DNA metabarcoding reveals diet diversity and niche partitioning by two sympatric herbivores in summer (#95199)

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DNA metabarcoding reveals diet diversity and niche partitioning by two sympatric herbivores in summer

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Background. Food provides the necessary energy for life activities, and dietary niche analysis can be used to explore foraging strategies and interspecific relationships among wildlife. The vegetation succession has significantly reduced understory forage resources available to sika deer (*Cervus nippon kopschi*). Little is known about the summer foraging strategies or the interspecific relationship between sika deer and Reeves' muntjac (*Muntiacus reevesi*). **Methods.** The present study used high-throughput sequencing and DNA metabarcoding techniques to investigate the feeding habits and interspecific relationships between sika deer and Reeves' muntjac in our study. **Results.** A total of 458 amplicon sequence variants (ASVs) were identified from fecal samples, with 88 ASVs (~19.21%) unique to sika deer and 52 ASVs (~11.35%) unique to Reeves' muntjac, suggesting the consumption and utilization of specific food items for the two species. The family Rosaceae was the most abundant for both species, especially *Rubus chingii* and *Smilax china*. Alpha diversity (local species richness) indicated that the dietary species richness of sika deer was higher than that of Reeves' muntjac, but the difference was not statistically significant. Sika deer also exhibited a higher evenness index ($J' = 0.514$) than Reeves' muntjac ($J' = 0.442$). Linear discriminant effect size analysis revealed significant differences in forage plants between the two herbivores. The niche breadths of sika deer and Reeves' muntjac were 11.36 and 14.06, respectively, and the dietary niche overlap index was 0.44. We concluded that sika deer and Reeves' muntjac exhibit nutritional partitioning in their diets and that resource competition was moderate for the two sympatric herbivores in summer. This study will provide a deeper insight into the diversity of foraging strategies and the coexistence of herbivores within the diet dimension.

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Abstract

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Results. A total of 458 amplicon sequence variants (ASVs) were identified from fecal samples, with 88 ASVs (~19.21%) unique to sika deer and 52 ASVs (~11.35%) unique to Reeves' muntjac, suggesting the consumption and utilization of specific food items for the two species. The family Rosaceae was the most abundant for both species, especially *Rubus chingii* and *Smilax china*. Alpha diversity (local species richness) indicated that the dietary species richness of sika deer was higher than that of Reeves' muntjac, but the difference was not statistically significant. Sika deer also exhibited a higher evenness index ($J' = 0.514$) than Reeves' muntjac ($J' = 0.442$).

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Keywords: DNA metabarcoding; herbivores; dietary partitioning; niche overlap; summer

Introduction

Food provides animals with the necessary energy and nutrients for their life activities and as such is a crucial resource for maintaining the survival and growth of populations (*Zhang et al., 2020a*). Due to habitat fragmentation, climate change, exotic invasive species, artificial disturbance, and other factors, large herbivorous animals are in decline, making them the most endangered group of vertebrates (*Kowalczyk et al., 2011*; *Atwood et al., 2020*). Some rare animal behaviors have been observed, e.g., wild Asian elephants migrating to the north from Xishuangbanna of Yunnan Province (China) to forage for food in 2021. As the first step in wildlife conservation, diet analysis can assess the nutritional intake, explore the relationship between foraging behavior and habitats, and clarify the effect of food on intra- and interspecific relationships through qualitative and quantitative analyses (*Kartzinel et al., 2015*). Such knowledge can further be used to reveal the adaptive mechanisms toward temporal and spatial variation in food availability or diet specialization (*Leigh et al., 2018; Zhang et al., 2018; Vesterinen et al., 2016*).

The sika deer (*Cervus nippon*), ~~belonging to the family Cervidae and genus *Cervus*,~~ is an endemic ungulate of the East Asian monsoon region. Natural populations of sika deer are distributed over northeastern Asia from the Ussuri region of Russia to mainland China, North Vietnam, Taiwan, and Japan (*Tamate et al., 1998*). It was classified in 2015 as a Least Concerned species by the International Union for Conservation of Nature (*Harris, 2015*). In Japan, the number of sika deer declined approximately tenfold from 1990 to 2014, with the current population estimated to be 3.05 million animals (*Kawarai et al., 2022*). Historically, there were six subspecies of wild sika deer in China that were widely distributed in northeastern, northern, central, southern, and southwestern China and the eastern parts of the Qinghai-Tibet Plateau (*Su et al., 2023; Guo and Zheng, 2000*). However, by the 1960s only three subspecies remained, the Sichuan sika deer (*Cervus nippon sichuanicus*), Dybowski's deer (*Cervus nippon hortulorum*), and the South China sika deer (*Cervus nippon kopschi*) (*Sheng, 1992*). Because the distribution areas are small and isolated, communication between populations is at a low level, and the numbers of sika deer have been decreasing to the point where the total number of wild sika deer in China is less than 2000, and the species has been classified as a national Class I protected animal (*Wemmer, 1998*; *Guo and Zheng, 2000*; *Zhang et al., 2016*). Taohongling National Nature Reserve (hereafter, TNNR) was established in 2001 to protect the South China sika deer. The vegetation succession has resulted in a significant reduction in understory forage resources and a limitation of the environmental carrying capacity. As a result, sika deer frequently forage beyond the reserve boundary, a behavior that poses challenges to wildlife conservation and management ~~of the~~ reserve.

The period from May to July of each year is the fawning season when sika deer require considerable energy to raise their offspring. Reeves' muntjac (*Muntiacus reevesi*) is a closely related species that coexists with sika deer in the TNNR. Reeves' muntjac reaches sexual maturity at 7–8 months of age and has a gestation period of 18 weeks. The females can conceive 3–4 days after giving birth, and lactation does not affect their ability to reproduce. Numerous monitoring surveys (i.e., using camera traps, vocalizations, and feces) have revealed a higher relative abundance index for Reeves' muntjac (39.59%) than those for sika deer (3.90%) and wild boar (*Sus scrofa*) (9.72%) in the TNNR (*Kong et al., 2024*). The previous population of sika deer comprised only 365 individuals, with a growth rate of 17% in 1983, which is currently less than 2% (*Jiang et al., 2012*).

Traditional diet analyses, including stomach content analysis, fecal microscopic analysis, indoor feeding experiments, direct tracking observation and indirect utilization, and stable isotope analysis, have been employed for the Eurasian badger (*Zhu et al., 2018*; *Choi et al., 2015*), Alpine musk deer (*Xu et al., 2018*), Tibetan antelopes (*Cao et al., 2008*), and spinner dolphins. However, as herbivorous ruminants, sika deer and Reeves' muntjac have long food retention times in the digestive system, an aspect that imposes certain limitations on the use of traditional analysis. DNA metabarcoding based on high-throughput sequencing allows simultaneous identification of mixed samples originating from multiple species (*Li et al., 2021*). The method involves extraction of total DNA from fecal and stomach content samples, The polymerase chain reaction (PCR) amplification of DNA barcode markers from food taxa of interest, and then DNA sequencing for taxonomic classification of the recovered sequences (*Deagle et al., 2019*). Thus, food items can be accurately classified to the species level, enabling the identification of degraded or mixed dietary samples (feces, food boluses, or stomach contents) (*Lenain et al., 2004*; *Barco et al., 2016*; *Zhang et al., 2020b*). Using high-throughput sequencing and DNA metabarcoding facilitates diet analysis, and the method can also compensate for the limitations of traditional methods in terms of qualitative and quantitative analyses (*Pompanon et al., 2012*).

