1.1"E	Gongju, Chungcheongnam-do
L4	2019/02/21	36°27'18.28"N	127°9'2.04"E	Gongju, Chungcheongnam-do
L5	2019/02/21	36°27'17.45"N	127°9'1.79"E	Gongju, Chungcheongnam-do
L6	2019/02/21	36°27'11.21"N	127°8'55.71"E	Gongju, Chungcheongnam-do
L7	2019/02/21	36°30'52.96"N	127°19'41.42"E	Yeongi-gun, Chungcheongnam-do
L8	2019/02/20	36°36'10.53"N	127°7'38.15"E	Gongju, Chungcheongnam-do
L9	2019/02/20	36°36'4.58"N	127°7'54.92"E	Gongju, Chungcheongnam-do
L10	2019/02/20	36°36'4.8"N	127°7'53.11"E	Gongju, Chungcheongnam-do
L11	2019/02/20	36°35'43.81"N	127°8'47.17"E	Gongju, Chungcheongnam-do
L12	2019/02/20	36°35'44.95"N	127°8'46.55"E	Gongju, Chungcheongnam-do
L13	2019/02/21	36°27'17.87"N	127°8'58.7"E	Gongju, Chungcheongnam-do
L14	2019/02/21	36°30'59.4"N	127°19'38.73"E	Yeongi-gun, Chungcheongnam-do
L15	2019/02/05	36°17'55.8"N	128°7'27.12"E	Sangju, Gyeongsangbuk-do
L16	2019/02/20	35°45'13.2"N	128°23'44.52"E	Dalseong-gun, Daegu
L17	2019/02/20	35°44'54"N	128°23'42.72"E	Dalseong-gun, Daegu
L18	2019/02/20	35°44'17.64"N	128°24'3.42"E	Goryeong, Gyeongsangbuk-do
L19	2019/02/20	35°44'18"N	128°24'3.12"E	Goryeong, Gyeongsangbuk-do
L20	2019/02/20	35°44'17.46"N	128°24'3.66"E	Goryeong, Gyeongsangbuk-do
L21	2019/02/20	35°42'52.5"N	128°25'54.36"E	Goryeong, Gyeongsangbuk-do
L22	2019/02/20	35°42'13.8"N	128°23'39.9"E	Goryeong, Gyeongsangbuk-do

S2.2 Table. Shotgun metagenomic sequencing statistics. The number of sequence reads that are thought to be derived from the diet of the leopard cat is shown [a].

Sample ID	Accession number [b]	No. reads assigned to Chordata [c]	No. reads assigned to Streptophyta	No. reads assigned to Arthropoda	Total
L3	SAMN22418688	18,465	1,364	1,708	21,537
L4	SAMN22418689	20,408	2,187	2,209	24,804
L5	SAMN22418690	5,353	12,235	3,107	20,695
L7	SAMN22418691	12,994	4,430	1,995	19,419
L9	SAMN22418692	3,047	4,611	1,234	8,892
L10	SAMN22418693	7,589	61,264	3,471	72,324
L11	SAMN22418694	6,102	35,360	2,695	44,157
L12	SAMN22418695	5,499	32,524	1,836	39,859
L13	SAMN22418696	26,513	8,840	4,218	39,571
L15	SAMN22418697	17,004	26,046	6,554	49,604
L22	SAMN22418698	2,744	5,347	3,092	11,183
Total		125,718	194,208	32,119	352,045

[a] As the phyla that are thought to be related to the diet of the leopard cat, Chordata, Streptophyta, and Arthropoda were selected.

[b] Raw data has been previously published by our study [111] under the accession number of PRJNA772888 of NCBI.

[c] The reads assigned to the family Felidae to which the leopard cat belongs were excluded because they are likely to be derived from the leopard cat itself (not from the diet).

S2.3 Table. Vertebrate mitochondrial 12S rRNA gene sequencing statistics.

Sample ID	Accession number	No. sequence reads
L1	SAMN29914670	103,904
L2	SAMN29914671	48,693
L3	SAMN29914672	90,343
L3_dup	SAMN29914672	134,637
L4	SAMN29914673	69,969
L4_dup	SAMN29914673	161,245
L5	SAMN29914674	92,925
L6	SAMN29914675	66,271
L7	SAMN29914676	86,932
L8	SAMN29914677	96,808
L9	SAMN29914678	75,460
L9_dup	SAMN29914678	140,001
L10	SAMN29914679	85,175
L11	SAMN29914680	97,166
L12	SAMN29914681	37,386
L12_dup	SAMN29914681	149,700
L13	SAMN29914682	78,576
L14	SAMN29914683	84,382
L15	SAMN29914684	80,200
L16	SAMN29914685	51,006
L17	SAMN29914686	129,413
L18	SAMN29914687	80,916
L19	SAMN29914688	89,688
L20	SAMN29914689	77,293
L20_dup	SAMN29914689	154,454
L21	SAMN29914690	70,430
L21_dup	SAMN29914690	143,366
L22	SAMN29914691	91,863
L22_dup	SAMN29914691	147,867
Total		2,816,069

S2.4 Table. Major Streptophyta genera identified by shotgun metagenomic sequencing and their inhabitation status in Korea. Fifteen most abundant genera detected are shown.

Genus	Mean relative abundance (%)	Inhabitation in Korea	Availability of genome
Ricinus	49.8	Confirmed [146]	*Ricinus communis* [147]
Arabidopsis	13.6	Not confirmed	*Arabidopsis thaliana* [148, 149]
Physcomitrella	12.6	Not confirmed	*Physcomitrella patens* [150]
Vitis	5.6	Confirmed [151]	*Vitis vinifera* [152]
Populus	5.3	Confirmed [153]	*Populus trichocarpa* [154]
Oryza	3.6	Confirmed [155]	*Oryza sativa* [156, 157]
Sorghum	3.5	Confirmed [158]	*Sorghum bicolor* [159]
Selaginella	1.2	Confirmed [151]	*Selaginella moellendorffii* [160]
Equisetum	0.4	Confirmed [151]	Not available
Oenothera	0.3	Confirmed [151]	*Oenothera biennis* [161, 162]
Syntrichia	0.3	Confirmed [163]	*Syntrichia caninervis* [164]
Marchantia	0.3	Confirmed [165]	*Marchantia polymorpha* [166]
Chara	0.3	Confirmed [167]	*Chara braunii* [168]
Mesostigma	0.2	Not confirmed	*Mesostigma viride* [169]
Spinacia	0.2	Confirmed [170]	*Spinacia oleracea* [171]

Chapter 3. Diversity of fecal parasitomes of wild carnivores inhabiting Korea, including zoonotic parasites and parasites of their prey animals, as revealed by 18S rRNA gene sequencing

3.1. Introduction

Parasites are one of the most understudied pathogens, despite the fact that large numbers of people are at risk of adverse health effects from their infections [172]. Furthermore, in addition to their impacts on human health, they cause pathological conditions in livestock, hinder food intake and growth, and reduce livestock productivity [173]. Additionally, parasite infections are impacting the health of wildlife, including threatened species, raising concerns about their population declines [174]. However, previous research on parasites has mainly focused on those that pose a high risk to humans and livestock [175], and their diversity, host relationships, and ecosystem roles are still poorly understood [175, 176]. For example, there are an estimated 100,000–350,000 species of endoparasites in vertebrates, of which 85%–95% are still scientifically undescribed [176]. In particular, as urbanization increases the risk of contact between humans and wildlife [177], there is a growing need for research on zoonotic parasites in wildlife from the perspective of One Health.

Parasite identification has historically been based primarily on morphological observations, and more recently by serological, molecular, and proteomics–based methods [178]. For example, morphological identification is conducted by observing the egg, cyst, or larval morphology unique to each parasite [179]. However, these methods have drawbacks, such as the need for specialized

knowledge of parasite morphology, the need for laborious observation, and the inability to objectively identify parasite species. On the other hand, among the newly adopted methods, the method using DNA barcodes has the advantages of being able to identify the species of parasites with high sensitivity and objectiveness, and to analyze their composition and diversity. As examples of DNA barcode markers for parasites, the mitochondrial cytochrome oxidase subunit I [180], the 18S ribosomal RNA (rRNA) gene [181], and the internal transcribed spacer 2 region [182] have been used. Recently, a method using multiple primer pairs targeting the 18S rRNA genes of a wide variety of parasites has also been introduced, enabling comprehensive characterization of the parasites in environmental samples [183].

Here, we aimed to characterize the diversity of parasitomes (collections of parasites) in feces of three species of wild carnivores inhabiting Korea, namely, the raccoon dog (*Nyctereutes procyonoides*), the leopard cat (*Prionailurus bengalensis*), and the Eurasian otter (*Lutra lutra*), including zoonotic parasites and parasites of their prey animals. In South Korea, the raccoon dog maintains decent population, but the leopard cat and the Eurasian otter are classified as endangered species [7]. To comprehensively detect species of parasites in carnivorous wild animals, we used the method reported by Cannon et al. [183], in which 13 primer pairs are used to amplify 18S rRNA genes of parasites belonging to nine different taxonomic groups: Amoebozoa, Apicomplexa, *Blastocystis*, Diplomonadida, Kinetoplastida, Microsporidia, Nematoda, Parabasalia, and Platyhelminthes. To our knowledge, few studies have investigated the parasitomes of those carnivorous wildlife in Korea.

3.2. Materials and methods

3.2.1. Fecal samples

Fecal samples previously collected for diet studies of the wild

animals [33, 109, 184] were used. A total of 40 fecal samples were collected, consisting of 11, 22, and 7 samples of raccoon dogs, leopard cats, and Eurasian otters, respectively. Sample metadata are listed in S3.1 Table and sample collection methods are reported in our previous studies [33, 109, 184]. Briefly, fecal samples of raccoon dogs and Eurasian otters were collected from a waterside area [109] and an estuary area [33], respectively. Fecal samples of leopard cats were collected in inland areas [184]. Approximately 10 g of each sample was collected with a sterile wooden spatula in the field. Each collected sample was placed in a 50 ml conical tube and transported to the laboratory with ice packs. In our previous studies [33, 40], the host of each fecal sample was confirmed by PCR specific for each animal. The transported samples were stored at −80° C until analysis.

3.2.2. DNA extraction

DNA was extracted from the collected fecal samples using a PowerMax® Soil DNA Isolation Kit (Mobio Laboratory, Inc., Carlsbad, CA, USA). Prior to DNA extraction, each sample was manually homogenized using 5 ml of ultra-pure water and a sterilized wooden spatula. Approximately 0.2 g of each homogenized sample was introduced into a 2 ml tube supplied with the kit. To enhance DNA extraction efficiency, 300 mg of 0.1 mm diameter glass bead and 100 mg of 0.5 mm diameter glass bead were added to the kit's 2 ml tube. Each sample was homogenized for 3 min using a bead beater (BioSpec Products, Inc., Bartlesville, OK, USA). Then, DNA was purified and eluted in 50 μl of Tris-EDTA buffer according to the kit's protocol. The extracted DNA was stored at −80℃ until analysis.

