Tibetan red deer (*Cervus elaphus wallichii*) diet composition patterns and associations during withered seasons in Tibet, China (#103213)

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Tibetan red deer (*Cervus elaphus wallichii*) diet composition patterns and associations during withered seasons in Tibet, China

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During withered seasons, Tibetan red deer (*Cervus elaphus wallichii*) in the high-altitude environment of the Qinghai-Tibet Plateau could face seasonal challenges from food shortages and nutritional deficiencies. We investigated diet composition patterns of a Tibetan red deer population distributed in Sangri Red Deer Reserve, Tibet Autonomous Region during the harsh withered season. In March 2021 and 2022, we searched for Tibetan red deer in the reserve and collected freshly defecated samples after they moved to another location. We collected 89 fecal samples from individuals in the reserve. Diet composition at the individual level was determined using micro-histological analysis followed by K-means clustering and co-occurrence network analysis to reveal population level diet composition patterns. Diet composition of Tibetan red deer included 14 and 19 plant species (or genera) in 2021 and 2022 respectively. K-means clustering indicated two distinct diet patterns within the population across both sampling periods. In 2021, *Salix* spp., *Juniperus pingii*, and *Rhododendron fragariiflorum* emerged as the top three most prevalent food items in both clusters, with proportions of each species varying greatly between clusters. In 2022, one cluster was similar to those in 2021, while *Rosa macrophylla*, *J. pingii* and *Koenigia tortuosa* were the most dominant species in the other. Results of co-occurrence networks showed positively associated food combinations of less dominant food items, with a staple food occurring in all food item pairs in both years. However, randomness accounted for 95.83% and 73.68% of all food item pairs in 2021 and 2022, respectively. The 2022 co-occurrence network displayed complex associations, while the 2021 network exhibited limited and simple associations. Our results suggest that Tibetan red deer fulfill their nutritional requirements by consuming high quantities of several food items or a balanced combination of foods with complex co-occurrence associations. The diets of large ungulates are influenced by the nutritional values and availabilities of food items as well as plant community structure. Therefore, multilayer networks containing additional information are necessary for a deeper understanding of nutritional strategies in high cost of reproduction habitats.

Tibetan red deer (*Cervus elaphus wallichii*) diet composition patterns and associations during withered seasons in Tibet

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Abstract

During withered seasons, Tibetan red deer (*Cervus elaphus wallichii*) in the high-altitude environment of the Qinghai-Tibet Plateau could face seasonal challenges from food shortages and nutritional deficiencies. We investigated diet composition patterns of a Tibetan red deer population distributed in Sangri Red Deer Reserve, Tibet Autonomous Region during the harsh withered season. In March 2021 and 2022, we searched for Tibetan red deer in the reserve and collected freshly defecated samples after they moved to another location. We collected 89 fecal samples from individuals in the reserve. Diet composition at the individual level was determined using micro-histological analysis followed by K-means clustering and co-occurrence network analysis to reveal population level diet composition patterns. Diet composition of Tibetan red deer included 14 and 19 plant species (or genera) in 2021 and 2022 respectively. K-means clustering indicated two distinct diet patterns within the population across both sampling periods. In 2021, *Salix* spp., *Juniperus pingii*, and *Rhododendron fragariiflorum* emerged as the top three most prevalent food items in both clusters, with proportions of each species varying greatly between clusters. In 2022, one cluster was similar to those in 2021, while *Rosa macrophylla*, *J. pingii* and *Koenigia tortuosa* were the most dominant species in the other. Results of co-occurrence networks showed positively associated food combinations of less dominant food items, with a staple food occurring in all food item pairs in both years. However, randomness accounted for 95.83% and 73.68% of all food item pairs in 2021 and 2022, respectively. The 2022 co-occurrence network displayed complex associations, while the 2021 network exhibited limited and simple associations. Our results suggest that Tibetan red deer fulfill their nutritional

requirements by consuming high quantities of several food items or a balanced combination of foods with complex co-occurrence associations. The diets of large ungulates are influenced by the nutritional values and availabilities of food items as well as plant community structure. Therefore, multilayer networks containing additional information are necessary for a deeper understanding of nutritional strategies in high cost of reproduction habitats.

Introduction

Generalized consumers show a combination of ranked and balanced preferences to food items when no single available food item fulfills their nutritional requirements (Begon et al. 2006). Nutritional requirements can only be satisfied by either eating large quantities of food to obtain enough of the nutrient in most limited supply, or by eating a combination of food items that together fulfill the consumer's requirements (Begon et al. 2006). Red deer (*Cervus elaphus*) are adaptable generalized browsers with a wide geographic range, complex subspecies taxonomy, and diets that vary by region and habitat (Gebert, and Verheyden-Tixier 2001, Kay and Staines 1981, Ludt et al. 2004). Of the many red deer subspecies some are abundant and well-studied, while others are imperiled and not well known. Understanding the diets of imperiled taxa is essential to their conservation, especially in environments that are harsh, changing, or where there are non-native potential competitors. One of the most imperiled and understudied red deer taxa, the Tibetan red deer (*C. e. wallichii*), has evolved survival strategies for high cost of reproduction habitats (Wei 2023). Their dietary composition is a direct reflection of adaption and acclimatization to their harsh, high altitude, environment (Wei 2023). However, research on the dietary patterns of Tibetan red deer during the withered seasons is limited (Wei, et al. 2023).

Tibetan red deer, once declared extinct in the wild by the World Wide Fund for Nature (WWF) in 1992, were rediscovered near Zengqi Township, Sangri County, China (Schaller, 1996). This area, located north of the Yarlung Tsangpo River in the Tibet Autonomous Region, is characterized by its high altitude, harsh environment, and prolonged withered seasons that can last seven to eight months (late October to May). A subsequent field survey conducted in 2005 estimated the population to be around 220 individuals (Shen, 2009). This population may face nutritional constraints from harsh seasons and competition from sympatric livestock necessitating adoption of special foraging strategies to maintain their nutritional requirements (Lv, 2020). While red deer populations on the Qinghai-Tibet Plateau may be increasing, due to regional climactic trends, habitat availability is projected to decrease over 40% while habitat overlap with livestock is projected to increase 60% by the 2050s, causing increased competition for food (Ye et al. 2023). This highlights the conservation importance of understanding the nutritional ecology of Tibetan red deer.

Understanding the processes of nutritional ecology for large browsing ungulates that are both rare and free ranging can necessitate research on diet composition and patterns at small temporal scales, such as feeding observations or fecal analysis. Diet composition observed from a fecal sample reflects the diet composition of an individual over several hours (depending on feeding and digestion time). While each fecal sample represents a singular temporal intercept of an individual's feeding ecology, data from fecal samples is often used to determine diet composition of populations over longer time periods (Davis and Pineda Munoz 2016). However, at any given sampling period, we cannot assume all individuals within a population have identical nutritional requirements or access to the same plants, which could cause different diet composition patterns in some individuals. To further understanding of the nutritional ecology of Tibetan red deer during the harsh withered season, we concentrated on collecting singular temporal intercepts (sampled feces) from multiple individuals on multiple dates to reveal diet composition patterns and characterized diet combinations. In this study, we (i) used fresh feces to determined diet composition patterns formed by Tibetan red deer during the harsh withered season in the Sangri Protection Area and (ii) used co-occurrence network analysis to determine patterns of co-occurring food items. Our research will increase understanding of the ecology of Tibetan red deer and help facilitate its conservation.

Materials & Methods

Study Area

The study area is located within the Sangri Red Deer Nature Reserve near Zengqi Village, in Sangri County, Shannan City, Tibet Autonomous Region, China (29° 22'47"-29° 38'10"N, 92° 09'54"-92° 33'11"E). The protected area is primarily characterized by high-mountain valleys, with altitude ranging from 4000-4900 m. The main vegetation types in this region are plateau shrublands and plateau meadows. Our study area experiences an average annual temperature of 8.2° C and receives an annual precipitation of 429mm (Shen, 2009). The reserve is home to various wildlife species, including white-lipped deer (*Przewalskium albirostris*), musk deer (*Moschus chrysogaster*), snow leopards (*Panthera uncia*), and brown bears (*Ursus arctos*) (Schaller, 1996), as well as domestic yak (*Bos grunniens*) and horses (*Equus caballas*).

Samples Collection

Fecal sample collection occurred using non-invasive sampling methods over a 24 day period in March of 2021 and 26 day period in March of 2022. Each day, we drove along roads in the nature reserve visually searching for Tibetan red deer. When living Tibetan red deer individuals were observed, we waited until they left the area and then went to collect freshly defecated fecal samples (15-20 fecal pellets from each fecal pile) and sample vegetation. For vegetation sampling, we utilized nested plot sampling techniques to determine the minimum plot size (Mueller-Dombois, 1974) and recorded plant species within plots and collected approximately 0.5 kg of sample material for each species. We only samples that could be reached by Tibetan red deer (up to 2.5 m high). For grasses and forbs, entire aboveground portions of plants were collected. For shrubs, branches approximately 15-20 cm long were collected. All collected plant specimens were identified to species and stored in paper document folders prior to processing.

