

Article

DNA Metabarcoding Reveals Seasonal Variations in Crop-Foraging Behavior of Wild Rhesus Macaques (*Macaca mulatta*)

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Abstract

The ecological drivers of wildlife crop-foraging behavior—whether as a compensatory response to natural resource scarcity or as opportunistic exploitation of anthropogenic food sources—remain poorly understood in human–wildlife conflict research. Traditional methodologies, which primarily rely on direct observation and morphological identification, have limitations in comprehensively quantifying wildlife dietary composition, particularly in accurately distinguishing between morphologically similar plant species and conducting precise quantitative analyses. This study utilized DNA metabarcoding technology (rbcL gene markers) to identify and quantify plant dietary components through fecal sample analysis, systematically investigating the dietary composition and patterns of agricultural resource utilization of wild rhesus macaques (*Macaca mulatta*) in human–wildlife interface zones of southwestern China. A total of 29 rhesus macaque fecal samples were analyzed (15 from spring and 14 from winter), identifying 142 plant genera, comprising 124 wild plant genera, and 18 crop genera. The results revealed distinct seasonal foraging patterns: crops accounted for 32.11% of the diet in winter compared to 7.66% in spring. Notably, rhesus macaques continued to consume crops even during spring when wild resources were relatively abundant, challenging the traditional hypothesis driven by resource scarcity and suggesting that crop-foraging behavior may reflect an opportunistic, facultative resource selection strategy. This study demonstrates the significant value of DNA metabarcoding technology in wildlife foraging behavior research, providing scientific evidence for understanding human–primate conflict ecology and developing effective management strategies.

Keywords: *Macaca mulatta*; DNA metabarcoding; seasonal variation; agricultural crops; human–wildlife conflict



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1. Introduction

The increasing interaction between wildlife and agricultural systems has made primate crop foraging a critical challenge in conservation biology. While such behavior is often interpreted as compensatory feeding during natural food shortages (H1: Nutritional Stress Hypothesis), growing evidence suggests that some species may strategically exploit cultivated resources as part of behavioral plasticity (H2: Opportunistic Optimization Hypothesis) [1]. Resolving this dichotomy is crucial—if crop raiding primarily stems from

habitat resource deficits, management should prioritize ecological restoration; conversely, if driven by facultative opportunism, solutions must focus on deterrence strategies [2,3].

The rhesus macaque (*Macaca mulatta*), the most widely distributed non-human primate globally, demonstrates a high degree of environmental adaptability. Its range spans tropical, subtropical, and temperate forests, and even extends into rural and urban areas [4]. This widespread distribution not only reflects the macaque's adaptability to diverse ecological environments but also positions it as a typical representative of human–wildlife conflict. Through flexible behavioral strategies—such as rapid learning of new skills and exploiting various food resources—the macaque has successfully adapted to environments ranging from forests to urban areas [5]. Despite having abundant natural food sources, macaques frequently “visit” farmlands, gardens, and even rummage through trash bins for food. This behavior has led to macaques being colloquially referred to as “garbage monkeys” in many regions of Asia. The macaque's ability to seamlessly transition between natural and human-dominated environments, coupled with its strong reproductive and dispersal capabilities, has led to its classification as a “weed species”—an organism that thrives in human-disturbed habitats [6]. Recent data shows that the macaque population in China has exceeded 200,000 individuals, with significant populations in regions such as Yunnan, Guizhou, Guangxi, and Guangdong. With ongoing ecological improvements in certain areas, macaque populations have rapidly increased in specific regions. However, the overlap between human activity and macaque habitats has also risen significantly, leading to frequent “human–macaque conflicts.” This issue is particularly evident in cases such as macaque damage to crops and their intrusion into human settlements. While the macaque's flexible feeding habits and strong environmental adaptability enhance its survival advantage, they also contribute to escalating conflicts with humans [7]. Crop raiding, particularly of calorie-dense foods, can lead to increased fertility and larger primate group sizes [8], which in turn results in more crop damage and higher instances of human–wildlife conflict (HWC). Therefore, in-depth studies on the feeding behaviors of “problem individuals” (i.e., “troublesome monkeys” or “nuisance macaques”), especially regarding the composition of their diets and the proportion of crops within them, are essential for developing scientifically grounded and effective management strategies.