An animal’s diet is an important attribute of its niche and affects its role in the ecosystem. As such, diet can be used to gauge interspecies relationships (*Du-Preez et al., 2017*). Sika deer and Reeves' muntjac are ruminants belonging to the Cervidae and may have similar diet selection requirements due to their evolutionary and physiological similarities (*Schaller, 2000; Lv et al., 2020*). Considered together with the fast reproductive cycle and dominant population of Reeves' muntjac, this exerts interspecific pressure and potential resource competition (i.e., for space and food). However, several significant differences between the species may facilitate their coexistence, even if resources are limited (*Glen and Dickman, 2008*). Classical ecological theory offers two principal explanations for the coexistence of species in a community: habitat differentiation and resource differentiation (*Shmida, 1984*). Species coexistence theory also emphasizes niche partitioning (*Chesson, 2000*), positing that interspecific competition typically arises when two or more species use the same resources, but the similarity of niches is limited (*Chu et al., 2017*). The strategic distribution of trophic resources plays a pivotal role in the mechanisms enabling the coexistence of sympatric herbivores with similar resource requirements (*Filella et al., 2024*). Therefore, we hypothesize that sika deer and Reeves' muntjac exhibit trophic niche partitioning in their diets and that this relaxes interspecific competition and serves as one of the mechanisms facilitating their coexistence. This hypothesis generates two predictions. (1) Differences will occur in the diet composition of the two sympatric species in summer when there is higher forage availability than in other seasons. (2) Species consuming adequate food resources would increase their niche breadth and weaken the degree of diet overlap. Our study aims to investigate the feeding habits and interspecific relationships between sika deer and Reeves' muntjac using high-throughput sequencing and DNA metabarcoding techniques. This information is significant to population conservation and management of sika deer and biodiversity monitoring.

Materials & Methods

Study area and sample collection

The TNNR is located on the south bank of the middle and lower reaches of the Yangtze River, Pengze, Jiangxi Province. The total area of the TNNR is 12,500 hm^2, and the reserve is divided into three zones. The core zone, with an area of 2,670 hm^2, is for conservation and allows only a few human activities. Most of the sika deer live in this area. An experimental zone is for human activities and regulated development. A buffer zone with an area of 8,000 hm^2 has some allowable human activities, thereby mitigating the effect of the human activity zone on the core zone (*Liu et al., 2008*). The TNNR is in a subtropical monsoon climate zone with four distinct seasons. Most plants begin to germinate during the spring. The summer vegetation type features mixed evergreen-deciduous broad-leaved forest, coniferous forest, mixed coniferousbroadleaved forest, broad-leaved forest, and bamboo, with abundant and nutrient-rich forage plants. The plant phenology enters a period of color change and leaf shedding in autumn. Especially in winter, deciduous broad-leaved forests become dormant; perennial and annual herbs wither, and plant community structure and the forest phase are prone to changes.

Based on previous camera trap surveys, our sampling sites were largely set in areas with frequent activity of sika deer, i.e., Nursery bases, XianLingAn, fir forests, WuGuiShi, NieJiashan, and the Bamboo Garden. Three to five transects (2 km surveyed per transect) were set up at each sampling site, and each transect was randomly positioned in the study area (Figure 1). To minimize the probability of multiple samples from the same individuals, all collected samples were separated by at least 30 m. To distinguish between the fecal pellets of sika deer and Reeves' muntjac, fecal pellet dimensions are usually the best guide (*Chapman, 2004*). The fecal pellet morphology of sika deer is similar to that of black peanuts, while for Reeves' muntjac, the fecal pellets are cylindrical and spherical with a smaller size (*Cao et al., 2024*). For samples collected from mixed-species flocks (including samples between adults of one species and juveniles of another), we used the COI gene fragment to identify the species. The fresh fecal samples were collected using sterile tweezers and transferred into sterile hermetically sealed bags, which were then transported at 4°C to the laboratory and stored at −80°C. A total of 60 fecal samples from two species (30 each) were collected in the summers of 2022 and 2023.

DNA extraction and trnL amplification

The host and fecal plant DNA were extracted with a QIAamp Power Fecal DNA Kit (Qiagen, Hilden, Germany) and plant genomic extraction kits (Omega Bio-Tek, USA) according to the manufacturer's guidelines. For DNA extraction in each round, negative controls (i.e., extraction without feces) were included to monitor for possible contamination. The DNA optical density value was measured using an ultraviolet spectrophotometer, and the A260/A280 ratio of most DNA extracts was between 1.70 and 2.21, indicating highly purified DNA. COI primers F: 5'-TTGGTGCCTGAGCAGGCATAGT-3' and R: 5'-GAGAACAAGTGTTGATATAGAAT-3' were used for amplifying, and species identification of herbivores was made using approximately 574 bp (*Zhang et al., 2011*). The metabarcoding universal primer sequences c: 5'-CGAAATCGGTAGACGCTACG-3' and h: 5'-CCATTGAGTCTCTGCACCTATC-3' were used to amplify an approximately 150 bp region of the chloroplast trnL intron (*Hou et al., 2021*). PCR amplifications were performed in a total volume of 25 µl of PCR mixture containing 12.5 µl of PCR mix (Tiangen, Beijing, China), 1 µl of DNA, 1 µl of each primer, and 9.5 µl of H_2O, with a PCR negative control. The reaction conditions were as follows: denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 30 sec, 56°C for 30 sec, and 72°C for 45 sec, with a final extension at 72°C for 10 min at and storage at 4°C for 10 h. A PCR blank was included as a negative control, and no contamination was detected. The PCR products were detected using agarose gel electrophoresis for subsequent high-throughput sequencing.

Bioinformatic and statistical analyses

The valid fecal amplicons were purified and pooled for sequencing by Shenzhen Microsun Technology Co., Ltd. Paired-end sequencing was performed using the Illumina HiSeq X Ten system (Illumina Inc., San Diego, CA, USA). The raw data were processed using Trimmomatic (v1.2.11) and Flash software (v0.33). The barcoding at the end and the primer sequence distinguished the samples to obtain an effective sequence and correct the sequence direction, resulting in optimized data. After quality inspection and control of the original data, demultiplexed sequences from each sample were quality filtered and trimmed, denoised, and merged, and any chimeric sequences were identified and removed using the QIIME2 dada2 plugin. Each generated unique sequence was referred to as an amplicon sequence variant (ASV) at the 100% threshold of similarity. Representative sequences of the ASVs were selected and compared with the Nucleotide Sequence Database (NT) using a 99% sequence similarity threshold to obtain species annotation information by using the QIIME2 software.

To test the first prediction, the read abundance data were converted to relative read abundance (RRA, i.e., proportional summaries of counts) of each food item (*Deagle et al., 2019*). We also analyzed the intra- and interspecific differences in diet composition. Alpha diversity refers to diversity on a local scale, describing the species diversity (richness) within a functional community (*Shannon, 1948*; *Andermann et al., 2022*). Indices of diversity, including Observed_species, Shannon's information index, Faith's phylogenetic diversity (Faith's_pd), and Pielou's index, were used in the QIIME2 plugin to calculate alpha diversity. Kruskal-Wallis and Wilcox tests implemented in the QIIME2 software were used after obtaining the overall alpha diversity indices for statistical analysis and visualization of significant differences between groups. To compare the differences in food composition structure between groups, a permutational multivariate analysis of variance (PERMANOVA) was employed using the "qiime diversity beta-group-significance" command in QIIME2.

To further validate our second prediction, dietary breadth was measured using Levins' index (*Levins, 1970*), and the dietary overlap of each species was calculated using Pianka's index (*Smith, 1982; Pianka, 1973*). Pianka's niche overlap index > 0.3 was considered a meaningful niche overlap between species, and a significant niche overlap was considered at a value >0.6 (*Sun et al., 2022*). We performed a nonmetric multidimensional scaling (NMDS) analysis based on the Bray-Curtis dissimilarity. Patterns of diet composition and dietary niche overlap of sika deer and Reeves' muntjac were visualized in two-dimensional space using the NMDS plots. Linear discriminant (LDA) effect size (LefSe) analysis was performed to obtain a ranking of abundant modules in the diet plant species for sika deer and Reeves' muntjac. A size-effect threshold of 4.0 on the logarithmic LDA score was used for discriminative functional biomarkers.