Diet composition was determined through fecal microhistological analysis. All plant samples and 10-15 fecal pellets from each fecal sample, were oven-dried at 105℃ until they achieved a consistent weight. The plant samples were subsequently ground using a grinder and sifted through an 80-100 mesh laboratory strainer. Fragments smaller than 80 mesh and larger than 100 mesh were stored in sealed bags as experimental samples. Fecal samples were pulverized using a mortar and pestle. Separate petri dishes were used to hold reference plants and fecal sample slides. Each dish was filled with. Both fecal and plant samples were individually bleached for 2-4 hours in 40 mL of a 10% sodium hypochlorite solution at room temperature, after which the thoroughly digested samples were placed on a microscope slide. Samples were then rinsed with distilled water 3-4 times before the slides were sealed with glycerol and Canada balsam. Five slides were prepared for each plant species as references and ten slides were prepared for each fecal sample. Finally, 300 plant fragments from each fecal sample were inspected under 100- or 200-times magnification and identified to the species or genus (Poaceae) level based on their epidermis and trichome morphology.

Mathematical Analysis

Identifiable cell wall fragments of each plant species were counted in each fecal sample and relative density (*RD*) was calculated using the formula:

$$RD = D_i / \sum D_i$$

Where D_i represents cuticle fragments of each species or genus and $\sum D_i$ represents the sum of all cuticle fragments (Johnson 1982). After dimension reduction of diet composition data by principal component analysis (PCA), we explored diet composition patterns of Tibetan red deer in two withered seasons within population using K-means clustering. Optimal number of clusters was determined using average silhouette method (MacQueen. 1967)

In order to observe characterized food item combinations, we filtered relative densities whose values were ten times lower than the highest in each food item from all fecal samples. We then calculated relative frequency of each plant species in both fecal samples and plots. Finally, we converted diet composition data into presence-absence form and performed co-occurrence network analysis, which is based on probability models of species co-occurrence (Veech.2013). Co-occurrence networks assume that co-occurrence of species is a random and independent event, and their co-occurrence is not influenced by other factors. The key concept of this model involves calculating the observed frequency (E_{ij}) and expected frequency (E_{ij}) of co-occurrence for each pair of species and comparing them to random co-occurrence. Co-occurrence probability (P_{ij}) represents the probability of observing the actual co-occurrence frequency or more extreme co-occurrence frequencies under random conditions.

$$P_{ij} = 1 - P(X \le O_{ij})$$

Where X is a random variable following the hypergeometric distribution (Johnson, et al. 1997), representing the number of times species i and j coexist simultaneously under random conditions, and $P(X \leq O_{ij})$ is the cumulative distribution function of the hypergeometric distribution. All statistical analysis were performed in R (Version 4.2.0)

Results

Diet composition

During the withered seasons of March 2021 and March 2022, a total of 39 and 50 fresh fecal samples were collected, respectively, within the boundary of the Sangri Tibetan Red Deer Nature Reserve in Sangri County. We examined 26,700 plant tissue fragments from fecal samples and 26,106 fragments were successfully identified. In March 2021, Tibetan red deer consumed a diverse array of 16 plant species or genera from 12 families. Among these, *Salix spp*. (46.53%), *Rhododendron fragariiflorum* (17.79%), and *Juniperus pingii* (13.21%) were the primary food items, collectively accounting for 77.53% of their diet. *Poaceae* (4.73%), *Dasiphora parvifolia* (2.66%), *Koenigia tortuosa* (2.58%), *Berberis temolaica*(2.37%) *Spiraea alpina* (2.12%) were less dominant food items. *Artemisia wellbyi, Ligularia rumicifolia, Anemone rivularis, Ceratostigma ulicinum*, *Rosa macrophylla*, *Carex littledalei*, *Betula costata*, *Sibiraea angustata* were occasional consumed food items and occupied less than 2% of diet composition each and less than 10% in total. In March 2022, Tibetan red deer consumed 19 species or genera from 14 plant families. *Salix spp*. (36.70%), *J. pingii* (13.53%), and *R. fragariflorum* (10.11%) remained the primary food items consumed, accounting for 60.34% of their diet collectively. Consumption of *Hippophae tibetana*, *Caragana versicolor*, *C. littledalei*, *B. temolaica*, *R. macrophylla*, Poaceae, *K. tortuosa*, and *Dasiphora parvifolia* accounted for 2%-5% of diet composition each and 30.75% combined. Occasional consumption of plants including *B. costata*, *S. angustata*, *L. rumicifolia*, *C. ulicinum*, *S. alpina*. *A. wellbyi, A. rivularis* and *Rhododendron nivale* comprised the rest of the diet (Figure1). Diet composition of Tibetan red deer exhibited consistency in the most dominant food items: *Salix spp.*, *R. fragariflorum*, and *J. pingii* were the most frequently consumed plant species. The population exhibits strong selectivity for shrubs and forbs, which together constitute most of the diet composition.

Comparative analysis focusing on the relative frequency of various plant species present in feces and plots over two consecutive years reveals a notable trend: the species that are most prevalent within the plots do not necessarily align with those that dominate the diet composition (Figure 2a, b). Specifically, Poaceae, *Salix* spp., and *D. parvifolia* emerged as the most commonly occurring species within the plots both years. In the year 2021, *Salix* spp. and *R. fragariflorum* were identified in every fecal sample collected, indicating their significant dietary presence (Figure2 a). The following year, 2022, marked a shift with *C. versicolor* being the sole species to appear in all fecal samples, suggesting a change in dietary preference or availability (Figure 2 b). During the fieldwork conducted in 2021, there were no *R. fragariflorum*, *C.*

ulicinum, and *A. rivularis* collected within the plots, highlighting a potential discrepancy between the observed diet and the available flora within the plots. To address this gap and enhance the accuracy of species identification, plant samples were collected in 2022 as a supplementary effort. This additional collection aimed to provide a more comprehensive understanding of the species composition and its variations over the years, thereby enriching the dataset for more accurate comparisons and analyses.

Comparisons between food item proportions demonstrate that there are exceptional cases, or outliers, in the distribution of all food items within the collected samples for each year under study. These outliers represent proportions that are different from the majority of diet composition. *Salix* spp. is the most dominant food item across most of the samples, with the median proportion being around 40%. This indicates that, on average, nearly half of the food composition in all samples was made up of *Salix* spp. However, a small number of samples exhibited a remarkably low proportion of Salix spp. in 2022, with the outliers constituting about 1-2% of the total food composition. This is a substantial deviation from the norm and suggests that in these specific instances, *Salix* spp. were consumed much less than usual. Additionally, in the year 2022, *R. macrophylla* accounted for a relatively minor portion of the overall food composition, typically less than 4%, and there was a contribution from just four fecal samples, where the proportion was more than 20%. This indicates that in these samples, *R. macrophylla* was consumed in much higher quantities than what was generally observed, highlighting a possible preference or increased availability of this food item in certain situations. The analysis further revealed that, with the exception of *J. pingii* and *C. versicolor*, all other food items in the study exhibited outlier proportions. This suggests that the consumption of these two food items was relatively stable across the samples, without the extreme variations in proportion that characterized the other food items (Figure 3).

Diet patterns

Results of PCA followed by k-means cluster showed that the food composition of Tibetan red deer during the withered seasons in both 2021 and 2022 could be clustered into two clearly distinguishable patterns. In 2021, the clustering outcomes unveiled two groups comprising 16 and 23 samples, respectively (Figure 4 a). Diet composition of both clusters was dominated in order of prominence by *Salix*. spp., *R. fragariiflorum*, and *J. pingii*, but their proportions varied greatly. The first cluster consumed *Salix*. spp. up to 58.49%, which was much higher than the second cluster (33.67%). In 2022, there were also two clusters, consisting of 46 samples and 4 samples, respectively (Figure 5 a). Unlike all other clusters, in the 4 samples cluster, *R. macrophylla* had the highest ranking and occupied 34.83% of diet composition. In the second cluster from 2022, *Salix.* spp. (39.39%) was the most consumed food item while ranking of *J. pingii* and *R. fragariiflorum* ranked third and second within cluster, respectively, which was different from 2021.

Results of silhouette coefficients for validation demonstrated that the silhouette coefficients for the food composition in 2021 and 2022 were 0.35 and 0.64, respectively (Figures 4 b, 5 b). These silhouette coefficients indicated that a substantial portion of the samples were effectively grouped into two distinct clusters. However, two samples were not similar to either of the two clusters (Figure 4 b).

