

## Article

# Spatial Variation in Leaf Stable Carbon Isotope Composition of Three *Caragana* Species in Northern China

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**Abstract:** Leaf stable carbon isotope ( $\delta^{13}\text{C}$ ) composition provides comprehensive insight into plant carbon cycles and water use efficiency and has also been widely used to evaluate the response of plants to environmental change. In the present study, leaf  $\delta^{13}\text{C}$  was analyzed in samples of *Caragana microphylla* Lam., *C. liouana* Zhao, and *C. korshinskii* Kom. from 38 populations. These species provide great environmental benefits and economic value and are distributed east to west continuously across northern China. We studied the relationship of  $\delta^{13}\text{C}$  to altitude, mean annual precipitation (MAP), mean annual temperature (MAT), mean annual relative humidity (RH), leaf nitrogen (N), and phosphorus (P) concentrations to examine the patterns and controls of leaf  $\delta^{13}\text{C}$  variation in each species. Results indicated that, across the three species, leaf  $\delta^{13}\text{C}$  significantly decreased with MAP, RH, and leaf N and P concentrations, while it increased with altitude and MAT. However, patterns and environmental controls of leaf  $\delta^{13}\text{C}$  varied proportionally with species. *C. korshinskii* was mainly controlled by MAP and leaf N concentration, *C. liouana* was controlled by both MAT and MAP, and *C. microphylla* was mainly controlled by MAT. Further analysis indicated significant differences in leaf  $\delta^{13}\text{C}$  between species, which tended to increase from *C. microphylla* to *C. korshinskii*. Overall, these results suggest that the three *Caragana* species may respond differently to future climate change due to different controlling factors on leaf  $\delta^{13}\text{C}$  variation, as well as differentiation in water use efficiency, which likely contributes to the geographical distribution of these species.

**Keywords:** *Caragana*; carbon isotope composition; mean annual precipitation; mean annual temperature; relative humidity; water use efficiency

## 1. Introduction

Leaf carbon isotope composition ( $\delta^{13}\text{C}$ ) provides comprehensive insight into how plants interact with and respond to their biotic and abiotic environments, and also enhances our understanding of ecosystem carbon cycles [1,2]. As such, being able to assess the spatial variability of leaf  $\delta^{13}\text{C}$  across environmental gradients and identifying the patterns and controls of leaf  $\delta^{13}\text{C}$  would improve our understanding of how individual plants and ecosystems may respond and adapt to future global

changes, including climate warming, atmospheric CO<sub>2</sub> enrichment, shifts in precipitation, and N deposition [2–5]. Studies on this topic have recently received increasing attention [6–10].

Leaf  $\delta^{13}\text{C}$  is largely related to the ratio of CO<sub>2</sub> partial pressure inside the leaf and ambient air ( $c_i/c_a$ ) [11,12], which is driven by stomatal conductance and photosynthetic processes [11,12]. It has been shown in several studies that there is a strong positive correlation between  $\delta^{13}\text{C}$  and plant water use efficiency (WUE) via  $c_i/c_a$  [11–13], which suggests that leaf  $\delta^{13}\text{C}$  can be measured as a proxy for plant WUE [14–21]. WUE reflects the balance between carbon fixation and the amount of water consumed by plants. Therefore, WUE is one of the most indicative traits of survival capacity during drought [11,12,14]. Previous studies have shown that the WUE and the ability to tolerate low soil availability or compete for such resources differ between plant species [9,10]. For example, plants with higher  $\delta^{13}\text{C}$  or WUE tend to experience greater survival during drought conditions than those with lower  $\delta^{13}\text{C}$  or WUE [10,16,19].

Any factor that affects stomatal aperture or carboxylation ultimately influences the amount of  $\delta^{13}\text{C}$  in tissue. In particular, climatic factors including precipitation and temperature have been regularly reported to show strong negative correlations with leaf  $\delta^{13}\text{C}$  [15,19,22–24]. Other studies report that plant  $\delta^{13}\text{C}$  correlates with altitude [10,14,18,25]. However, the response of plant  $\delta^{13}\text{C}$  to altitude remains controversial and unresolved [22,25–28], with studies having found that leaf  $\delta^{13}\text{C}$  increases [28], decreases, or remains constant [27] with increasing altitude. That leaf  $\delta^{13}\text{C}$  varies nonlinearly with altitude has also been reported [25].