A network analysis was performed using *igraph* packages in the R software (version 4.3.2) to reflect the interactions of species enriched in each sample group.

Results

High-throughput sequencing of trnL metabarcoding

The Gel electrophoresis analysis revealed that four samples with low concentrations and weak bands were unusable for further ~~experiments.~~ Therefore, this study focused on analyzing a total of 56 samples from sika deer (Group 1 = 28 samples) and Reeves' muntjac (Group 2 = 28 samples). The 56 samples produced 1,339,361 valid amplified sequences by high-throughput sequencing, with an average of 23,917 valid sequences per sample. The total number of valid bases was 192,872,294, with the shortest sequence being 120 bp, the longest average read being 338 bp, and the total average length being 144 bp. The ASVs common to two sample sets as well as those specific to each species were identified to reflect the compositional similarity and differences at the ASV level. A total of 458 ASVs were identified; the sika deer group had 88 unique ASVs accounting for approximately 19.21%, while the Reeves' muntjac group had 52 unique ASVs, accounting for 11.35%. The species shared 318 ASVs, accounting for approximately 69.43% of the total.

Alpha diversity and inter-group differences

The Observed species and Chao1 indices reflected the richness of ASVs in the samples. The highest community richness values were 99.46 for sika deer and 71.21 for Reeves' muntjac. The average Chao1 for the sika deer group was 121.59, while for the Reeves' muntjac group, the average was 87.09. The Shannon and Simpson indices showed that higher community diversity was observed for sika deer than for Reeves' muntjac (Shannon index: sika deer = 2.81 and Reeves' muntjac = 2.38, on average). Faith's_pd is a diversity index calculated based on a phylogenetic tree. The index uses representative sequences of ASVs within each sample to calculate the distances used in constructing the phylogenetic tree. The average Faith's_pd for the sika deer group was 4.73, while for the Reeves' muntjac group, this was 3.74. Pielou's index reflects the species evenness; the averages were 0.51 for sika deer and 0.44 for Reeves' muntjac (Supplementary material Table S1 and S2). The species-based rarefaction curves reached plateaus as the sample sequencing reads increased (Figure 2).

Diet composition

Both "occurrence" (i.e., presence/absence of taxa) and "RRA" approaches are semi-quantitative surrogates for the true diet. The error associated with weighted occurrence data stems from overestimating the abundance of rare items (*Deagle et al., 2019*). We used RRA, which provides a more accurate view of species' diet than the frequency of occurrence, to summarize the dietary data (*Hou et al., 2021*). Ultimately, a total of 160 food items were identified in the feces of sika deer, comprising 149 genera in 79 families. A total of 155 food items comprising 146 genera in 76 families were identified for Reeves' muntjac. The top 10 most abundant unique forage plants detected in sika deer were *Zygnema* sp., *Trapa natans*, *Acer amplum*, *Syzygium grijsii*, *Citrus reticulata*, *Campylopus* sp., *Oplismenus* sp., *Kadsura longipedunculata*, *Hypericum* sp., and *Hibiscus syriacus* (Table 1). In contrast, the top 10 most abundant unique forage plants among the Reeves' muntjac samples were *Morus alba*, *Picrasma quassioides*, *Strobilanthes* sp., *Perilla frutescens*, *Ailanthus altissima*, *Juglans* sp., *Clerodendrum cyrtophyllum*, *Pinus thunbergii*, *Staurastrum* sp., and *Patrinia villosa* (Table 2). For the common forage plants consumed by sika deer and Reeves' muntjac, the top 10 species with the highest relative abundance at the species level were *Smilax china*, *Rubus chingii*, *Loropetalum chinense*, *Sassafras tzumu*, *Phyllostachys edulis*, *Cunninghamia lanceolata*, *Alangium chinense*, *Rumex acetosa*, *Rhododendron simsii*, and *Rhus chinensis* (Supplementary material Table S3).

Dominant genera and species in the complete diet spectra

Due to point mutations, multiple ASV representative sequences may belong to the same species, and these need to be merged into unique sequences. At the genus level, the dominant genera in both the sika deer and Reeves' muntjac groups were *Smilax* (15.19%), *Rubus* (10.89%), *Dicranum* (8.74%), *Loropetalum* (5.97%), and *Sassafras* (4.74%) (Figure 3). At the species level, the most dominant food item in the feces of sika deer is *Smilax china* (RRA = 24.45%), followed by *Rubus chingii* (~7.24%), *Loropetalum chinense* (~5.72%), *Pohlia elongata* (~5.07%), *Cunninghamia lanceolata* (~4.29%), and *Rhododendron simsii* (~3.68%). *Rubus chingii* (~14.75%), *Dicranum scoparium* (~14.45%), *Sassafras tzumu* (~9.44%), *Loropetalum chinense* (~6.50%), and *Phyllostachys edulis* (~5.12%) were the dominant food items for Reeves' muntjac (Table 3). The distribution histograms of the top 20 species in the sika deer and Reeves' muntjac groups are shown in Figure 4. LEfSe analysis revealed the significant differences in forage plants between sika deer and Reeves' muntjac (LDA score > 2.0, $P < 0.05$). Among those, three orders (Bryales, Asterales, and Liliales) and three families (Bryaceae, Asteraceae, and Smilaceae) were enriched in sika deer. Four orders (Cornales, Lamiales, Laurales, and Saxifragales) and five families (Dicranaceae, Cornaceae, Lauraceae, Moraceae, and Hamamelidaceae) occurred in Reeves' muntjac (Figure 5).

Interspecific niche partitioning and network analysis

Based on the NMDS analysis at the ASV level, the stress value of 0.208 indicated a good fitness of the NMDS model. There was a certain degree of partitioning in dietary habits between sika deer and Reeves' muntjac. Each point in the plot represents a sample, and points shown in different colors belong to different sample sets. The distance between points represents the degree of community difference between samples. The closer the distance between two points, the higher the similarity in community structure and the smaller the difference. PERMANOVA detected significant differences between the food composition of sika deer and Reeves' muntjac (PERMANOVA, p = 0.001, Pseudo-F = 5.17, df = 1), supporting the results of the NMDS analysis. The niche breadth of a species reflects its degree of specialization. The wider the niche, the less specialized the species, indicating a tendency toward being a generalist. Conversely, a narrower niche indicates a tendency toward being a specialist. The niche breadths of sika deer and Reeves' muntjac were 11.36 and 14.06, respectively. The dietary overlap index of the niches between sika deer and Reeves' muntjac was 0.44, indicating that they share some food resources and have a certain diet overlap in summer (Figure 6a). The nutrients and plant secondary metabolites in forage plants (i.e., species and abundance) likely act in concert to alter the feeding habits of herbivores and foraging strategies (*Villalba et al., 2002*). Network tests showed the forage plant abundance between sika deer and Reeves' muntjac at the genus level. Among these, *Smilax* was significantly correlated with *Persea*, *Cinnamomum*, and *Alangium* ($p < 0.05$). *Rubus* was significantly negatively correlated with *Rhus* (Figure 6b).

