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Palatability and Livestock Preferences of Restored Plants in Steppe Restoration Areas, Hulunbuir, Inner Mongolia, China

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ABSTRACT

Hulunbuir steppe, one of the four largest steppes in China, has experienced rapidly progressing desertification partly due to overgrazing by livestock. The objective of this study was to investigate the effectiveness of various vegetation recovery methods, including the selection of unpalatable plants less affected by grazing livestock. To determine livestock grazing preferences at Hulunbuir restoration sites, we used DNA barcoding methods to analyze fecal materials of horses and cattle grazing on four restored plants: two trees (*Pinus sylvestris* L. var. *mongolica* and *Populus canadensis*) and two shrubs (*Caragana microphylla* and *Corethrodendron fruticosum*). Neither of the two tree species were detected in livestock feces, whereas both shrub species were detected at lo w frequencies. There were no significant differences in compositions of species consumed by horses and cattle except that Asteraceae species were more often consumed by cattle. Our results showed that the four plants used for restoration may be classified as unpalatable or less palatable species in the Hulunbuir restoration area. Our results may help inform restoration strategies implemented in restoration areas, especially regarding negative effects of livestock grazing during the initial stage of restoration in Hulunbuir.

Keywords: Desertification, Dietary analysis, DNA barcoding, Fecal material, Hulunbuir

Introduction

Desertification has become a global issue, causing serious environmental and socioeconomic problems worldwide. Earth's surface consists of 41% drylands, and approximately 25% of drylands are under threat of desertification (D'Odorico *et al*., 2013; Reynolds *et al*., 2007). China is one of countries most seriously affected by desertification due to rapid industrialization in the past decades (Wang *et al*., 2013; Zhang & Huisingh, 2018). Most desert-affected lands are located in the northern part of China, with the Inner Mongolia region being one of areas most seriously damaged by desertification (Lyu *et al*., 2020; Wang *et al*., 2013; Wang *et al*., 2020). The Chinese government has taken various measures to address and combat desertification (Zhang & Huisingh, 2018).

Hulunbuir is a prefecture-level city in northeastern Inner Mongolia, China. The Hulunbuir steppe, a wellknown steppe, is one of the four largest grasslands. It is famous as an important base of livestock husbandry in China (Nie *et al*., 2005; Zhang & Huisingh, 2018). Hulunbuir is also one of the four most famous sandy lands in China (Zhang

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& Huisingh, 2018). The geographical footprint of sandy land in Hulunbuir is increasing rapidly due to fast population growth, over-cultivation, over-grazing of livestock, and so on (Dong & Ya, 2002; Nie *et al*., 2005). In contrast, steppe coverage in Hulunbuir decreased by up to 35% in the 2000s (Park *et al*., 2013).

Many projects have been launched to mitigate land degradation, such as the National Action Programme (NAP), the Three-North Shelterbelt Programme (TNSP), the Grain to Green Programme (GGP), and so on (Wang *et al*., 2020; Zhao *et al*., 2010). Under these policies, various studies have been conducted to increase effects of vegetation restoration (Lyu *et al*., 2020). Adaptation capabilities of restored plants are important factors for the success of vegetation restoration. Tree and shrub species used for restoration have been selected as suitable species for environmental conditions of restoration regions as they are highly tolerant to drought, cold, and wind erosion (Wang *et al*., 2020; Zhao *et al*., 2007). After restoration, land enclosure is conducted using fences to prevent human disturbance and grazing of livestock, thus helping the natural reestablishment of vegetation (Gao *et al*., 2002; Park *et al*., 2013). Nevertheless, enclosures do not completely prevent the grazing of livestock in restoration areas for a sufficient period to allow for rehabilitation (Verdoodt *et al*., 2010). Furthermore, in consideration of local herders' economic interests, local governments often allow livestock grazing at certain time. Livestock grazing can produce changes in grassland attributes, including net primary production, plant species composition, and community structure (Anderson & Briske, 1995). Several studies conducted in Inner Mongolia have reported preferences of grazing livestock and their effects on plant community changes (Wang *et al*., 2003; Wang *et al*., 2014).

The objective of this study was to examine the effect of livestock grazing on restored plants at vegetation restoration sites in Hulunbuir, Inner Mongolia, China. This study sought to answer the following questions: (1) whether livestock would feed on restored plants during the growing season, (2) whether there were differences in feeding preferences according to livestock species, and (3) whether there were differences in grazing frequency according to the extent of restoration.