Nitrogen (N) and phosphorus (P) are essential elements for plants to function. For example, enzymes such as RuBP carboxylase contain large quantities of N [29]. An increase in photosynthesis might induce less discrimination against  $\delta^{13}\text{C}$  and therefore lead to higher  $\delta^{13}\text{C}$ . Consequently, a considerable number of studies have found a positive correlation between leaf N concentration and  $\delta^{13}\text{C}$  [30–34]. However, negative correlations and no correlation have also been reported [35–38]. P indirectly affects plant photosynthesis and subsequently leaf  $\delta^{13}\text{C}$  because P is one of the key components in enzymes associated with the Calvin cycle and the synthesis of protein, RNA, and DNA [34–38]. However, research relating leaf P to  $\delta^{13}\text{C}$  is limited and the findings are inconsistent. Studies have found that the correlation between P and  $\delta^{13}\text{C}$  can be positive [39], negative [40,41], or completely unrelated [42]. Further, studies have also indicated that the patterns of leaf  $\delta^{13}\text{C}$  across environmental gradients differ with plant genus [9], species [8,25], and plant functional group [14,19,28,43,44]. Therefore, these conflicting results suggest that patterns of leaf  $\delta^{13}\text{C}$  are variable with respect to leaf nutrients, and climatic and geographic variables, and that further research is needed to understand the interactions between plant  $\delta^{13}\text{C}$  and environmental factors for a single genus or species.

In arid and semi-arid regions, the availability of water, nutrients, and temperature are crucial for determining plant performance, abundance, and distribution [1,2]. Particularly, regions in northern China that are characterized by drastic changes in precipitation, temperature, and soil nutrient availability from east to west offer an ideal ‘natural experiment’ to explore spatial patterns and environmental drivers of leaf  $\delta^{13}\text{C}$  in local plant species. In these regions, the genus *Caragana* exemplifies a combination of important environmental benefactors and valued economic resources for its role in sand fixation and as fodder [45–50]. *Caragana microphylla* Lam., *C. liouana* Zhao, and *C. korshinskii* Kom. are three of the most common species in the desert region of the Inner Mongolian Plateau of northern China. Interestingly, these three species have been identified as being derived from the same ancient species, undergoing divergent speciation during the Qinghai-Tibetan Plateau uplift and the Asian interior aridification [45]. To date, these three species have dominantly occupied distinct desert regions on the Inner Mongolian Plateau and form obvious spatially distinct distributions from east to west [45,46]. Previously, we have found that these three species diverge substantially in growth and physiological parameters in response to drought stress during a controlled experiment. *C. korshinskii* appeared to be the most drought-tolerant species with a superior growth rate and water use efficiency compared to the other two species [51,52]. As such, evaluating the patterns and environmental controls of  $\delta^{13}\text{C}$  in these three species will not only help predict how these species

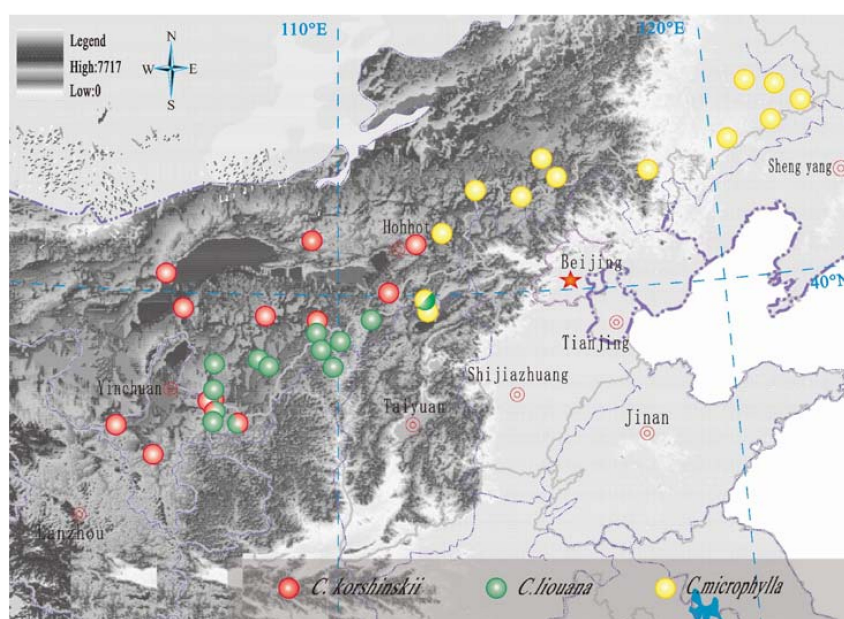
respond to future climate change, but will also provide evidence for the physiological mechanisms underlying the distribution pattern of these three closely related *Caragana* species.

