DNA metabarcoding analysis of the North China and Amur Leopards' feeding habits

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Abstract

Leopards *Panthera pardus* are top predators that play a crucial role in maintaining ecosystem balance and stability by exerting top-down control on prey species populations through direct predation. Despite their wide distribution, in recent years leopard populations have become endangered in many regions due to increasing anthropogenic activity. Studying the feeding habits of endangered species provides insight into their survival from the perspective of their trophic niche and can help identify factors contributing to their decline, aiding in the development of targeted conservation strategies. In this study, the North China leopard (P. p. japonensis) and Amur leopard (P. p. orientalis) were selected as the study objects. A total of 97 fecal samples were collected throughout their typical habitats, 83 of which were confirmed as originating from leopards using molecular identification. DNA metabarcoding identified 13 prev species for the North China leopard and 15 for the Amur leopard, with small- and medium-sized prey (< 37 kg) comprising over 75% of biomass intake. Among all the prey species, roe deer (*Capreolus pygargus*) provided the highest biomass contribution—41.69% for the North China leopard and 29.2% for the Amur leopard. In addition, the frequent occurrence of domestic dogs (*Canis familiaris*) in their diet (>5.8%) highlights the impact of anthropogenic activities on leopard feeding habits. To support leopard conservation, it is recommended to protect all prey species within reserves and reduce anthropogenic interference in these habitats.

Key words: Carnivore, DNA barcode, diet analysis, biomass

Graphical abstract



The dietary surveys of the North China and Amur leopards showed that small and medium-sized prey were their main prey, and that domestic dogs were also a major food source, suggesting that anthropogenic disturbances have affected the feeding habits of leopards.

Large carnivores play a key role in shaping ecosystem structure and function through their effects on lower trophic levels (Hoeks et al. 2020; Ripple et al. 2014). As such, rehabilitating populations of endangered large carnivores is essential for repairing damaged ecosystems (Rode et al. 2021). Prey resources are a primary requirement for large carnivores and a key factor influencing their population size and distribution (Stepkovitch et al. 2022; Wilkinson et al. 2020). To that end, a significant driver of endangerment for large carnivores worldwide is the scarcity of prey (Fernandez-Sepulveda and Martín 2022). Therefore, identifying the prey composition of endangered large carnivores and prioritizing these prey in recovery plans are crucial steps for large carnivore conservation. As one of the world's most adaptable large carnivores, leopards (*Panthera pardus*) have historically been widely distributed across many regions of Asia and Africa, playing a key role in maintaining ecosystem balance and stability (Farhadinia et al. 2021; Searle et al. 2020). In recent years, anthropogenic activities—especially hunting and habitat destruction— have led to a sharp decline in leopard populations and their range. Historical data shows that leopard habitat have shrunk by 63-75% over the past century, surpassing the average habitat loss of other large carnivores (Laguardia et al. 2017). Of the nine current leopard subspecies, five are classified as 'threatened' by the International Union for Conservation of Nature (IUCN), with three subspecies considered critically endangered (Vitekere et al. 2021). China, which hosts the highest number of leopard subspecies, is home to four subspecies of leopards: the Amur leopard (P. p. orientalis), North China leopard (P. p. japonensis), Indian leopard (P. p. fusca) and Indo-Chinese leopard (P. p. delacouri) (Jacobson et al. 2016; Yin et al. 2023). The Amur leopard inhabits Northeast China, including Heilongjiang and Jilin Provinces; the North China leopard has a broader range across North, Central, and South China; while the Indo-Chinese and Indian leopards are found in Southwest China. There is notable lack of studies on the specific diet and prey composition of leopards in China, which is hindering effective conservation and management efforts.

Determining the prey composition of endangered animals requires comprehensive dietary studies (Dou et al. 2023; Mcinnes et al. 2017). Common methods for studying the diets of large carnivores in the wild include direct observation, food trace analysis, fecal content analysis, and DNA metabarcoding (Van Der Heyde et al. 2021). However, for leopards, direct observation is challenging due to their scarcity and secretive nature. Macroscopic fecal content analysis also has limitations, as it requires expertise and may not reliably identify rare prey species (Shao et al.