Co-occurrence network of food items

Co-occurrence network analysis showed only positive associations in 2021 and both positive and negative associations in 2022. In the network, nodes had no associations with the other means and their associations were random. In the heatmap, species were removed if all associations to the other species were random (Figure 6 b). The co-occurrence network and heatmap of food items in 2021 (Figure 6 a, b) showed that 10 food items had nonrandom associations with at least one of the other food items. In 2021, three shrub food items including (*D. parvifolia*, *B. temolaica*, *R. macrophylla*) had positive associations with forb species, including *L. rumicifolia*, *A.rivularis* and *B. costata*. Poaceae was positively connected with *C. littledalei*. Each of these species except Poaceae occupied less than 2% of total food composition, and no associations with any of the three most dominant food items were found in 2021 (Figure 6 a, b)

Compared to 2021, the co-occurrence network and heatmap of food items in 2022 (Figure 7 a, b) showed higher complexity. Thirteen food items had positive or negative associations with other food items and most associations were among shrub species. The only association between herbaceous plants in 2022 was a positive association between Poaceae and *L. rumicifolia*. Among all food items, *Salix*. spp and *R. fragariiflorum* were two of the three most dominant food items in cluster 1 (section 3.2). *R. macrophylla* had negative associations with three shrub species (*D. parvifolia*, *Salix*. spp, and *R. fragariiflorum*), suggesting that when Tibetan red deer fed on *R. fragariiflorum,* they did not simultaneously feed on those species. *Salix*. spp had positive associations with *R. fragariiflorum* (>10% of total diet composition) and *S. alpina*, which occurred in 84% and 62% of fecal samples, respectively (Figure 2 b).

The percentage of total pairings for each species showed that when considering all food item (or species) pairs, randomness occupied 95.83% (Figure 8 a) and 73.68% (Figure 8 b) in 2021 and 2022 respectively. Tibetan red deer did not show many consistent food item combination patterns in 2021, except for 6 food items (Figure 8 a). *Salix* spp. had a relative occurrence frequency of 100% in 2021 (Figure 2 a) meaning it co-occurred with all other food items in all fecal samples. However, combinations of food items were more obvious in 2022, especially for the most frequently consumed food items (Figure 8 b).

Discussion

Prolonged and harsh climate conditions during withered seasons on the Qinghai-Tibet Plateau potentially leads Tibetan red deer to suffer from limited food resources and decreased forage quality causing nutritional constraints. Diet composition during the withered seasons in both of our study years indicate that Tibetan red deer rely on *Salix spp*., *J. pingii*, and *R. fragariflorum* as staple food to meet their basic nutritional needs. We infer that Tibetan red deer satisfy most nutritional requirements by eating large quantity of the three top ranking food items. However, a boxplot for both years showed most food items exhibited several outliers in composition. For example, a high proportion of *R. macrophylla* and low proportion of *Salix* spp. This phenomenon indicated that that one or more individuals within the population exhibited distinct diet preferences and did not rely solely on staple food species to meet their nutritional requirements. This suggested that even at short temporal scales of 2-3 weeks, there are hidden diet composition patterns at the individual level and within the Tibetan red deer population.

Though, to some extent arbitrary, two distinct diet composition patterns have emerged during the withered seasons in both years. Therefore, we can infer that Tibetan red deer have diet composition preferences, but they vary within the population as well as in proportional amount. Samples within clusters have similar intake amounts of each dietary component and display preferences to certain food items. Two samples did not group with the two clusters in 2021, suggesting the existence of additional diet composition patterns. A high proportion of *R. macrophylla* in four samples in 2022 suggests that *R. macrophylla* was a staple food of some individual(s), but not others. Regardless of whether the four samples were from the same individual or not, we can infer that at times some individual(s) have relatively different diet compositions and probably different nutritional intake patterns from those who take *Salix* spp. and *R. fragariiflorum* as staple food. It is likely that not all individuals have the same nutritional requirements at any given time, and each may adjust their dietary intake accordingly.

Causes of the different diet patterns could be various, including food resources availability, intra- and interspecific competition, nutritional content (Agetsuma, et al. 2019; Hobbs, et al.1991; Murray, et al. 2016), habitat differences (Araujo, et al. 2011; Bolnick, et al. 2003; Proulx, et al. 2005), and herbivore-plant interactions (Perkovich and Ward. 2022). Vegetation types in the Sangri protected area are primarily composed of alpine shrub meadows where the staple foods of the Tibetan red deer are relatively abundant (Hu. 2002, Lv, et al. 2020). Grazing livestock (yak and horse) that primarily consume herbaceous plants from the Cyperaceae and Poaceae families (Lv. 2020) are presant on the Sangri Red Deer Nature Reserve. Although Tibetan red deer are typical mixed browsers, grazing livestock could compete with them for certain plants (Ye et al. 2023) or hinder access to certain feeding areas. In other areas, dietary overlap between deer and cattle (*Bos taurus*) may increase when forage availability decreases during the winter (Ortega et al. 1997; Thrill and Martin. 1989) and there is evidence that livestock can alter the feeding behavior of deer or displace deer from foraging areas (Chaikina and Ruckstuhl 2006, Stewart et al. 2002, Weiss et al. 2022). More investigation is needed to determine if free-ranging livestock impact the foraging behavior of Tibetan red deer.

Ungulates regulate selection and consumption patterns of plant species with limited availability and form diet combinations of available vegetation (Provenza et al. 2003, Ortíz-Domínguez et al. 2022). When characterizing diet combinations from co-occurrence networks, lack of associations does not indicate lack of co-occurrences. In 2021, though the network did not reveal associations for *Salix* spp., *Juniperus pingii*, and *Rhododendron fragariiflorum*, this did not indicate lack of co-occurrence with any other plant species. On the contrary, they co-occurred in almost all fecal samples which made them appear to have no co-occurrence associations and apparently random. Positively connected food items appeared to play a role of complementing unsatisfied nutritional requirements, as all positively connected food item accounted for less than 5% of diet composition each (Mark Hebblewhite. 2002). Co-occurrence network in 2022 exhibited higher complexity. It was interesting that *R. macrophylla* accounted for less than 5% of diet composition but was the most dominant food item in cluster 2 and had negative associations with another three shrub species (*D. parvifolia*, *Salix*. spp, and *R. fragariflorum*). Two of them (*Salix*. spp, and *R. fragariflorum*) are considered as staple food in the other 3 clusters in both 2021 and 2022. This suggests that such diet combinations might meet a different nutritional requirement of some deer or are caused by other ecological associations.

The large proportion of randomness observed in the co-occurrence network should not be ignored. Positively co-occurring combinations make up only a small fraction of all possible combinations. For any complex ecological system, randomness is crucial for stability (Barbier et al, 2021, Meena et al 2023). The presence of this randomness reflects the diversity and adaptability of the Tibetan red deer's diet in a variable resource environment. Such dietary diversity and adaptability could help Tibetan red deer populations survive projected habitat changes (i.e. Ye et al. 2023). Additionally, characterized diet combinations and patterns of ungulate browsing in small temporal scales can be influenced by community composition and structure.

Different proxies for an animal's diet can represent different temporal periods and scales, and sometimes yield different information (Davis and Pineda Munoz 2016). Field surveys for plants with evidence of browsing by Tibetan red deer that occurred at the same time and region as our study identified somewhat different dietary compositions (Wei et al. 2023). The surveys identified *Salix* spp., *Rosa macrophylla*, and *Dasiphora parvifolia* as comprising >50% of Tibetan red deer diet, whereas our fecal analysis identified *Salix* spp., *R. fragariiflorum*, and *J. pingii* as the primary components of diet. Furthermore, the browsing evidence surveys showed that diet varied with altitude and plant community, however other than *Salix* spp., plants identified as primary diet components were only minor components of the diet composition we identified from feces. It is possible that feeding on *R. fragariiflorum* and *J. pingii* occurred at other locations or evidence of feeding on these species was not as readily identifiable as on other species. Digestion times for ruminants, such as red deer (Picard et al. 2015), also means that deposited feces might not contain forage eaten at the location of deposition and could be composed of forage from multiple locations. This highlights why determining diet for large highly mobile browsing ungulates can be complex and benefit from multiple methods of investigation.

Our co-occurrence network analysis revealed dietary associations in the feces of Tibetan red deer, however, the possible reasons behind these associations within this complex ecological system require further investigation. More information is needed about the nutrient contents of dietary items, as well as how plant community structure is related to food availability and dietary choices of Tibetan red deer to better understand this complex network.

Conclusions

In withered seasons, Tibetan red deer predominantly feed on *Salix* spp., *J. pingii*, and *R. fragariflorum* in most circumstances. More attention should be given to diet compositions that have outliers, as they might imply different nutritional requirements or environment change in a small temporal scale. Diet composition patterns within the population exhibit variability in proportion and rankings of dominant food items across k-means clustering of food composition. Positive associations in co-occurrence networks suggest Tibetan red deer select some food combinations to complement unsatisfied nutritional requirements in addition to consuming staple foods. Negative associations represent different diet composition patterns during the withered season, but the reasons for such associations are unknown and need further investigation. Additionally, high randomness between many dietary item co-occurrences across individual and population levels should not be ignored as it might represent adaptation to a complex and changing environment.

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References

Agetsuma N.; Agetsuma-Yanagihara, Y.; Takafumi, H.; Nakaji, T. 2019. *Plant constituents affecting food selection by sika deer* **Journal of Wildlife Management** *83* (3), 669-678. DOI: 10.1002/jwmg.21615.

Araujo, M. S.; Bolnick, D. I.; Layman, C. A. 2011. *The ecological causes of individual specialisation* **Ecology Letters** *14* (9), 948-958. DOI: 10.1111/j.1461-0248.2011.01662.x.

Barbier, M, Mazancourt, Claire, Loreau, M, et al.2021. Fingerprints of high-dimensional coexistence in complex ecosystems. Physical Review X 11(1),011009. DOI:10.1103/PhysRevX.11.011009.