Traditional dietary analysis methods, such as direct observation and fecal microscopic histological analysis, face numerous challenges in practical applications. These methods are not only labor-intensive and resource-demanding but also exhibit significant limitations in precision, characterized by difficulties in tracking highly vigilant animal individuals and low efficiency in distinguishing morphologically similar food types, particularly in identifying highly digestible animal food components [9,10]. In recent years, with the rapid development of molecular biology techniques, DNA metabarcoding technology, as an emerging molecular analytical method, has provided an effective approach for analyzing animal dietary composition [11–13]. Compared to traditional methods, metabarcoding technology demonstrates distinct advantages: first, it employs non-invasive sampling methods, minimizing disturbance to animals [14]; second, it conducts species identification at the species or genus level based on DNA sequences, with identification efficiency and accuracy significantly higher than traditional methods [15]; additionally, species identification through barcode sequence similarity effectively eliminates subjective interference [16]. Based on these technical advantages, DNA metabarcoding technology is increasingly being applied to dietary studies of various wildlife species [17–19]. For example, Portugal-Baranda et al. used metabarcoding to reveal the highly diversified diet of red-legged partridge, primarily composed of grains, weeds, and legumes [20]. Zhang et al. revealed the winter dietary composition of Guizhou golden monkeys (*Rhinopithecus brelichi*) through this technology [21].

This study investigates the wild rhesus macaque (*Macaca mulatta*) population inhabiting the karst ecosystem of Guizhou Province, a region characterized by frequent human–macaque conflicts. By collecting and analyzing fecal samples from macaques in the Qiannan Buyi and Miao Autonomous Prefecture and employing chloroplast gene *rbcL*-based metabarcoding, the study aims to (1) systematically document the seasonal variations in the macaques' plant-based dietary composition, quantitatively distinguishing between necessity-driven (winter) and opportunistic (spring) crop utilization patterns [14]; (2) assess the degree of macaques' dependence on agricultural resources and evaluate their potential socio-ecological consequences, thereby elucidating the underlying mechanisms of human–macaque conflicts and informing evidence-based mitigation strategies; and (3) determine the optimal timing for implementing deterrent measures versus alternative provisioning approaches based on empirical data.

2. Materials and Methods

2.1. Study Area and Sample Collection

Fecal samples were collected in early January 2024 (winter) and late April 2024 (spring) from areas where wild macaque habitats overlap with human activities in Qiannan Buyei and Miao Autonomous Prefecture, Guizhou Province, China. Prior to sampling, different social groups within the study area were identified and distinguished through field surveys based on macaque territorial distribution and social structure. A total of 29 fresh fecal samples from wild macaques were collected from multiple social groups, comprising 14 winter samples and 15 spring samples (Supplementary Table S1). To ensure sample representativeness and independence, sampling followed the following criteria: (1) within the same social group, sampling points were spaced ≥ 1 m apart to avoid repeated sampling from the same individual; (2) sample origin was determined based on morphological characteristics including fecal size, shape, and color; (3) GPS coordinates of each sampling point were recorded. During sample collection, researchers wore disposable sterile gloves and placed fresh fecal samples into 50 mL centrifuge tubes pre-filled with absolute ethanol for fixation. Samples were immediately transported to the laboratory and stored at -80°C .