2021; Yang et al. 2022). A comparative study of all four research methods conducted by Kress et al. (2015) indicated that DNA metabarcoding is particularly suitable for elusive species like leopards, which have diverse diets and are difficult to track. Recently, DNA metabarcoding, which leverages high-throughput sequencing technology, has become one of the common methods for wildlife dietary surveys, owing to its ease of use, high identification efficiency, and accuracy (Ando et al. 2020; Sousa et al. 2019).

To clarify the feeding habits of the North China and Amur leopards in China, and to support effective conservation and management of these species, fecal samples were systematically collected from their typical habitats. By utilizing DNA metabarcoding data, this study aimed to address the following questions: 1) What is the diet and prey composition of these leopards? 2) Which prey species are their primary food sources? and 3) What are the similarities and differences in the feeding habits of the two leopard species and what factors contribute to these patterns?

Materials and Methods

Study sites and sample collection

In 2017-2018, North China leopard and the Amur leopard fecal samples were collected from the typical distribution areas in China. For the North China leopard, samples were mainly gathered from the Loess Plateau, including three nature reserves: Tieqiaoshan (LPS) in Shanxi, Ziwuling (ZWL) in Shaanxi, and Liupanshan (LPS) in Ningxia (Fig. 1). The Loess Plateau, one of China's four major plateaus, has an altitude range of 800-3000 m and is characterized by mountainous, hilly terrain, sloping downward from northwest to southeast (Fu et al. 2023). The region experiences a warm temperate continental monsoon climate, with average annual temperatures between 3.6 and 14.3 °C.

Historically significant as the birthplace of Chinese civilization and one of the most densely populated regions, the Loess Plateau has faced large-scale deforestation. Combined with loose soil, this deforestation has caused severe soil erosion, with heavy rainfall carrying sediment into the Yellow River, making it one of the world's most sediment-laden rivers. Since the Chinese government launched the Natural Forest Protection Project in 1999, vegetation on the Loess Plateau has gradually recovered, and wildlife populations are slowly rebounding. Recent studies

indicate that the Loess Plateau now holds the largest and densest leopard populations in China (about 130 individuals) (Yang et al. 2021; Zhu et al. 2021).

The Amur leopard feces collection area was primarily located in northeastern China within the Northeast Tiger and Leopard National Park (Fig. 1). Established in 2021, this national park spans 14,600 km² in the southern part of Laoyeling, along the border between China's Jilin and Heilongjiang Provinces. It borders Russia's Leopard National Park in Primorsky Krai to the east and southeast, and North Korea across the Tumen River to the south-west (Li et al. 2023). The park's terrain is dominated by low to medium altitude mountains, canyons, and hills, with elevations generally below 1,000 m that gradually decline from the park's center outward. Situated in a continental humid monsoon climate zone, the park has an average annual temperature of 5 °C Rich in wildlife, the park is home to the Amur tiger (*P. p. altaica*) and Amur leopard, along with 10 national-level protected species, and 43 national second-level protected species. Recent studies confirm that the leopard population in the park has now reached 80 individuals (Wang et al. 2023).

Fresh feces were collected in the field, with the source of each sample identified based on morphology and signs of leopard activity (tracks, scrapes, etc.) in the area. To avoid duplicate sampling, scats were collected at least 5 km apart and placed in sterile 50 ml sample tubes, with the time and geographical coordinates recorded. In the laboratory, the samples were stored in a freezer at -80°C until DNA was extracted.

DNA extraction and identification of samples source

Six pellets were collected from different areas of the surface and interior of each fecal sample, with a total sample weight not exceeding 220 mg per sample. DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Inc., Germany), with a blank negative control included. Most of the extraction procedure followed the kit instructions, with a modification in the preliminary step: after incubation at 70 °C for 15 min with added lysis buffer, the samples were ground for 1 min in a high-throughput tissue mill (Scientz-950E; Xinzhi, Inc., China).