In this study, we collected leaf samples of three *Caragana* species along an east to west transect in northern China and measured leaf  $\delta^{13}\text{C}$ . Our objectives were to (1) quantify the variation in leaf  $\delta^{13}\text{C}$  of the three species along the environmental gradients and determine the patterns and environmental controls of leaf  $\delta^{13}\text{C}$ ; and (2) test whether there were differences in leaf  $\delta^{13}\text{C}$  between the three species and whether these differences corresponded with their geographical distributions.

## 2. Materials and Methods

### 2.1. Study Area

This study was conducted along an east to west transect in arid and semi-arid regions of northern China, starting in Bayannaoer City and ending in the Khorchin District in the Nei Mongol Autonomous Region (Figure 1). The longitude of the transect covered approximately  $16^\circ$ , ranging from  $104^\circ 57' \text{ E}$  to  $122^\circ 28' \text{ E}$ , and the latitude ranged from  $37^\circ 20' \text{ N}$  to  $43^\circ 63' \text{ N}$ . Altitude varied from 227 to 1789 m above sea level. The climate was characterized as predominantly arid and semi-arid continental. Mean annual precipitation (MAP) ranged from 87.2 to 434.4 mm and mean annual air temperature (MAT) ranged from 1.4 to  $8.4^\circ \text{C}$  (in supplementary file, Table S1).



**Figure 1.** Locations of the sampling populations of three different *Caragana* species (*C. microphylla* Lam., *C. liouana* Zhao and *C. korshinskii* Kom.) in arid and semi-arid regions of northern China.

### 2.2. Sampling and Measurements

In August 2015, leaf samples were collected from 38 populations (12 for *C. korshinskii*, 13 for *C. liouana*, and 13 for *C. microphylla*) along the east to west transect in northern China (Figure 1). At each sampling site, fully expanded sun-exposed leaves were collected from four different individual plants 5 m apart from each other and pooled into one sample, which means that three replicates were collected for each population. All samples were collected from robust mature plants that grew in unshaded habitats. Plant samples collected at each site were placed in paper envelopes, rinsed with deionized water to remove dust particles, and dried at  $65^\circ \text{C}$  for 48 h to a constant weight in the oven upon returning to the laboratory. After grinding with a ball mill, they were stored in plastic bags until further analysis. Leaf  $\delta^{13}\text{C}$  values were determined from 5–6 mg of homogeneously ground material

from each replicate using an isotope ratio mass spectrometer (Finnigan MAT-253, Thermo Electron, Gormley, ON, Canada). The stable carbon isotope ratio ( $\delta^{13}\text{C}$ , ‰) of each sample was calculated as  $\delta^{13}\text{C}$  (‰) =  $(R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$ , where  $R_{\text{sample}}$  and  $R_{\text{standard}}$  represent the  $^{13}\text{C}/^{12}\text{C}$  ratio in the leaf sample and standard, respectively. Nitrogen (N) concentrations in leaf samples were simultaneously determined. Leaf phosphorus concentrations were measured colorimetrically using molybdenum blue spectrophotometry (6505 UV spectrophotometer, Jenway, Stone, UK).

### 2.3. Meteorological Data

The latitude, longitude, and altitude of each sampling location was recorded using a GPS (Global Positioning System) (eTrex Venture, Garmin, Olathe, KS, USA). MAP, MAT, and mean annual relative humidity (RH) of each sampling site were provided by the IWMI (International Water Management Institute) online climate summary service portal (<http://wcatlas.iwmi.org/Default.asp>), based on geographical coordinates (latitude and longitude). Detailed geographical and environmental information is presented in Table S1.