Begon, Michael ; Townsend, R Colin and Harper, L John. 2006. *Ecology from individual to ecosystem* 4th ed. Blackwell Publishing UK

Bolnick, D. I .; Svanback, R.; Fordyce, J. A.; Yang, L. H.; Davis, J. M.; Hulsey, C. D.; Forister, M. L. 2003. *The ecology of individuals: Incidence and implications of individual specialization* **Amercian Naturalist**161 (1), 1-28. DOI: Doi 10.1086/343878.

Chaikina N A, Ruckstuhl KE. 2006. *The effect of cattle grazing on native ungulates: the good, the bad, and the ugly*. Rangelands 28(3):8-14 DOI: 10.2111/1551-501X(2006)28[8:TEOCGO]2.0.CO;2

Davis M., Pineda M S. 2016. The temporal scale of diet and dietary proxies. **Ecology and Evolution**, 6(6), 1883-1897. DOI: 10.1002/ece3.2054

Gebert C.,Verheyden T H. 2001. *Variations of diet composition of red deer (Cervus elaphus L.) in Europe*. Mammal Review 31(3-4):189-201. DOI:10.1111/j.1365-2907.2001.00090.x

Mueller D D , Ellenberg H .1974. Aims and Methods of Vegetation Ecology: John Wiley & Sons.

Hobbs, N. T.; Schimel, D. S.; Owensby, C. E.; Ojima, D. S. 1991. *Fire and Grazing in the Tallgrass Prairie - Contingent Effects on Nitrogen Budgets* **Ecology** *72* (4), 1374-1382. DOI: Doi 10.2307/1941109.

Hu, F. B. (2002) *Dietary pattern analysis: a new direction in nutritional epidemiology* **Current Opinion in Lipidology** 13(1)), 3-9. DOI:10.1097/00041433-200202000-00002

Johnson, M. K. *Frequency Sampling for Microscopic Analysis of Botanical Compositions*. Journal of Range Management: 1982; 35(4):541-542. DOI: 10.2307/3898628

Johnson, N. L.; Kotz, S.; Balakrishnan, N. 1997. *Discrete multivariate distributions*, Wiley.

Kay, R. N. B., Staines, B. W. 1981. *The nutrition of the red deer (Cervus elaphus)*. Nutrition Abstractsand Reviews B 51:601-622.

Ludt C. J., Schroeder W., Rottmann, O., Kuehn R. 2004. *Mitochondrial DNA phylogeography of red deer (Cervus elaphus)*. Molecular phylogenetics and evolution, 31(3):1064-1083. DOI:10.1016/j.ympev.2003.10.003

Lv, Z.H.2020. Foraging Strategy, Habitat Selection and Assessment of Tibetan Red deer (*Cervus wallichii*) in Grass Period under the Influence of Interspecific Competition. D.Phil. Thesis, Northeast Forestry University.

Lv, Z.H.; Zhang, W. Q.; Liu, H.; Zhang, M. H. et al. 2020. *Comparison on feeding habits of Cervus wallichii and sympatric ungulates and domestic animals in green grass period* **Chinese Journal of Applied Ecology** *31* (2), 651-658. DOI: 10.13287/j.1001-9332.202002.002.

MacQueen, J. B. 1967. *Some Methods for classification and Analysis of Multivariate Observations* **In Proceedings of the Fifth Berkeley Symposium on Mathematical Statistics and Probability** (1), 281-297.

Mark Hebblewhite, Daniel HP. 2002. *Effects of elk group size on predation by wolves* **Canadian Journal of Zoology** 80(5), 800-809. DOI:10.1139/z02-059

Meena C, Hens C, Acharyya S et al.2023. Emergent stability in complex network dynamics. Nature Physics, 19(7): 1033-1042. DOI: 10.1038/s41567-023-02020-8.

Murray, B. D.; Webster, C. R.; Jenkins, M. A.; Saunders, M. R.; Haulton, G. S. 2016.*Ungulate impacts on herbaceous-layer plant communities in even-aged and uneven-aged managed forests* **Ecosphere** 7 (6). DOI:10.1002/ecs2.1378.

Ortíz-Domínguez GA., Marin-Tun CG et al.. 2022. Selection of Forage Resources by Juvenile Goats in a Cafeteria Trial: Effect of Browsing Experience, Nutrient and Secondary Compound Content. Animals 12(10):1317. DOI:10.3390/ani12101317

Perkovich C., Ward D. (2022) *Differentiated plant defense strategies: Herbivore community dynamics affect plant-herbivore interactions* **Ecosphere** *13* (2):e3935. DOI: 10.1002/ecs2.3935.

Picard M., Papaïx J., Gosselin F., Picot, D., Bideau E., Baltzinger C. 2015. *Temporal dynamics of seed excretion by wild ungulates: implications for plant dispersal*. Ecology and Evolution, 5(13):2621-2632. DOI:10.1002/ece3.1512

Provenza, F. D., J. J. Villalba, L. E. Dziba, S. B. Atwood, R. E. Banner. 2003. Linking herbivore experience, varied diets, and plant biochemical diversity. Small Ruminant Research 49(3):257-274.DOI: 10.1016/S0921-4488(03)00143-3

Proulx, S. R.; Promislow, D. E. L.; Phillips, P. C. (2005) *Network thinking in ecology and evolution* **Trends in Ecology& Evolution** 20 (6):345-353. DOI:10.1016/j.tree.2005.04.004.

Schaller G., Liu W., Wang X. 1996 *Status of Tibet red deer* **Oryx** *30* (4), 269-274. DOI: 10.1017/S0030605300021761.

Shen, G.S.2009. *Food composition, feeding and nutritional adaption strategies of Tibetan red deer (*Cervus elaphus wallichi*) during the green grass period in Tibet*. Master,Thesis, Northeast Forestry University.

Stewart, K. M., Bowyer, R. T., Kie, J. G., Cimon, N. J., Johnson, B. K. 2002. *Temporospatial distributions of elk, mule deer, and cattle: resource partitioning and competitive displacement*. Journal of Mammalogy 83(1):229-244.DOI: 10.1644/1545-1542(2002)083<0229:TDOEMD>2.0.CO;2

Veech, J. A. (2013) *A probabilistic model for analysing species co-occurrence* **Global Ecology Biogeography** 22 (2), 252-260. DOI: 10.1111/j.1466-8238.2012.00789.x.

Wei, K. 2023. *Comparison of Nutritional Strategies of Tibetan Red Deer During Withered Grass Period and Green Grass Period*. Master thesis, Northeast Forestry University.

We K.; Liang X.; Yu J.; Wang L.; Zhou L.; Lyu Z.; Zhang M.; Zhang W. 2023.*Effects of plant community and altitude on food composition of Cervus elaphus wallichii during the withered grass period on the Tibetan Plateau,China* **Chinese Journal of Applied Ecology** 34 (4), 1117-1122,DOI:10.13287/j.1001-9332.202304.028.

Wei Z.; Yang T. J.; Friman V P.; Xu Y C.; Shen Q R.; Jousset A. 2015. *Trophic network architecture of root-associated bacterial communities determines pathogen invasion and plant health* **Nature Communications** 6. DOI: 10.1038/ncomms9413.

Weiss F., Michler F. U., Gillich B., Tillmann J., Ciuti S., Heurich M., Rieger S. 2022. *Displacement effects of conservation grazing on red deer (Cervus elaphus) spatial behaviour*. Environmental Management 70(26):763-779. DOI: 10.1007/s00267-022-01697-6

Ye X., Guo K., Li. X, Wu. Q., Zhang M., Li. Ming, Zhao X. 2023 *Climate change results in* imbalance population growth and change in suitable habitat for red deer in the Qinghai-Tibet Plateau: a case study in the Leiwuqi National Nature Reserve. Acta Theriologica Sinica 43:149-156. DOI:10.16829/j.slxb.150706

Figure 1

Figure 1 Diet composition of Tibetan Red Deer (*Cervus wallichii*) during the withered season at the Sangri Red Deer Nature Reserve 2021 (Blue) and 2022 (Red)