Materials and Methods

Study site and taxonomic survey of plants

This study was conducted from July 2014 to August 2014 in a restoration area of a grassland in Hulunbuir steppe, Inner Mongolia Autonomous Region, China (48°2312″ N, 118°1000″ E; Fig. 1, 2). The grassland is located at the boundary of semi-humid and semi-dry climatic regions. The average annual temperature in Hulunbuir steppe is between -2.5°C and 0°C. The area experiences severe temperature changes of -49°C in winter and 40°C in summer. The average annual rainfall is between 270 mm and 370 mm, with more th an 70% of the rainfall occurring in July and August. Restoration areas were separated according to the year of restoration. Four restoration sites (restored in 2005, 2008, 2011, and 2012) were selected for monitoring and sampling. All restored sites were surrounded by wire-entangled fences of 1.5 m high around these sites to protect planted vegetation from grazing of livestock. Part of the enclosure could only undergo grazing once or twice a year by local herders (Park *et al*., 2013). The non-restoration area (NR), where sand dunes were exposed, was selected as a reference site.

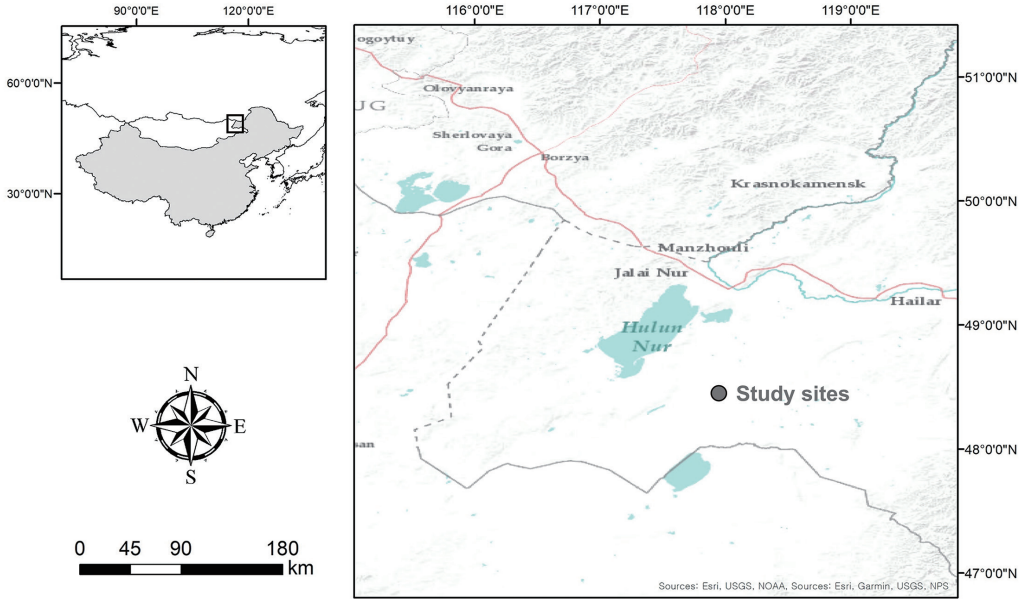

Fig. 1. Map showing the location of the study restoration sites in Hulunbuir, Inner Mongolia, China.

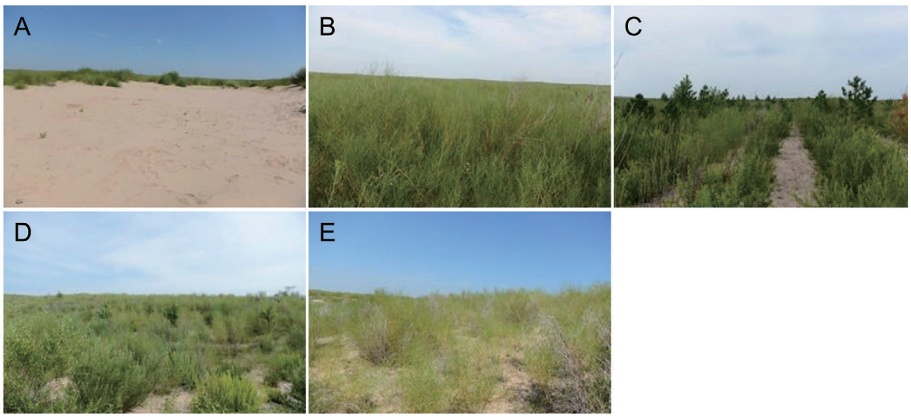

Fig. 2. Photographs of study sites in Hulunbuir. A. Non-restoration site; B. Site restored in 2005; C. Site restored in 2008; D. Site restored in 2011; E. Site restored in 2012.