2.2. DNA Extraction, PCR Amplification, and Sequencing

DNA was extracted using the OMEGA Soil DNA Kit (D5635-02) (Omega Bio-Tek, Norcross, GA, USA). The extracted DNA was assessed for molecular size using 0.8% agarose gel electrophoresis, and its concentration and purity were determined with a NanoDrop spectrophotometer, ensuring the A260/A280 ratio was close to 1.8, meeting purity standards. Samples with a DNA concentration ≥ 100 ng/ μL and acceptable purity were used for subsequent PCR amplification. The target gene region was amplified using the *rbcL* gene barcode primers Z1aF (5'-ATG TCA CCA CCA ACA GAG ACT AAA GC-3') and hp2R (5'-CGT CCT TTG TAA CCA TCA AG-3') [22]. PCR products were analyzed by 2% agarose gel electrophoresis, and the target fragments of expected size were excised, then recovered and purified using the Axygen Gel Recovery Kit. The PCR products were then quantified using the Quant-iT PicoGreen dsDNA Assay Kit on a Microplate Reader, and samples were pooled in equal amounts based on the results. Library construction was performed using the Illumina TruSeq Nano DNA LT Library Prep Kit (Illumina, San Diego, CA, USA), completing end repair, A-tailing, adapter ligation, and purification of the PCR products. After library construction, the library fragments were again selected and purified by 2% agarose gel electrophoresis. Quality control was performed using the Agilent High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA) to check the fragment length and quality of the library, followed by precise quantification on the Promega QuantiFluor system using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo

Fisher Scientific, Waltham, MA, USA). Qualified libraries were sequenced on the Illumina NovaSeq 6000 platform using PE250 paired-end sequencing.

2.3. Statistical Methodology

The raw sequencing data underwent a comprehensive bioinformatics pipeline to ensure data quality and accurate taxonomic identification. Initially, primer sequences were trimmed using Cutadapt (v.2.3) [23], followed by quality control and sequence processing in QIIME2 (v.2019.4) [24]. The DADA2 (v.1.8) module [25] was employed for denoising, paired-end read merging, and removal of chimeric sequences, resulting in high-quality representative sequences and an amplicon sequence variant (ASV) feature table. Taxonomic annotation was performed by aligning the representative sequences against the NT database using blastn (v.2.16.0) [26], with subsequent taxonomic assignment implemented through BASTA. To specifically investigate macaque crop-foraging behavior, sequences corresponding to crop species were identified and extracted based on the annotation results and the known crop types in the sampling areas.

To investigate seasonal variations in crop-foraging behavior of wild rhesus macaque (*Macaca mulatta*), we performed a χ^2 test of independence comparing agricultural and wild plant read counts, applying Bonferroni correction ($\alpha = 0.05$) for multiple comparisons. Crop resource diversity (α -diversity) during spring and winter was quantified using Shannon and Simpson indices, with seasonal differences assessed via Wilcoxon rank-sum tests. To visualize differences in agricultural crop utilization patterns by wild macaques between spring and winter seasons, non-metric multidimensional scaling (NMDS) ordination analysis was performed. Bray–Curtis distance matrices were constructed based on crop utilization frequency data, and NMDS analysis was conducted using the vegan package (v.2.7.1) in R software (v.4.4.1), with stress values < 0.2 considered acceptable. Clustering heatmaps were generated using the pheatmap package in R with complete linkage clustering and Bray–Curtis distance.

3. Results

3.1. Overview of Sequencing Data

The rarefaction curve illustrates the variation in the number of observed species in each sample as sequencing depth (sequence count) increases. By randomly sampling sequences and calculating the number of species, the rarefaction curve reflects the accumulation of species and is used to evaluate whether the sequencing depth is sufficient to capture most of the species in the sample. The results indicate that as sequencing depth increases, the number of observed species gradually rises and eventually plateaus, suggesting that the current sequencing depth has captured the majority of species in the samples (Figure 1A). A total of approximately 4,105,267 raw reads were obtained from 29 samples. After merging, filtering, and chimera removal, 2,973,971 valid sequences were obtained (Supplementary Table S2). After denoising and filtering out low-abundance OTUs (Operational Taxonomic Units), 1,137 OTUs were identified, with Winter1 containing the fewest OTUs (25) and Spring11 containing the most OTUs (142) (Figure 1B).