Discussion

Multiple ASV representative sequences can be assigned to the same species based on the NT database, suggesting that there may be point mutations or next-generation sequencing errors among individuals within the plant species. Therefore, quantitative analysis of forage plants should be performed by merging and accumulation. For DNA identification of plants, researchers have proposed several combinations of DNA regions, i.e., rbcL + trnH-psbA, rbcL + ITS2, rpoC1 + matK + trnH-psbA, and rpoC1 + matK + rpoB (*Pennisi, 2007*). Additionally, the two-locus combination of rbcL + matK represents a pragmatic solution to a complex trade-off between sequence quality, discrimination, universality, and cost (*Hollingsworth et al., 2009*). However, despite a high separation rate obtained compared with a single gene barcode, only a plateau in resolution of ~70% was achieved from the plant dataset in combination (*Fazekas et al., 2009*). The rate of successful identification with ITS2 was 92.7% for medicinal plants, but the resolution of closely related species is still limited, especially within the species level (*Chen et al., 2010*).

The chloroplast trnL (UAA) gene selected was highly conserved in this study, and the amplification system and primers were robust and relatively specific, indicating a relatively good quantitative assessment of diet within and between species (*Mallott et al., 2018*). However, some results obtained by alignment based on public databases are controversial. Interspecific hybridization and gene flow are quite common in plants, and some sequences may be difficult to identify to genus or species levels. Integrating the compound barcoding of trnL can improve the accuracy of species identification, i.e., the combination of trnL-trnF + ITS barcodes (*Liu et al., 2018*). In the field of dietary research, no universal primers are suitable for all taxonomic ranks due to varying recognition capacity, universality of DNA barcoding, and sequence variation across different plant taxa. Simultaneously, a local reference database of potential forage plants should be considered and constructed to provide sequence alignment resources and improve species identification derived from the reserve.

A previous study found that the diet of the South China sika deer comprised 37 plant species, containing 21 herbaceous and 16 woody species such as *Smilax china*, *Rubus chingii*, *Rhododendron simsii*, *Rhus chinensis*, and *Cunninghamia lanceolata* (*Jiang, 2009*). *Smilax* and *Rubus* were the dominant genera foraged by the two herbivores in this study. *Smilax china* is rich in nutrients, containing amino acids, fats, and organic acids, while extracts or active substances from *Rubus* spp. are also reported to have various pharmacological properties. Both of these plants are widely used in traditional Chinese medicine (*Wang et al., 2023*; *Sheng et al., 2020*). The functions of the nutritional and pharmacological components consumed from forage and their effects on the ruminants' physiology need to be further explored. Additionally, more bryophytes were consumed by the two cervid species in summer, a finding that may be attributed to their preference for moist and shady valley habitats. In-depth monitoring is needed to confirm this intriguing phenomenon. To sum up, both South China sika deer and Reeves' muntjac showed a preference for lianas and herbaceous plants. It has been speculated that different utilization patterns and co-evolution of food resources occur during long-term animal-plant interactions but not to the exclusion of the vegetation differences resulting from the subtropical and temperate marine climate (i.e., Japanese sika deer).

We detected interspecific differences in diet composition for sika deer and Reeves' muntjac. The data supported our first prediction: the perennial vine *Smilax china* dominated in sika deer diet (24.45% RRA) but was just 4.50% RRA for Reeves' muntjac. *Rubus* spp. and *Dicranum scoparium* together comprised 29.20% RRA for Reeves' muntjac but just 10.26% for sika deer; *Sassafras tzumu* comprised 9.44% RRA for Reeves' muntjac but just 0.11% for sika deer. Pansu's study refers to differences in dietary species composition as stabilizing, because that is their only plausible effect on coexistence, and the effect of differences in diet composition can be relaxed during interspecific competition relative to the scenario in which all herbivore species eat the same plant taxa (*Pansu et al., 2022*). The bison in Spain consumed significantly more graminoids (21%), whereas legumes were more present in the fallow deer diet (32%), indicating a distribution of trophic resources between the two species that may facilitate their coexistence (*Filella et al., 2024*). Similarly, the selection of different food types by alpine musk deer, red serow, and white-lipped deer helps avoid conflicts resulting from resource competition (*Luo et al., 2024*). In total, it is clear that sika deer and Reeves' muntjac had a wide selection at the dietary level. Although these species consumed common food items, differences in proportions occurred between the two species; furthermore, each species had exclusive plant species in summer, and the specific ASVs in sika deer were distinguished from those of Reeves' muntjac.

Dietary selection and foraging strategies are affected by seasonal shifts, as animals consume different plants due to temporal and spatial changes (i.e., forest types, aspects, and physiognomy) in different seasons. In winter, Taohongling sika deer ~~is~~ predominantly foraged on *Rubus* spp., *L. chinense*, and *Eurya japonica*, accounting for 75.30%; Reeves' muntjac consumed mostly *Rubus* spp., *E. japonica*, and *Euonymus grandiflorus*, accounting for 68.80%, for niche breadths of 4.53 and 3.44, respectively (*Wang et al., 2023*). For comparison, both sika deer and Reeves' muntjac in Taohongling exhibited relatively broad niches (B = 11.36 and 14.06, respectively), and the diet breadth ~~was~~ significantly increased in summer. When forbs and new grasses are available to foraging deer, they would be expected to broaden their dietary niches to include forbs and thereby improve the diet quality (*Nicholson et al., 2006*). Our observations support this hypothesis and also indicate that diet selection and foraging strategies varied with food resource abundances and seasonal shifts (*Nisha et al., 2019*).

Sympatric species may use the same resources such as food and space to survive, resulting in dietary niche overlap or potential competition (*Du-Preez et al., 2017*). A high diet overlap, together with a lack of habitat segregation, indicated potential competition for resources between collared (*Dicrostonyx groenlandicus*) and brown lemmings (*Lemmus trimucronatus*) (*Soininen et al., 2015*). Inter-specific competition may restrict the growth of the population, and sympatric species can achieve coexistence through niche separation to relieve substantial or potential competition (*Lear et al., 2021*). The dietary overlap of the yellow mongoose (*Cynictis penicillatta*) and the slender mongoose (*Galerella sanguinea*) was ~~the greatest~~ in summer. Nonetheless, the specialized slender mongoose diet and the generalist yellow mongoose diet potentially facilitated their coexistence (*Cronk and Pillay, 2019*). Similarly, the diets of roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), and moose (*Alces alces*) varied in the proportion of each food type, despite a 52.6% dietary niche overlap (*Czernik et al., 2013*). In the TNNR, two herbivores coexist sympatrically and share environmental resources, along with a short reproductive cycle and dominant population of Reeves' muntjac, factors that may promote potential resource competition (i.e., space and food) for sika deer. However, this study found that the dietary niche overlap index was 0.44 in summer, as further evidence of diet differences between sika deer and Reeves' muntjac based on the similarity of diets at the species level of plant composition was extremely low, indicating moderate competition between the two species in this season, consistent with our predictions.

Competitive interactions are predicted to be severe between species that have the same feeding style and similar body weights among herbivores; nevertheless, species may partition resources by size and energy requirements when body weights are different (*Prins and Olff, 1998*; *Ritchie and Olff, 1999*). Reeves' muntjac is slightly smaller than sika deer, and as the two species have

~~been co-existing~~ in the reserve for several decades, we speculate that body size is also one of the reasons for dietary partitioning. However, quantitative analysis of the dietary richness and quality between different body sizes has not yet been performed; thus, this surmise must be interpreted cautiously. Optimal resource utilization strategy facilitates maintaining the coexistence of sika deer and Reeves' muntjac, reflecting interspecific niche partitioning and specific resource utilization.