### 2.4. Data Analysis

Statistical analyses were carried out using the SPSS 16.0 package (IBM, Chicago, IL, USA). Due to the difference in sample size between the three species, we performed a standardized z-score normalization for the climate and leaf trait data before it was analyzed with the Pearson correlation analysis, regression analysis, and one-way analysis of variance (ANOVA). The normal distribution of measured variables was tested and the data with a normal distribution was analyzed using ANOVA and Tukey's post hoc test, while the non-normal distribution data was analyzed using the non-parametric Mann-Whitney test to determine the difference in leaf  $\delta^{13}\text{C}$  between species. The regression analysis was applied to investigate the relationship between leaf  $\delta^{13}\text{C}$  and variables including climatic factors, altitude, and leaf N and P concentrations. Multiple regressions, using a stepwise selection procedure based on Akaike information criteria (AIC), were applied to define the contribution of altitude, MAP, MAT, RH, and N and P concentrations to the variation in leaf  $\delta^{13}\text{C}$  among the three *Caragana* species. A *p*-value of <0.05 was considered statistically significant.

## 3. Results

### 3.1. Variation across the Sites in Climate and Leaf Nutrient Composition of Three *Caragana* Species

Climatic variables varied significantly among populations and among species (Table 1, Table S1). *C. korshinskii* showed a significantly higher mean population MAP than *C. liouana* or *C. microphylla*. The mean population MAT of *C. microphylla* appeared to be significantly lower than for *C. korshinskii* and *C. liouana*, but RH was significantly higher. In addition, mean population leaf N and P concentrations of *C. korshinskii* exhibited significantly higher values than those of *C. liouana* and *C. microphylla* (Table 1).

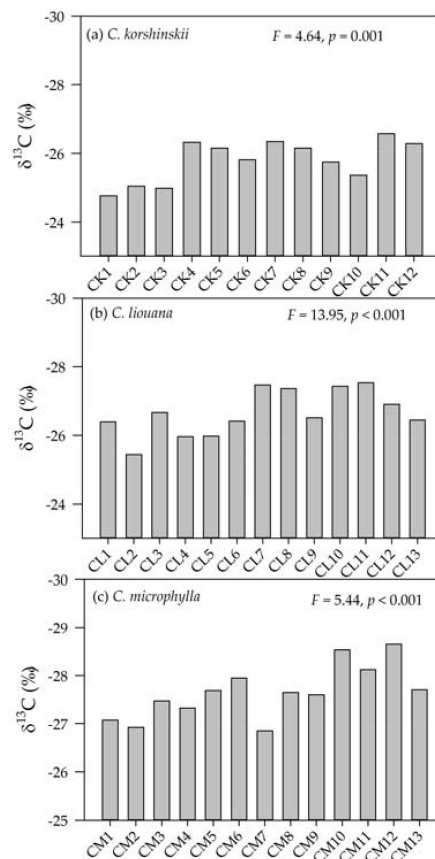
**Table 1.** Mean values of geographical and environmental variables and leaf nutrient concentrations across sites for each *Caragana* species. Mean values followed by different letters indicate significant differences between means in each row (*p* < 0.05).

Variables	<i>C. korshinskii</i>	<i>C. liouana</i>	<i>C. microphylla</i>
	Mean ± SD	Mean ± SD	Mean ± SD
Altitude (m)	1368.8 ± 173.1 a	1293.2 ± 142.9 a	971.9 ± 612.7 b
MAP (mm)	232.9 ± 81.6 b	330.2 ± 69.1 a	372.9 ± 40.0 a
MAT (°C)	7.2 ± 1.5 b	7.2 ± 0.9 b	4.6 ± 2.2 a
RH (%)	55.5 ± 0.6 b	56.0 ± 0.9 b	59.3 ± 3.3 a
Leaf N (mg·g <sup>-1</sup> )	32.62 ± 4.59 b	38.32 ± 1.89 a	39.76 ± 2.6 a
Leaf P (mg·g <sup>-1</sup> )	1.38 ± 0.23 b	1.70 ± 0.17 a	1.71 ± 0.24 a

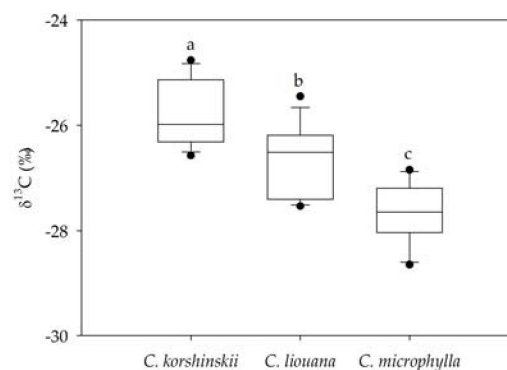
MAP: mean annual precipitation; MAT: mean annual temperature; RH: mean annual relative humidity; SD: standard deviation.