We used a two-step approach to identify faecal source species. The first step was to amplify and sequence the fecal DNA using the 16S-F/R primer (Xiong et al. 2016), a universal vertebrate primer (Table 1), to identify the suspected leopard samples by sequence alignment. Considering that there are other cats besides leopards in the study area, in the second step, we amplified and sequenced the DNA of the suspected leopard samples using leopard-specific primers Ppo-CbF/R (Table 1) to determine whether the samples were from leopards or not (Sugimoto et al. 2006). The PCR amplification system was 20 μ L, including 10 μ L of dNTP Mixture (TransGen Biotech, Beijing, China), 0.1 μ g/ μ L of BSA, 0.6 μ L each of forward and reverse primers, and 2 μ L of DNA template, and the remaining system was supplemented with ddH₂O. The PCR program started with an initial denaturation step of 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C (16S-F/R)/ 57 °C (Ppo-CbF/R), and 30 s at 72 °C, and a final elongation stepof 10 min at 72 °C. Nucleotide sequences were obtained with an ABI PRISM 3130xl Genetic Analyser (Applied Biosystems). Forward and reverse sequences were spliced into complete sequences using DNAMAN software (https://www.lynnon.com/dnaman.html). Final contigs were compared against data in Genbank using blast, and species identity was assigned based on matches with a similarity of 95%-100%.

Library preparation and amplicon sequencing

For DNA samples derived from leopard scats, PCR amplification was performed using the vertebrate mitochondrial universal primer 12sV5 (Riaz et al. 2011). Additionally, a leopard-specific block primer was designed (Table 1). A 7-10 base barcode was added to the forward primer to distinguish between samples within the same library. The PCR system was prepared with a total volume of 25 μ l, including 0.25 μ l Q5 high-fidelity DNA polymerase (NEB, inc., USA), 5 μ l of 5×Reaction Buffer, 5 μ l of 5×High GC Buffer, 2 μ l dNTP (10 mM), 2 μ l template DNA, 1 μ l of forward and reverse primers, respectively (10 μ M), with the remainder comprised of ddH₂O. The PCR protocol consisted of an initial pre-denaturation at 98°C for 5 min, followed by 25 cycles of 98°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 5 min, then storage at 12°C. Three negative controls, replacing DNA with water, were included in the experiment. Amplification products were analyzed via 2% agarose gel electrophoresis, after which target fragments were excised and recovered using the Axygen Gel Recovery Kit (Axygen, Inc., USA). The recovered PCR products were subsequently purified using AMPure XP SPRI beads (Beckman Coulter, Brea, CA, USA) according to the manufacturer's protocol.

PCR products were quantified using a Microplate reader (FLx800; BioTek, Inc., USA) with the Quant-iT PicoGreen dsDNA Assay Kit (Thermo, Inc., USA). Library construction was performed with the TruSeq Nano DNA LT Library Prep Kit (Illumina, Inc., USA). For quality control, 1 μ l of the library was analyzed using the Agilent High Sensitivity DNA Kit (Agilent, Inc., USA). Qualified libraries were sequenced using 2×250 bp paired end sequencing on Illumina NovaSeq, with the NovaSeq 6000 SP Reagent Kit (500 cycles) (Illumina, Inc., USA). The library was diluted to 2nM, then mixed in proportion to the required data yield. Prior to sequencing, the mixed library was denatured to single-strands using 0.1 N NaOH for on-line sequencing.

Sequence analysis and taxon assignment

Raw sequence data were processed with the QIIME2 plug-in *demux* (https://qiime2.org) for decoding, and quality filtering and chimera removal were conducted using the *DADA2* plugin (Callahan et al. 2016). Processed sequences were then analyzed using the OBITools program (Boyer et al. 2016). Paired-end reads were assembled with the *Illuminapairedend* command, sequence similarity was checked with the *stats* command, and sequences with an overall score above 0.8 were retained using the *grep* command. Sample specific sequences were assigned with the *Ngsfilter* program, and reads with fewer than 50 occurrences and shorter than 80 bp were removed. Erroneous sequences were detected and eliminated using *obiclean*.

The processed sequences were then compared to the NCBI Nucleic Acids Database to identify operational taxonomic units (OTUs) (Shao et al. (2021). Raw sequence reads are archived on the NCBI Sequence Read Archive under BioProject PRJNA1179321, BioSamples SAMN44486419–SAMN44486487, and SRA accessions SRR31146160–SRR31146092.