3.2.3. DNA sequencing

The method reported by Cannon et al. [183] was used. Briefly, for each sample, 13 different primer pairs were used to amplify the 18S rRNA genes of nine different groups of parasites: Amoebozoa, Apicomplexa, *Blastocystis*, Diplomonadida, Kinetoplastida,

Microsporidia, Nematoda, Parabasalia, and Platyhelminthes. PCR primers and reaction conditions were according to the method reported by Cannon et al. [183]. These PCR assays used primers with the adapter sequences of Illumina MiSeq (Illumina, Inc., San Diego, CA, USA). PCR products were checked on an agarose gel with SYBR™ Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and those with unintended lengths were excluded. PCR products amplified with 13 different primer pairs derived from each fecal sample were purified by AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA). The purified DNA samples were quantified using a DS-11 FX (DeNovix, Inc., Wilmington, DE, USA), normalized to the lowest concentration among the purified DNA samples, and diluted with 10 mM Tris pH 8.5. The normalized DNA samples from each fecal sample were pooled into a single sample. Each pooled sample was tagged by index PCR using a Nextera XT Index Kit v2 (Illumina). After index PCR, the products were purified with AMPure XP beads (Beckman Coulter). The purified DNA samples were quantified using a Quant-iT PicoGreen dsDNA reagent kit (Life Technologies, Carlsbad, CA, USA). The samples were diluted with 10 mM Tris pH 8.5 and normalized to 4 nM. The normalized DNA samples were loaded onto a v3 600 cycle-kit reagent cartridge (Illumina) with 30% PhiX internal control for 2 × 300 bp paired-end sequencing on an Illumina MiSeq system. Negative controls were included in all PCRs and had no amplification.

3.2.4. Data processing and analysis

Trimming adapter and index sequences and removing reads with a quality score <20 were performed using MiSeq Reporter version 2.5 (Illumina). Additionally, low-quality reads with >1.0 expected errors and reads with less than 150 bp in length were removed using USEARCH version 11.0.667 [85]. Then, the UNOISE algorithm [185] was used to identify zero-radius operational taxonomic units (ZOTUs) and remove chimeric reads. Additionally, if the forward and reverse primer sequence pairs contained in the

ZOTU sequences were from PCRs used to detect different parasite groups, they were determined to be chimeric and manually excluded. Each of resultant ZOTUs was taxonomically assigned by BLASTN version 2.14.0+ against the NCBI nucleotide collection (nt) database in May 2023. Taxonomic assignments were performed based on the lowest E-value, with cut-off values for E-value and alignment identity set at $<1e^{-10}$ and >99%, respectively. For species-level identification, only cases where the top hit of each ZOTU was assigned to only one species were allowed, and all other cases were regarded ambiguous. If multiple species belonging to the same genus, family, order, or class were assigned as top hits, they were considered ambiguous at the species level and assigned to a taxon that was unambiguous at the taxonomic level higher than the species level. Unintended reads from non-parasites such as fungi, bacteria, and arthropods that might have been amplified due to the inadequate primer specificity [183] were excluded. In addition, phylogenetic tree analysis was performed to confirm the BLASTN results. Specifically, the sequences of the top 100 BLASTN hits of each ZOTU were aligned with those of the ZOTUs by the MUSCLE [186] and the phylogenetic tree was constructed with the neighbor-joining method [187] for each parasite species.

3.2.5. Statistical analysis

Statistical analysis was performed using the phyloseq package [45] and vegan package [46] on R version 4.1.0. The diversity of parasites within (α diversity) and between (β diversity) samples were analyzed. For α diversity, the Chao1 richness estimator and Shannon index were calculated to characterize parasite richness and diversity within each sample, respectively. Kruskal–Wallis and *post hoc* Wilcoxon rank-sum tests were performed to compare these α diversity metrics between the host animals. For β diversity, the Jaccard index and Bray–Curtis dissimilarity were calculated to characterize differences in parasite membership and structure between samples, respectively. Permutational multivariate analysis of variance (PERMANOVA) was performed to compare differences

in parasite membership and structure between the host animals.

3.3. Results

3.3.1. Sequencing statistics

A total of 2,093,268 high-quality sequence reads were generated from a total of 39 fecal samples (S3.2 Table). One of the leopard cat samples (L10) was excluded because the target organisms were not amplified. Of these sequence reads obtained, approximately 43.6% (912,196 reads) were assigned to the target parasites. The remaining reads were excluded from subsequent analyses, either because they were not classified into the targeted parasites or did not meet criteria of e-value and/or alignment identity.

3.3.2. Diversity

Statistical differences were found in the Chao1 estimator and Shannon index, which represent within-sample diversity (α diversity) of parasite ZOTUs, between the host animals ($p < 0.05$; Kruskal Wallis rank sum test) (Fig 3.1A), indicating that the parasite richness in the samples are different between the host animals. Similarly, differences were identified in between-sample diversity (β diversity), such as structure (Bray-Curtis dissimilarity) and membership (Jaccard index) of parasite ZOTUs, between the host animals (r^2 = 0.146 and 0.248, respectively, $p < 0.001$; PERMANOVA) (Fig 3.1B), indicating that the parasite species that constitute the parasitome are different for each host animal.

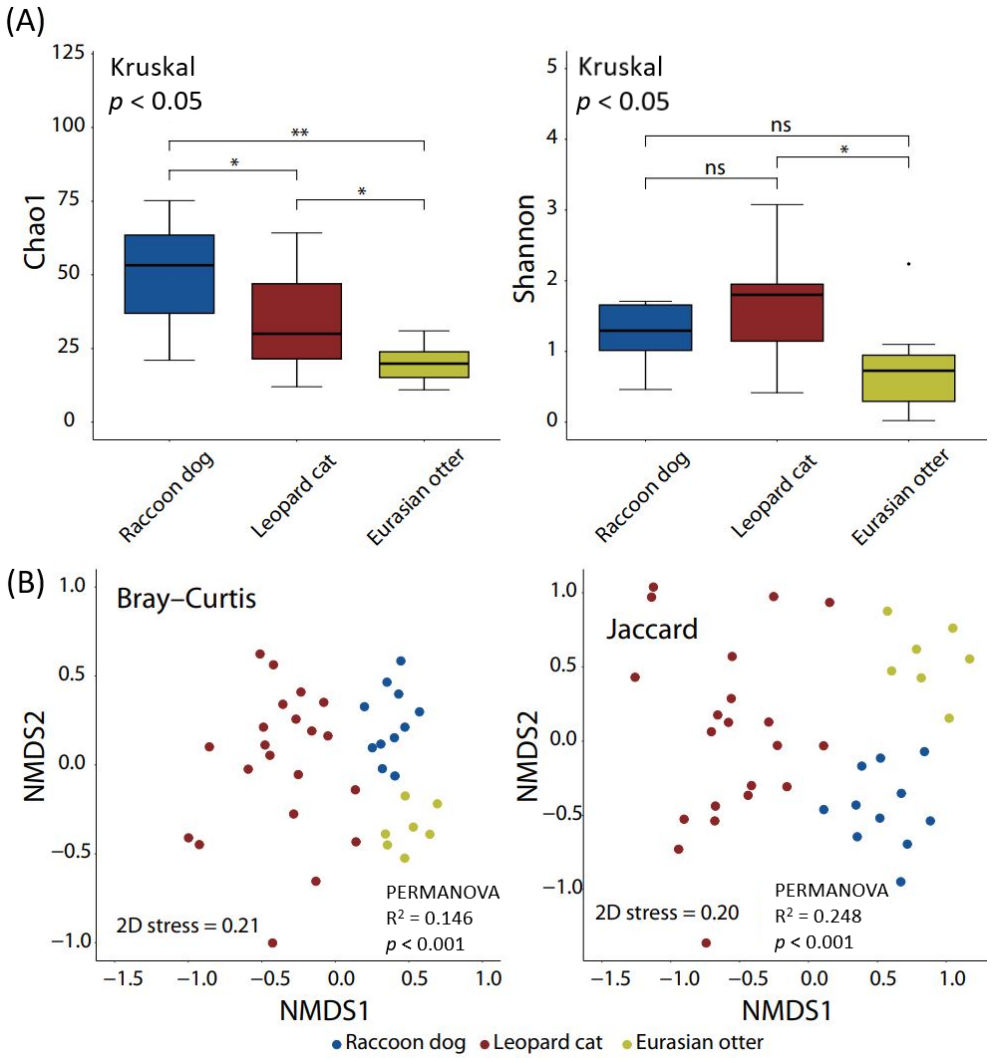


Fig. 3.1. Diversity of fecal parasitomes of wild carnivores in Korea. The results shown are based on the diversity of zero-radius operational taxonomic units (ZOTUs) that were taxonomically assigned to parasites. (a) Comparison of richness and diversity of parasite ZOTUs between host animals estimated by the Chao1 estimator and Shannon index, respectively. (b) Non-metric multidimensional scaling (NMDS) plots showing the structure and membership of parasite ZOTUs represented by the Bray–Curtis dissimilarity and Jaccard index, respectively. In the panel (a), one asterisk (*) and two asterisks (**) represent $p < 0.05$ and $p < 0.01$, respectively, by the *post hoc* Wilcoxon rank-sum test. The abbreviation “ns” represents no statistical difference.

3.3.3. Taxonomic composition

Numerous parasites belonging to eight target groups, excluding Diplomonadida, were detected (Fig 3.2). A total of 35 parasite genera, including parasites of prey animals of the carnivorous animals investigated in this study, were identified, i.e., 18 from raccoon dogs, 22 from leopard cats, and 11 from Eurasian otters (S3.3 Table). Among Apicomplexa genera, *Goussia*, *Eimeria*, and *Sarcocystis* were abundantly detected. In particular, *Goussia* was detected in large amounts in the feces of Eurasian otters and raccoon dogs. *Eimeria* and *Sarcocystis* were abundantly detected in the feces of leopard cats. Among Nematoda genera, *Heterakis* was abundantly detected in the feces of leopard cats. In addition, *Philometra* was detected in a large amount in a fecal sample of the Eurasian otter. Among Platyhelminthes genera, *Neodiplostomum* was abundantly detected in fecal samples of leopard cats. Information on parasites identified at the species level and their known hosts is provided in S3.4 and S3.5 Tables.

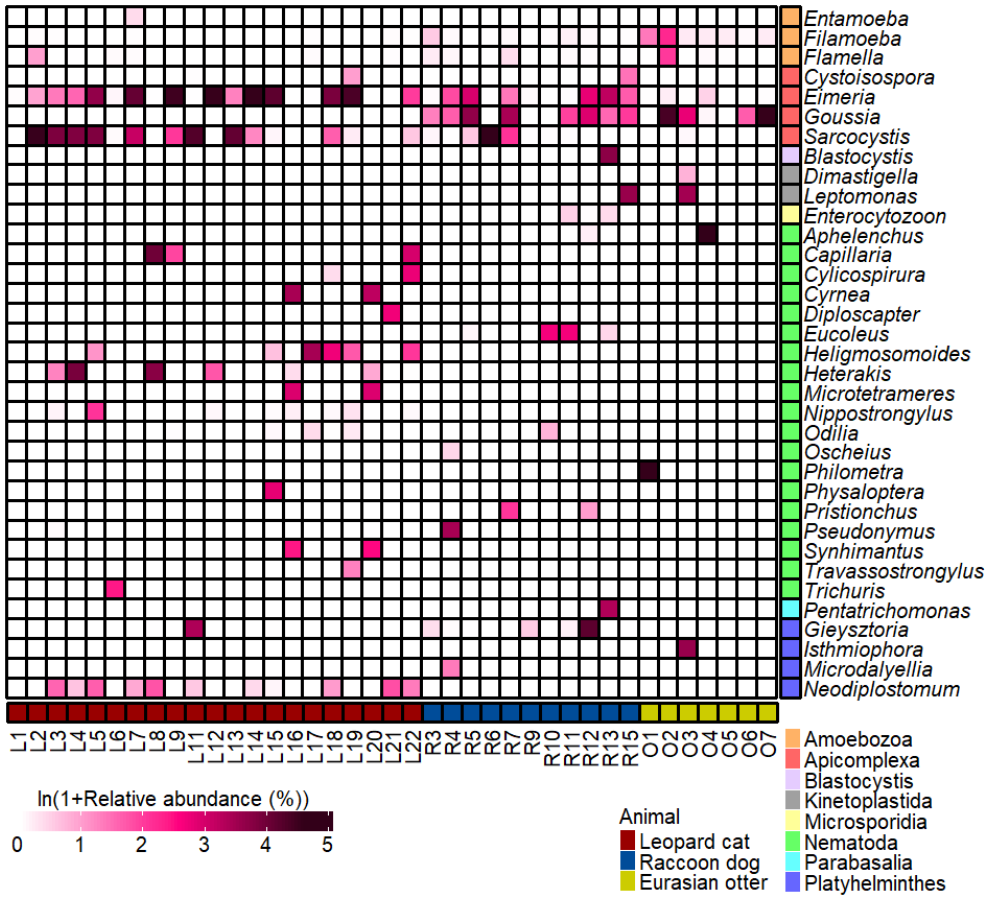


Fig 3.2. Relative abundance of all parasite genera detected from fecal samples of wild carnivores in Korea. The relative abundance of each parasite is defined as the ratio of the number of sequence reads assigned to that parasite to the total number of sequence reads assigned to all target parasites.

