


Decoding the DNA of scat and the application of genetic methodologies to understanding carnivore diet

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Abstract: Carnivore species are vital to ecosystem function and maintenance. One key component to understanding carnivore ecology and the most effective means of management is knowledge of dietary resource use. Traditional methods used to study carnivore diet, such as microhistology, have several technical and logistical shortcomings. These have hindered the quality and quantity of data that shape understanding of how carnivores exploit prey. Advances in genetic methodologies and their application to wildlife biology has transformed the manner in which information about species can be gained. DNA metabarcoding is one such example. With this approach, genetic sequences present in scat can be determined via next-generation sequencing and matched to reference databases, revealing the carnivore that deposited the scat and the prey it consumed. DNA metabarcoding has the ability to overcome many of the previous challenges associated with dietary analysis and works to advance and inform current knowledge surrounding carnivore ecology, predator-prey relationships, conflicts between carnivores and humans, and potential adaptability to large-scale landscape shifts. Its use has provided novel insights on numerous carnivore species to help inform research priorities and wildlife policies, including those living in unique fragile environments such as the Qinghai-Tibetan Plateau of China. The continued development and increased capacity of molecular dietary analysis via DNA metabarcoding has the promise to grossly improve carnivore conservation management strategies on a global scale.

Keywords: Carnivore, Conservation Genetics, DNA Metabarcoding, Molecular Dietary Analysis, Next-Generation Sequencing, Predator

Introduction

Carnivores play key roles in the functional maintenance of the ecosystems they occupy. They influence herbivore populations which in turn controls vegetation (Ripple et al., 2014), impact disease dynamics and parasite transmission (Terborgh & Estes, 2010), and provide carrion for scavengers (Wilmers et al., 2003). Successful habitat conservation and management may hinge on the holistic understanding of carnivore species in a given area, and necessitates the obtainment of information on their ecology, natural history, and diet.

The importance of understanding carnivore diet

Knowledge of carnivore diet can inform the accessibility and availability of prey, the degree of

competition between different carnivore species and how resources are partitioned, the extent of livestock loss and its potential economic impact on human livelihoods, as well as the adaptive potential of a species and how it may respond to large-scale environmental changes (Symondson et al. 2002; Foster et al., 2013; Hacker et al., 2022).

Traditional methods used for carnivore diet analysis

The dominant method for understanding carnivore diet has historically been microhistology. With this approach, a sample, typically scat, is non-invasively collected in the habitat where the carnivore of interest is found. The scat sample is then dried, and digested hairs or bones are extracted for

comparison to the reference material of already-known species (Pompanon et al., 2012). Another method, albeit most commonly used to assess live-stock predation rather than native prey, is the interviewing of local community members sharing landscapes with carnivores (Meena et al., 2011; Oli et al., 1994). Carnivores outfitted with global positioning systems (GPS) have also been used as a way to find kill sites and subsequently identify prey species (Bacon et al., 2011; Johansson et al., 2015), as has the examination of carnivore stomach contents (Balesrieri et al., 2011).

Limitations and disadvantages of traditional methods of analysis

Each of the above methods has drawbacks that limit the ability to accumulate large amounts of accurate data. Microhistology is a tedious process that can be time intensive and subject to misidentification errors and inter-observer bias (Pompanon et al. 2012). Biological reference material may not be available and the host carnivore thought to have deposited the scat may be erroneously identified (Weiskopf et al., 2016). With interviews, respondents may incorrectly recall predation events, misidentify the carnivore responsible, are subject to social pressure and bias, and may experience fatigue or disinterest if the interview is too long (De Vaus & De Vaus, 2013; Fisher 1993; Oli et al., 2014). Use of GPS coordinates to identify kill sites requires the carnivore to be GPS collared and the kill site to be physically accessible to researchers (Johansson et al., 2015). Lastly, the analysis of stomach contents requires either euthanasia of the animal, or dependence on fresh carcasses resulting from natural and non-natural mortality events, such as roadkill.