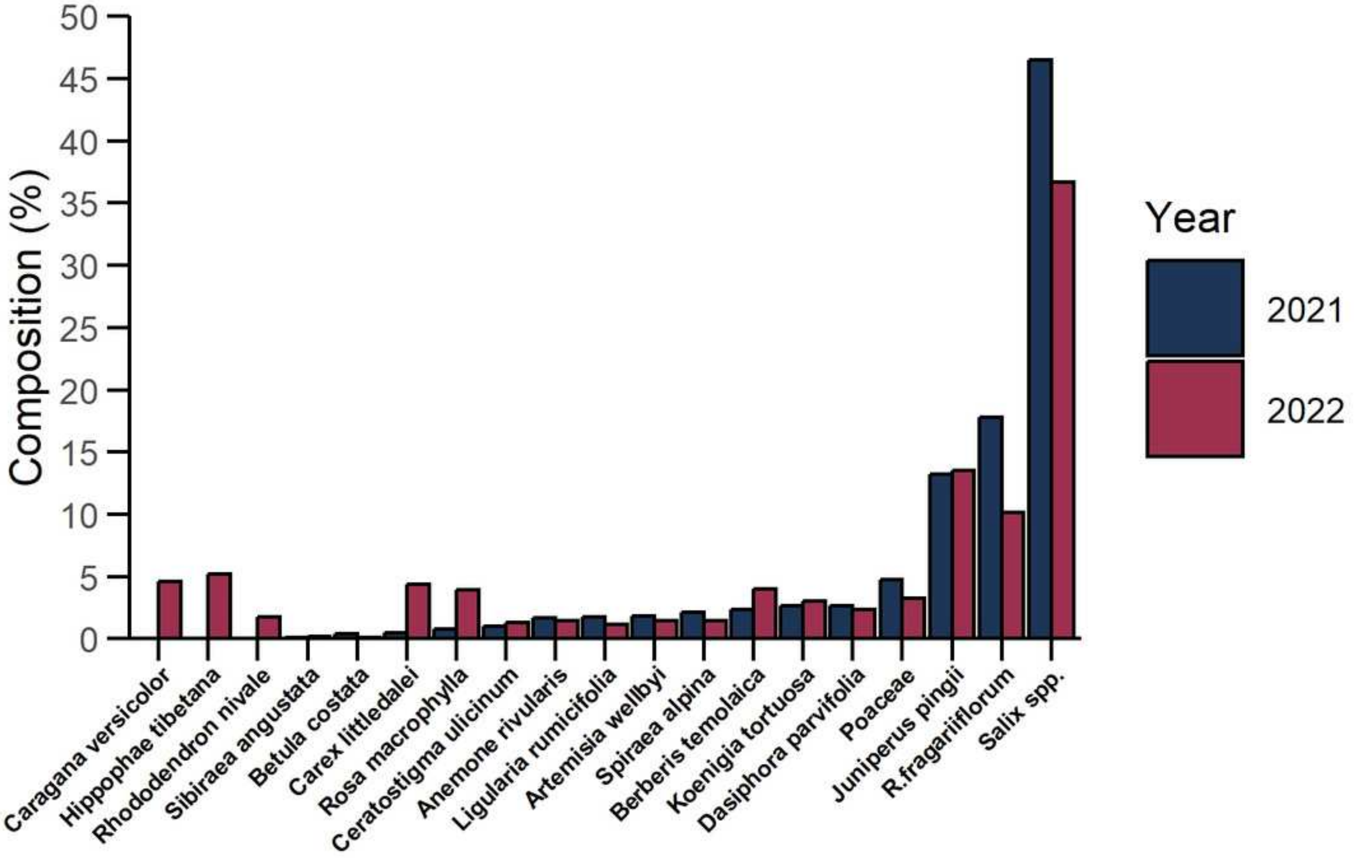


Figure 2

Figure2 Comparison of relative frequency of plant species between Tibetan Red Deer (*Cervus wallichii*) feces and vegetation plots during the withered season in 2021 (a) and 2022 (b).

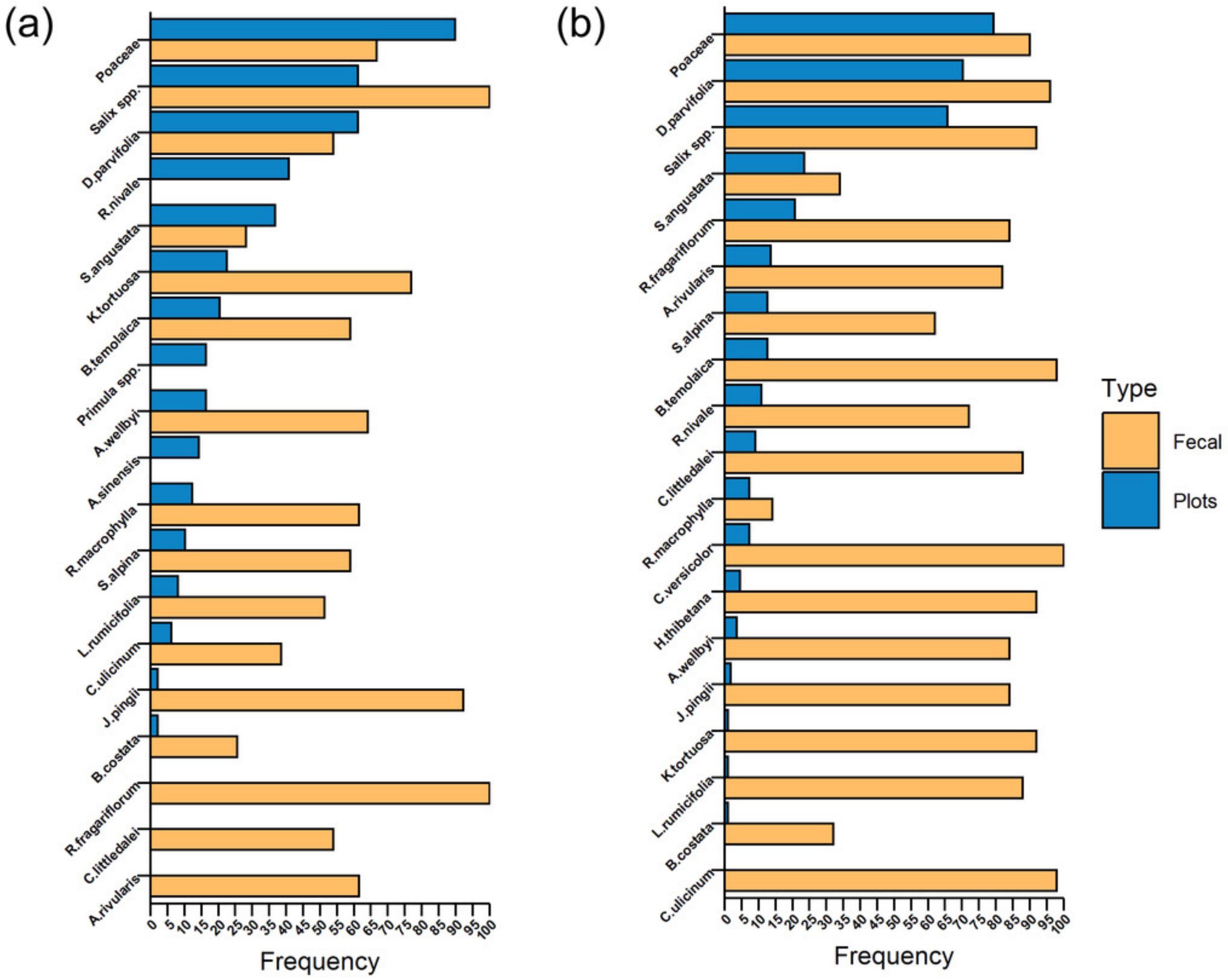


Figure 3

Figure3 Boxplot of Tibetan red deer diet composition during the withered season in March 2021 (blue) and 2022 (red).