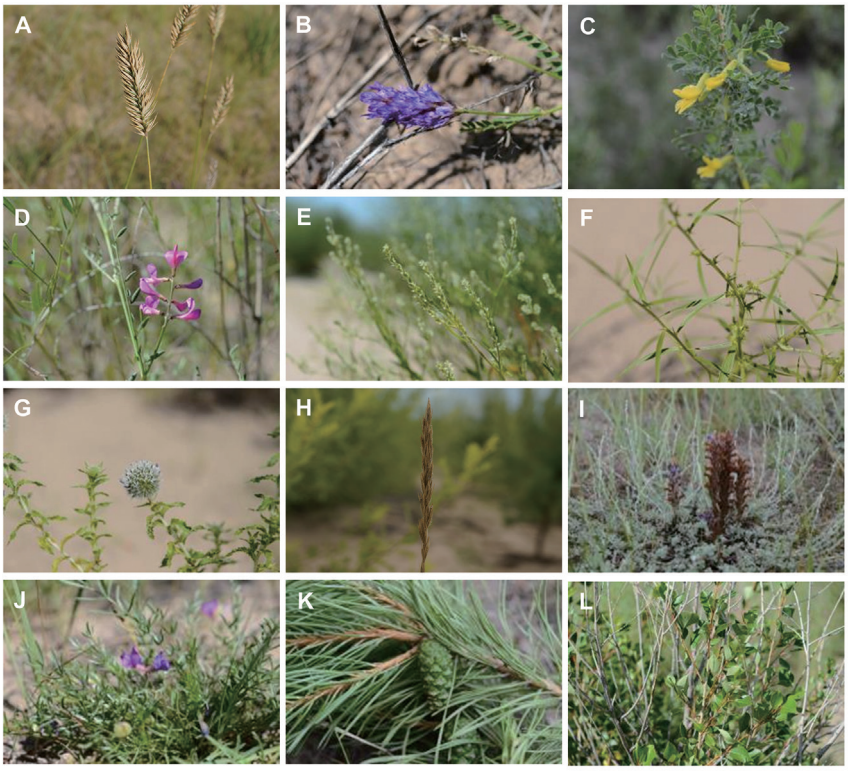

Fig. 3. Photographs of restored and native plants at study sites: A. *Agropyron cristatum* (L.) Gaertn.; B. *Astragalus adsurgens* Pall; C. *Caragana microphylla* Lam.; D. *Corethrodendron fruticosum* (Pallas) B.H. Choi & H. Ohashi; E. *Corispermum hyssopifolium* L.; F. *Corispermum squarrosum* (L.) Moq.; G. *Echinops gmeliniTurcz*.; H. Leymus secalinus (Georgi) Tzvelev; I. O*robanche coerulescens* Stephan ex Willd.; J. *Oxytropis racemosa* Turcz.; K. *Pinus sylvestris* var. *mongolica* Litv.; L. *Populus canadensis Moench*.

Four different plants: two shrub species (*Caragana microphylla* Lam. and *Corethrodendron fruticosum* (Pallas) B.H. Choi & H. Ohashi) and two tree species (*Pinus sylvestris* L. var. *mongolica* Litv. and *Populus canadensis* Moench) were used for restoration by planting (restored in 2008), seeding (restored in 2005, 2012), and mixing (restored in 2011) (Fig. 3).

A taxonomic field survey was conducted for the restoration area and a non-restoration area. The identification of plant species was based on the flora of China (Wu *et al*., 2010), the flora of grassland in China (Lu *et al*., 2012), and the flora of Korea (Lee, 2003).

Sample preparation and DNA extraction

We counted the number of livestock feces detected in each restoration site and NR based on line-transect surveys. Some fecal samples were collected to analyze feeding preferences of livestock. A total of 800 m transects (200 m each, 4 replicates) were randomly arranged in each restoration area. All feces of livestock were classified as either cow or horse feces according to their morphological characteristics. While collecting feces, the outer portion of feces was eliminated. Only the inner part of feces was collected and transferred individually into polyethylene bags to avoid sample contamination. All collected fecal samples

were delivered to the laboratory and stored at -80°C until DNA extraction.