Novel approaches to dietary analysis

Advancements in genetic methodologies have resulted in the application of novel approaches to replace the methods described above. Next-generation sequencing (NGS) platforms, such as Illumina, now allow scientists to quickly and accurately determine the order of nucleotides that comprise DNA (A, C, T, G), resulting in terabytes of data in the form sequencing reads. Online databases such as NCBI GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and BOLD (<http://www.boldsystems.org/>) have become reservoirs of uploaded genetic sequences for species genes and genomes. These known sequences serve as references by which sequencing

reads obtained by NGS can be uploaded, matched, and compared for identification purposes.

The method of DNA metabarcoding

NGS technology is leveraged to study carnivore diet via DNA metabarcoding (Figure 1). With this method, researchers collect scat in the field and then bring it to a laboratory. The DNA present in the scat is then non-specifically extracted from the sample and subjected to PCR (polymerase chain reaction) with a pair of universal primers. Universal primers are less-specific and smaller stretches of pre-made genetic sequences that attach to target areas of the animal's DNA. These primers then "run" across the DNA to make copies of a specific gene segment of interest via a series of pre-programmed cycles in a thermocycler. The selection of the segment of DNA to be amplified and sequenced is important. It must be conserved enough so that all possible species possess it, but different enough in its nucleotide sequence to tell them apart (Valentini et al., 2009). For example, mitochondrially encoded 12S ribosomal RNA (MT-RNR1) is found in all vertebrate species, but may not be different, or different enough, between all vertebrate species (Shehzad et al., 2012).

The resulting genetic material undergoes another round of PCR to attach index tags to each individual sample so that they can be multiplexed into one tube and later "pulled out" of the mix for analysis. The final product of pooled samples with index tags is pipetted onto a flow cell and placed on the sequencer. Once finished, data files of returned sequences and other documents, such as a quality control report, are available for retrieval and analysis using bioinformatics.

Bioinformatic tools allow researchers to analyze and process complex and voluminous genetic data in a streamlined, collaborative, and efficient manner (Gauthier et al., 2018). Researchers load returned sequences onto a computer, and use bioinformatic pipelines and software programs to match returned sequences to reference sequences. Parameters and other predetermined metrics are used to help ensure confidence in the determination of the host species as well as the prey item.

There are a number of key benefits to DNA metabarcoding, many of which overcome the limitations associated with more traditional dietary analysis approaches. DNA metabarcoding allows for multiple samples to be processed rapidly in tandem, resulting in large amounts of data in a

relatively short period of time (Shokralla et al., 2012). It also eliminates issues surrounding human-observer bias and produces more accurate information (Pompanon et al., 2012; Valentini et al., 2009). Further, DNA metabarcoding requires only a small amount of scat as starting material. Alternatively,

microhistology may necessitate collection of the entire sample, which takes up more space, challenges transportation logistics and costs, and fully removes potentially important chemosensory information from the environment.