Our study used RRA to reflect the ~~quantitative level;~~ however, this method is still controversial. One reason is that herbivores have relatively long gut transit times that can impede DNA fragment amplification (*Sakaguchi, 2003*). An additional complicating factor is that herbivore guts have different digestion abilities for different plants. Woody stems contain more indigestible material than leaves or buds, and the plants or plant tissues that are more thoroughly digested may result in more thoroughly degraded DNA and therefore be underrepresented in the resulting sequence counts (*Shipley et al., 1999*; *Stapleton et al., 2022*). The continual advancement of sequencing technology may further improve the ability of metabarcoding to accurately assess diet composition. More studies on captive herbivores fed a known diet may also better explain sources of bias in sequence counts and refine ways to alleviate these effects.

Conclusions

High dietary niche overlap is often interpreted as indicating intense interspecific competition. Our study indicated that the dietary overlap and competition were moderate for two sympatric herbivores in summer. Niche partitioning must consider the abundance and proportions of common foods and the number of specific foraging plants. Subsequent efforts should establish a complete local barcoding database, enhance the investigation of available foraging plants (especially Rosaceae and Smilacaceae), assess the biomass of foraging plants, and strengthen dynamic monitoring of herbivores. Additionally, artificial cultivation of preferred forage, habitat improvement, and reserve boundary adjustments should be considered when necessary.

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Author contributions

R.L. and D.W. performed the experiments, analyzed the data, prepared figures and tables, and/or approved the final draft. Z.C., Y.L., W.W. and W.L. collected the samples, Z.B., X.H. and C.G. assisted in conducting the experiments, and Y.X. conceived and designed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.

Ethical statement

No animals were captured, and fecal sample analyses were performed based on the noninvasive principle.

Data availability

Raw sequence data are archived in the NCBI Short Read Archive (http://www.ncbi.nlm.nih.gov/sra) as BioProject PRJNA1110641. It is also available at Figshare: https://doi.org/10.6084/m9.figshare.25026785.v5.

Conflict of Interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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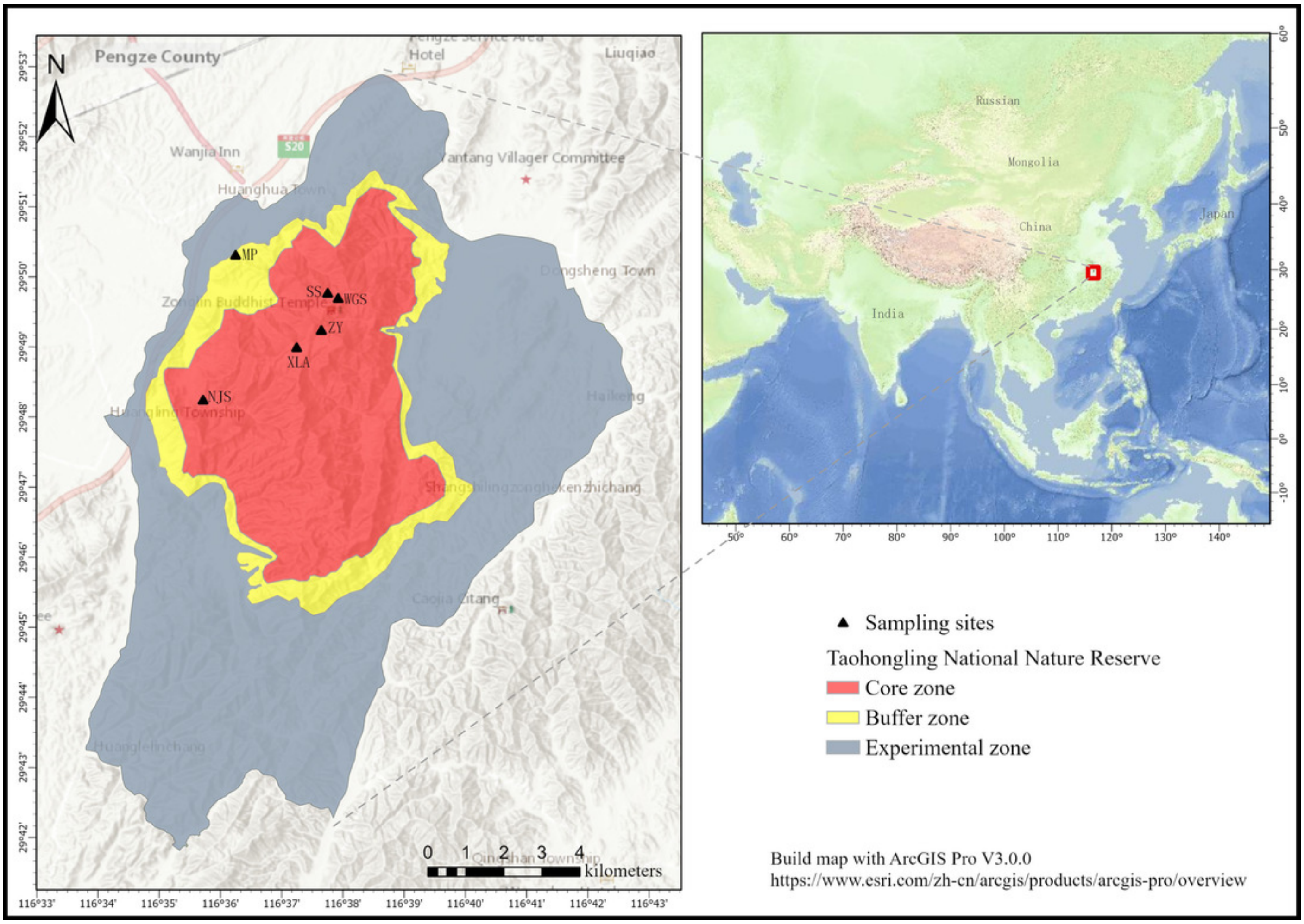
Figure 1

Sampling sites at the Taohongling Sika Deer National Nature Reserve. (MP: Nursery bases; XLA: XianLingAn; SS: Fir forests; WGS: WuGuiShi; NJS: NieJiashan; ZY: Bamboo garden).

(MP: Nursery bases; XLA: XianLingAn; SS: Fir forests; WGS: WuGuiShi; NJS: NieJiashan; ZY: Bamboo garden).



Build map with ArcGIS Pro V3.0.0
https://www.esri.com/zh-cn/arcgis/products/arcgis-pro/overview

Figure 2

(a) Box-plot of the alpha diversity index using Kruskal-Wallis and Wilcox tests.

In each panel, the abscissa is the group, and the ordinate is the value of the corresponding alpha diversity index. Alpha rarefaction curves: (b) Observed species index, (c) Shannon index, (d) Faith_pd index.