Dietary analysis

To characterize the prevalence of prey species in the diet of predators, the frequency of prey occurrence in fecal samples was calculated as the relative frequency of occurrence (%POO) (Klare et al. 2011), using the following formula:

$$\% POO_i = N_i / \sum N_i \times 100\% \tag{1}$$

Due to differences in prey weight, smaller prey produce more feces than larger prey for the same amount of biomass consumed (Chakrabarti et al. 2016; Wachter et al. 2012). Consequently, using the frequency method in carnivore dietary studies can lead to an underestimation of the

relative importance of large prey and an overestimation of small prey. Therefore, this method alone does not accurately represent the significance of each prey species in a carnivore's diet. To accurately quantify the relationship between fecal counts and prey body weight (biomass), it is necessary to apply the Correction factor (*Y*) equation for the target species obtained through feeding experiments. In this study, the correction equation applicable to felines was utilized (Chakrabarti et al. 2016), and *Y* was calculated as follows:

$$Y_i = \left(0.033 - 0.025 \exp^{-4.284 X i/Z}\right) * Z \qquad (2)$$

where Y_i denotes the actual biomass of prey *i* consumed by the predator in a single fecal sample, X_i denotes the weight of prey *i*, and Z denotes the weight of the predator. Predator and prey weights were obtained from the literature (Dou et al. 2023; Dou et al. 2019; Smith et al. 2010) as detailed in Supplementary Tables 3 and 4).

To address the misestimation of prey importance in predation caused by the frequency method, the concept of relative proportion of biomass consumed (D) was introduced. This concept represents the proportion of biomass that a specific prey species contributes to the predator's diet (Ramesh et al. 2009). It is calculated using the following formula:

$$D = \frac{Y_i \times A_i}{\sum_{i=1} (Y_i \times A_i)} \times 100$$
(3)

where Y_i denotes the correction factor for prey species *i* and A_i denotes the relative frequency of occurrence of prey species *i* in feces (%*POO*_{*i*}).

%POO and %RM data were analyzed separately using the R package *spaa* (Zhang 2016) to calculate dietary parameters related to species diversity and ecological niche occupancy in leopards' diets. These parameters included Shannon's Diversity Index (H), Peilou' s J, Levin's niche breadth (B), and standardized niche breadth (B_s). The Pianka index (O) was also calculated to estimate the degree of prey overlap between the diets of the North China and Amur leopards, with confidence intervals obtained from 1,000 bootstrap samples using the same package. Additionally, species rarefaction and extrapolation curves were generated to estimate the total number of prey species potentially consumed by these leopards. These curves were calculated and plotted using the R package *iNEXT* (Hsieh et al. 2016), with the number of extrapolation

samples set to 100, the number of nodes set to 20, and 95% confidence intervals derived from 1,000 bootstraps.

Results

Summary of sequence data

In this study, 42 fecal samples were collected from the North China leopard distribution area on the Loess Plateau. Of these, 35 samples were successfully amplified and identified as originating from leopards (Table 2). Four amplifications failed, one sample had a lower sequence count than the control, and 30 samples were ultimately used for dietary analysis (Table 2). Additionally, 55 fecal samples were collected in the Northeast Tiger and Leopard National Park, with 48 identified as leopard-origin. Among these, three samples failed to amplify, six had lower sequence counts than the control, and 39 were successfully used for dietary analysis (Table 2).

The sequencing run generated a total of 1,963,574 raw sequence reads, which were trimmed, merged, and filtered by length, resulting in 615,079 sequences. After removing chimeras and redundancy through clustering, 614,045 reads remained, with an average read count of 8,528 per sample (including controls). Of these 613,799 reads (99.96%) were successfully assigned a taxonomic rank (see Supplementary Tables 1 and 2). Read lengths for each negative control PCR were typically below 100 bp, indicating minimal contamination.

Taxon assignment

Sequences of all species exhibited more than 99% similarity to corresponding species in the NCBI Nucleic Acids Database, allowing all prey to be identified at the species level. From the feces of the North China leopard, 13 prey animal OTUs were identified, encompassing 12 genera, 10 families, and 6 orders (Fig. 2, Supplementary Table 1). Each fecal sample contained between 1 and 4 prey species $(1.61 \pm 0.87, \bar{X} \pm SD)$. In contrast, a total of 15 prey animal OTUs were identified in the Amur leopard feces, representing 14 genera, 10 families, and 6 orders (Fig. 2, Supplementary Table 2). Each scat included between 1 and 5 prey species $(1.64 \pm 1.07, \bar{X} \pm SD)$. Comparative analysis of species composition revealed that both North China and Amur leopards share eight prey species: domestic mouse (*Mus musculus*), brown rat (*Rattus norvegicus*), dog (*Canis familiaris*), leopard cat (*Prionailurus bengalensis*), hare (*Lepus mandshuricus*), cattle (*Bos taurus*), wild boar (*Sus scrofa*) and roe deer (*Capreolus pygargus*).