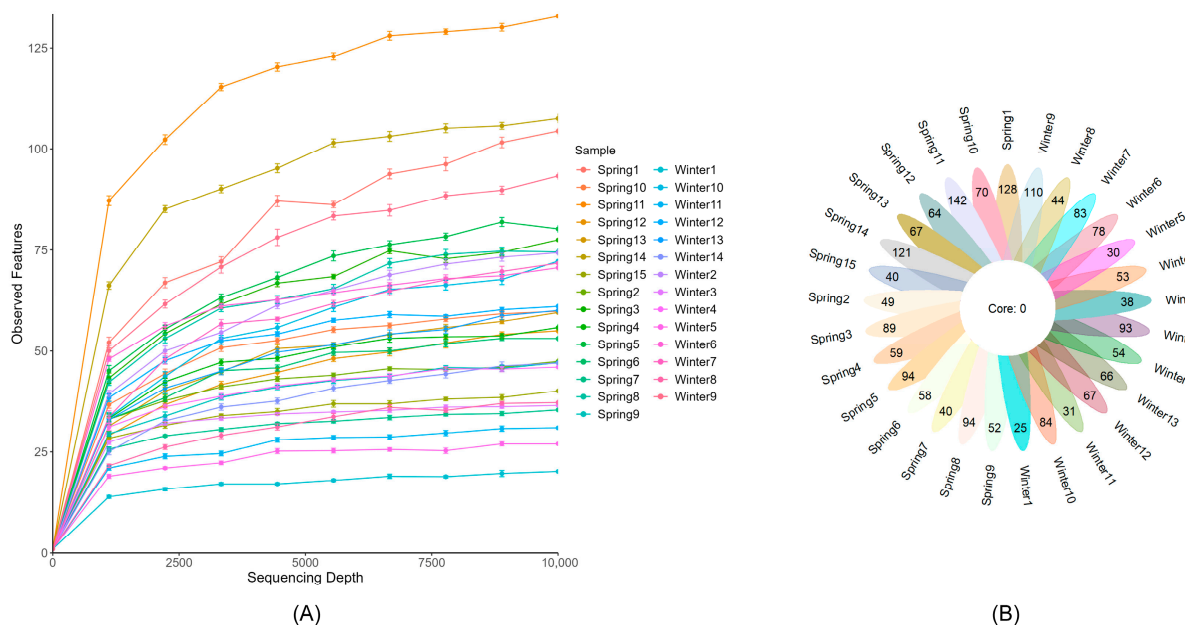


Figure 1. (A) Rarefaction curves of observed OTUs (Operational Taxonomic Units); (B) The flower plot displays the number of OTUs common to all pools (located in the center) and the number of OTUs specific to each sample (located in the petals).

3.2. Seasonal Dietary Composition and Variations

In this study, the wild macaques' preferred food was covering both wild plant-based foods and crop-related foods. The results showed that the main food types of macaques involved 5 classes, 40 orders, 89 families, and 142 genera. Among these, wild plant-based foods were distributed across 5 classes, 39 orders, 85 families, and 124 genera, with the most abundant genera being *Fallopia* (9.85%), *Dioscorea* (9.28%), *Jasminum* (7.13%), *Styphnolobium* (3.12%), and *Wisteria* (1.85%). Crop-related foods were distributed across 1 class, 10 orders, 13 families, and 18 genera, with the most abundant genera being *Ficus* (3.94%), *Solanum* (3.57%), *Vicia* (3.31%), *Lactuca* (1.33%), and *Zea* (0.59%) (Figure 2A). Among these genera, 10 crop species were further identified, including *Allium tuberosum*, *Brassica oleracea*, *Brassica rapa*, *Ipomoea batatas*, *Lablab purpureus*, *Nicotiana tabacum*, *Phaseolus vulgaris*, *Prunus mume*, *Pueraria montana*, and *Zea mays*.

DNA metabarcoding of fecal samples revealed stark seasonal contrasts in agricultural resource exploitation. This seasonal divergence was statistically robust ($\chi^2 = 77,667.85$, $p < 0.005$). During winter, crop-associated reads accounted for 32.11%, dominated by calorie-rich genera *Solanum* (12.36%) and *Ficus* (11.34%), and supplemented by *Vicia* (3.95%), *Lactuca* (1.72%), and *Zea* (1.66%). Meanwhile, the wild plants, however, made up 67.89%, with *Dioscorea* (28.72%) and *Fallopia* (21.04%) as the core sources, and *Taxillus* (5.76%), *Jasminum* (5.29%), and *Wisteria* (3.83%) as important supplementary sources.