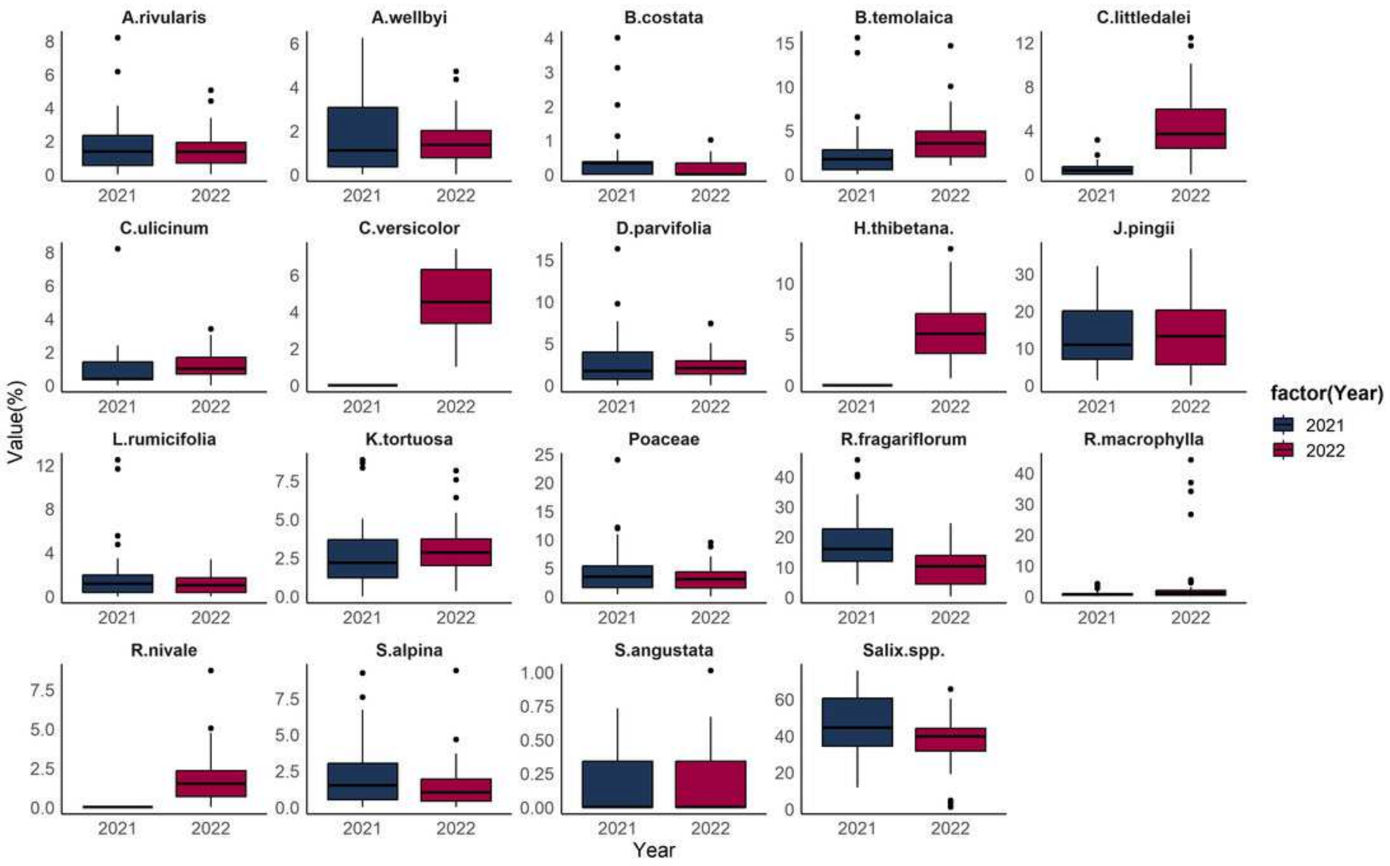
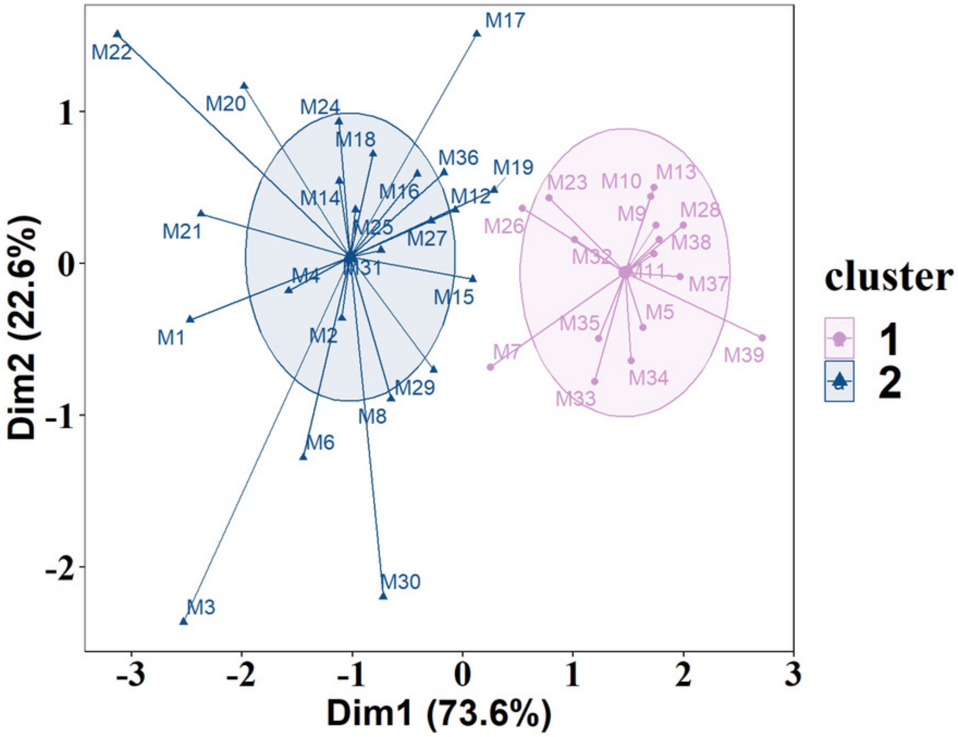


Figure 4

Figure 4. (a) K-means clustering plot of Tibetan red deer (*Cervus wallichii*) food composition in fecal pellets in March 2021. (b) Silhouette validations for k-means cluster plots in 2021.

The samples were clustered into two groups (blue triangles and purple circles).

(a)


M17
M22
M20
M24
M18
M36
M19
M14
M16
M12
M25
M27
M21
M4
M31
M15
M1
M2
M29
M8
M6
M30
M3
M23
M10
M13
M9
M28
M26
M38
M32
M11
M37
M35
M5
M7
M39
M34
M33
1
0
-1
-2
-3
-2
-1
0
1
2
3
Dim1 (73.6%)
Dim2 (22.6%)
cluster
1
2

(b)

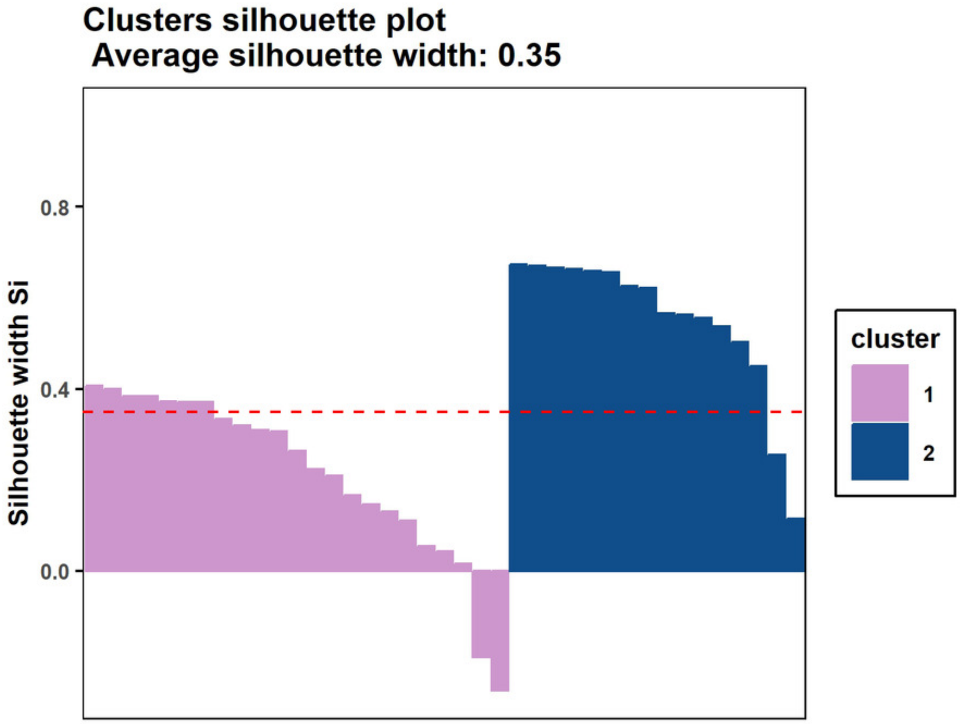

Clusters silhouette plot
Average silhouette width: 0.35
0.8
0.4
0.0
Silhouette width Si
cluster
1
2

Figure 5

(a) K-means clustering plot of Tibetan red deer (*Cervus wallichii*) food composition in fecal pellets in March 2022. (b) Silhouette validations for k-means cluster in 2022.

The samples were clustered into two groups (blue triangles and purple circles).