Relative frequency of occurrence

Relative frequency of occurrence analysis showed that roe deer is the most frequent prey of the North China leopard (27.1%), followed by red fox (12.5%) (Fig. 2). House mouse and domestic dogs are the third most frequent prey items, each with a frequency of 10.4%. Wild boar, leopard cat, and hare each account for more than 5% of its diet. Prey items with the lowest frequency of occurrence include domestic chicken (*Gallus domesticus*), long-eared owl (*Asio otus*), and badger (*Meles meles*), suggesting these three prey contribute minimally to the biomass intake of the North China leopard.

For the Amur leopard, roe deer had the highest frequency of occurrence (23.8%), followed by merganser (15.9%) and domestic dogs (14.3%) (Fig. 2). Additionally, the occurrence frequency of house mouse, brown rat, and wild boar each exceeded 5%.

Relative biomass contribution of prey

Analysis of relative biomass contribution (%*RM*) indicated that roe deer is the primary food source for the North China leopard, contributing 41.69%. Domestic dog depicts the highest biomass contribution (14.38%), followed by wild boar at 9.56% (Fig. 3, Supplementary Table 3). Together, these species account for over 65% of the total biomass contribution. Furthermore, the relative biomass contributions of red fox, cattle, and leopard cat each exceeded 5%. The long-eared owl has the lowest biomass contribution, and its presence in feces was recorded only once, suggesting it is an incidental prey item for the North China leopard.

For the Amur leopard, the top three biomass contributors are roe deer (29.2%), sika deer (19.75%), and domestic dog (16.2%), which collectively account for 65.15% of all prey biomass. In addition, wild boar (9.88%) and cattle (5.92%) were the only other prey species contributing more than 5% to the relative biomass (Fig. 3, Supplementary Table 4).

Prey diversity and niche width

The Shannon diversity index and Peilou's evenness for prey in the diets of North China and Amur leopards, which were based on %*POO* and %*RM* data, showed no significant differences (Table 3). However, despite their standardized niche width being smaller, Amur leopards exhibited a slightly larger dietary niche than North China leopards. The degree of dietary overlap between the two species was high for both methods (%*POO*: *Pinka' O* = 0.78, 95% *CI* 0.74-

0.92; %*RM*: *Pinka'O* = 0.77, 95% *CI* 0.77-0.98) indicating a high degree of similar prey composition between the two.

The Rareness/Extrapolation (R/E) curves indicate that prey item richness for both leopard populations has not yet reached a stable level given the current sample size (Fig. 4). Data extrapolation showed that 13 prey species (95% *CI* 10.03-15.96) were identified from 30 North China leopard fecal samples, representing 85% (95% *CI* 0.92-0.93) of all prey species. Increasing the sample size to 54 would capture 90% of the North China leopard's prey species with an estimated 15.15 species (95% *CI* 10.41-19.90) (Supplementary Table 5). For the Amur leopard, 39 fecal samples identified 15 prey species (95% *CI* 0.93-0.98). Increasing the sample size to 66 would capture 90% of prey species for Amur leopards, estimated at 17.62 species (95% *CI* 12.36-22.88) (Supplementary Table 5).