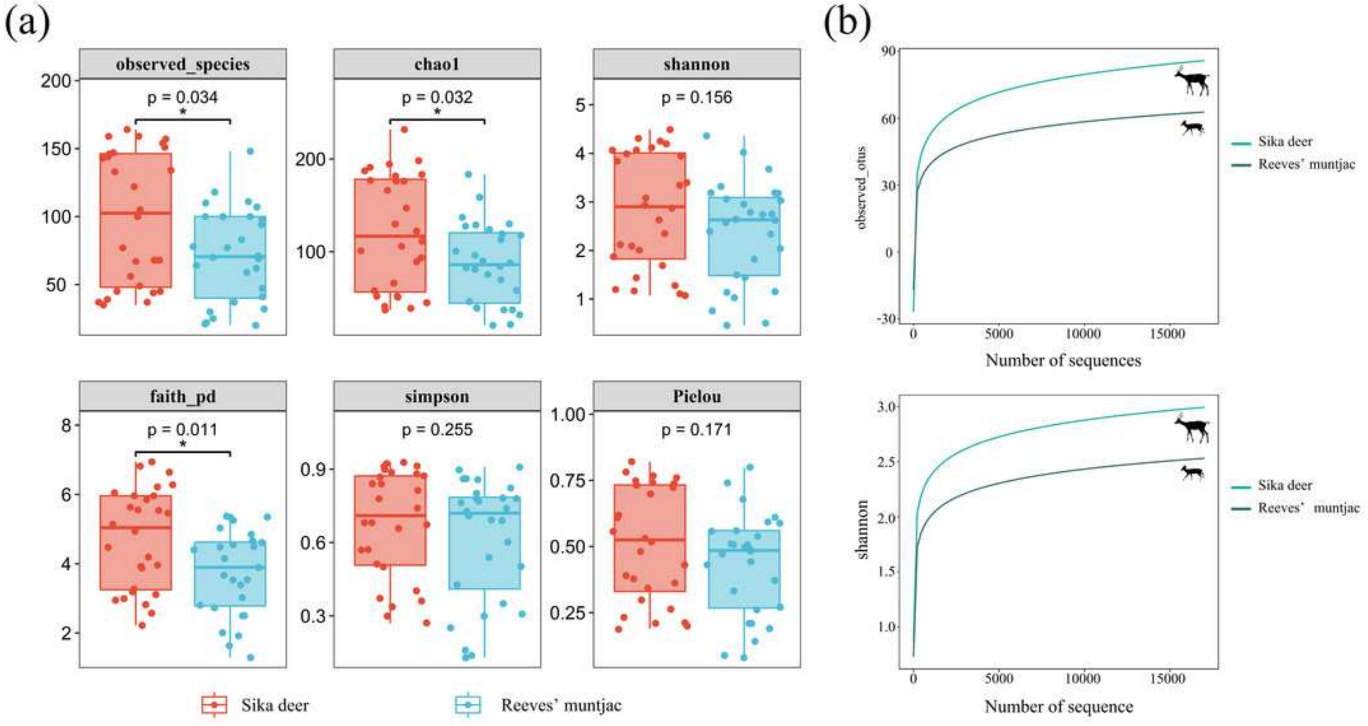


Figure 3

The bar chart distribution of the dominant forage plant in sika deer and Reeves' muntjac groups at the genus level. The x-axis stands for individual samples.

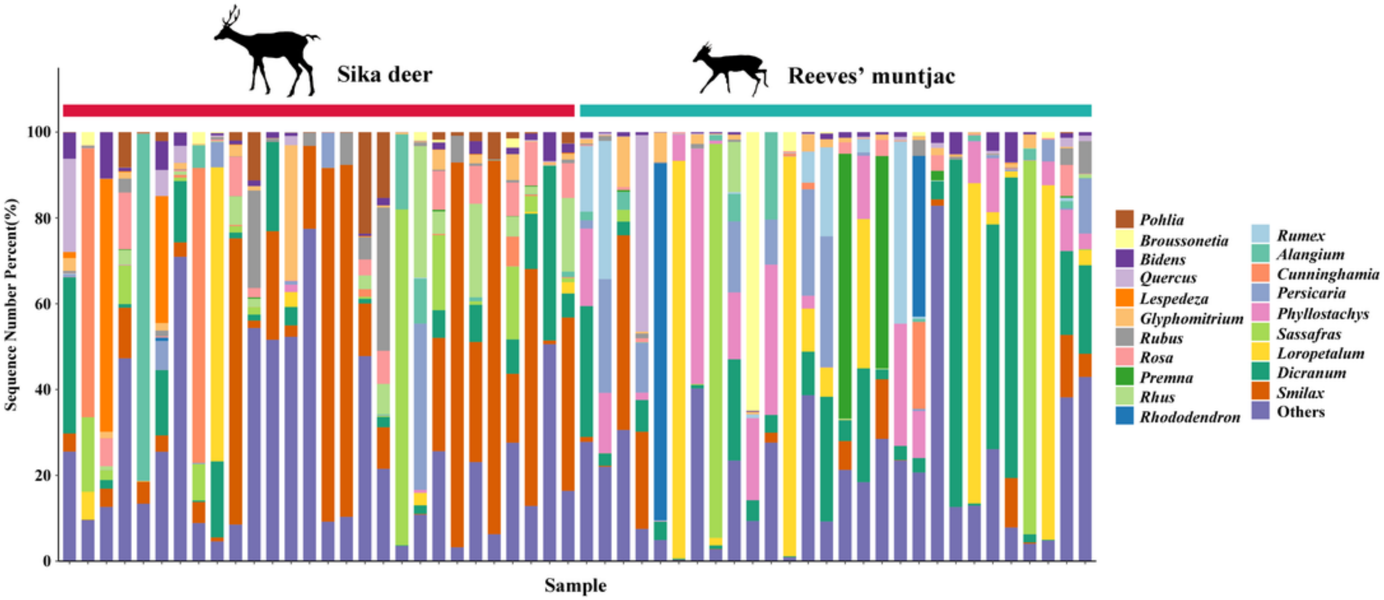


Figure 4

Top 20 forage plants with the highest proportions in sika deer and Reeves' muntjac groups at the species level. The x-axis stands for individual samples.

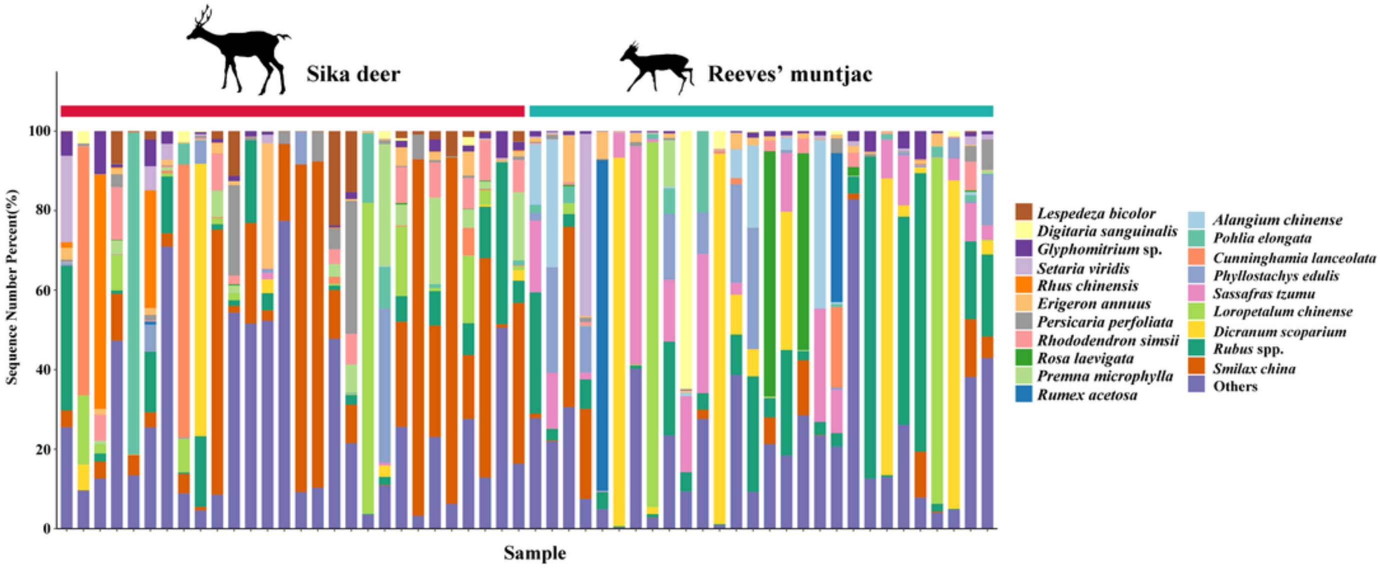


Figure 5

(a) Cladogram based on LEfSe analysis, showing ASVs with the significance of 2 herbivores (green: sika deer; dark green: Reeves' muntjac). (b) Log10-transformed LDA scores for ASVs, i.e., with a threshold value > 4.0.