3.3.4. Zoonotic parasites

Some of the detected DNA had sequences identical or highly similar to 18S rRNA gene sequences of zoonotic parasites known to parasitize humans. Table 3.1 shows 5 zoonotic parasite species with sequences identical or highly similar to those of ZOTUs detected in this study. The five species are *Enterocytozoon bieneusi*, *Capillaria hepatica*, *Trichuris vulpis*, *Pentatrichomonas hominis*, and *Isthmiophora hortensis*, ZOTUs assigned to those species were unambiguously assigned only as those species without being top-hit with other species at the same E-value. Their phylogenetic trees are shown in S3.1–3.5 Figs, and information on the DNA sequences of ZOTUs assigned to these zoonotic parasites is provided in S3.1 Data.

Table 3.1. Zoonotic parasites detected from fecal samples of wild carnivores in Korea. Phylogenetic trees and sequences of ZOTUs assigned to these zoonotic parasites are provided in S3.1–3.5 Figs and S3.1 Data, respectively.

Group	Species	Number of samples detected			Definitive and/or intermediate hosts	Ref.
		Raccoon dog (n = 11)	Leopard cat (n = 21)	Eurasian otter (n = 7)		
Microsporidia	*Enterocytozoon bieneusi*	3	n.d.	n.d.	Primates, pigs, cattle, horses, llamas, kudus, dogs, cats, foxes, raccoon, otters, guinea pig, beavers, rabbits, muskrats, falcons, and other birds	[188]
Nematoda	*Capillaria hepatica*	n.d.	3	n.d.	Rodents and mammals	[189, 190]
	Trichuris vulpis	n.d.	1	n.d.	Canine	[191]
Parabasalia	*Pentatrichomonas hominis*	1	n.d.	n.d.	Mammals	[192]
Platyhelminthes	*Isthmiophora hortensis*	n.d.	n.d.	1	Rats, dogs, cats, freshwater snails, loaches, and freshwater fish	[193]

Abbreviation: n.d., not detected.

3.4. Discussion

In this study, we successfully characterized the fecal parasitomes of raccoon dogs, leopard cats, and Eurasian otters inhabiting Korea by 18S rRNA gene sequencing using multiple primer pairs. We found that the richness, structure, and membership of parasites varied greatly between the host animals. Furthermore, we confirmed the detection of DNA sequences identical or highly similar to the 18S rRNA gene sequences of 5 zoonotic parasites known to parasitize humans. The method used in this study has the advantage of being able to detect a wide range of parasite groups from a sample compared to the traditional methods. For example, the method we used can detect not only host-specific parasites but also non-host-specific parasites. Therefore, a comprehensive understanding of parasites can be achieved without being bound by *a priori* assumptions. Furthermore, sequencing, such as bacterial 16S rRNA gene sequencing, generally proceeds to one region as a target and uses a single primer pair, but the method of pooling each PCR product with multiple primer pairs has an advantage in terms of sequencing cost.

Differences in parasitomes between the host animals are most likely due to their dietary content. For example, genera containing fish parasites, such as the apicomplexa *Goussia* [194] and *Philometra* [195], were abundantly detected in the feces of raccoon dogs and/or Eurasian otter. Our dietary research revealed that fish is the main food of raccoon dogs inhabiting the waterside area [109] and Eurasian otters inhabiting the estuary area [33] in Korea. In addition, in the feces of leopard cats, genera containing species whose intermediate and/or definitive hosts are small mammals such as rodents and/or birds, e.g., the apicomplexa *Eimeria* [196] and *Sarcocystis* [197], the nematodes and *Heterakis* [198, 199], and the platyhelminths *Neodiplostomum* [200], were abundantly detected. Our dietary study revealed that murids and birds are the main prey of leopard cats in the inland areas [184]. It is well known

that parasites pass from prey to predators, in which they are digested to death or an infection is established [201]. We consider that the observed differences in parasitomes are due to each host preying on different animals and thus concomitantly taking up different species of parasites.

We also detected DNA with sequences identical or highly similar to those of zoonotic parasites that can parasitize humans. For example, DNA with sequences highly similar to those of the microsporidia *Enterocytozoon bieneusi* was detected in some fecal samples of raccoon dogs. The hosts of *E. bieneusi* are wide-ranging, including canids, felids, and primates including humans [188]. It has been reported that the DNA of *E. bieneusi* was detected in the feces of raccoon dogs in Korea [202]. DNA with a sequence identical to those of the parabasalid *Pentatrichomonas hominis* was also detected from a fecal sample of raccoon dog. *P. hominis* is considered zoonotic and is known to parasitize mammals such as humans, monkeys, cats, dogs, and rats [192]. It has been reported that *P. hominis* was detected in farmed raccoon dogs in China [203].

Capillaria hepatica, a zoonotic nematode, was detected in some leopard cat feces. *C. hepatica* is known to have a wide range of hosts including rodents [189] and other mammals, such as domestic cats [190]. There have been no reports that *C. hepatica* has been detected in the leopard cat, but it has been reported that it was detected in the Sunda leopard cat (*Prionailurus javanensis*), a close relative of the leopard cat, in the Philippines [204]. A zoonotic nematodes *Trichuris vulpis*, which is known to have canids as their definitive hosts [191, 205], was also detected from a fecal samples of leopard cats. Few cases of the detection of *T. vulpis* from felines have been reported, and we do not know the reason. One hypothesis is that the leopard cat concomitantly preyed on the dog parasitized with *T. vulpis*, because our dietary study identified the dog as a dietary item of leopard cats in our study areas [184].

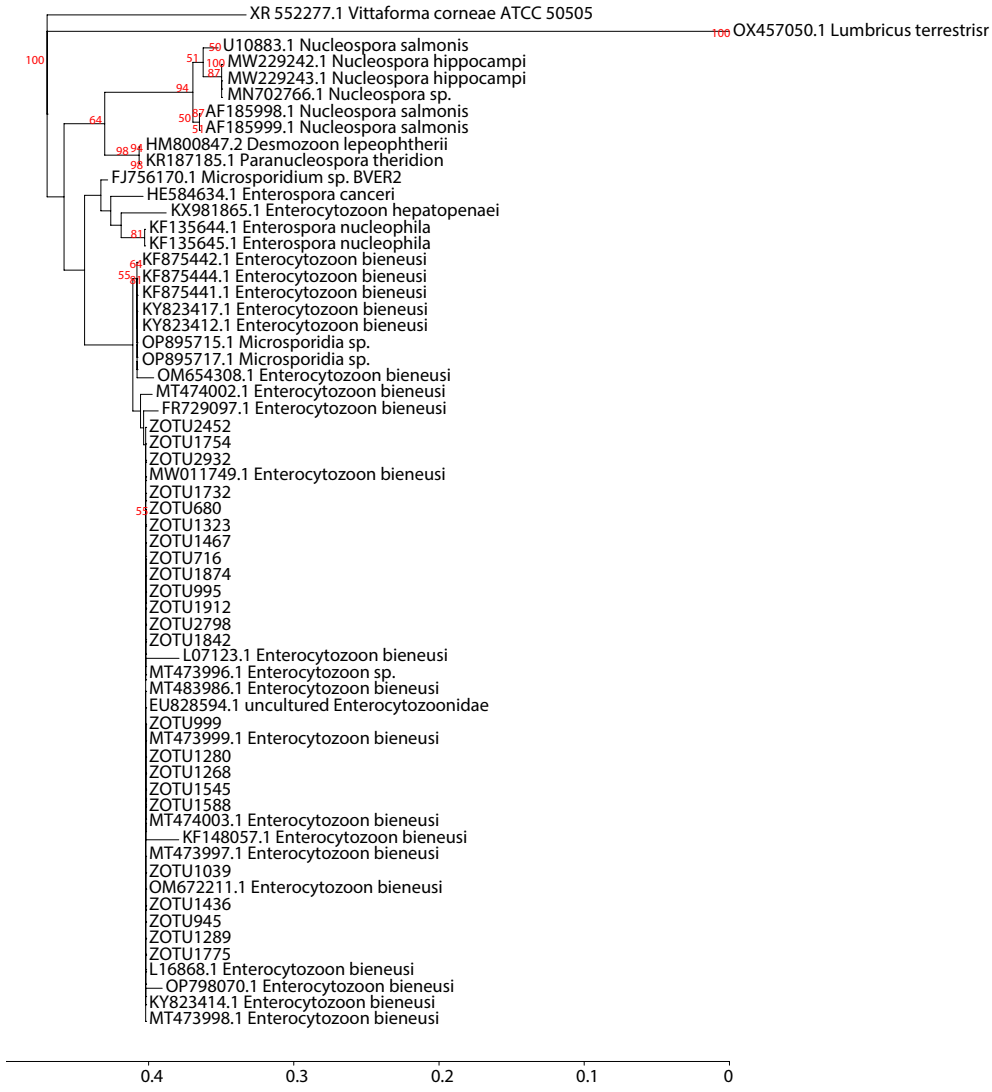
From a fecal sample of Eurasian otter, four ZOTUs with DNA sequences similar to the DNA sequence of the platyhelminth *Isthmiophora hortensis* (S3.1 Data), but well-separated phylogenetically (S3.5 Fig), were detected. These ZOTUs may be derived from a novel species of the *Isthmiophora* genus, but further studies are needed to prove this. The detection of *Isthmiophora* species seems reasonable because the *Isthmiophora* genus includes fish parasites [193]. In fact, it has been reported that several species of *Isthmiophora* have been detected in carcasses of Eurasian otters, such as *Isthmiophora inermis* in Korea [206] and *Isthmiophora melis* in Denmark [207]. Additionally, the *Isthmiophora* genus, including *I. hortensis*, has been reported to be prevalent in wildlife, especially mustelids, in Korea [206].

Finally, we note that analysis of sequencing data, especially removal of chimeras, requires careful attention. Some of our ZOTUs consisted of sequences from different pairs of forward and reverse primers, which we manually removed. In addition, we also note that sequencing that includes the PCR process cannot be free from PCR bias, and in particular, PCR bias can make it difficult to detect taxa that exist in small quantities [141, 183].

