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# Postprint: Application of High-Throughput Sequencing Technology in Wildlife Diet Analysis

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## Abstract

Diet research constitutes an important topic of considerable interest in animal ecology, and diet analysis methods are continuously being improved and updated due to limitations in technology and applicability. With the development of high-throughput sequencing technology, this technique has gradually been extended to diet analysis of wildlife, greatly enhancing the efficiency of diet analysis and broadening its application scope. Although the advantages of applying high-throughput sequencing to diet analysis in terms of data volume, sensitivity, and resolution are relatively obvious, the application currently remains a relatively under-researched field due to the numerous steps involved and complex influencing factors. This paper outlines the basic workflow of applying high-throughput sequencing technology to diet analysis, summarizes research trends of this technology in food composition analysis, intra- and interspecific dietary relationships, and relationships between food, habitat, and behavior, analyzes the impacts of PCR, contamination, and quantitative analysis on the applicability of this technology, proposes corresponding solutions and recommendations, and provides an outlook on its application prospects.

## Full Text

### The Application of High-Throughput Sequencing Technologies to Wildlife Diet Analysis

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## Abstract

Diet studies are a critical focus in animal ecology, and diet analysis methods have been continuously improved and updated due to technical and applicability limitations. With the development of high-throughput sequencing (HTS) technology, this technique has gradually been extended to wildlife diet analysis, greatly improving efficiency and broadening application scope. Although HTS offers obvious advantages in data volume, sensitivity, and resolution for diet analysis, it remains a relatively weak research field because it involves many steps and is influenced by complex factors. This study summarizes the general strategies adopted for diet analysis using HTS, reviews current progress in analyzing diet components, and elucidates intra- and inter-specific dietary relationships and the relationship between food resources and habitat and behavior. We discuss the influences of PCR bias, contamination, and quantitative analysis on accurate diet assessment, propose ways to improve the technique, and provide prospects for future applications.

**Keywords:** high-throughput sequencing; fecal DNA; diet analysis; interspecific relationship; intra-specific relationship; foraging behavior

## 1. The Workflow of Fecal DNA and High-Throughput Sequencing for Diet Analysis

The basic workflow for animal diet analysis based on high-throughput sequencing involves sample collection, DNA extraction, selection of high-resolution barcode primers (which can be tagged with oligonucleotide labels), high-throughput amplicon sequencing, and bioinformatics analysis to identify food species corresponding to each sequence [17].

### 1.1 Sample Collection

In most studies using high-throughput sequencing for diet analysis, feces serve as the primary sample material, though a few studies on rodents and grasshoppers have analyzed stomach contents [18-19], and some avian diet studies have used pellets [20]. Feces are particularly suitable as non-invasive samples for studying wildlife, especially rare and endangered species. DNA extraction efficiency and sequencing success depend on fecal freshness, which is the key factor determining sample quality. Collection location also matters; mixing the middle layer and outer skin of fecal pellets or scat can significantly improve detection rates, especially for rarely consumed foods. Regarding sampling quantity, Erickson et al. [22] conducted multiple high-throughput sequencing runs on the same fecal sample and found that intra-sample variation was significantly smaller than inter-sample variation, indicating that replicate sampling of the same fecal specimen is unnecessary.

The effects of preservation and extraction methods on molecular markers have been well documented in conservation genetics studies [23], but their impact

on diet analysis has not yet been examined. Preservation method, extraction method, and their interaction determine the quantity of food residues in collected feces. Common fecal preservation methods in diet analysis include silica drying (used for brown bears [25] and rodents [24]), buffer preservation (used for lizards [26] and bats [28]), ethanol preservation (used for seals [29] and great bustards [30]), and freezing (used for bats [31]). Some studies have employed two-step preservation methods (ethanol followed by silica) for leopard cats [32]. Researchers must also consider the feasibility of preservation methods in field conditions and the convenience of sample transport.

Most studies use common commercial fecal DNA extraction kits, though exceptions exist [33]. For example, the QIAamp DNA Stool Mini Kit contains potato adsorbents that can contaminate extractions, causing potato to appear in the diets of animals that do not consume it. When analyzing herbivore diets, researchers should avoid such kits [34]. The extraction efficiency of the QIAamp DNA Stool Mini Kit was significantly lower than that of the Zymo Soil/Fecal DNA MiniPrep Kit for western bluebirds (Sialia mexicana) [35]. When selecting extraction methods, researchers can reference literature on species with similar diets but must also develop appropriate protocols through experimentation.

### 1.2 DNA Extraction, Amplification, and High-Throughput Sequencing

Animal diets can generally be classified as herbivorous, carnivorous, or omnivorous. DNA extracted from diet samples is typically degraded to some degree. While overly long fragments amplify poorly, excessively short fragments reduce resolution. The read length of high-throughput sequencing is well-suited for diet analysis. Different dietary characteristics require selection of different DNA barcodes or metabarcodes. The greatest advantage of high-throughput sequencing for diet analysis is the ability to pool multiple PCR products and obtain massive numbers of reads in a single sequencing reaction. By adding oligonucleotide tags to primer ends, sequences can be traced back to individuals after sequencing. The number of bases in the oligonucleotide polymer depends on the number of pooled individuals; more bases allow more samples to be pooled but can affect PCR efficiency. Octamers are typically sufficient [39], with differences greater than 2 bp between octamers.