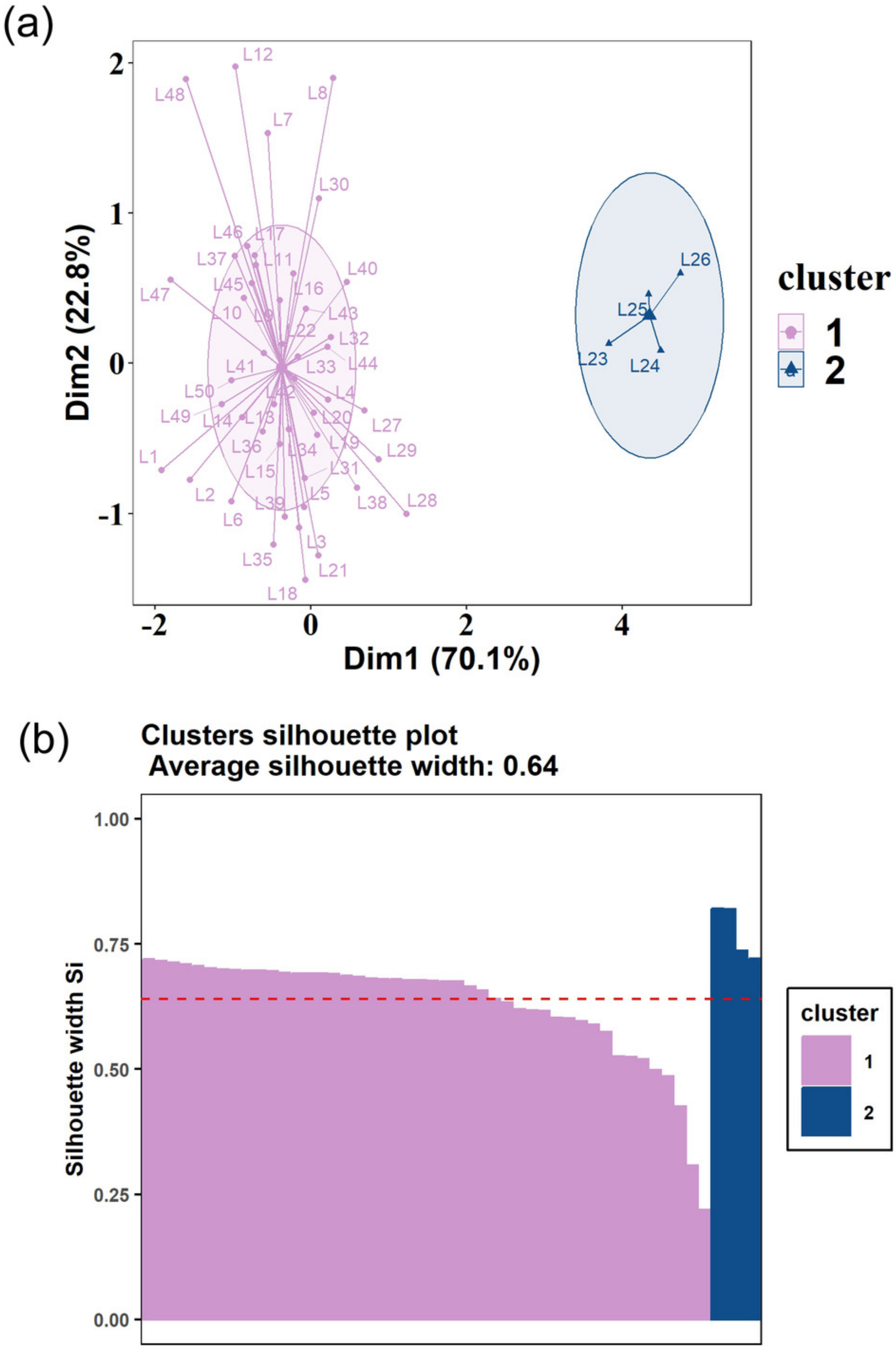

(a)
Dim2 (22.8%)
Dim1 (70.1%)
-2
0
2
4
2
1
0
-1
L12
L48
L8
L7
L30
L46
L17
L37
L11
L40
L47
L45
L16
L10
L9
L43
L22
L32
L44
L41
L33
L50
L42
L4
L49
L14
L13
L20
L27
L36
L34
L19
L29
L1
L15
L31
L2
L39
L5
L38
L28
L6
L3
L35
L21
L18
L26
L25
L23
L24
cluster
1
2
(b)
Clusters silhouette plot
Average silhouette width: 0.64
Silhouette width Si
1.00
0.75
0.50
0.25
0.00
cluster
1
2

Figure 6

Figure 6. (a) Co-occurrence network of food items in Tibetan red deer (*Cervus wallichii*) fecal in March 2021. (b) Heat map showing the positive species association.

（a）Edges between nodes represent significant co-occurrence relationships among plant species in 2021, solid lines represent positive associations.(b) Species names are positioned to indicate the columns and rows that represent their pairwise relationships with other species in 2021.

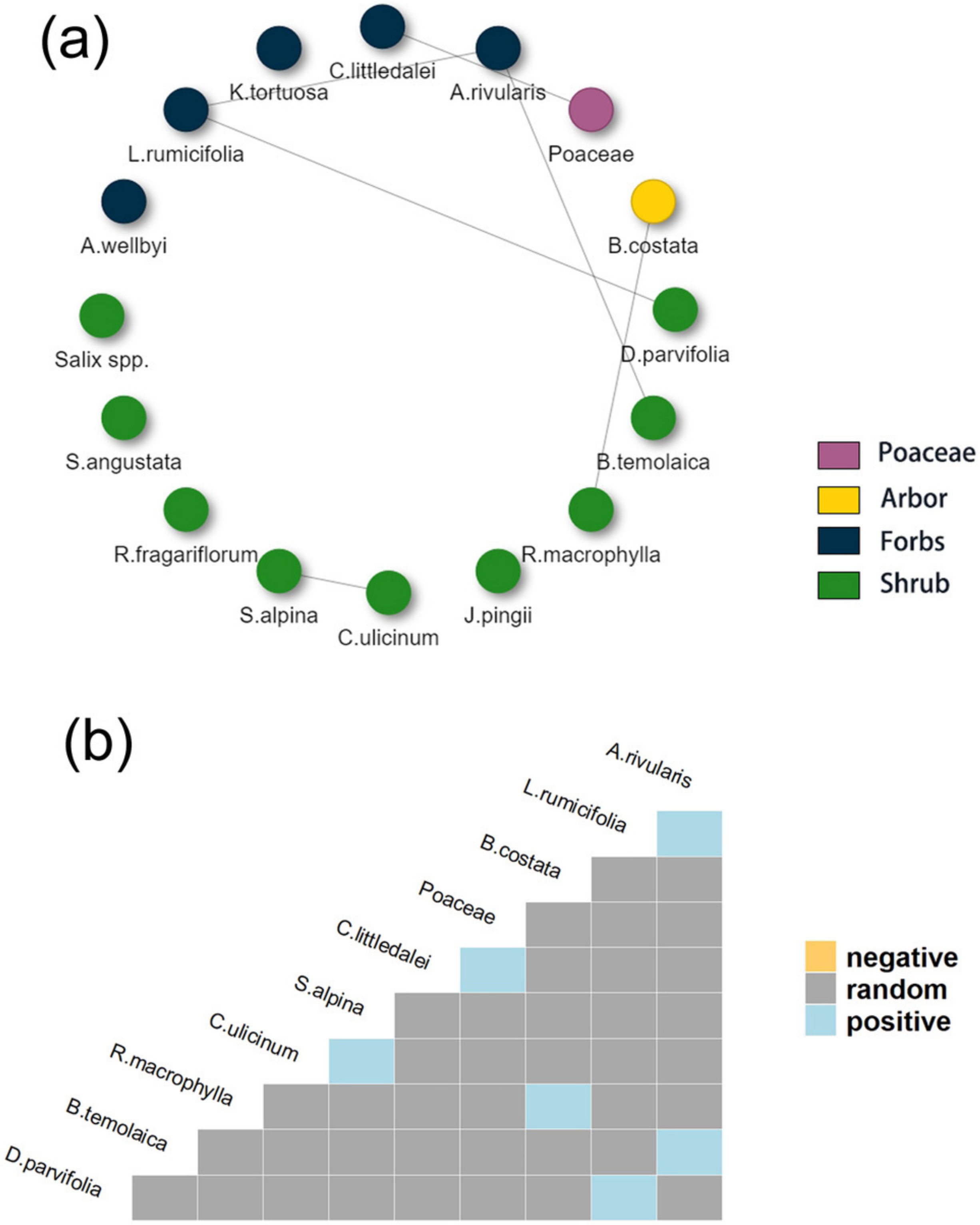

(a)
C.littledalei
K.tortuosa
A.rivularis
L.rumicifolia
Poaceae
A.wellbyi
B.costata
Salix spp.
D.parvifolia
S.angustata
B.temolaica
R.fragariflorum
R.macrophylla
S.alpina
C.ulicinum
J.pingii
Poaceae
Arbor
Forbs
Shrub
(b)
A.rivularis
L.rumicifolia
B.costata
Poaceae
C.littledalei
S.alpina
C.ulicinum
R.macrophylla
B.temolaica
D.parvifolia
negative
random
positive

Figure 7

(a) Co-occurrence network of food items in Tibetan red deer (*Cervus wallichii*) fecal pellet in March 2022. (b) Heat map showing the positive and negative species associations

(a)Edges between nodes represent significant co-occurrence relationships among plant species in 2022; (b)solid lines represent positive associations and dashed line represent negative associations., species names are positioned to indicate the columns and rows that represent their pairwise relationships with other species in 2022.