Genomic DNA was extracted from feces using the QIAamp® DNA Stool Mini Kit (Qiagen, USA) following the manufacturer's protocols excluding the lysis step. To ensure sufficient homogenization during the lysis step, we added two 5-mm stainless steel beads to samples and mixed them by shaking on a mixer mill (Retsch, Germany) at 26 Hz for 1 min (Joo *et al*., 2014). Extracted DNA was eluted in 200 μL of ATE buffer and stored at -20°C until further analyses.

Polymerase chain reaction (PCR) amplification, cloning, and sequencing

We selected the internal transcribed spacer 2 (ITS2) gene region for the identification of plant species in fecal samples. Target genes were amplified using primer set ITS_S2F and S3R (Chen *et al*., 2010). PCR was conducted in a 25-μl reaction volume, containing 1 μl of extracted DNA, 1× Ex *Taq* Buffer, 1.5 mM of MgCl2, 0.2 mM of dNTPs, 0.2 μM of each primer, and 1 U of Ex *Taq* DNA polymerase (Takara, Japan). PCR was performed in a thermal cycler (Applied Biosystems 2720, USA) using the following conditions: a n initial denaturation at 94°C for 5 min, 45 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 45 s, and a final extension step at 72°C for 10 min. PCR products were purified using an AccuPrep® PCR Purification Kit (Bioneer, Korea). Purified PCR products were ligated into the pGEM®-T Easy Vector (Promega, USA) according to the manufacturer's protocols and transformed into DH5α chemically competent cells. Cells were plated in Luria–Bertani agar + ampicillin medium with X-gal solution (2% w/v) for antibiotic selection and blue-white screening.After the cloning step, white colonies were selected and amplified using M13F and M13R primers for colony PCR. PCR conditions were as follows: an initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 1 min, and a final extension step at 72°C for 7 min. Sequencing was conducted using a commercial sequencing service company (Genotech, Korea). Each DNA sequence was identified using BLASTN searches of the GenBank database. Sequence alignments and length calculations were conducted using the MEGA 5 program (Tamura *et al*., 2011).

Diversity of plant species detected in feces

The diversity of plant species detected in feces was determined using both the Shannon-Wiener diversity index (H′) and Simpson's index (D) as follows:

$$H' = -\sum p_i \log p_i \quad (1)$$

where p_i was the frequency of occurrence, which was the percentage of samples in which a food species was found, and

$$D = \sum (n_i / N)^2 \quad (2)$$

where n was the total number of each species, and N was the total number of all species (Shannon & Weaver, 1949; Simpson, 1949). Simpson's index was transformed to *1–D*, with values ranging from 0 to 1. A diversity index was derived for the diet detected from feces using molecular approaches.

Statistical analysis

Analysis of variance (ANOVA) was used to test statistically significant differences in the density of livestock feces detected at each study site, and post hoc tests were performed using Tukey's method ($P < 0.05$). Fisher 's exact test was used to assess differences in feeding frequency between livestock species. All statistical analyses were performed with S-Plus 8 for Windows (Insightful Corp., USA) and R program (R Core Team, 2013).

Results

Flora of study sites

A total of 41 plants were identified at the study sites. Aside from restored plants, main native plants growing in the restoration sites were *Corispermum squarrosum* (L.) Moq., *Corispermum hyssopifolium* L., *Astragalus adsurgens* Pall., *Artemisia desertorum Spreng., Orobanche coerulescens* Stephan ex Willd., *Oxytropis racemosa* Turcz., *Agropyron cristatum* (L.) Gaertn., *Echinops gmelini* Turcz., and so on (Fig. 3). The largest number of plant species was observed at the 2008 restoration site. The number of plant species tended to increase in areas restored long time ago (7 species in NR, 10 species in 2012, 11 species in 2011, and 19 species in 2005; Table 1).

Species diversity detected in feces and the mean fecal density of livestock

A total of 102 livestock feces samples were collected from the four different restoration areas and nonrestoration sites (Table 2). The species diversity index of plants detected in livestock feces differed according to the study site (*H'*: 1.88–2.18, *1-D*: 0.79–0.90, Table 2). The restoration site with the highest species diversity was the 2008 site (*H′*: 2.48, *1-D*: 0.90), while the site with the lowest species diversity was the 2005 site (*H′*: 1.88, *1-D*: 0.79).