Discussion

In this study, roe deer emerged as the most frequently occurring prey and the highest contributor to biomass (%POO: 27.08; %RM: 37.87) in the North China leopard's diet, which is consistent with findings from morphological analysis of fecal contents (Zhao 2022). A similar trend was observed for the Amur leopard (%POO: 23.08; %RM: 29.20). Previous studies conducted in the Northeast Tiger and Leopard National Park show that Amur tigers primarily prey on larger and more abundant sika deer and wild boar, while Amur leopards focus on the relatively smaller and slightly less abundant roe deer—likely due to the competitive pressures from tigers (Dou et al. 2019; Yang et al. 2018). In contrast, on the Loess Plateau, where stronger competitors are absent, roe deer still dominate the North China leopard's diet. This suggests that interspecific competition may not be the primary factor driving Amur leopard's preferences for roe deer. Studies on leopard predation in other parts of the world (Forbes et al. 2024; Havmøller et al. 2020; Hussain et al. 2019) suggest that prey size significantly influences leopard predation patterns, as smaller ungulates, like roe deer, provide optimal energy returns to both leopard species, indicating that leopards may have evolved to target these animals preferentially.

With respect to larger prey (> 80 kg), wild boar is the primary large prey for North China leopards, contributing 9.56% of biomass (%*RM*: 9.56); while sika deer, wild boar, and equids contribute up to 33.58% of the Amur leopard's diet (%*RM*: 33.58). Previous studies have shown that predators over 21.5 kg can tackle prey exceeding 45% of their own body weight (Carbone et

al. 1999). This theory suggests that both leopard species may target vulnerable individuals among larger prey, such as those that are sick, injured, or young. As *%RM* values were calculated using average body weights of wild adult individuals, the actual biomass contribution from large prey may be overestimated.

Excepting roe deer, small- to medium-sized animals (< 37 kg) accounted for the majority (> 75%) of biomass consumed by both leopard species in this study. The North China leopard's prey included small wild carnivores, such leopard cats, red foxes, and badgers, although badgers were detected in only one scat. Similarly, the Amur leopard prey included leopard cats, raccoons, and weasels, with weasels appearing in only one scat. Although birds, rodents, hares, and northeastern forest frogs (*Rana dybowskii*), are frequently found in leopard feces, their small body size limits their contribution to biomass. In some mountainous areas of Kenya, leopards have a high proportion of small mammals, like rodents, in their diet (Rödel et al. 2004), due to limited local food resources and scarcity of small- and medium-sized ungulates. In contrast, food resources at the current study site are relatively abundant, suggesting that leopard predation on smaller animals here may be due to random encounters or specific nutritional requirements.

The leopard's flexibility in prey selection as an opportunistic predator contributes to its broad global distribution (Balme et al. 2020). Variations in habitat type and prey availability lead to considerable regional and habitat-specific differences in its diet (Surve et al. 2022). Therefore, leopard diet composition can provide insights into habitat conditions. In this study, domestic dogs constituted a significant portion of the diet of both leopard species (% RM: 14.38 - North China leopard; 16.20 - Amur leopard), indicating a degree of human disturbance at the study site. Similar findings have been reported in other regions of the world. In Nepal, Kandel et al. (2020) found that cattle (% RM: 19.9) were the primary leopard prey outside the Terai Plains Reserve, while dogs ranked as the fourth largest relative biomass contributor (% RM: 6.1). In north-east India, Kshettry et al. (2018) observed that domestic animals dominated leopard diets in human-impacted landscapes (80.30%), with cattle contributing 48.22% of the biomass.

In habitats that have been disturbed by anthropogenic activity, limited wild prey resources may compel leopards to hunt domestic animals to meet their nutritional needs. In addition, studies have shown that the free range model for domestic dogs, which is practiced in the study area (Consolee et al. 2020; Wang et al. 2023), offers them unrestrained and unsupervised access to the forest, thus increasing the likelihood of encounters with leopards. Since domestic dogs are

similar in size to roe deer, they may become preferred prey for leopards. This observation is supported by infrared camera footage of North China leopards chasing domestic dogs in the Tieqiaoshan Nature Reserve in Shanxi Province (see Attachment Video).

Most studies on the feeding habits of large carnivores rely on traditional morphological analysis of undigested remains (e.g., hair, bone, claws, etc.). However, this method has several drawbacks—including that it may misidentify feces from sympatric carnivores as those of target species. For example, Lu et al. (2021) reviewed snow leopard (*Panthera uncia*) diet analyses and found there is a high probability of misidentifying feces belonging to other carnivores as snow leopard feces (33-79%). In this study, ~10 feces samples were shown to belong to other animals, such as red foxes and dogs. Additionally, the traditional method can overestimate the occurrence of smaller prey because they leave more remains per unit of biomass consumed than larger prey. Furthermore, this approach heavily relies on the investigator's subjective experience, making data accuracy and consistency harder to secure.