Redundant sequences generated during PCR amplification and high-throughput sequencing can be filtered and removed using appropriate programs [26, 32].

## 2. Construction of a Local Potential Food Source Barcode Database

Sequences generated by high-throughput sequencing platforms must be compared against databases to identify food taxa [40]. Because geographic distributions of animals and plants vary greatly, public databases (NCBI, EMBL, DDBJ) that only contain locally uploaded sequences are far from meeting the

needs of diet analysis researchers [17]. With numerous barcode types and varying resolution, different barcode combinations must be selected based on animal diets. If public databases lack such barcode data, classification accuracy decreases, affecting the application of diet results to endangered species conservation. Researchers must therefore construct a comprehensive local potential food source barcode database based on selected barcodes. This involves collecting tissue materials from potential food sources within the target animal' s activity and foraging areas, conducting taxonomic identification by specialists, synthesizing appropriate primers, and performing Sanger sequencing to build the database for subsequent comparison with high-throughput sequences to identify food sources at the species level.

For herbivore analysis, building a local database or increasing barcode types can significantly improve species-level identification. For example, when comparing bat diet high-throughput data, sequences could be identified to species or genus level when a local database was available, but the proportion identified to species decreased dramatically without a local database [37]. More importantly, a well-constructed local barcode database can itself be used for biodiversity assessment and monitoring [41].

## 3. High-Throughput Sequencing Data Analysis

When comparing sequences against local and public databases, species assignment is based on sequence similarity, but threshold setting remains controversial. Some researchers use relaxed similarity thresholds for species-level classification [25, 37, 42], while others recommend stricter thresholds [18, 31]. Some suggest setting different thresholds based on specific barcodes and research objectives. Although clustering methods (MOTUs, Molecular Operational Taxonomic Units) can be used for diet composition analysis, building local databases generally improves taxonomic assignment, clarifies specific food items consumed, and facilitates application of diet research results to species conservation practice [17].

## 4. Applications in Food Composition Analysis

Determining animal food composition is fundamental to diet research. High-throughput sequencing can identify food types and proportions far beyond what morphological identification can achieve. Brown et al. [43] used high-throughput sequencing to study frog consumption by brown rats (Rattus norvegicus) and house mice (Mus musculus), finding that detection rates for frogs in stomach contents increased compared to microscopic analysis. Egeter et al. [33] studied the diet of the smooth snake (Coronella austriaca) in Austria, discovering for the first time that some small mammals are major prey items for snakes. Leray et al. [45] analyzed the diet of coral-dwelling predatory fish using high-throughput sequencing, identifying 46 operational taxonomic units (OTUs), most of which could be identified to species level. High-throughput sequencing has even been

applied to invertebrate diet analysis [46].

## 5. Food Effects on Intra- and Interspecific Relationships

Food provides the energy and nutrients required for animal survival and reproduction, and food relationships reflect fundamental interspecific interactions. Diets differ among species. Sympatric species may evolve different foraging strategies to avoid competition, such as selecting different microhabitats, consuming different foods, or foraging at different times to meet energy and nutritional needs [47]. Food factors play important roles in species coexistence and competition [48], and diet studies are prerequisites for understanding food selection mechanisms. Within a species, dietary differences may exist between sexes due to different reproductive tasks, or among individuals due to personality factors [49].

Lopes et al. [24] used high-throughput sequencing to analyze and compare the diets of two tuco-tuco species (Ctenomys), finding significant dietary differences between them. Soineinen et al. [50] compared winter diets of two sympatric lemming species in the Arctic and, combining high-throughput data with food resource surveys, found high dietary overlap. However, abundant local resources may reduce apparent competition. Diet studies using high-throughput sequencing provide new tools for investigating relationships between cryptic species and resources, helping reveal mechanisms of species coexistence and food overlap. Studies on bat diets from high-throughput sequencing show that sympatric, morphologically similar, closely related insectivorous bats exhibit differentiation in specialized prey despite overlap in primary foods, confirming resource partitioning as a coexistence mechanism [51]. Burgar et al. [37] subsequently analyzed diets of additional bat species, finding that greater morphological differences correlate with more significant heterogeneity in resource utilization, which also relates to developmental stage and sex.

## 6. Diet-Behavior Relationships

Animal migration timing and destination are controlled by genetic factors but also influenced by environmental factors like food [52]. Migration demands high energy, and animals adapt to environmental changes through physiological, behavioral, and dietary shifts. Fecal microhistology revealed similar diets for Nathusius' s pipistrelle (Pipistrellus nathusii) during autumn migration and summer residence, but higher-resolution high-throughput sequencing showed significant differences: forest insects dominated in summer while wetland insects predominated in autumn, indicating different foraging strategies to adapt to local resource changes [42]. Birds typically supplement energy and nutrients efficiently before migration [53], and research on the lesser kestrel (Falco naumanni) confirmed this strategy—narrowing diet breadth and consuming higher-energy Orthoptera to prepare for migration [53].