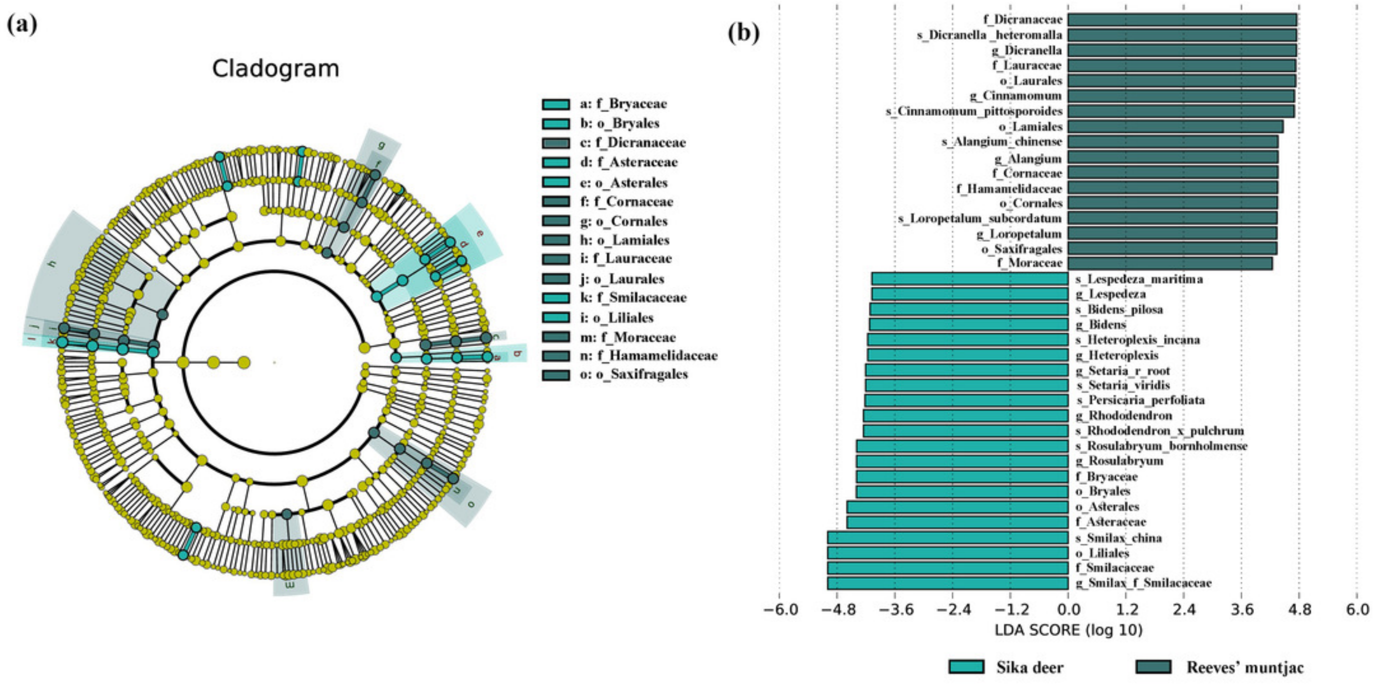


Figure 6

(a) NMDS analysis of sika deer and Reeves' muntjac with 95% confidence ellipse. (b) Network analysis of forage plants.

The size of the circles represents relative abundance, the lines indicate a significant correlation between two species ($p < 0.05$). Red lines mean positive correlations and blue means negative correlations.

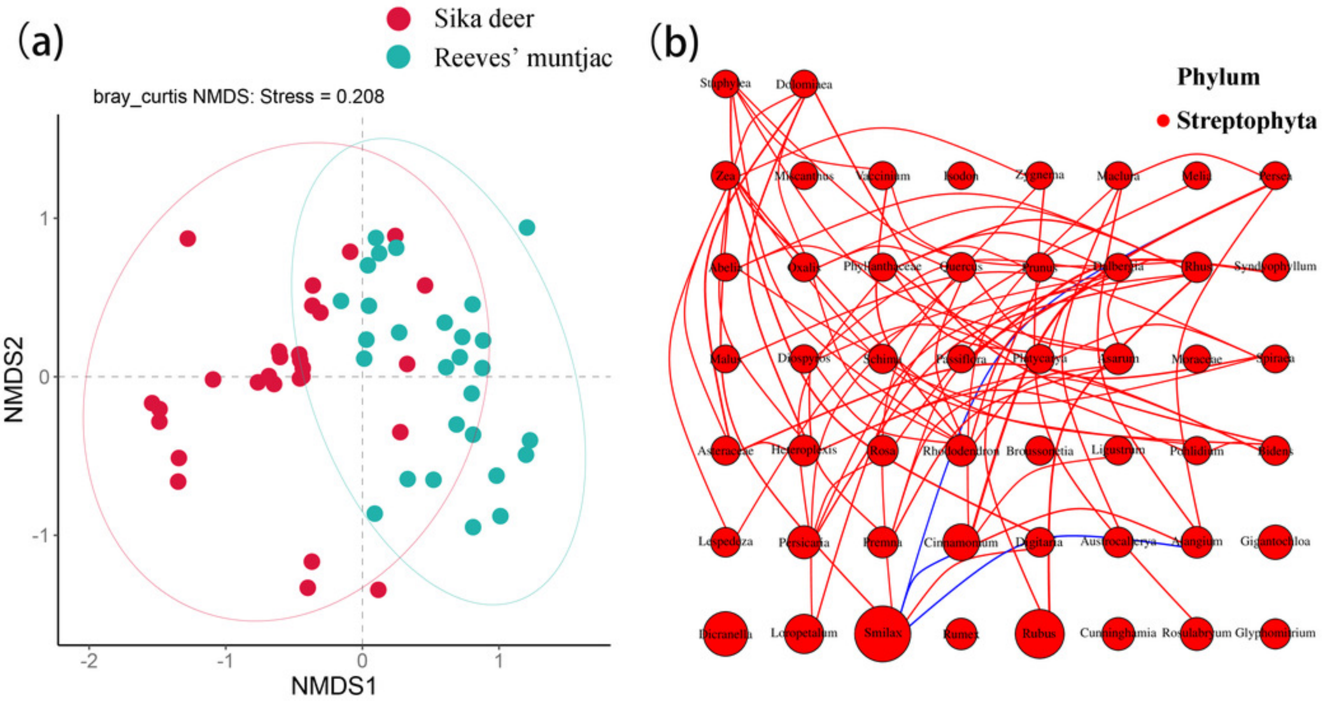


Table 1(on next page)

Annotation information of specific diet ASVs including ASVs ID, abundance, order, family, genus, and species for Sika deer

Table 1 Annotation information of specific diet ASVs including ASVs ID, abundance, order, family, genus, and species for Sika deer