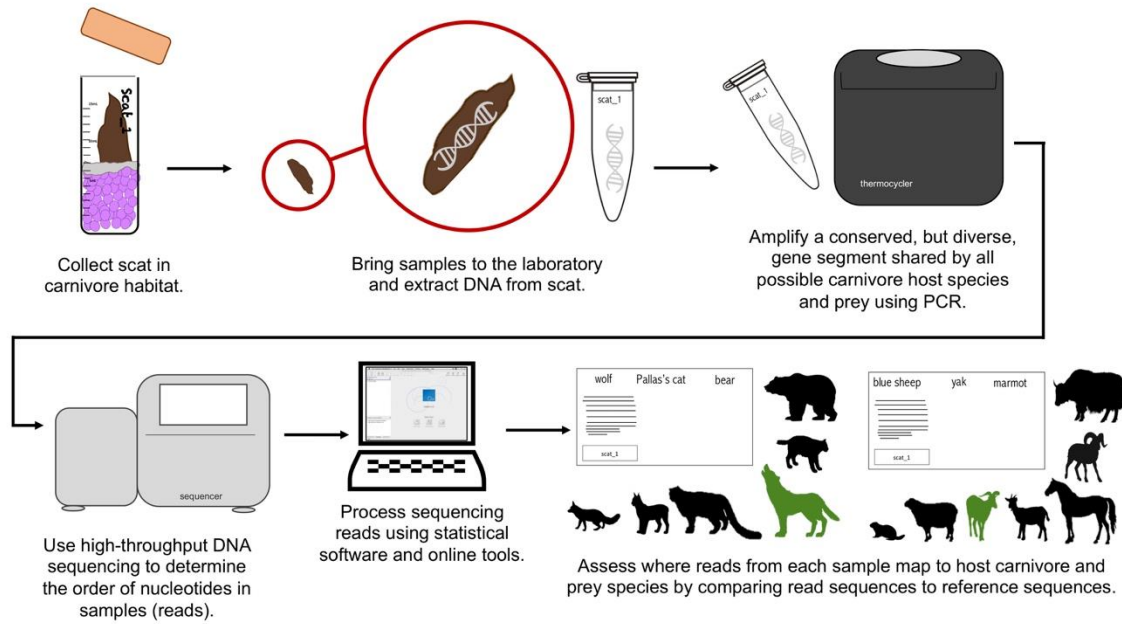


Figure 1: A workflow example showing the application of DNA metabarcoding to determining carnivore diet.

Using DNA metabarcoding to study carnivore diet in China

One area where DNA metabarcoding has been heavily leveraged to understand carnivore diet is the Qinghai-Tibetan Plateau (QTP) of China. The QTP is a 2.5 million km² landscape with an average altitude of 4,500 m above sea level (Zhang et al., 2002). The relatively recent and rapid uplift of the plateau has led to numerous ecosystems and endemic species found nowhere else in the world (Chang, 1981). Carnivores on the QTP include the Tibetan wolf (*Canis lupus*), Tibetan brown bear (*Ursus arctos pruinosus*), snow leopard (*Panthera uncia*), Eurasian lynx (*Lynx lynx*) (Xu et al., 2008), Tibetan fox (*Vulpes ferrilata*), red fox (*V. vulpes*), Pallas’s cat (*Otocolobus manul*), and Asian badger (*Meles leucurus*), among others. Native prey species include blue sheep (*Pseudois nayaur*), Tibetan gazelle (*Procapra picticaudata*), argali (*Ovis ammon*), white-lipped deer (*Cervus albirostris*), marmot (*Marmota* spp.), chukar partridge (*Alectoris chukar*), and pika

(*Ochotona* spp.), among others (Jackson, 2012; Schaller et al., 1988). Pastoralism remains heavily practiced in the area by local Tibetans and a number of livestock that may serve as food for wild carnivores are also present. These include domestic yak (*Bos grunniens*), goat (*Capra aegagrus hircus*), sheep (*Ovis aries*), and horse (*Equus caballus*).

Livestock depredation causes financial burdens on herders, their families, and the local economy, promotes negative attitudes towards carnivores, is emotionally traumatizing, can create tension between residents and agencies seeking to protect at-risk species, and can lead to retaliatory killings. Herders who retaliate may also sell body parts to recuperate their financial losses depending on the carnivore killed, which can contribute to the illegal wildlife trade. Understanding the use of livestock as a food source is needed for carnivore conservation in areas dominated by pastoralism, as it is necessary for constructing tailored mitigation strategies, validating herder knowledge to outside

entities, and designing assistance programs such as livestock insurance .

In an effort to better understand the role that different carnivore species play in livestock loss on the QTP, DNA metabarcoding was used to determine the livestock species in 147 red fox, 25 Tibetan fox, 153 Tibetan wolf, 191 snow leopard, and 72 Eurasian lynx scats collected from September 2017 to July 2018 in Qinghai and Gansu Provinces (Hacker et al., 2022). As expected via previous interviews with local herders (Hacker et al., 2020), the Tibetan wolf had the highest percentage of livestock in their diet at 21.7% of total prey counts, followed by red fox at 5%, snow leopard at 4.9%, Tibetan fox at 4%, and Eurasian lynx at 3.6%. Domestic yak were most often identified, followed by sheep, and then goat. Interestingly, two isolated occurrences of horse and pig (*Sus scrofa*) were identified as being consumed by snow leopard, a species that is often described as a near-specialist (Lyngdoh et al., 2014). The degree of livestock present in the diets of smaller carnivores such as the red and Tibetan fox was unexpected, but may be attributable to scavenging or the depredation of newborns. Unfortunately, DNA metabarcoding is unable to reveal the life stage of the prey identified and cannot confirm if the host carnivore killed the species it ultimately consumed.