As high-throughput sequencing improves diet analysis precision, it challenges

some theories established through behavioral observation or morphological identification. Optimal foraging theory suggests that specialist species should shift to less-preferred foods when resources decline. However, high-throughput sequencing analysis of Daubenton's bat (Myotis daubentonii) found no diet broadening even under high energy demands before hibernation, indicating that food preferences may be independent of spatiotemporal constraints [31]. For sexually dimorphic species like the brown anole (Anolis sagrei), females often show higher dietary diversity than males [26].

## 7. Diet-Habitat Relationships

Habitats provide essential food resources, and unique environments may evolve specific food selection behaviors. Habitat changes also alter food availability and diversity. Studying diet-habitat relationships provides insights into foraging strategies and habitat selection. High-throughput sequencing enables efficient diet analysis across large spatial scales. Clare et al. [28] analyzed diets of the little brown bat (Myotis lucifugus) across different habitats, proposing bat food quality as an environmental quality indicator. Trevelline et al. [54] used high-throughput sequencing to analyze Louisiana waterthrush (Parkesia motacilla) diet, finding terrestrial insects comprised a large proportion, contrary to previous beliefs about preference for aquatic insects from polluted environments.

Herbivores may strongly influence natural plant community formation and distribution, even affecting invasive species. Erickson et al. [22] used high-throughput sequencing to analyze white-tailed deer (Odocoileus virginianus) diet, finding they selected native plants as primary foods while promoting invasive plant expansion. Khanam et al. [19] analyzed diets of four rodent species, providing scientific basis for biological pest control by identifying their plant and invertebrate prey.

## 8. Contamination Effects

Contamination represents a non-negligible error source in high-throughput sequencing diet analysis. Sample processing and PCR bias can individually or interactively affect final results. Both feces and stomach contents exhibit varying degradation. To reduce costs, oligonucleotide tags are added to primers for multiplexing, but errors introduced during PCR are amplified during high-throughput sequencing. Copy number differences among food DNA templates can further affect results, as high-efficiency amplification may suppress low-efficiency amplification, leading to underestimation of suppressed foods [25]. Given that high-throughput sequencing can simultaneously assay multiple food barcodes in a single reaction, it is essential to include positive controls and replicates, especially for omnivores.

Environmental contamination captured during highly sensitive sequencing can overestimate food diversity or affect diet difference analyses. When analyzing Egyptian mongoose (Herpestes ichneumon) diets, cross-contamination between

samples was detected [57]. Another contamination source is ecological: when prey containing food residues are consumed by higher-level predators, analyzing the predator's diet inevitably introduces contamination—a issue requiring careful attention in complex food chain studies [17, 58]. When analyzing carnivore diets, predator DNA can also be amplified, interfering with sequencing and consuming resources; blocking oligos can prevent primer binding to predator templates [25, 59].

## 9. Quantitative Analysis

Beyond food types and diversity, researchers are particularly interested in the proportion of specific foods consumed and dietary preferences. Whether high-throughput sequencing data quantitatively reflects actual consumption remains controversial [56]. Foods with high sequence counts may be consumed only occasionally, while those with low counts may be primary or preferred foods [17]. This bias stems from biological factors (different cell numbers in food tissues, digestion rates) and technical factors (amplification efficiency differences, primer tag errors, mismatching, redundancy filtering parameters).

Control experiments feeding penguins fish found that high-throughput sequencing data matched quantitative PCR results, suggesting sequence counts can reflect consumption quantities—higher counts indicating greater consumption [60]. However, studies on harbor seals (Phoca vitulina) found that fed fish proportions did not match sequence count proportions [56]. To ensure reliable qualitative and quantitative data, parallel application of another diet analysis method within the same study effectively validates accuracy [38]. Srivathsan et al. [38] used shotgun metagenomics to quantitatively analyze the diet of the Indochinese grey langur (Pygathrix nemaeus), finding that while shotgun sequencing yielded higher dietary diversity than amplicon sequencing, most sequences were microbial, making amplicon sequencing more accurate [22].

## 10. Conclusions and Prospects

High-throughput sequencing technology offers unique advantages in data volume and species-level identification that other diet analysis methods cannot match. As the technology matures and researchers better control for error factors, it will see broader and deeper application. Currently, most animal diets remain roughly described qualitatively. As costs decrease, more zoologists are expected to use this technology to address complex questions: exploring relationships between herbivory and plant pollination/seed dispersal, identifying which pollination processes require animal mediation, determining ecological roles in food webs [61], and studying how climate change affects dietary preferences and shifts to predict adaptation to global warming [62].

High-throughput sequencing's strength in identifying food types and differences, combined with stable isotope analysis of food sources and energy flow, provides complementary approaches for complex food web research [46]. As quantitative

analysis matures, integration with nutritional geometry will deepen understanding of animal nutritional needs and foraging strategies [63].

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