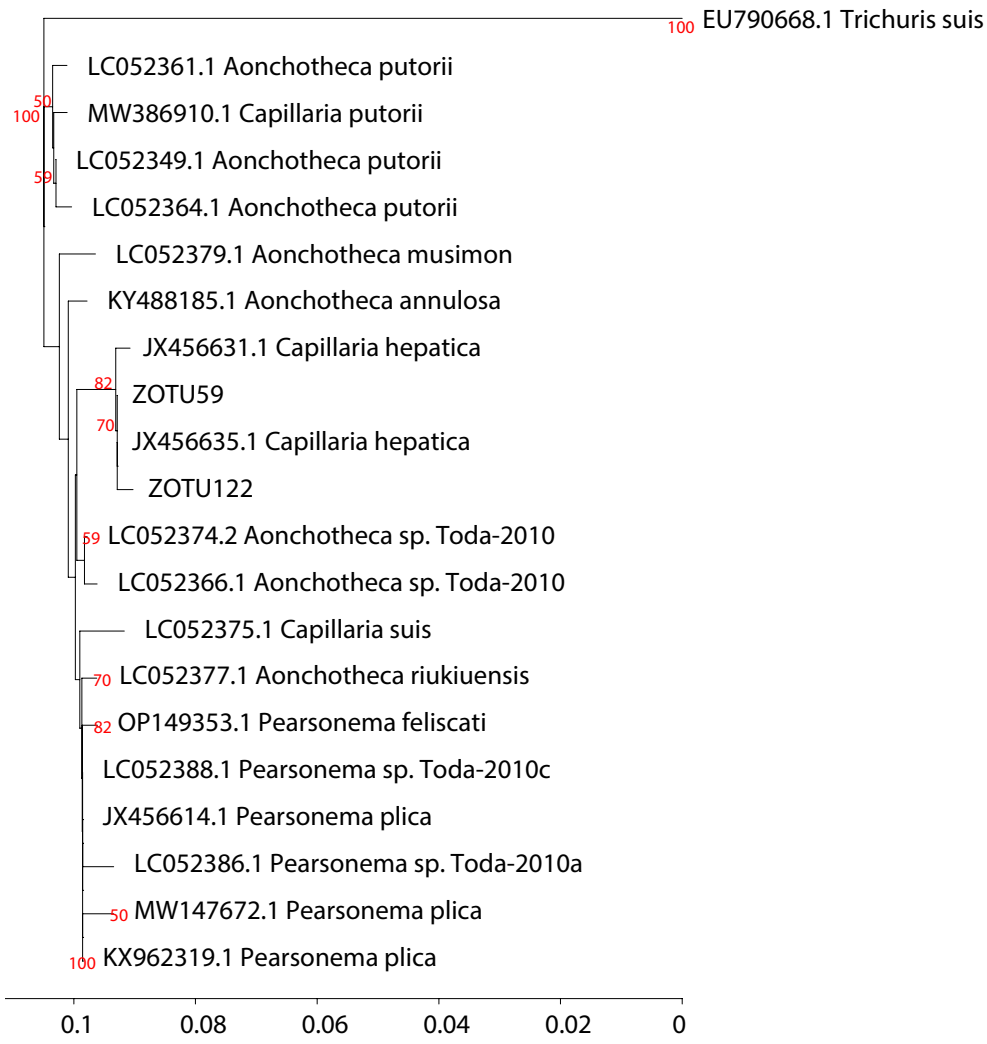
3.5. Conclusion

In Korea, an increase in wildlife-associated diseases has been reported in recent decades [4]. Therefore, it is imperative to monitor zoonotic pathogens, including zoonotic parasites, harbored by wildlife. In this study, we investigated the diversity of parasitomes, including zoonotic parasites, associated with the carnivorous wildlife in Korea by using 18S rRNA gene sequencing with multiple primer pairs. We found that the parasitome richness, structure, and membership differed between the host animals, and that the detected zoonotic parasites were plausible in light of the animal species known to be their hosts, as well as the predatory propensities of the host animals. In the future, it is expected that

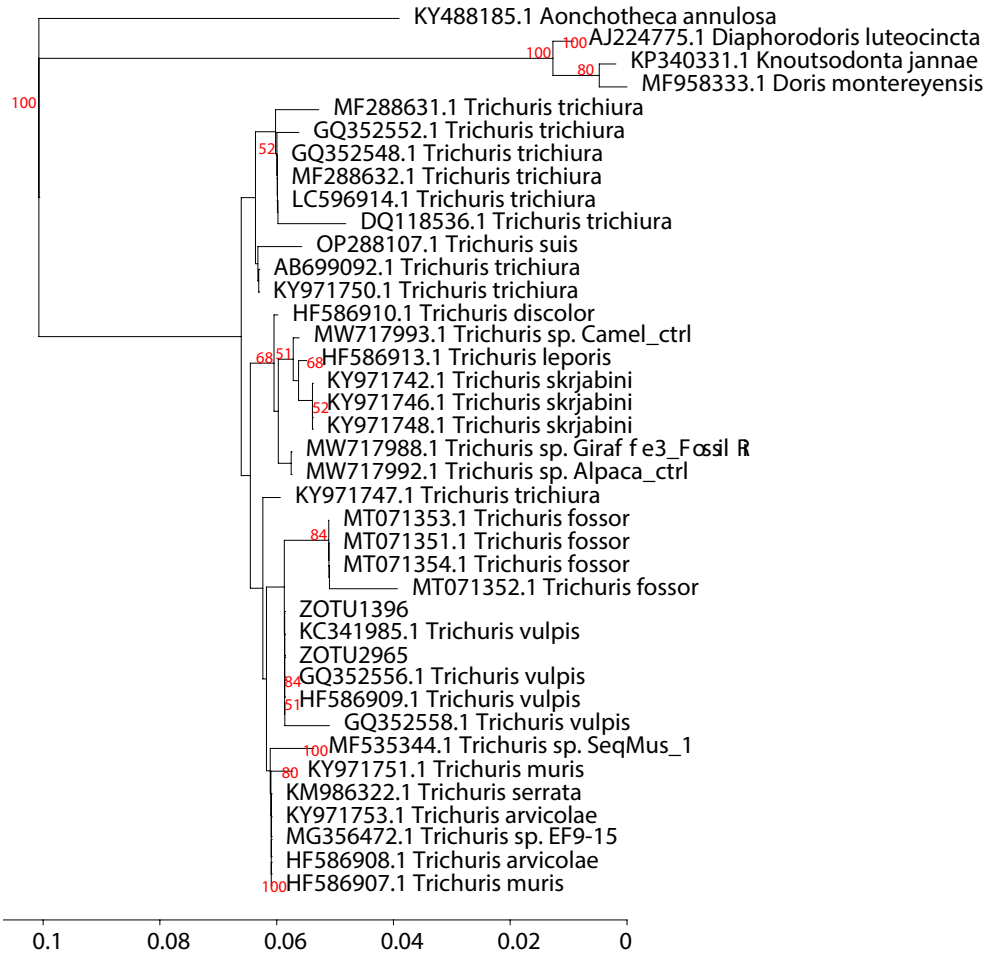
zoonotic diseases will further increase due to the increased proximity between humans and wildlife due to urbanization [208]. Vigilance may need to be continuously exercised, such as by monitoring parasites in the feces of wildlife, as was done in this study.



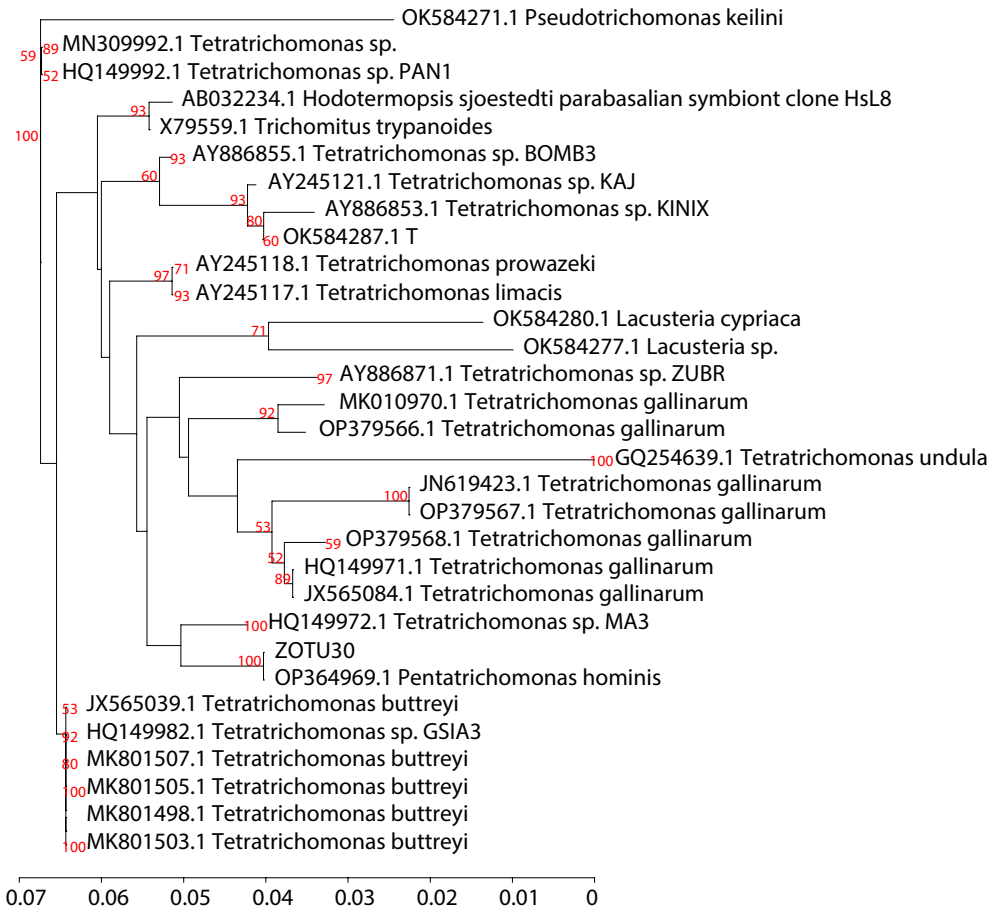
S3.1 Fig. Phylogenetic tree of the 23 ZOTUs taxonomically assigned as *Enterocytozoon bieneusi* and the top 100 BLASTN hits of the ZOTUs. Aligned sequences to ZOTUs with 100% similarity were merged, and multiple sequence alignment was performed using the MUSCLE [186]. Phylogenetic tree construction was performed based on the neighbor-joining method [187] on the ape package [209] of R version 4.1.0. The values in red represent percentages of bootstrap values based on 1000 resamplings. The sequence of *Vittaforma corneae* is used as the root.



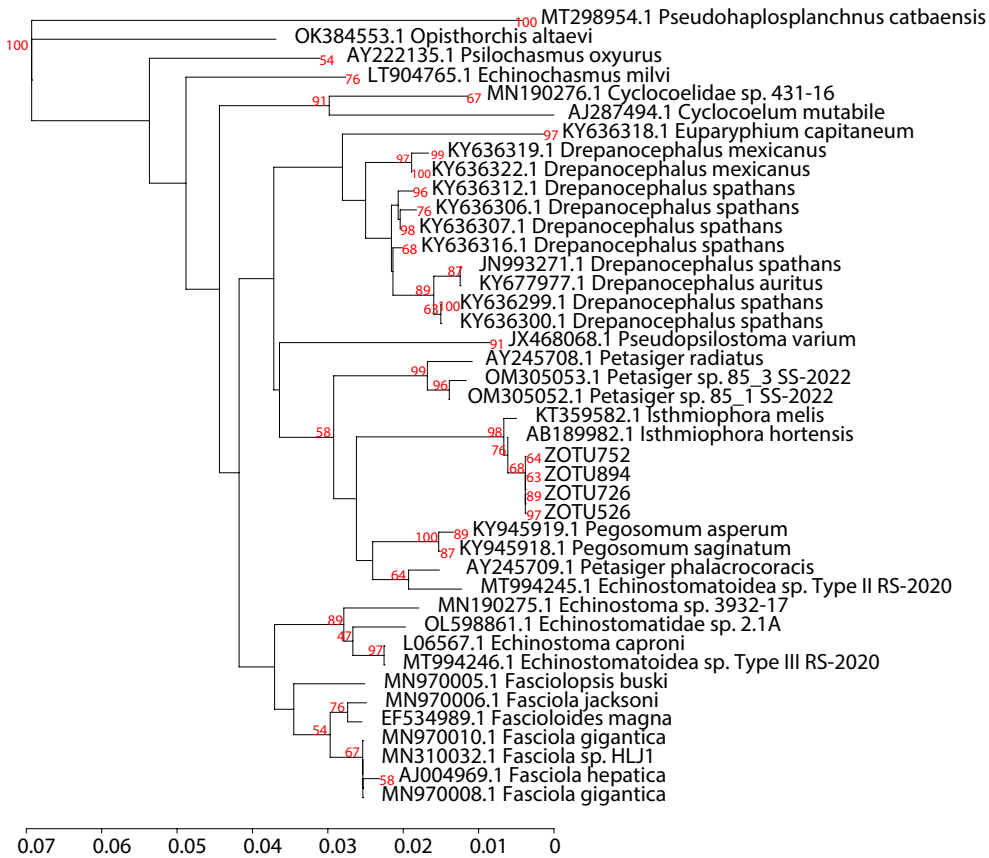
S3.2 Fig. Phylogenetic tree of the 2 ZOTUs taxonomically assigned as *Capillaria hepatica* and the top 100 BLASTN hits of the ZOTUs. Aligned sequences to ZOTUs with 100% similarity were merged, and multiple sequence alignment was performed using the MUSCLE [186]. Phylogenetic tree construction was performed based on the neighbor-joining method [187] on the ape package [209] of R version 4.1.0. The values in red represent percentages of bootstrap values based on 1000 resamplings. The sequence of *Trichuris suis* is used as the root.