Table 1. List of plant species identified in Hulunbuir study area

No.	Species	2005	2008	2011	2012	NR
1	*Agropyron cristatum* (L.) Gaertn.	+				
2	*Allium condensatum* Turcz.	+	+			
3	*Artemisia argyi* H. Lév. & Vaniot			+		
4	*Artemisia capillaris* Thunb.	+				
5	*Artemisia desertorum* Spreng.		+	+	+	+
6	*Arundinella* sp.1		+			
7	*Astragalus adsurgens* Pall.	+	+	+	+	+
8	*Calamagrostis pseudophragmites* (Haller f.) Koeler	+				
9	*Caragana microphylla* Lam.	+	+	+	+	+
10	*Carex duriuscula* C.A. Mey.	+				
11	*Chenopodium ficifolium* Sm.	+				
12	*Cleistogenes squarrosa* (Trin.) Keng	+				
13	*Corispermum hyssopifolium* L.	+	+		+	+
14	*Corispermum squarrosum* (L.) Moq.		+	+	+	
15	*Cuscuta australis* R. Br.	+				
16	*Echinops gmelini* Turcz.		+	+	+	
17	*Epilobium* sp.1		+			
18	*Euphorbia humifusa* Willd.	+				
	Hedysarum fruticosum Pall.					
19	*(Corethrodendron fruticosum* (Pallas) B.H. Choi & H. Ohashi)	+	+	+	+	+
20	*Inula britanica* L.		+			
21	*Juncus gracillimus* (Bunchenau) V. Krecz. & Gontsch.		+			
22	*Lappula myosotis* V. Wolf	+				
23	*Leymus secalinus* (Georgi) Tzvelev		+			
24	*Medicago polymorpha* L.	+				
25	*Olgaea lomonosowii* (Trautv.) Iljin.	+				
26	*Orobanche coerulescens* Stephan ex Willd.			+	+	+
27	*Oxytropis racemosa* Turcz.		+	+	+	+
28	*Phragmites australis* (Cav.) Trin. ex Steud.		+			
29	*Pinus sylvestris* L. var. *mongolica* Litv.		+			
30	*Plantago* sp.1		+			
31	*Populus canadensis* Moench		+	+		
32	*Potentilla acaulis* L.		+			
33	*Salix cheilophila* C.K. Schneid.		+			
34	*Setaria viridis* (L.) P. Beauv.	+	+		+	
35	*Silene jenisseensis* Willd.		+			
36	*Sonchus arvensis* L.		+			
37	*Sophora flavescens* Aiton		+			
38	*Suaeda glauca* (Bunge) Bunge	+				
39	*Taraxacum asiaticum* Dahlst.		+			
40	*Thymus mongolicus* (Ronniger) Ronn.	+				
41	*Ulmus pumila* L.		+	+		

There was no significant difference in the species diversityof food sources by livestock species, although more plant species were detected in cow feces (Table 2).

Table 2. Amplification success rate and diversity index of plants by study site and livestock

	No. of feces	Amplification success (%)	Shannon index (H')	Simpson's index (1-D)
Study site				
2005	33	20(60.6)	1.88	0.79
2008	24	19(79.2)	2.48	0.90
2011	15	13(86.7)	2.14	0.86
2012	12	9(75.0)	1.89	0.82
NR (non-restored)	18	16(88.9)	2.18	0.86
Livestock				
Cow	38	27(71.1)	2.07	0.79
Horse	64	50(78.1)	2.79	0.92
Total	102	77(75.5)	1.44	0.91

Mean (± SE) fecal densities ranged from 3.0 ± 1.4 feces/replicate at the 2012 site to 8.3 ± 0.6 feces/replicate at the 2005 site (Fig. 4). The mean fecal density at NR sites was 4.5 ± 0.65, which was higher than those at the two restored areas in recent years. The mean fecal density was positively correlated with the restoration period. It was significantly different between the 2005 site and the 2011 or 2012 site ($P < 0.05$) (Fig. 4).