By spring, crop utilization plummeted to 7.66%, replaced by wild plant genera such as *Pueraria* and *Quercus*. The proportion of crops decreased to 7.66% (Figure 2B), with *Vicia* (4.34%) becoming the dominant component, while *Lactuca* (1.67%) and *Ficus* (1.31%) saw notable declines. *Zea* (0.21%) and *Prunus* (0.05%) were present in trace amounts. Meanwhile, the proportion of wild plants increased to 92.34%, with *Jasminum* (11.19%) replacing *Dioscorea* as the primary source. Secondary components included *Fallopia* (7.53%) and *Styphnolobium* (6.24%), while the proportions of *Dioscorea* (1.95%) and *Senna* (1.69%) showed substantial reductions.

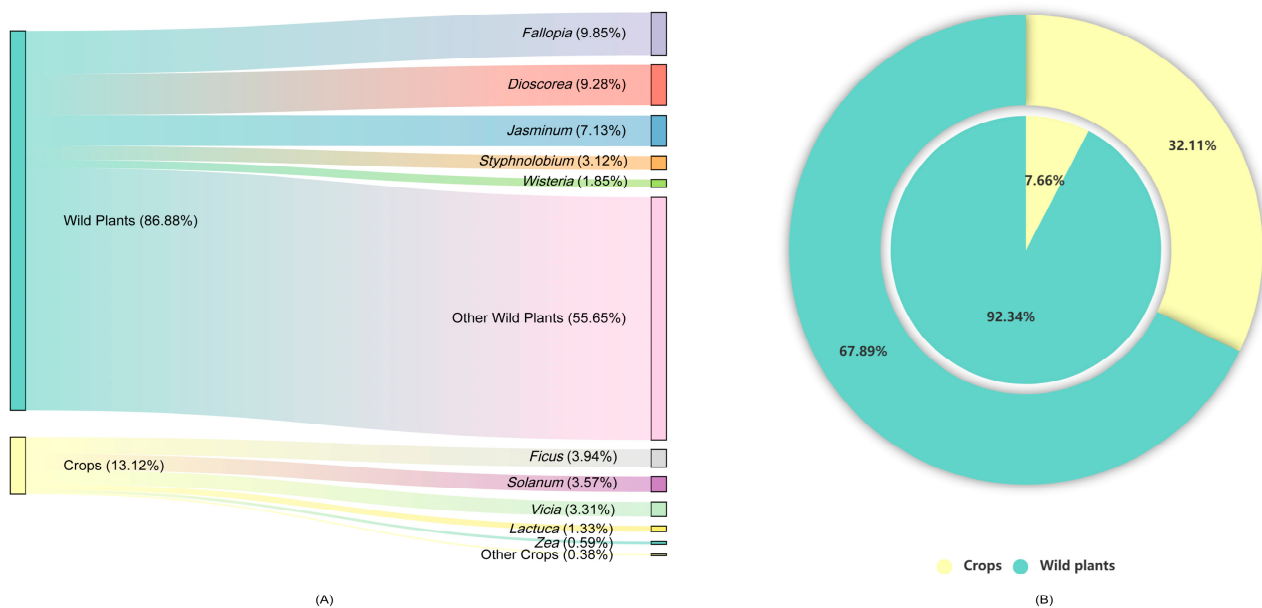


Figure 2. (A) Proportional composition of wild plant versus crop food items in the macaque diet (pooled data from spring and winter); (B) Seasonal shift in dietary resource allocation between wild plants and crops, with inner ring representing spring and outer ring indicating winter.