S3.3 Fig. Phylogenetic tree of the 2 ZOTUs taxonomically assigned as *Trichuris vulpis* and the top 100 BLASTN hits of the ZOTUs. Aligned sequences to ZOTUs with 100% similarity were merged, and multiple sequence alignment was performed using the MUSCLE [186]. Phylogenetic tree construction was performed based on the neighbor-joining method [187] on the ape package [209] of R version 4.1.0. The values in red represent percentages of bootstrap values based on 1000 resamplings. The sequence of *Aonchotheca annulosa* is used as the root.



S3.4 Fig. Phylogenetic tree of the ZOTU taxonomically assigned as *Pentatrichomonas hominis* and the top 100 BLASTN hits of the ZOTU. Aligned sequences to ZOTU with 100% similarity were merged, and multiple sequence alignment was performed using the MUSCLE [186]. Phylogenetic tree construction was performed based on the neighbor-joining method [187] on the ape package [209] of R version 4.1.0. The values in red represent percentages of bootstrap values based on 1000 resamplings. The sequence of *Pseudotrichomonas keilini* is used as the root.



S3.5 Fig. Phylogenetic tree of the 4 ZOTUs taxonomically assigned as *Isthmiophora hortensis* and the top 100 BLASTN hits of the ZOTUs. Aligned sequences to ZOTUs with 100% similarity were merged, and multiple sequence alignment was performed using the MUSCLE [186]. Phylogenetic tree construction was performed based on the neighbor-joining method [187] on the ape package [209] of R version 4.1.0. The values in red represent percentages of bootstrap values based on 1000 resamplings. The sequence of *Pseudohaplosplanchnus catbaensis* is used as the root.

S3.1 Table. Sample metadata.

Sample ID	Sampling date (YYYY/MM/DD)	Latitude	Longitude	City or Gun
R3	2017/05/21	36°40'34.80"N	126°28'10.85"E	Seosan, Chungcheongnam-do
R4	2017/05/21	36°40'07.82"N	126°28'40.65"E	Seosan, Chungcheongnam-do
R5	2017/05/21	36°39'59.61"N	126°28'46.67"E	Seosan, Chungcheongnam-do
R6	2017/05/21	36°40'18.71"N	126°29'13.25"E	Seosan, Chungcheongnam-do
R7	2017/05/21	36°40'15.01"N	126°30'25.57"E	Seosan, Chungcheongnam-do
R9	2017/05/21	36°39'39.54"N	126°27'49.62"E	Seosan, Chungcheongnam-do
R10	2017/05/21	36°39'41.34"N	126°27'55.72"E	Seosan, Chungcheongnam-do
R11	2017/05/21	36°39'45.32"N	126°28'08.19"E	Seosan, Chungcheongnam-do
R12	2017/05/21	36°39'47.84"N	126°28'16.84"E	Seosan, Chungcheongnam-do
R13	2017/05/21	36°37'50.97"N	126°29'28.88"E	Seosan, Chungcheongnam-do
R15	2017/05/21	36°37'44.85"N	126°28'37.22"E	Seosan, Chungcheongnam-do
L1	2019/02/20	36°36'1.83"N	127°8'2.71"E	Gongju, Chungcheongnam-do
L2	2019/02/20	36°34'32.91"N	127°9'43.39"E	Gongju, Chungcheongnam-do
L3	2019/02/21	36°27'19.5"N	127°9'1.1"E	Gongju, Chungcheongnam-do
L4	2019/02/21	36°27'18.28"N	127°9'2.04"E	Gongju, Chungcheongnam-do
L5	2019/02/21	36°27'17.45"N	127°9'1.79"E	Gongju, Chungcheongnam-do
L6	2019/02/21	36°27'11.21"N	127°8'55.71"E	Gongju, Chungcheongnam-do
L7	2019/02/21	36°30'52.96"N	127°19'41.42"E	Yeongi-gun, Chungcheongnam-do
L8	2019/02/20	36°36'10.53"N	127°7'38.15"E	Gongju, Chungcheongnam-do
L9	2019/02/20	36°36'4.58"N	127°7'54.92"E	Gongju, Chungcheongnam-do
L10	2019/02/20	36°36'4.8"N	127°7'53.11"E	Gongju, Chungcheongnam-do
L11	2019/02/20	36°35'43.81"N	127°8'47.17"E	Gongju, Chungcheongnam-do
L12	2019/02/20	36°35'44.95"N	127°8'46.55"E	Gongju, Chungcheongnam-do
L13	2019/02/21	36°27'17.87"N	127°8'58.7"E	Gongju, Chungcheongnam-do
L14	2019/02/21	36°30'59.4"N	127°19'38.73"E	Yeongi-gun, Chungcheongnam-do
L15	2019/02/05	36°17'55.8"N	128°7'27.12"E	Sangju, Gyeongsangbuk-do

L16	2019/02/20	35°45'13.2"N	128°23'44.52"E	Dalseong-gun, Daegu
L17	2019/02/20	35°44'54"N	128°23'42.72"E	Dalseong-gun, Daegu
L18	2019/02/20	35°44'17.64"N	128°24'3.42"E	Goryeong, Gyeongsangbuk-do
L19	2019/02/20	35°44'18"N	128°24'3.12"E	Goryeong, Gyeongsangbuk-do
L20	2019/02/20	35°44'17.46"N	128°24'3.66"E	Goryeong, Gyeongsangbuk-do
L21	2019/02/20	35°42'52.5"N	128°25'54.36"E	Goryeong, Gyeongsangbuk-do
L22	2019/02/20	35°42'13.8"N	128°23'39.9"E	Goryeong, Gyeongsangbuk-do
O1	2017/06/06	37°16'14.43"N	126°50'22.48"E	Ansan, Gyeonggi-do
O2	2017/06/06	37°16'14.83"N	126°50'22.78"E	Ansan, Gyeonggi-do
O3	2017/06/06	37°16'16.26"N	126°50'25.74"E	Ansan, Gyeonggi-do
O4	2017/06/06	37°16'14.82"N	126°50'27.78"E	Ansan, Gyeonggi-do
O5	2017/06/06	37°16'14.97"N	126°50'31.24"E	Ansan, Gyeonggi-do
O6	2017/06/06	37°16'14.23"N	126°50'31.32"E	Ansan, Gyeonggi-do
O7	2017/06/06	37°16'22.62"N	126°50'24.70"E	Ansan, Gyeonggi-do

S3.2 Table. DNA sequencing statistics.

Sample ID	Accession number	No. reads assigned to targeted organisms [a]	No. reads assigned to non-targeted organisms	Total
R3	SAMN33268131	4,382	49,833	54,215
R4	SAMN33268132	18,191	30,243	48,434
R5	SAMN33268133	23,724	29,979	53,703
R6	SAMN33268134	59,077	14,359	73,436
R7	SAMN33268135	2,691	47,266	49,957
R9	SAMN33268136	3,920	34,519	38,439
R10	SAMN33268137	6,718	23,117	29,835
R11	SAMN33268138	3,663	36,037	39,700
R12	SAMN33268139	30,380	37,578	67,958
R13	SAMN33268140	44,376	17,394	61,770
R15	SAMN33268141	27,547	44,980	72,527
L1	SAMN33268142	9,450	48,104	57,554
L2	SAMN33268143	11,365	52,076	63,441
L3	SAMN33268144	28,694	22,802	51,496
L4	SAMN33268145	37,452	31,361	68,813
L5	SAMN33268146	24,802	43,453	68,255
L6	SAMN33268147	1,941	32,293	34,234
L7	SAMN33268148	33,651	26,997	60,648
L8	SAMN33268149	11,284	25,246	36,530
L9	SAMN33268150	22,730	52,975	75,705
L11	SAMN33268151	25,448	37,701	63,149
L12	SAMN33268152	64,890	27,637	92,527
L13	SAMN33268153	38,356	32,502	70,858
L14	SAMN33268154	49,404	24,791	74,195
L15	SAMN33268155	38,200	19,310	57,510
L16	SAMN33268156	43,497	13,626	57,123
L17	SAMN33268157	17,862	18,369	36,231
L18	SAMN33268158	39,550	12,838	52,388
L19	SAMN33268159	46,837	14,341	61,178
L20	SAMN33268160	28,162	17,501	45,663
L21	SAMN33268161	23,494	22,836	46,330
L22	SAMN33268162	26,215	5,771	31,986
O1	SAMN33268163	9,351	37,045	46,396
O2	SAMN33268164	511	39,426	39,937
O3	SAMN33268165	7,264	27,706	34,970
O4	SAMN33268166	5,014	42,901	47,915
O5	SAMN33268167	18,806	23,461	42,267
O6	SAMN33268168	17,151	19,453	36,604
O7	SAMN33268169	6,146	43,245	49,391
Total		912,196	1,181,072	2,093,268

[a] Targeted organism are Amoebozoa, Apicomplexa, *Blastocystis*, Diplomonadida, Kinetoplastida, Microsporidia, Nematoda, Parabasalia, and Platyhelminthes.

S3.3 Table. List of detected parasite genera and number of samples detected.

Group	Genus	Raccoon dog (n = 11)	Leopard cat (n = 21)	Eurasian otter (n = 7)
Amoebozoa	*Entamoeba*	n.d.	1	n.d.
	Filamoeba	10	9	7
	Flamella	8	8	3
Apicomplexa	*Cystoisospora*	1	2	n.d.
	Eimeria	10	20	5
	Goussia	8	n.d.	5
	Sarcocystis	9	15	1
Blastocystis	*Blastocystis*	1	n.d.	n.d.
Kinetoplastida	*Dimastigella*	n.d.	n.d.	1
	Leptomonas	1	n.d.	1
Microsporidia	*Enterocytozoon*	3	n.d.	n.d.
Nematoda	*Aphelenchus*	1	n.d.	1
	Capillaria	n.d.	3	n.d.
	Cylicospirura	n.d.	3	n.d.
	Cyrnea	n.d.	4	n.d.
	Diploscapter	n.d.	1	n.d.
	Eucoleus	4	n.d.	n.d.
	Heligmosomoides	n.d.	8	n.d.
	Heterakis	n.d.	7	n.d.
	Microtetrameres	n.d.	2	n.d.
	Nippostrongylus	n.d.	8	n.d.
	Odilia	1	6	n.d.
	Oscheius	1	n.d.	n.d.
	Philometra	n.d.	n.d.	1
	Physaloptera	n.d.	2	1
	Pristionchus	2	n.d.	n.d.
	Pseudonymus	1	2	n.d.
	Synhimantus	n.d.	2	n.d.
	Travassostrongylus	n.d.	2	n.d.
	Trichuris	n.d.	1	n.d.
Parabasalia	*Pentatrichomonas*	1	n.d.	n.d.
Platyhelminthes	*Gieysztoria*	8	5	n.d.
	Isthmiophora	n.d.	n.d.	1
	Microdalyellia	1	n.d.	n.d.
	Neodiplostomum	n.d.	11	n.d.