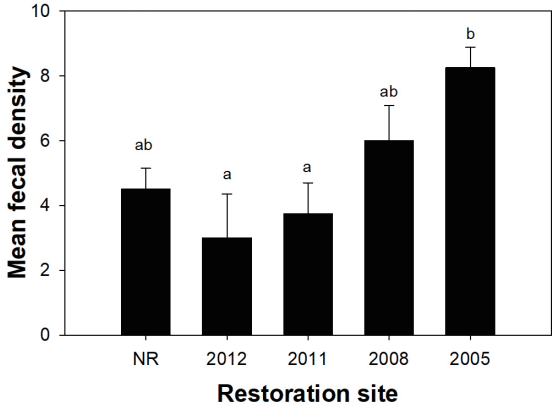

Fig. 4. Mean density of livestock feces at study sites. Mean fecal density represents the mean number of livestock feces found in 800 m transect (200 m each, 4 replicates). Different small alphabets on the bar means statistically different among sites ($P < 0.05$).

Feeding preference of livestock

We detected plant species genes from 77 (75.5%) out of 102 fecal samples collected from the study sites. The amplification success rate ranged from 60.6% to 88.9%, depending on the year of restoration. It showed no significant difference between livestock species (Table 2).

A total of 35 different sequences were detected via DNA barcoding. Plant species were identified at the species or genus level by comparison with GenBank (Table 3). Most sequences showed similarities of more than 98%. A total of 17 potential plant species were detected in comparison with results of the vegetation survey at the study sites (Table 3). Two shrub species, *C. microphylla* Lam. and *C. fruticosum* Pall., were detected in the feces of livestock. However, *Caragana* sp. was only detected at a low frequency (< 0.2) in livestock feces collected at the 2008 restoration site. In contrast, *Corethrodendron* sp. was detected with a frequency from 0.05 to 0.25 in most restored and non-restored sites, excluding the 2012 site (Fig. 5). In most restoration sites, three families, Amaranthaceae, Asterceae, and Fabaceae, comprised a large portion of total detected plant species. However, plant species belonging to family Amaranthaceae were not detected at the 2012 restoration site. In the case of plant species belonging to family Fabaceae, the frequency of occurrence was lower (0.05) in the 2005 restoration site than in other restoration sites (0.22 –0.47). In particular, Fabaceae showed the highest frequency (0.69) in the NR site. The frequency of plant species detected in livestock feces was similar for horses and cattle, although plant species belonging to Asteraceae were detected at significantly higher rates in cow feces than in horse feces (P = 0.00053, Fig. 6).

Discussion

For successful restoration, it is necessary to select appropriate species that are well adapted to local ecological environment conditions. It is also important to meet socioeconomic needs of local residents (Sacande *et al*., 2020). In Inner Mongolia, animal husbandry plays an important role in rural economies. The density of grazing livestock in Inner Mongolia is much higher than in other regions of the Mongolian Plateau (Angerer *et al*., 2008). In particular, in Inner Mongolia, socioeconomic factors such as total population, gross domestic product, fiscal revenue, production values, cropland area, and number of livestock affect the desertification more than climate factors (Liu *et al*., 2020). Fecal analysis of livestock in this study showed that livestock fed on *C. microphylla* Lam. and *C. fruticosum,* Pall., two of the four plant species used for restoration in Hulunbuir. However, they did not prefer them in the summer growing season. Most plant species detected in livestock feces were species found near the restoration site or naturally introduced by vegetation recovery. In addition, there were no significant differences in livestock preference among plant species except for family Asteraceae, although we did find some differences in diet compositions detected in livestock feces depending