### 3.2. Variation in Leaf $\delta^{13}\text{C}$ within and Among Species

Leaf  $\delta^{13}\text{C}$  showed substantial variability among populations for each of the three species, with *C. korshinskii* ranging from  $-26.57$  to  $-24.77\text{‰}$ , *C. liouana* from  $-27.53$  to  $-25.45\text{‰}$ , and *C. microphylla* from  $-28.65$  to  $-26.85\text{‰}$  (Figure 2,  $p < 0.001$ ). Differences in  $\delta^{13}\text{C}$  between species were also significant, with the highest mean  $\delta^{13}\text{C}$  ( $-25.79 \pm 0.61\text{‰}$ ) found in *C. korshinskii*, *C. liouana* having an intermediate mean ( $-26.66 \pm 0.65\text{‰}$ ), and the lowest mean found in *C. microphylla* ( $-27.66 \pm 0.56\text{‰}$ ) (Figure 3).



**Figure 2.** Population variation in leaf  $\delta^{13}\text{C}$  for each of the three *Caragana* species (a), *C. korshinskii*, CK1-CK12; (b) *C. liouana*, CL1-CL13; (c) *C. microphylla*, CM1-CM13.

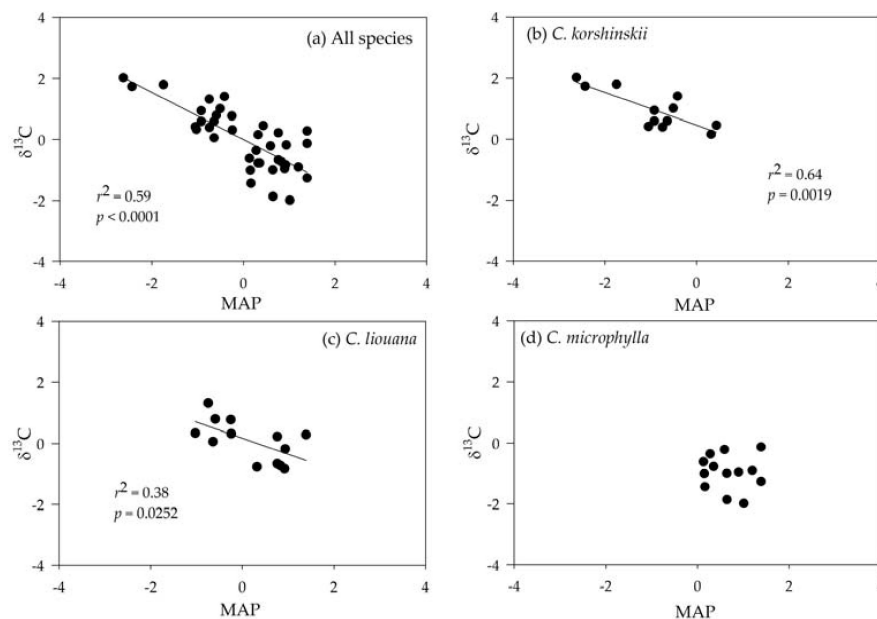


**Figure 3.** Difference in leaf carbon isotope composition ( $\delta^{13}\text{C}$ ) between three *Caragana* species collected along environmental gradients in northern China. Different letters indicate significant differences between means ( $p < 0.05$ ).