Abbreviation: n.d., not detected.

S3.4 Table. Parasite species detected from fecal samples of the wild carnivores in Korea that are known or likely to be host-specific for that animal.

Group	Species	Number of samples detected			Host specificity	Definitive and/or intermediate hosts, or sources of isolation	Reference(s)
		Raccoon dog (n = 11)	Leopard cat (n = 21)	Eurasian otter (n = 7)			
Microsporidia	*Enterocytozoon bieneusi*	3	n.d.	n.d.	Identified	Primates, pigs, cattle, horses, llamas, kudus, dogs, cats, foxes, raccoons, otters, guinea pigs, beavers, rabbits, muskrats, falcons, and other birds	[188, 202]
Nematoda	*Capillaria hepatica*	n.d.	3	n.d.	Identified in relative species	Rodents and mammals	[189, 190, 204]
	Cylicospirura petrowi	n.d.	3	n.d.	Identified in relative species	Cats	[210]
Parabasalia	*Pentatrichomonas hominis*	1	n.d.	n.d.	Identified	Mammals	[192, 203]
Platyhelminthes	*Isthmiophora hortensis*	n.d.	n.d.	1	Possible, other species of the same genus have been detected	Rats, dogs, cats, freshwater snails, loaches, and freshwater fish	[193, 206]

Abbreviation: n.d., not detected.

S3.5 Table. Parasite species detected from fecal samples of the wild carnivores in Korea that are not host-specific for that animal and likely from their prey animals.

Group	Species [a]	Number of samples detected			Definitive and/or intermediate hosts, or sources of isolation	Reference(s)
		Raccoon dog (n = 11)	Leopard cat (n = 21)	Eurasian otter (n = 7)		
Amoebozoa	*Entamoeba bovis*	n.d.	1	n.d.	Cattle, sheep, and reindeer	[211]
Apicomplexa	*Eimeria adenoeides*	3	13	1	Turkeys	[212]
	Eimeria leucisci	5	3	n.d.	Cyprinid fish	[213, 214]
Kinetoplastida	*Dimastigella trypaniformis*	n.d.	n.d.	1	Isoptera	[215]
	Leptomonas jaculum	1	n.d.	1	*Nepa cinerea*	[216]
Nematoda	*Aphelenchus avenae*	n.d.	n.d.	1	Plant-pathogenic fungi and *Agaricus bisporus*	[217]
	Cyrnea leptoptera	n.d.	4	n.d.	*Milvus migrans* and *Falco tinnunculus*	[218, 219]
	Heligmosomoides thomomyos	n.d.	8	n.d.	Western pocket gophers	[220]
	Heterakis gallinarum	n.d.	2	n.d.	Gallinaceous birds	[198]
	Heterakis spumosa	n.d.	7	n.d.	Rats	[199]
	Microtetrameres cloacitectus	n.d.	2	n.d.	*Accipiter gentilis* and *A. nisus*	[218]
	Nippostrongylus brasiliensis	n.d.	8	n.d.	Rats	[221]
	Pseudonymus islamabadi	1	2	n.d.	Water beetles	[222]
	Synhimantus laticeps	n.d.	2	n.d.	Birds, terrestrial isopods, odonate, and dermapteran insects	[200]
	Trichuris vulpis	n.d.	1	n.d.	Canine	[223]
Platyhelminthes	*Neodiplostomum attenuatum*	n.d.	11	n.d.	Birds, amphibians, reptiles, and freshwater snails	[200]

[a] Species whose sources were environmental such as soil and water were excluded. In addition, free-living species, species for which host information has not been established, and species for which relevant literature was not found were excluded.
Abbreviation: n.d., not detected.

S3.1 Data. Information on sequence reads of ZOTUs assigned to these zoonotic parasites.

Enterocytozoon bieneusi (Microsporidia)
>ZOTU680
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Chapter 4. Diversity of fecal viromes, including zoonotic viruses, of wild carnivores in Korea revealed by metagenomic sequencing

4.1. Introduction

Viruses, one of the representative pathogens of emerging infectious diseases, are known to have profound effects on humans, livestock, crops, and wildlife. Viruses have adversely affected human health, dramatically changed people's lifestyles, and caused huge economic losses to related workers by reducing the population of livestock and crops and reducing productivity [224]. In addition, viruses can cause declines in wildlife populations, which can pose a great threat to endangered species in particular [225]. However, the diversity and identity of viruses, estimated at about 10^{31} and regarded omnipresent and plentiful in a variety of environments, are not fully understood [226]. Considering that about 60% of emerging pathogens are zoonotic and more than 71% of these pathogens originate from the wildlife [227], the increased contact frequency between humans and wild animals due to urbanization [208] may pose a new threat to public health. Therefore, the need for research on viruses in wild animals is growing from the perspective of One Health.

Viral research has historically focused on pathogenic viruses and viruses that can be cultured in cells [228], but the recent introduction of high-throughput sequencing and bioinformatic tools have made it possible to study viral communities in a variety of environments, such as ocean [229], soil [230], sewage sludge [231]. In addition, viral metagenomics has enabled the discovery of various viral communities and new viruses in wild animals, such as rodents [228], carnivores [232], birds [233] and bats [234]. Meanwhile, since the results of viral metagenomics can vary

depending on the protocol of isolating, purifying, and amplifying viruses from samples, various viral metagenomic preparation protocols have been implemented and evaluated [235–238]. Recently, a reproducible and optimized protocol has been introduced, enabling comprehensive identification of the virome in fecal sample [239].

This study aimed to investigate the composition and diversity of the viromes of three medium–sized carnivores, the raccoon dog (*Nyctereutes procyonoides*), the leopard cat (*Prionailurus bengalensis*), and the Eurasian otter (*Lutra lutra*), in Korea. The raccoon dog has a moderate population in South Korea, but in the case of the leopard cat and Eurasian otter, they are managed as endangered species by the Korean government [7]. For comprehensive identification of viromes of three wild animals, we adopted the method reported by Conceição–Neto et al. [239]. As far as we know, there are few studies investigating the virome of the three wild animals in Korea. In conclusion, our study introduces viral metagenomics to compare and analyze the viral composition and diversity of three medium–sized carnivores, and to identity potential public health risks.

4.2. Materials and methods

4.2.1. Fecal samples

The number of fecal samples of raccoon dogs, leopard cats, and Eurasian otter used in this study was 11, 22, and 7, respectively, and the detailed method of collecting fecal samples was described in our previous studies [33, 109, 184]. Briefly, fecal samples of raccoon dogs, leopard cats, and Eurasian otter were collected from the waterside area, inland area, and estuary area, respectively. Each of sample was collected with a sterile wooden spatula in a 50 ml sterile tube at each sample collection point and transported to the laboratory with ice packs. Host information for each fecal sample was confirmed by animal–specific PCR in our previous

studies [33, 40]. The collected fecal samples were stored at −80℃ until subsequent analysis. Additional information on each fecal sample is listed in S3.1 Table.

4.2.2. Virome library preparation and sequencing

Viral DNA and RNA extraction from each fecal sample was performed according to NetoVIR (Novel Enrichment Technique of Viromes) [239]. Briefly, each fecal sample was placed in a 2 ml tube without beads and homogenized for 3 min using a bead beater (BioSpec Products, Inc., Bartlesville, OK, USA). Each of homogenized fecal samples was centrifuged at 17,000 g for 3 min and then filtered through a 0.8 μm PES membrane filter. 1μl of micrococcal nuclease (New England Biolabs, Inc., Ipswich, MA, USA), 2 μl of benzonase (Millipore®, Burlington, MA, USA), and 7 μl of buffer (1M Tris, 100 mM $CaCl_2$ and 30 mM $MgCl_2$, pH 8) were added to the filtered solution of each sample and treated at 37℃ for 2 hours. Viral DNA and RNA were extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) without the addition of carrier RNA and then randomly amplified with the Whole Transcriptome Amplification Kit 2 (Sigma−Aldrich®, Burlington, MA, USA). For each randomly amplified sample, a sequencing library was prepared with the Nextera XT DNA Library Preparation Kit (Illumina, Inc., San Diego, CA, USA) and Nextera XT Index Kit v2 (Illumina). Indexed libraries were quantified and normalized using 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) and Quant−iT PicoGreen dsDNA reagent kit (Life Technologies, Carlsbad, CA, USA). Normalized libraries were subjected to 2 × 300 bp paired−end sequencing on the Illumina MiSeq system.

4.2.3. Data processing and analysis

Index sequences have been removed from MiSeq Reporter version 2.5 (Illumina). Adapter sequences, reads with quality scores less than 20, and reads less than 50 bp were removed by Trimmomatic version 0.39 [43] (ILLUMINACLIP:NexteraPE−PE.fa:2:30:10

LEADING:15 TRAILING:15 SLIDINGWINDOW:4:20 MINLEN:50). Deduplication was performed on the forward and reverse reads of each sample in FastUniq version 1.1 [240]. Singletons from each deduplicated forward and reverse read were removed with fastq-pair version 1.0-2 [241]. The processed forward and reverse reads were merged using FLASH [113]. Merged reads were aligned against non-redundant protein database of NCBI (downloaded in March 2023) based on the default settings of DIAMOND version 2.1.4 [242] and BLASTX. Taxonomic binning was performed using MEGAN version 6.24.21[243], and reads not classified as viruses were excluded from further analysis. Reads classified as viruses were assembled into viral contigs using the *de novo* assembly algorithm and the meta option of SPAdes version 3.15.5 [244]. Based on a viral database extracting only viral sequences from the non-redundant protein database, taxonomic binning of viral contigs was performed with DIAMOND [242], MEGAN [243] and an e-value cut-off of e^{-10}. Information related to the host of the virus was obtained from the International Committee on Taxonomy of Viruses (ICTV) [245].

4.2.4. Statistical analysis

Statistical analysis was conducted based on R version 4.1.0 with the phyloseq package [45] and vegan package [46]. To analyze the diversity of viral contigs, two types of diversity metrics were analyze: α diversity (within samples) and β diversity (between samples). To characterize viral richness and diversity within each sample, the Chao1 richness estimator and Shannon index were calculated, respectively. Comparison of α diversity metrics between host animals was performed with the Kruskal–Wallis test. To characterize viral membership and structure between samples the Jaccard index and Bray–Curtis dissimilarity were calculated, respectively. Comparison of β diversity metrics between host animals was performed with the permutational multivariate analysis of variance (PERMANOVA).

4.3. Results

4.3.1. Sequencing statistics

A total of 2,377,982 sequence reads were taxonomically binned using DIAMOND [242] and MEGAN [243], and approximately 23.3% (553,370 sequence reads) of them were identified as viral reads. The number of contigs de novo assembled with SPAdes [244] and viral reads was 7,489 in total (S4.1 Table).

4.3.2. Diversity

Comparisons of the Chao1 richness estimator and Shannon index, which represent within sample diversity (α diversity) of viral contigs between host animals, were found to be statistically insignificant ($p > 0.05$; Kruskal–Wallis test) (Fig 4.1A). However, the differences in the β diversity of the viral contigs confirmed by structure (Bray–Curtis dissimilarity) and membership (Jaccard index) (r^2 = 0.206 and 0.216, respectively, $p < 0.001$; PERMANOVA) (Fig 4.1B), indicating that viruses constituting the virome in each animal sample is statistically different for each animal.