3.3. Analysis of Seasonal Foraging Diversity for Crops

This study employed clustering analysis to explore the seasonal differences in agricultural crop-foraging behavior of wild rhesus monkeys during spring and winter. The outcomes demonstrated a tendency for separation between spring and winter samples in the dendrogram, implying potential disparities in foraging behavior between the two seasons. Heatmap analysis further elucidated the relative abundance patterns of various crop genera across these seasons, with *Ficus*, *Vicia*, and *Lactuca* exhibiting higher relative abundances in multiple samples from both seasons (Figure 3A). Although the Shannon diversity index was marginally higher in spring, which may suggest a more equitable species distribution within the plant food community during this period, this difference was not statistically significant ($p = 0.31$). Similarly, a slightly elevated Simpson index in spring indicated a modest improvement in species evenness; however, the difference compared to winter was also statistically non-significant ($p = 0.23$). Collectively, while there were fluctuations in the α -diversity indices of agricultural crop foods between spring and winter, these variations were not statistically significant (Figure 3B). Furthermore, β -diversity analysis utilizing Non-metric Multidimensional Scaling (NMDS) ordination, with a stress value of 0.137, indicated a good representativeness of the ordination results. The ordination plot revealed a certain degree of separation in the distribution of spring and winter samples within a two-dimensional space, suggesting possible structural differences in the composition of agricultural crop foraging between the two seasons (Figure 3C).