From these findings, mitigation measures that work in the interest of local herders and at-risk carnivore species could be recommended. For example, the dominance of yak in Tibetan wolf diet helped to validate herder knowledge, and assisted in leveraging support for yak protection practices specifically tailored to wolf ecology. In addition, the diversity of livestock in snow leopard diet provided insight into the potentially opportunistic and adaptable nature of the species. All herders may want to take protective measures if a snow leopard is in the area regardless of livestock species kept. However, the overall low percentage of livestock in snow leopard diet suggests that losses caused by this carnivore are relatively uncommon.

Continued dietary work on the QTP will be imperative given the fragile nature of its unique ecosystems and endemic species present. Climate change, rapid urban development, and shifts in traditional practices will continue to impact the region, including the human livelihoods and wildlife that reside there. Carnivores in shifting landscapes may find themselves overlapping with previously

unencountered species that outcompete them, causing an increased pursuit of livestock. Dietary information can be a helpful indicator of ecosystem modifications that alter the distribution and abundance of species as well as predicting how carnivores and prey may adapt to future changes.

Application of DNA metabarcoding for species in other parts of the world

DNA metabarcoding has also elucidated the feeding habits of mammalian carnivores in other areas of the world. The analysis of 96 scat samples belonging to Tasmanian devils (*Sarcophilus harrisii*) introduced to Maria Island, Tasmania revealed the first instance of domestic cat (*Felis catus*) (McLennan et al., 2022). It also confirmed that the drastic decreases in short-tailed shearwater (*Puffinus tenuirostris*) and little penguin (*Eudyptula minor*) populations were likely due to Tasmanian devils (McLennan et al., 2022). Eurasian otters (*Lutra lutra*) were found to have a potentially much more flexible feeding strategy than previously known in western China (Wang et al., 2022). The examination of red fox scats in Scotland found a curiously high percentage of domestic dog (*C. l. familiaris*) DNA. Researchers linked this finding to coprophagia, which may help to sustain red fox populations when their natural prey base fluctuates (Waggershauser et al., 2022). Coyotes (*C. latrans*) in New York City were expected to have human food in their diet, but DNA metabarcoding showed that domestic chicken (*Gallus gallus*), presumably intended for human consumption, comprised nearly half of dietary counts (Henger et al., 2022).

An evaluation of DNA metabarcoding

Despite the vast information and novel findings generated by DNA metabarcoding, it remains an intensive and sensitive procedure that is expensive, requires access to advanced laboratory equipment and computers, and demands rigorous training in molecular techniques and bioinformatics. Even minor changes in laboratory practices or protocols can cause differences in data when compared between labs. Contamination risk is also high (Hacker et al., 2021; Pompanon et al., 2012). The molecular marker, or genetic segment, selected must be one that can differentiate carnivores and prey down to a level appropriate for the research question to be answered (Hacker et al. 2021). Further, scientists

must ensure that uploaded sequences to be used as references are available and accurate .

Conclusion

Advancements in the molecular dietary analysis of carnivores will continue to move forward as genetic methodologies improve and become more accessible. Recent notable insights into the ecology and behavior of various species demonstrates the ability of such technology to shift understanding

and apply novel information to wildlife management plans. Continued capacity in DNA metabarcoding has the potential to improve our understanding of many more traditionally difficult-to-study carnivores, ultimately enhancing the conservation outcomes that impact them and the humans they share landscapes with.

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Conflict of Interest

No conflicts of interest to declare.