Kress et al. (2015) reviewed the dietary analysis methods and suggested that traditional morphological methods are more suitable for large carnivores with simple prey profiles, while molecular techniques are better suited for species with diverse diets, such as leopards. The results of this study support this conclusion, as small prey (e.g., frogs, birds, and mice) not previously detected by traditional methods were identified, and all OTUs were identified to the species level. Herein, seven sample amplification failures were attributed to severe degradation of fecal DNA, highlighting the importance of collecting fresh scat when using metabarcoding for dietary analysis.

Due to sample size limitations, the prey species identified in this study may not capture all the prey of the two leopard species. However, data extrapolation indicates that over 85% of the prey categories were captured (Fig. 4). Increasing the number of samples in future studies will improve results accuracy. In addition, collecting detailed environmental data (i.e., prey species and abundance, habitat type, distance from settlements, and season, etc.) and target species biological information (i.e., identity, sex, and age, etc.) could provide a more comprehensive understanding of leopard diet composition and predatory behavior.

In current studies on wild carnivore feeding, including this study, wildlife weights are typically substituted by zoo animal weights to calculate relative biomass contribution (Fløjgaard et al. 2022; Greenspoon et al. 2023). This simplified substitution is scientifically limiting. In the future, the development of real-time weighing equipment for wild animals, combined with infrared cameras to capture details—such as species, sex, group size, and body size, etc.—will enable a more accurate biomass assessment.

Anthropogenic factors can influence changes in prey communities (Wilson et al. 2020). Poaching and habitat modification reduce prey availability, posing a major threat to carnivore populations. Inadequate food availability forces large carnivores to hunt domestic animals near settlements, leading to human-animal conflicts, which have become a significant cause of large carnivore deaths (Schell et al. 2021). Leopard predation on domestic animals has also been common in the study area in recent years (Consolee et al. 2020; Wang et al. 2024), and effectively addressing this problem has become an urgent concern for wildlife conservation managers. Additionally, domestic animals can carry diseases, and their predation increases the risk of disease transmission to large carnivores (Gilbert et al. 2020; Seimon et al. 2013). Moving forward, managers should strengthen control over domestic animals near protected areas to prevent close contact with leopards. At the same time, monitoring wild ungulates populations and minimizing human interference will help ensure the stability and health of leopard populations.

In conclusion, the present study reconfirmed that small- and medium-sized animals (10-40 kg) are the primary prey of leopards, as previously reported (Hayward et al. 2006). Comparative dietary analyses revealed that roe deer contributed significantly more biomass to North China leopards than Amur leopards, which compensated for this gap by preying on larger ungulates (sika and red deer). This difference may be attributed to variations in ungulate composition in their respective environments. In addition, the diet analysis revealed the presence of a variety of domestic animals in leopard feces, which contribute substantial biomass, suggesting that anthropogenic disturbance has directly impacted the leopards' diets. Based on these findings, it is recommended that management focus on strengthening the monitoring of wild ungulate populations, while implementing measures to strictly control anthropogenic activities within the reserve.

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Conflict of Interest

The authors declare no conflict of interest.

Ethics Statement

The samples collected for the study were faeces, obtained by non-invasive methods, and no animals were captured or biological tissues collected. The team obtained approval from the nature reserve management department before entering the study area, and the sampling process was carried out in strict accordance with the 'Technical Procedures for the Investigation of Terrestrial Wildlife and their Habitats (GB/T 37364.3-2024)' issued by China's National Standardisation Administration, with no interference with the normal life of wildlife.