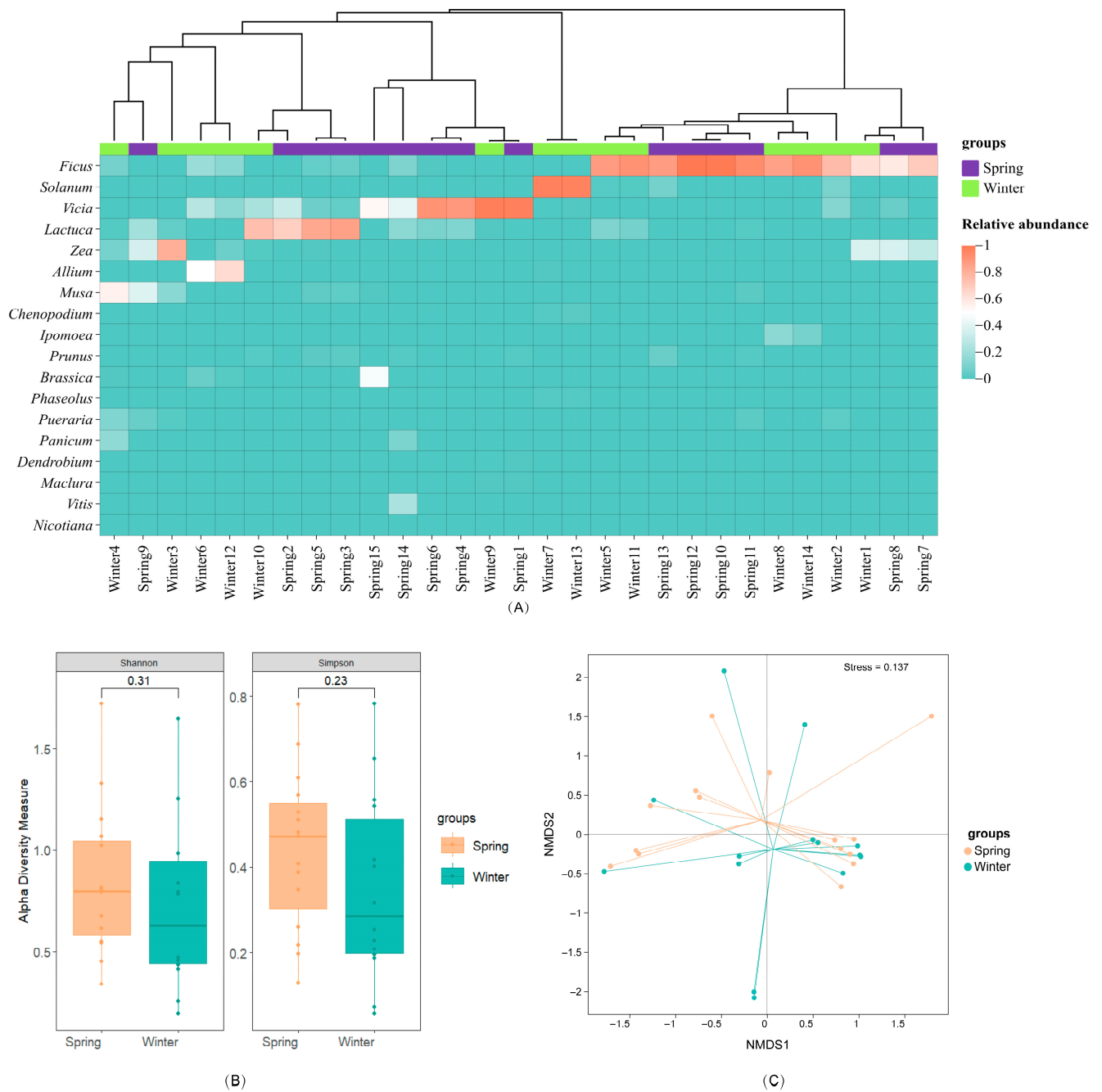


Figure 3. (A) Heatmap of relative abundance of crop genera and hierarchical clustering analysis based on Bray-Curtis distance, with colored strips indicating sample groupings for spring (purple) and winter (green) seasons; (B) Comparison of alpha diversity indices for crop feeding between spring and winter seasons, including box plots of Shannon diversity index and Simpson diversity index; (C) Non-metric multidimensional scaling (NMDS) ordination analysis based on crop feeding composition differences, showing community structure separation patterns between spring and winter season samples.

4. Discussion

This study employed DNA metabarcoding technology to systematically analyze the dietary composition and seasonal dynamics of wild macaques in human–wildlife interface zones of southwestern China. It reveals a systematic shift in their agricultural dependence between resource-scarce and resource-abundant seasons. The findings demonstrate that wild macaques consume multiple crop species during both spring and winter seasons, fully

reflecting their dietary adaptability in human-modified landscapes. This phenomenon is highly consistent with behavioral patterns observed in other crop-consuming primates [8].

During winter, wild macaques enhance their dependence on agricultural resources, with crops occupying a substantial proportion of their dietary composition. This dietary shift is primarily dominated by energy-dense plants from the *Solanaceae* and *Moraceae* families, conforming to the “energy maximization strategy” of primates. To cope with elevated metabolic demands under cold stress, carbohydrate-rich crops are preferentially selected to meet these requirements [27]. Supplementary consumption of *Lactuca* and *Zea* provides carbohydrates while supplying essential fiber, demonstrating compensatory foraging behavior during wild resource scarcity. This is similar to baboons (*Papio ursinus*) utilizing cultivated fruits during dry seasons [28]. This dependence reflects not merely opportunistic exploitation but strategic adaptation during periods of seasonal caloric insufficiency. When wild plant biomass declines below critical thresholds, agricultural resources maintain their energy requirements [29–31]. The nutritional components of crops, particularly their concentrated sugars and starches [8], provide thermoregulatory advantages during winter, while their spatial availability compensates for declining wild food diversity. This behavioral plasticity highlights macaques’ capacity to dynamically adjust foraging strategies in response to environmental resource fluctuations and elucidates the universal strategy of primates adapting to human-modified landscapes under resource pressure.

Compared to winter, macaque plant dietary composition undergoes changes during spring. With increased spring plant resources, macaque food diversity improves, with wild plant food sources increasing substantially while crop proportions correspondingly decrease. This transition indicates that during spring, macaque food selection becomes more dependent on wild plant resources, particularly from *Jasminum* and *Styphnolobium* species. Spring plant foods are typically rich in sugars, vitamins, and minerals, helping meet macaques’ higher metabolic demands and energy expenditure [32–34]. Macaque diet shifts from winter high-energy tubers toward spring nutrient-rich leaves, flowers, and fruits, corresponding to seasonal resource abundance while demonstrating flexibility in food selection. Specifically, *Jasminum* plants, with their tender leaves and fruits rich in easily digestible sugars and secondary metabolites, become important energy sources for spring macaques. Despite abundant wild plant resources in spring, macaques continue consuming certain proportions of crops, indicating that their crop consumption stems not solely from resource scarcity but may represent an opportunistic foraging strategy. Under specific conditions, compared to wild plants, crops such as *Ficus*, *Solanum*, and *Vicia* may provide more accessible food sources, especially in agricultural areas where macaques more easily locate fruits or leaves of these plants [35]. This suggests that macaque food selection strategies are influenced not only by food resource availability but also by energy requirements and foraging efficiency. However, this study’s sample size was relatively limited; future research requires expanded sample sizes to obtain more reliable and representative conclusions.