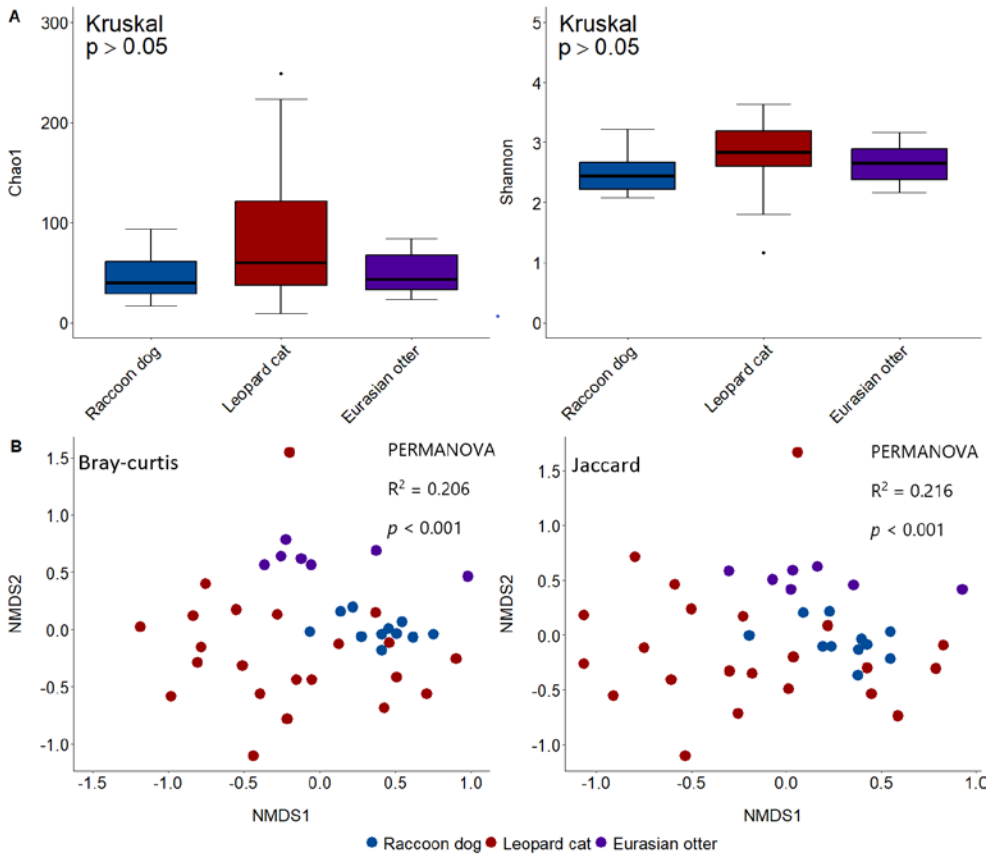

Fig. 4.1. Diversity of fecal viromes of wild carnivores in Korea. The results shown are based on the diversity of viral contigs. (A) Comparison of richness and diversity of viral contigs between host animals estimated by the Chao1 estimator and Shannon index, respectively. (B) Non-metric multidimensional scaling (NMDS) plots showing the structure and membership of viral contigs represented by the Bray–Curtis dissimilarity and Jaccard index, respectively.

4.3.3. Taxonomic composition of viral contigs

Fig 4.2 shows the taxonomic composition in the family level of the viral contigs identified in each animal. Viruses that host bacteria, i.e., Microviridae, Autographiviridae, and Zobellviridae, were most frequently detected in fecal samples, except for viruses that were not taxonomically established in the family level. Furthermore, viruses that host fungi, protists, and plants, i.e., Totiviridae, Partitiviridae, and Virgaviridae were detected. In addition, Parvoviridae, which mainly use animals as hosts, were detected, and Genomoviridae, which use various groups of organisms as hosts, were detected.

In the genus level, 32 viral genera using bacteria as hosts were abundantly detected (Fig 4.3). Chloriridovirus, Protoambidensovirus, and Ambidensovirus, which host inverterbrates, were detected. Furthermore, Etatorquevirus, Drosmacovirus, Gyrovirus, and Chaphamaparvovirus, which host vertebrates, were identified. In addition, 5 viral genera have been identified that contain virus species and/or strains capable of infecting humans (Table 4.1).

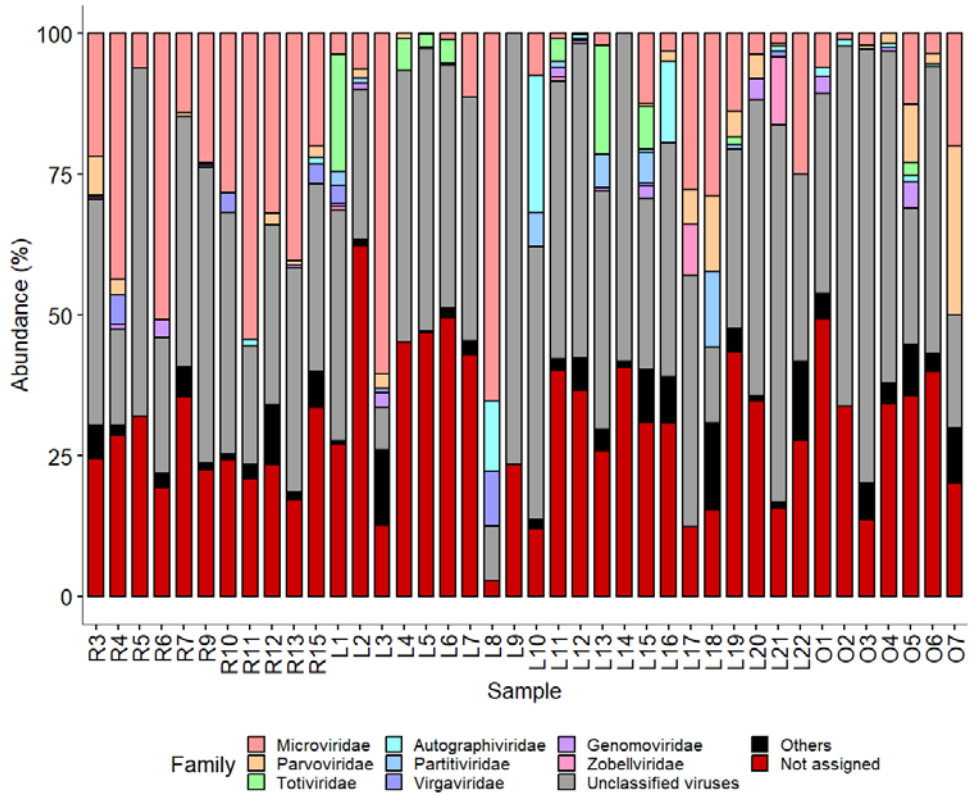

Fig 4.2. Relative abundance of identified viral families detected from fecal samples of wild carnivores in Korea. The relative abundance of each viral family is defined as the ratio of the number of viral contigs assigned to that viral family to the total number of detected viral contigs. R, L, and O mean raccoon dog, leopard cat, and Eurasian otter, respectively, and each number means a sample ID.

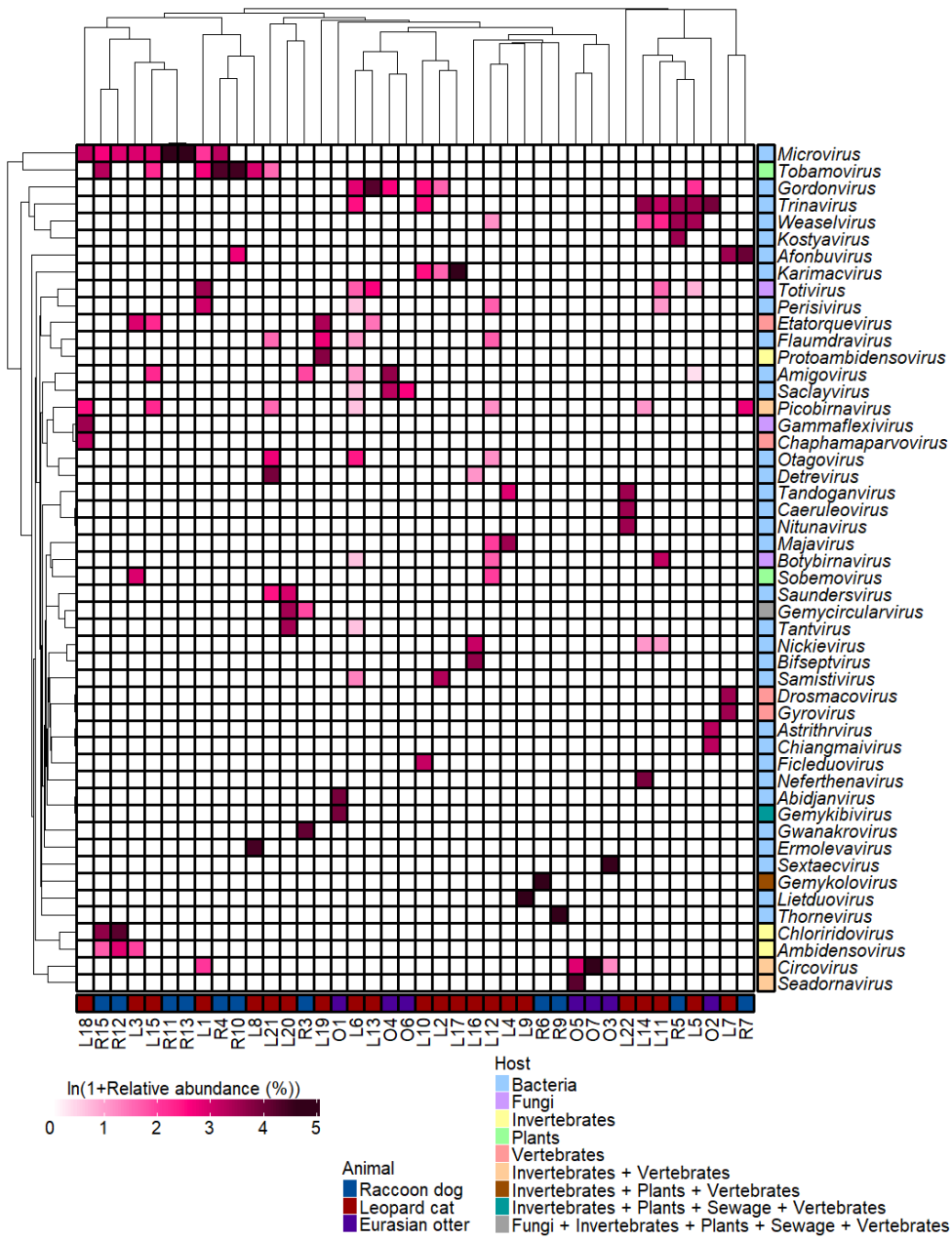

Fig 4.3. Relative abundance of the top 50 most abundant viral genera detected from fecal samples of wild carnivores in Korea. The relative abundance of each virus is defined as the ratio of the number of viral contigs assigned to that viral genus to the total number of detected viral contigs, excluding viruses for which there is no genus level information. The cluster dendrograms are based on Euclidean distances calculated from the compositional similarity of log-transformed relative abundances. Information on the host of each viral genera were based on the International Committee on Taxonomy of Viruses (ICTV).

Table 4.1. A list of genera that contain viral species capable of infecting humans among top 50 viral genera.