Table 3. BLAST search results of plants detected in livestock feces

No.	BLAST results					Occurrence of identified taxon on Hulunbuir
	Description	Query coverage	E value	Identities	Accession	
1	*Allium anisopodium*	100%	2E-169	99%	KF143822	*A. condensatum* Turcz.
2	*Allium* sp.	100%	1E-165	99%	GQ412199	
3	*Allium splendens*	100%	2E-169	99%	GQ412241	
4	*Amaranthus retroflexus*	100%	7E-167	100%	KF493799	
5	*Ambrosia trifida*	100%	7E-167	100%	DQ005970	
6	*Artemisia annua*	99%	2E-159	98%	KF866382	*A. desertorum* Spreng.
7	*Artemisia japonica*	100%	2E-169	99%	GU724289	*A. argyi* H. Lèv. & Vaniot
8	*Artemisia rupestris*	100%	9E-168	99%	AJ297261	*A. capillaris* Thunb.
9	*Artemisia sericea*	100%	9E-163	98%	EF577290	
10	*Astragalus* sp.	100%	9E-168	99%	KJ999345	*A. adsurgens* Pall.
11	*Bassia dasyphylla*	100%	4E-171	100%	AY489195	
12	*Betula* sp.	100%	9E-168	99%	JN998976	
13	*Boehmeria* sp.	100%	4E-171	100%	KP093179	
14	*Caragana* sp.	100%	7E-169	99%	AB262534	*C. microphylla* Lam.
15	*Carex gynocrates*	100%	9E-158	98%	JN999047	*C. duriuscula* C.A. Mey.
16	*Carex maritima*	100%	4E-166	99%	JN999057	
17	*Chenopodium album*	100%	2E-154	97%	FN561549	*C. ficifolium* Sm. *C.acuminatum* Willd.
18	*Citrus* sp.	100%	1E-164	99%	JQ990174	
19	*Corethrodendron multijugum*	91%	5E-155	100%	AB854479	*C. fruticosum* Pall.
20	*Corispermum* sp.	95%	3E-162	100%	JF792752	*C. hyssopifolium* L. *C. squarrosum* (L.) Moq.
21	*Cucumis* sp.	100%	2E-169	99%	KF963130	
22	*Elaeocarpus* sp.	94%	7E-154	98%	DQ448689	
23	*Humulus* sp.	100%	3E-165	99%	KC539581	
24	*Ipomoea trifida*	100%	4E-164	99%	KC621852	
25	*Luffa* sp.	96%	3E-162	99%	KF487334	
26	*Oxytropis* sp.	100%	4E-171	100%	KJ143722	*O. racemosa* Turcz.
27	*Phaseolus* sp.	100%	7E-169	99%	JN617200	
28	*Platycodon grandiflorus*	100%	3E-165	99%	KP058319	
29	*Puccinellia* sp.	100%	9E-163	98%	KJ598984	
30	*Rubus crataegifolius*	100%	9E-153	97%	GU980782	
31	*Salix* sp.	100%	4E-164	99%	KM978952	*S. cheilophila* C.K. Schneid.
32	*Salsola* sp.	89%	9E-148	99%	HM131659	*S. collina* Pall.
33	*Setaria italica*	100%	2E-169	99%	KF012851	*S. viridis* (L.) P. Beauv.
34	*Suaeda corniculata*	94%	4E-161	100%	FJ449820	*S. glauca* (Bunge) Bunge
35	*Suaeda maritima*	100%	2E-164	99%	KF866386	