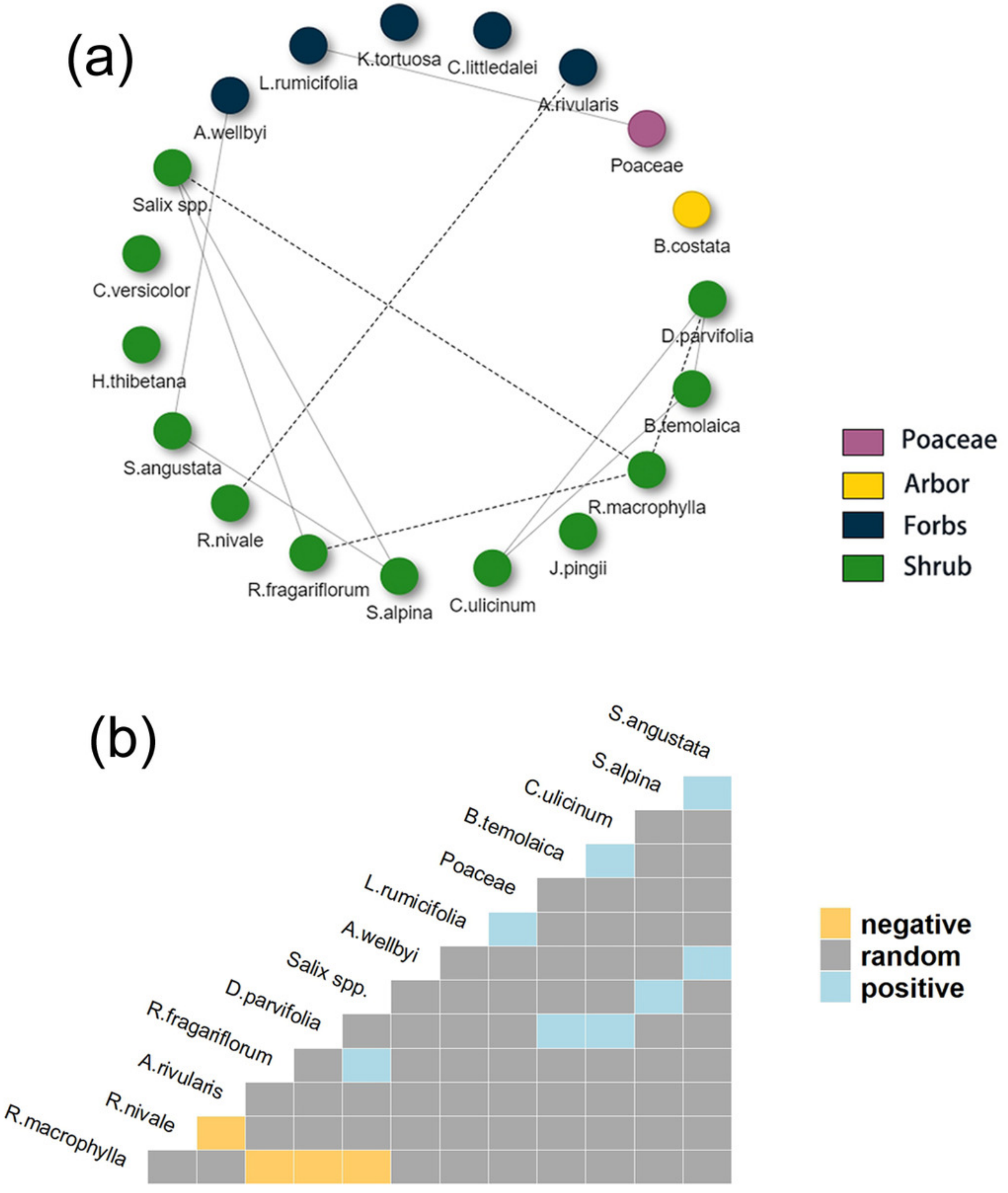
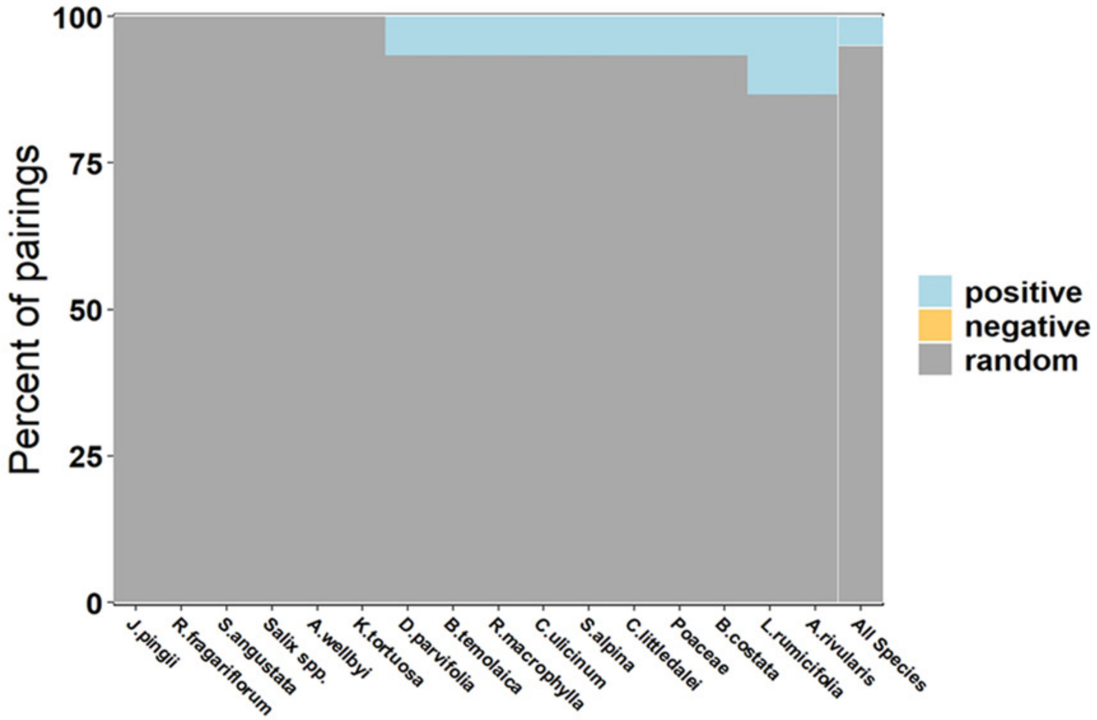

(a)
K.tortuosa
C.littledalei
L.rumicifolia
A.wellbyi
A.rivularis
Poaceae
B.costata
Salix spp.
C.versicolor
H.thibetana
D.parvifolia
B.temolaica
S.angustata
R.macrophylla
R.nivale
J.pingii
R.fragariflorum
S.alpina
C.ulicinum
Poaceae
Arbor
Forbs
Shrub
(b)
S.angustata
S.alpina
C.ulicinum
B.temolaica
Poaceae
L.rumicifolia
A.wellbyi
Salix spp.
D.parvifolia
R.fragariflorum
A.rivularis
R.nivale
R.macrophylla
negative
random
positive

Figure 8

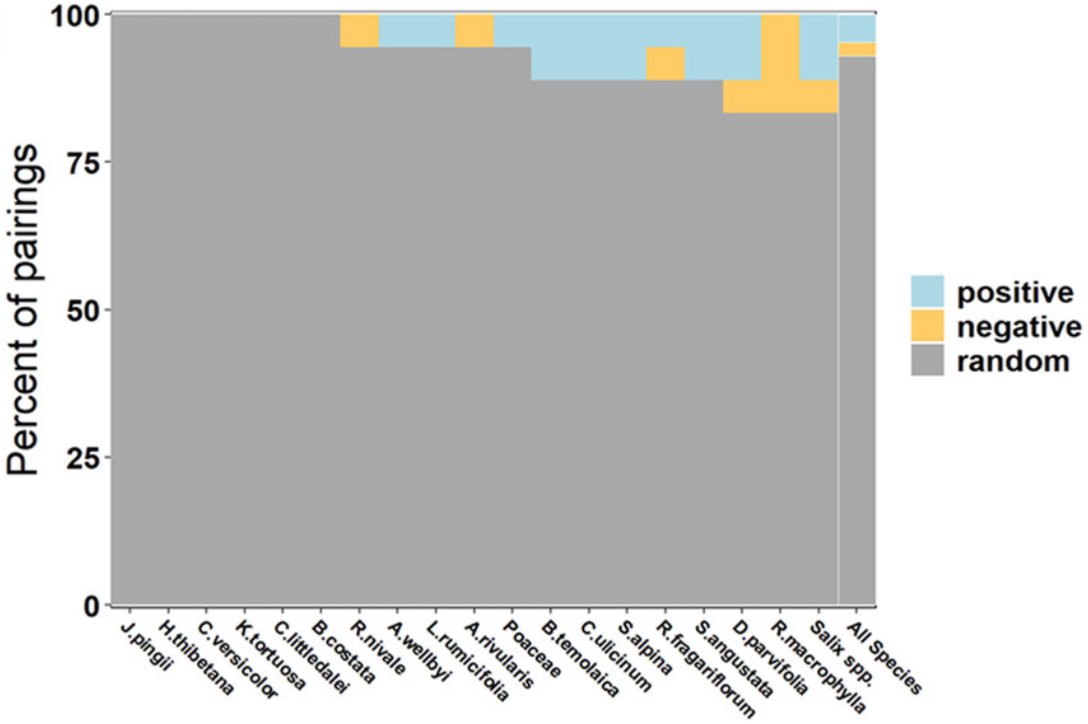
Boxplot showing the percent of total pairings for each species found in Tibetan red deer fecal pellets that are positive, random, or negative in March 2021 (a) and 2022 (b).

Species are ordered by increasing number of total associations. The right-most bar, outlined in white, represents the assemblage-wide percentages.

(a)


100
75
50
25
0
Percent of pairings
J.pingii
R.fragariflorum
S.angustata
Salix spp.
A.wellbyi
K.tortuosa
D.parvifolia
B.temolaica
R.macrophylla
C.ulicinum
S.alpina
C.littledalei
Poaceae
B.costata
L.rumicifolia
A.rivularis
All Species
positive
negative
random

(b)


100
75
50
25
0
Percent of pairings
J.pingii
H.thibetana
C.versicolor
K.tortuosa
C.littledalei
B.costata
R.nivale
A.wellbyi
L.rumicifolia
A.rivularis
Poaceae
B.temolaica
C.ulicinum
S.alpina
R.fragariflorum
S.angustata
D.parvifolia
R.macrophylla
Salix spp.
All Species
positive
negative
random