Genus	Number of samples from each animal in which viral contigs were detected			Host or isolation source	Ref.
	Raccoon dog (n = 11)	Leopard cat (n = 22)	Eurasian otter (n = 7)		
Circovirus (ssDNA virus)	0	1	3	Mammals including human, birds, freshwater fish, and invertebrates	ICTV[1]
Gemykibivirus (ssDNA virus)	0	0	1	Vertebrates including human, invertebrates, plants, and sewage	ICTV[1]
Gyrovirus (ssDNA virus)	0	1	0	Chicken and human	[246]
Picobirnavirus (dsRNA virus)	1	6	0	Mammals including human, birds, reptiles, and sewage	ICTV[1]
Seadornavirus (dsRNA virus)	0	0	1	Human and mosquito	ICTV[1]

[1] International Committee on Taxonomy of Viruses (ICTV)

4.4. Discussion

In this study, we successfully characterized the fecal viromes of raccoon dogs, leopard cats, and Eurasian otters inhabiting Korea by viral metagenomic sequencing. We found that the structure and membership of viruses varied greatly between the host animals. In addition, we detected 5 viral genera that contain zoonotic viruses known to infect humans. The method used in this study has the advantage of being able to detect a wide range of virus from a sample compared to target-specific identification method. For example, the method we used can detect not only viruses that can infect each wild animal but also viruses that can infect bacteria. Therefore, a comprehensive understanding of viruses can be achieved without being bound by *a priori* assumptions.

The viruses most abundantly detected in fecal samples of wild carnivores were identified as bacteriophages that infect bacteria (Fig 4.2 and Fig 4.3). For some detected bacteriophages in our study, the taxonomic lineage was not established, but they were abundantly detected than viruses infecting other organisms. The high detection of bacteriophages in fecal samples of animals is regarded to be because bacteria exist in large quantities in various environments including the intestine, and a large number of viruses have evolved to target and infect bacteria [226].

Differences in viromes between the host animals (Fig 4.1) are likely due to their gut microbiome and/or dietary contents. For example, the most frequently detected bacteriophages in fecal samples of leopard cat were Trinavirus, Karimacvirus, Gordonvirus, and Lietduovirus, which were classified as *Rhodococcus* phage, *Streptomyces* phage, *Arthrobacter* phage, and *Escherichia* phage, respectively. On the other hand, the most frequently detected bacteriophages in fecal samples of raccoon dog were Thornevirus, Gwanakrovirus, Kostyavirus, and Trinavirus, which were classified into *Bacillus* phage, *Erwinia* phage, *Mycobacterium* phage, and

Escherichia phage, respectively. Sextaecvirus, Abidjanvirus, Trinavirus, and Amigovirus were the most frequently detected bacteriophages in fecal samples of Eurasian otter, and they were classified into *Staphylococcus* phage, *Pseudomonas* phage, *Rhodococcus* phage, and *Arthrobacter* phage, respectively.

We detected 5 viral genera that contain zoonotic viruses known to infect humans. From the fecal samples of leopard cat, Circovirus, Gyrovirus, and Picobirnavirus were detected (Table 4.1). Reports that these three viruses were detected in leopard cats were not easily found, but they are known to have a variety of hosts, including avian species [247, 248]. Given that three viruses hosts avian species and that birds make up a significant portion of the leopard cat's diet we have identified [184], it is likely that the three viruses originated from the diet of leopard cat. From the fecal samples of raccoon dog, Picobirnavirus were detected (Table 4.1). Similar to our detection, Picobirnaviridae have been detected in the fecal virome of raccoon dogs in China [249]. From the fecal samples of Eurasian otter, Circovirus, Gemykibivirus, Seadornavirus were detected (Table 4.1). Circoviruses has been identified in diverse groups of wild animals including Eurasian otters [232, 250]. However, previous studies reporting that Gemykibivirus and Seadornavirus were detected in Eurasian otter were not easily found. In addition, it is not easy to know why these two viruses were detected in the Eurasian otter only with the information of the known hosts of these two viruses. Therefore, further studies are needed to determine whether these two viruses infect the Eurasian otter or are derived from its diet.

4.5. Conclusion

In Korea, an increase in wildlife-associated diseases has been reported in recent decades [4]. Therefore, it is imperative to monitor zoonotic pathogens, including zoonotic viruses, harbored by wildlife. In this study, we investigated the diversity of viromes,

including zoonotic viruses, associated with the carnivorous wildlife in Korea by using metagenomic sequencing. We found that the virome structure and membership differed between the host animals likely due to their gut microbiome and/or dietary contents. In the future, it is expected that zoonotic diseases will further increase due to the increased proximity between humans and wildlife due to urbanization [208]. Vigilance may need to be continuously exercised, such as by monitoring parasites in the feces of wildlife, as was done in this study.

S4.1 Table. Sequencing statistics.

Sample ID	No. viral contigs	No. reads assigned to virus	No. reads assigned to non-viral organisms	Total reads
R3	250	13,560	50,833	64,393
R4	119	8,086	29,581	37,667
R5	99	7,073	27,627	34,700
R6	128	14,551	37,385	51,936
R7	294	16,864	35,671	52,535
R9	447	20,988	30,359	51,347
R10	200	19,981	36,468	56,449
R11	85	14,572	28,925	43,497
R12	48	3,963	21,415	25,378
R13	153	8,736	38,180	46,916
R15	283	27,314	10,551	37,865
L1	167	12,271	18,946	31,217
L2	265	4,333	20,148	24,481
L3	122	20,632	12,584	33,216
L4	107	1,902	33,051	34,953
L5	621	24,940	22,250	47,190
L6	811	15,467	37,448	52,915
L7	255	31,262	18,855	50,117
L8	74	59,073	10,411	69,484
L9	30	31,179	17,846	49,025
L10	68	1,798	42,937	44,735
L11	306	13,777	25,472	39,249
L12	334	7,182	54,995	62,177
L13	189	6,719	34,840	41,559
L14	116	6,545	24,390	30,935
L15	190	14,323	25,343	39,666
L16	170	2,993	28,508	31,501
L17	66	21,195	18,805	40,000
L18	53	3,037	50,735	53,772
L19	226	20,641	39,884	60,525
L20	158	5,307	30,459	35,766
L21	219	15,898	31,687	47,585
L22	39	3,243	67,871	71,114
O1	65	2,118	52,793	54,911
O2	92	8,125	38,507	46,632
O3	144	20,895	44,335	65,230
O4	166	10,058	39,327	49,385
O5	91	22,539	16,216	38,755
O6	227	8,440	21,956	30,396
O7	12	1,790	43,648	45,438
Total	7,489	553,370	1,271,242	1,824,612

Limitation

There are several limitations in this study. First, collected fecal samples are limited to one season and one sampling site. The fecal samples of racoon dog were collected in May in Seosan, Chungcheongnam-do. The fecal samples of leopard cat were collected in February in Chungcheongnam-do and Gyeongsangbuk-do. The fecal samples of Eurasian otter were collected in Ansan, Gyeonggi-do in June. Due to this limitation, it is difficult to say that the results of this study represent the entire population of wild raccoon dogs, leopard cats, and Eurasian otters living in Korea. Second, the scope of zoonotic pathogens in this study was limited to parasites and viruses. Approximately 800 zoonotic pathogens can be classified into viruses, bacteria, fungi, protozoa, and helminthes [251], but this study was limited to a few groups of zoonotic pathogens. Therefore, the results of this study did not identify all hazards inherent in three wild carnivores.

However, this study introduced high-throughput sequencing to comprehensively identify the composition of the diet, parasites, and viruses of three wild animals through their feces. Therefore, the significance of this study is that it introduces a relatively new and non-invasive methodology, although the spatial and temporal representativeness of the collected fecal samples is poor. In addition, various parasite groups such as fungi (Microsporidia), protozoa (Apicomplexa, Amoebozoa, and Parabasalia), Nematode, and Platyhelminthes and viruses were examined in Chapter 3 and Chapter 4, respectively. Therefore, it can be regarded that the identification of zoonotic pathogens has been carried out in a wide range.

Conclusion

In this study, the research goals corresponding to each chapter were successfully achieved through a sequencing-based approach. Chapter 1 confirmed that the diet of raccoon dogs mainly consisted of fish, amphibians, and insects. Additionally, the efficiency of RacBlk, a blocking oligonucleotide developed to inhibit DNA amplification of raccoon dog, was also statistically verified. In Chapter 2, vertebrate 12S rRNA gene sequencing and shotgun metagenomic sequencing were used to reveal that the diet of leopard cats mainly consisted of small rodents such as mouse and rat. Chapter 3 confirmed that the composition of parasitomes in the feces of raccoon dogs, leopard cats, and Eurasian otters was different, which is thought to be related to each animal's diet. Additionally, 5 zoonotic parasites were detected in the fecal samples. In Chapter 4, fecal viromes of raccoon dogs, leopard cats, and Eurasian otters were characterized by shotgun metagenomic sequencing. It was confirmed that the viromes of each animal had a different composition, which is thought to be related to the gut microbiome of each animal. Additionally, five different viral genera containing zoonotic viral species were detected in the animal feces.

As wildlife-associated disease cases increase, continuous vigilance and zoonotic disease research, such as monitoring parasites and viruses in wildlife feces, may be necessary. Besides, from a One Health perspective, continuous monitoring can provide information for managing wildlife and their environment in a healthy manner.

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국문 초록

환경 분변의 high-throughput sequencing을 활용한 한국에 서식하는 야생동물의 생태와 인수공통감염 병원체의 규명

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중심어 : 야생동물; 식이; 기생충; 바이러스; 인수공통감염병; 원헬스

학번 : 2018-36209

도시화, 산림 파괴 등의 인위적 요인으로 가속화된 야생동물의 서식지 파괴는 야생동물과 인간 간의 물리적 거리를 줄어들게 만들었다. 이러한 상황과 맞물려 야생동물에게서 유래하는 인수공통감염 병원체는 공중 보건학적으로 심각한 위협을 끼칠 수 있다. 이 연구에서는 한국에 서식하는 야생동물 3종인 너구리 (*Nyctereutes procyonoides*), 삵 (*Prionailurus bengalensis*), 수달 (*Lutra lutra*)의 분변을 활용하여 야생동물의 식이와 인수공통감염 병원체를 조사하는 것을 목표로 하였다. High-throughput sequencing을 기반으로 충청남도 서산에 서식하는 너구리는 주로 어류나 양서류, 곤충 등을, 충청남도와 경상북도 등지에 서식하는 삵은 소형 설치류나 조류를 섭취하는 것을 확인하였다. High-throughput sequencing을 기반으로 야생동물 3종의 분변에서 검출된

기생충군은 야생동물에 따라 서로 다른 구성을 가지고 있었으며 이는 각 야생동물의 식이에 영향을 받은 것으로 판단된다. 또한, 인수공통감염을 일으킬 수 있는 기생충 5종을 검출하였다. Metagenomic sequencing을 기반으로 야생동물 3종의 분변에서 검출된 바이러스군은 야생동물에 따라 서로 다른 구성을 가지고 있었으며 이는 각 야생동물의 장내 미생물총 혹은 식이에 영향을 받은 것으로 판단된다. 또한, 인수공통감염을 일으키는 것으로 알려진 바이러스가 포함된 5개의 바이러스 속을 검출하였다. 도시화로 인하여 촉진된 인간과 야생동물 간의 접근성 증가로 인하여 인수공통감염병의 출현은 보다 자주 일어날 것으로 예상된다. 이 연구에서 수행된 것처럼 야생동물의 분변에 있는 인수공통감염 병원체를 지속적으로 모니터링하며 주의를 기울여야 할 것으로 판단된다. 또한 One Health의 관점에서, 지속적인 모니터링으로 얻을 수 있는 정보는 야생동물과 주변 환경을 건강하게 관리할 수 있는 기초 자료로 활용될 수 있을 것으로 여겨진다.