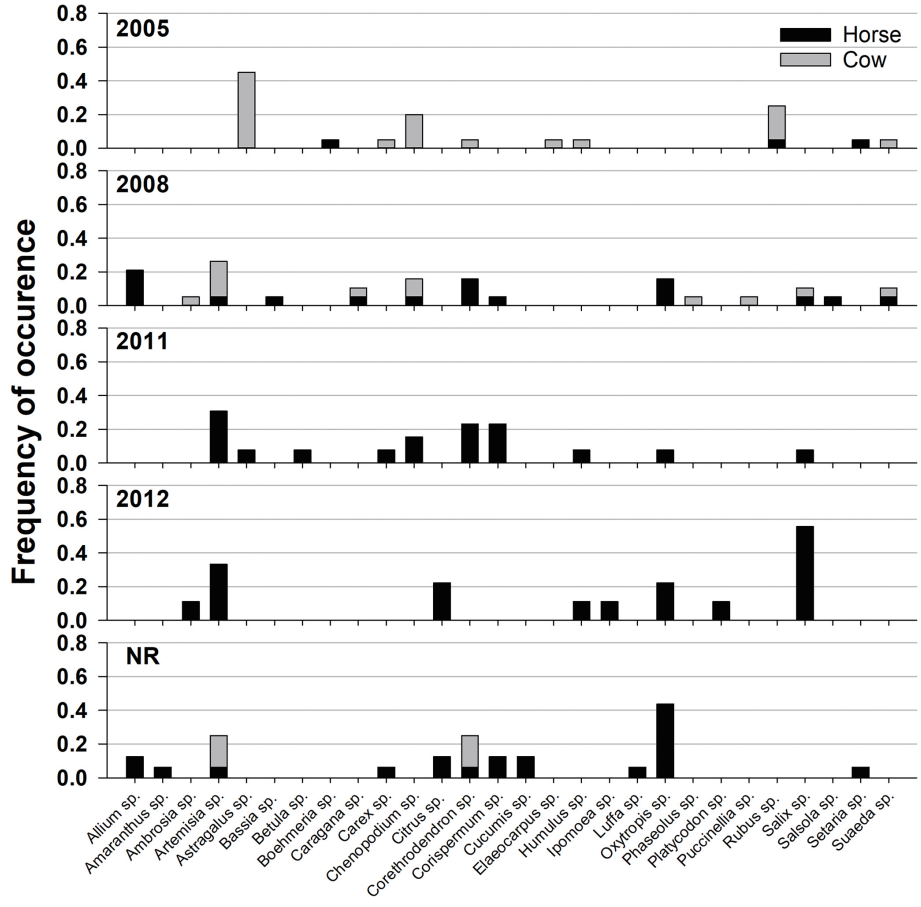

Fig. 5. Detection frequencies of plant genera in livestock feces.

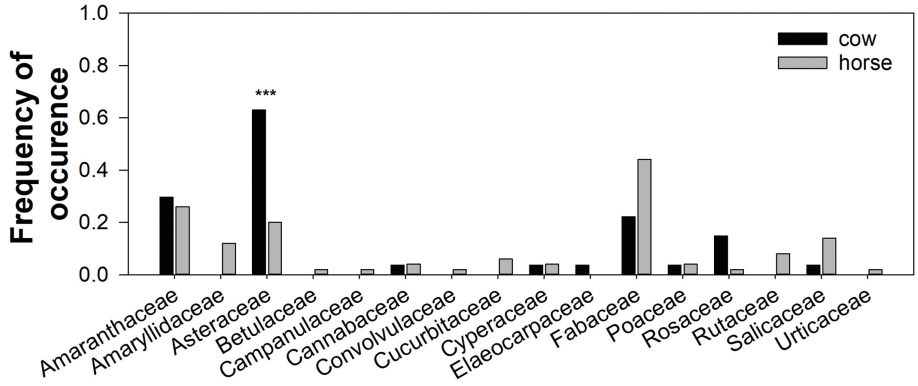

Fig. 6. Detection frequencies of plant families in livestock feces. Asteraceae species were detected at significantly higher rates in cow feces than in horse feces (***, $P < 0.001$).

on the restoration site. Our results suggest that livestock grazing in the restoration areas during the summer growth period might not have a significant impact on the survival of restored plants.

Most previous studies have focused on restoration of degraded ecosystems and harsh environments via vegetation recovery in desertified areas (Liao *et al*., 2019). The selection of restored plants is often focused on plants' ad-

aptability to climate and their ability to improve soil microbial communities (Yan & Feng , 2020). Shrub species are commonly planted to combat desertification. Legumes are potential nurse plants that can improve the survival and growth of other species due to their ability to fix nitrogen (Ren *et al*., 2008; Reynolds *et al*., 1999; Zhao *et al*., 2007). Unpalatable native species that could be planted in heavily grazed sites are recommended as nurse plants as they can help create microhabitats for the rehabilitation of other plants (Ren *et al*., 2008; Smit *et al*., 2006). Two shrub species, *C. microphylla* and *C. fruticosum*, were classified as unpalatable or less palatable species in this study. They might be helpful in increasing the survival and growth of other plants in restoration areas. We noted that at the 2008 site, the number of plant species increased to 26 species during the enclosure periods despite the grazing of livestock in restoration sites.

The Chinese government has implemented several policies and programs to restore grassland ecosystem functions by regulating and controlling grazing pressure, including enforcing partial or total rests from grazing for several years (Zhang *et al.*, 2015). Previous studies have reported that enclosures have positive effects on vegetation recovery and succession in an enclosed area of a grassland where dese rtification is in progress (Park *et al*., 2013). However, enclosure may not be suitable for severely degraded grasslands (Jones & Carter, 2016; Zhang e*t al*., 2015). For successful restoration, it is necessary to consider both the improvement of soil fertili ty and the economic demands of local herders (Anderson & Briske , 1995). In addition, it would be desirable to set a value of herbage mass that needs to be reached before grazing is allowed, rather than setting a time limit for rests (Zhang *et al*., 2015). Our results suggest that the four plant species used for restoration in Hulunbuir steppe are less affected by livestock grazing during the summer growing season and that the current restoration method may be positive and suitable for the initial stage of restoration in Hulunbuir.

Conflict of Interest

The authors declare that they have no competing interests.

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