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| Prim | Target | Primer Sequence 5'–3' | Prod. | Refere |
|------|----------|------------------------------------|-------|---------|
| er | Mitochon | - | Leng | nce |
| Nam | drial | | th | |
| e | Gene | | (bp) | |
| 16s | 16S rRNA | | 380 | Xiong |
| | | Fwd: GAGAAGACCCTATGGAGC | bp | et al. |
| | | Rev: AIAGAAACCGACCIGGAI | | 2016 |
| Ppo- | Cytb | | 156 | Sugim |
| Cb | | Fwd: GTAAATTATGGCTGAATTATCCGG | bp | oto et |
| | | Rev: CATAACCGTGAACAATAATACGAC | | al., |
| | | | | 2006 |
| 1.0 | 12S rRNA | | 150 | Riaz et |
| 128 | | Fwd: TAGAACAGGCTCCTCTAG | 150 | al. |
| V 3 | | Rev: HAGAIACCCCACIAIGC | вр | 2011 |
| Pard | | Block: | | |
| В | | CTATGCTTAGCCCTAAACCTAGATAGTTAGCCCA | | |
| 8 | | | | |

Table 1 Primers and blocking nucleotides utilized in this study.

| Locations | Samples No. | No. of Leopard scats | No. of successfully sequenced samples |
|--|-------------|----------------------|---------------------------------------|
| Liupanshan Nature Reserve | 2 | 2 | 1 |
| Ziwuling Nature Reserve | 7 | 4 | 2 |
| Tieqiaoshan Nature Reserve | 33 | 29 | 27 |
| Northeast Tiger and Leopard National Park | 55 | 48 | 39 |
| Total | 97 | 83 | 69 |
| | | | |

 Table 2 Collected vs Successfully Sequenced Samples.

| | Calculated at % POO | | Calculated at % RM | | |
|----------------------|---------------------|------------------|--------------------|------------------|--|
| Metrics | Р. | P. p. orientalis | Р. | P. p. orientalis | |
| | p. japonensis | | p. japonensis | | |
| Shannon's | 2.26 | 2.29 | 1.98 | 2.07 | |
| Diversity Index | | | | | |
| (H) | 0.00 | 0.05 | 0.77 | | |
| Peilou's J | 0.88 | 0.85 | 0.77 | 0.76 | |
| Levin's niche | 7.48 | 7.65 | 4.97 | 5.81 | |
| breadth (<i>B</i>) | 0.54 | 0.40 | 0.54 | 0.24 | |
| standardized niche | 0.54 | 0.48 | 0.54 | 0.34 | |
| breadth (B_s) | | | | | |
| | | | | | |
| | | NO. | | | |
| | | 6. | | | |
| | |) | | | |
| | XO | | | | |
| | Q | | | | |
| C | 0 | | | | |
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| | | | | | |
| | | | | | |

Table 3 Prey taxa diversity and niche width statistics of North China and Amur leopards.

Figure Legends

Figure 1 Location of leopard fecal samples collected in the Loess Plateau Nature Reserves, as well as Northeast Tiger and Leopard National Park. Grey shading indicates the Loess Plateau in China. Red areas on the left side indicate the geographical locations of the nature reserves and national parks involved in this study. The right side shows four areas: (A) Liupanshan Nature Reserve, (B) Ziwuling Nature Reserve, (C) Tieqiaoshan Nature Reserve, and (D) Northeast Tiger and Leopard National Park. The black dotted lines indicate the boundaries of the protected areas and national parks. The plus sign indicates the geographic location where leopard faeces were collected. Map was downloaded from the Resource and Environment Science and Data Center, China (http://www.resdc.cn/) and were mapped by Esri's software ArcGIS 10.6 (https://www.esri.com/en-us/home), US.

Alt Text: Pictures show the geographical locations where leopard faeces are collected. In particular, Amur leopard faeces are collected in the Northeast Tiger and Leopard National Park of China. North China leopard faeces are collected in three nature reserves on the Loess Plateau in China.

Figure 2 Relative occurrence frequency of prey in the diets of North China and Amur leopards (%*POO*).

Alt Text: Bar graphs showing the relative occurrence frequency of various prey items in the diets of the North China leopard and the Amur leopard, respectively.

Figure 3 Comparison of relative biomass contribution (*%RM*) in North China and Amur leopard diets.

Alt Text: Bar charts showing the relative biomass contribution (%*RM*) of various prey items in the diets of the North China leopard and the Amur leopard, respectively.

Figure 4 Rarefaction and extrapolation curves generated for North China and Amur leopards using *iNEXT*.

Alt Text: Rarefaction/extrapolation (R/E) curves produced for the scats of North China and Amur leopard using *iNEXT*. The trend of the curves shows that species diversity and coverage tend to stabilise as the number of samples increases.

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