Through systematic analysis of macaque dietary selection behavior, this study confirms that the Opportunity Optimization Hypothesis (H2) provides a more robust explanatory framework for their crop utilization compared to the Nutritional Stress Hypothesis (H1). Results reveal that during winter wild resource scarcity, crops occupied a substantial proportion of macaque diet, primarily consisting of high-energy-density plants from the *Solanum* and *Morus* genera. This foraging strategy aligns with expectations driven by seasonal resource scarcity. However, during spring wild resource recovery, despite continued crop availability, macaques markedly shift toward wild plant-dominated diets, particularly preferring nutrient-rich leaves and flowers from *Jasminum* and *Styphnolobium* [32]. This non-linear dietary transition contradicts H1 predictions while fully supporting H2’s

threshold-driven foraging model, indicating macaques prioritize energy efficiency over mere accessibility. In human-modified landscapes, crops provide macaques with supplemental energy opportunities while not hindering their optimization pursuit of wild resources. This flexible strategy is documented in other primate species adapted to habitat fragmentation [36]. This behavioral plasticity aligns with their adaptive utilization patterns in fragmented habitats and contrasts sharply with more rigid dietary patterns observed in folivorous colobines [34].

Informed by the research findings, this study proposes an Integrated Management Strategy (IMS) to construct a human–macaque coexistence framework across three key dimensions. First, a community co-management mechanism is established through Participatory Rural Appraisal (PRA), enhancing community participation and accountability in macaque conservation. Second, non-lethal deterrent measures are implemented to effectively reduce crop losses and human–macaque conflicts, thereby safeguarding farmers' interests. Third, in accordance with the principles of landscape ecology, Seasonal Vegetation Buffer Zones (SVBZs) are constructed during critical crop-growing seasons (November to February). These zones are designed to meet key ecological parameters, including tree-shrub cover and Normalized Difference Vegetation Index (NDVI) [35,37,38]. These strategies directly respond to ecosystem protection requirements in the UN Sustainable Development Goals (SDG 15.1–15.5), including sustainable forest management, mountain ecosystem protection, habitat degradation prevention, and land restoration, while indirectly promoting rural poverty reduction (SDG 1.4) and food security (SDG 2.4) through reduced crop losses, providing a scientifically viable practical pathway for achieving the 2030 Biodiversity Framework goal of “living in harmony with nature”.

5. Conclusions

This study used DNA metabarcoding to investigate the seasonal foraging strategies of wild rhesus monkeys in human–wildlife conflict zones in southwestern China at the molecular level. Monkeys consumed more high-calorie crops (*Solanum* and *Ficus*) in winter when resources are scarce but shifted to natural vegetation (*Jasminum* and *Sophora*) in spring while maintaining limited agricultural intake. This reveals a seasonal trade-off between energy acquisition and risk avoidance. The findings challenge the Nutritional Stress Hypothesis (H1) and support the Opportunity Optimization Mechanism (H2) as the key driver of primate foraging decisions. This study provides a significant example of applying DNA metabarcoding in wildlife ecology and offers a scientific basis for developing human–wildlife conflict management strategies based on behavioral ecology.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/d17080517/s1>, Supplementary Table S1: Sample Information. Supplementary Table S2: Sequencing Data Summary for Sample Processing Stages.

Author Contributions: Y.W. and H.L. collected and analyzed the data, and wrote the original manuscript. G.S., H.C. and M.H. revised the manuscript. H.S. designed the study and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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