ASVs ID	Abundance	Order	Family	Genus	Species
OTU240	3003	-	-	-	*Zygnema* sp.
OTU56	1831	Myrtales	Lythraceae	*Trapa*	*Trapa natans*
OTU71	1345	Sapindales	Sapindaceae	*Acer*	*Acer amplum*
OTU32	684	Myrtales	Myrtaceae	*Syzygium*	*Syzygium grijsii*
OTU30	220	Sapindales	Rutaceae	*Citrus*	*Citrus reticulata*
OTU246	212	Archidiales	Leucobryaceae	*Campylopus*	*Campylopus* sp.
OTU54	123	Poales	Poaceae	*Oplismenus*	*Oplismenus* sp.
OTU95	121	Oxalidales	-	-	-
OTU256	77	Archidiales	Leucobryaceae	*Campylopus*	*Campylopus* sp.
OTU124	68	Austrobaileyales	Schisandraceae	*Kadsura*	*Kadsura longipedunculata*
OTU223	60	Malpighiales	Hypericaceae	*Hypericum*	*Hypericum* sp.
OTU57	53	Malvales	Malvaceae	*Hibiscus*	*Hibiscus syriacus*
OTU136	49	Poales	Cyperaceae	*Carex*	*Carex gibba*
OTU150	46	Proteales	Sabiaceae	*Meliosma*	*Meliosma cuneifolia*
OTU168	46	Saxifragales	Haloragaceae	*Gonocarpus*	*Gonocarpus* sp.
OTU117	41	Boraginales	Boraginaceae	*Lithospermum*	*Lithospermum erythrorhizon*
OTU35	37	Araucariales	Podocarpaceae	*Podocarpus*	*Podocarpus neriifolius*
OTU158	27	Poales	Poaceae	*Digitaria*	*Digitaria* sp.
OTU91	27	Rosales	Rosaceae	*Sibbaldianthe*	*Sibbaldianthe* sp.
OTU194	26	Pottiales	Bruchiaceae	*Trematodon*	*Trematodon longicollis*
OTU174	23	Poales	Poaceae	*Eleusine*	*Eleusine indica*
OTU1	21	Fabales	Fabaceae	*Hylodesmum*	*Hylodesmum podocarpum*
OTU20	17	Rosales	Rosaceae	*Duchesnea*	*Duchesnea indica*
OTU161	15	Cornales	Cornaceae	*Alangium*	*Alangium* sp.
OTU212	14	Myrtales	Lythraceae	*Lagerstroemia*	*Lagerstroemia indica*
OTU140	14	Malvales	Malvaceae	*Melochia*	*Melochia corchorifolia*
OTU92	12	Asterales	Asteraceae	*Sonchus*	*Sonchus asper*
OTU10	8	Lamiales	Lamiaceae	*Phlomoides*	*Phlomoides umbrosa*
OTU99	6	Oxalidales	Oxalidaceae	*Oxalis*	*Oxalis* sp.
OTU28	5	Gentianales	Apocynaceae	*Trachelospermum*	*Trachelospermum jasminoides*
OTU55	5	Fabales	Fabaceae	*Lotus*	*Lotus* sp.
OTU276	5	Sapindales	Anacardiaceae	-	-
OTU173	5	-	-	-	Unknown phycophyta
OTU123	4	Sapindales	Sapindaceae	*Koelreuteria*	*Koelreuteria paniculata*
OTU73	4	Asterales	Asteraceae	-	-
OTU258	4	Malpighiales	Euphorbiaceae	*Mallotus*	*Mallotus* sp.
OTU5	3	Cornales	Cornaceae	*Cornus*	*Cornus macrophylla*
OTU107	3	Eubryales	Bryaceae	-	-

OTU217	2	Fagales	Fagaceae	*Quercus*	*Quercus variabilis*
OTU113	2	Malvales	Bixaceae	*Bixa*	*Bixa* sp.

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Table 2(on next page)

Annotation information of specific forage plants ASVs including ASVs ID, abundance, order, family, genus, and species for Reeves’ muntjac

Table 2 Annotation information of specific forage plants ASVs including ASVs ID, abundance, order, family, genus, and species for Reeves' muntjac

ASVs ID	Abundance	Order	Family	Genus	Species
OTU377	1503	Rosales	Moraceae	*Morus*	*Morus alba*
OTU288	333	Sapindales	Simaroubaceae	*Picrasma*	*Picrasma quassioides*
OTU314	255	Lamiales	Acanthaceae	*Strobilanthes*	*Strobilanthes* sp.
OTU326	207	Lamiales	Lamiaceae	*Perilla*	*Perilla frutescens*
OTU287	144	Sapindales	Simaroubaceae	*Ailanthus*	*Ailanthus altissima*
OTU296	133	Fagales	Juglandaceae	*Juglans*	*Juglans* sp.
OTU311	95	Lamiales	Lamiaceae	*Clerodendrum*	*Clerodendrum cyrtophyllum*
OTU492	74	Pinales	Pinaceae	*Pinus*	*Pinus thunbergii*
OTU497	62	-	-	-	*Staurastrum* sp.
OTU337	21	Dipsacales	Caprifoliaceae	*Patrinia*	*Patrinia villosa*
OTU520	19	Fabales	Fabaceae	*Amphicarpaea*	*Amphicarpaea edgeworthii*
OTU346	11	Euphorbiales	Euphorbiaceae	-	-
OTU350	9	Urticales	Moraceae	-	-
OTU371	8	Ranunculales	Ranunculaceae	*Clematis*	*Clematis florida*
OTU419	6	Ranunculales	Lardizabalaceae	*Sargentodoxa*	*Sargentodoxa cuneata*
OTU489	6	Lamiales	Scrophulariaceae	*Buddleja*	*Buddleja lindleyana*
OTU380	5	Ranunculales	Ranunculaceae	*Ranunculus*	*Ranunculus japonicus*
OTU463	5	Caryophyllales	Caryophyllaceae	*Pseudostellaria*	*Pseudostellaria heterophylla*
OTU500	4	Gentianales	Rubiaceae	*Damnacanthus*	*Damnacanthus indicus*
OTU498	4	-	-	-	Unknown phycophyta
OTU323	3	Lamiales	Oleaceae	*Osmanthus*	*Osmanthus fragrans*
OTU376	3	-	-	-	Unknown bryophytes
OTU324	2	Asparagales	Amaryllidaceae	*Allium*	*Allium sativum*
OTU315	2	Ranunculales	Papaveraceae	*Corydalis*	*Corydalis balansae*
OTU375	2	Cucurbitales	Cucurbitaceae	-	-

Table 3(on next page)

Relative read abundance (RRA; %) of food item in the diets of Sika deer and Reeves' muntjac (Top 20)

Table 3 Relative read abundance (RRA; %) of food item in the diets of Sika deer and Reeves' muntjac (Top 20).

Number	Food items	Sika deer	Food items	Reeves' muntjac
1	*Smilax china*	24.45%	*Rubus* spp.	14.75%
2	*Rubus* spp.	7.24%	*Dicranum scoparium*	14.45%
3	*Loropetalum chinense*	5.72%	*Sassafras tzumu*	9.44%
4	*Pohlia elongata*	5.07%	*Loropetalum chinense*	6.50%
5	*Cunninghamia lanceolata*	4.17%	*Phyllostachys edulis*	5.12%
6	*Rhododendron simsii*	3.77%	*Smilax china*	4.50%
7	*Persicaria perfoliata*	3.25%	*Alangium chinense*	4.44%
8	*Dicranum scoparium*	3.02%	*Rumex acetosa*	4.31%
9	*Erigeron annuus*	3.16%	*Premna microphylla*	4.08%
10	*Rhus chinensis*	3.15%	*Glyphomitrium* sp.	2.61%
11	*Setaria viridis*	2.82%	*Wisteria sinensis*	1.92%
12	*Digitaria sanguinalis*	2.29%	*Broussonetia papyrifera*	1.79%
13	*Phyllostachys edulis*	2.19%	*Platycarya strobilacea*	1.83%
14	*Lespedeza bicolor*	2.40%	*Schima superba*	1.83%
15	*Persicaria maculosa*	2.15%	*Ligustrum quihoui*	1.55%
16	*Rosa laevigata*	2.18%	*Ligustrum quihoui*	1.46%
17	*Bidens pilosa*	2.16%	*Cunninghamia lanceolata*	1.45%
18	*Rubus coreanus*	1.84%	*Prunus mume*	1.38%
19	*Carpesium abrotanoides*	1.85%	*Rubus coreanus*	1.15%
20	*Oxalis corniculata*	1.72%	*Lophatherum gracile*	1.00%