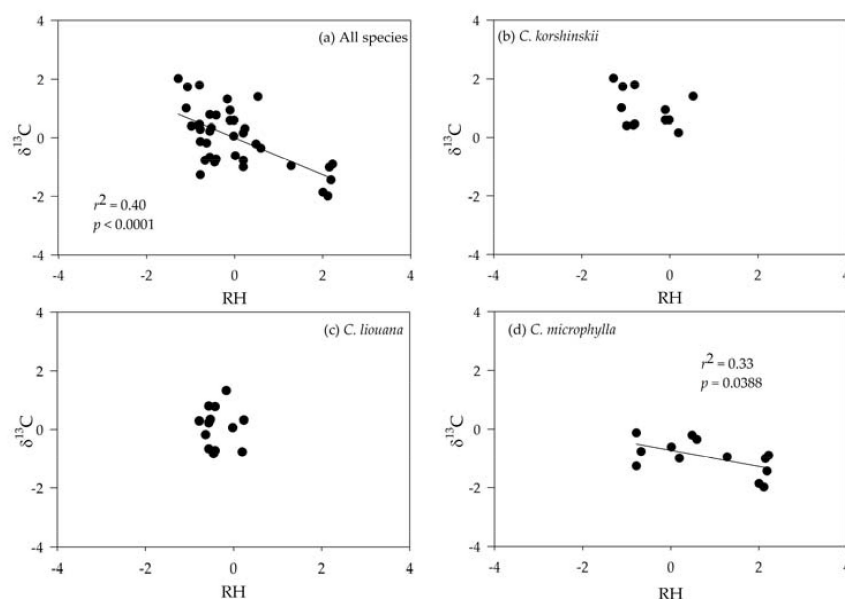


### 3.3. Correlating Leaf $\delta^{13}\text{C}$ with Climatic Variables

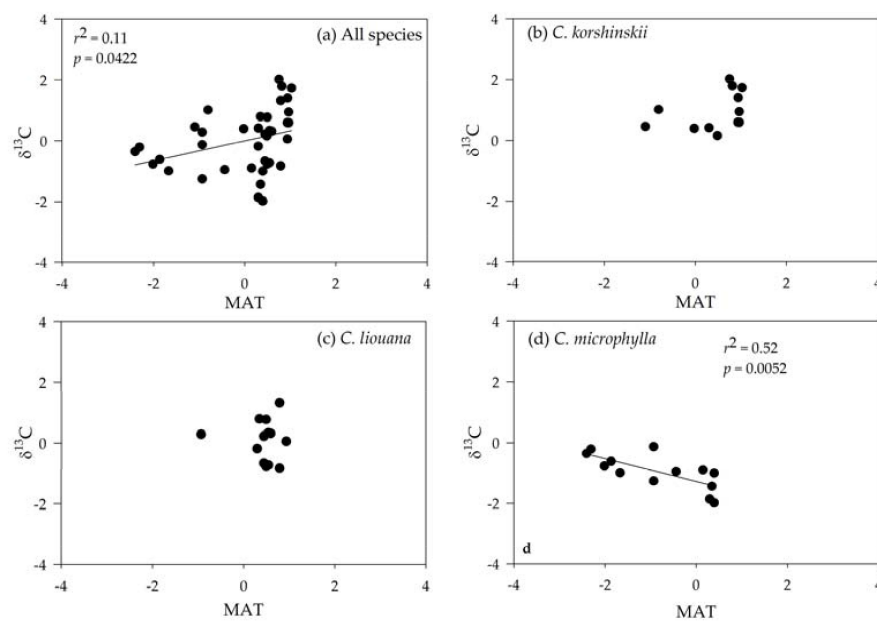
For all leaf samples, leaf  $\delta^{13}\text{C}$  was negatively correlated with climatic variables, i.e., MAP and RH (Figures 4 and 5), but positively related to MAT (Figure 6). However, the specific patterns of leaf  $\delta^{13}\text{C}$  varied with species. A significant negative correlation between MAP and leaf  $\delta^{13}\text{C}$  was only observed in *C. korshinskii* and *C. liouana* (Figure 4b,c), while MAT and RH negatively correlated with  $\delta^{13}\text{C}$  only in *C. microphylla* (Figures 5d and 6d). When examining the relationship between RH and leaf  $\delta^{13}\text{C}$  in all three species, the relationship was only significant for *C. microphylla* (Figure 5d).



**Figure 4.** Relationship between leaf  $\delta^{13}\text{C}$  and mean annual precipitation (MAP) for all species (a), *C. korshinskii* (b), *C. liouana* (c), and *C. microphylla* (d). Linear fits, correlation coefficients, and  $p$ -values are shown.



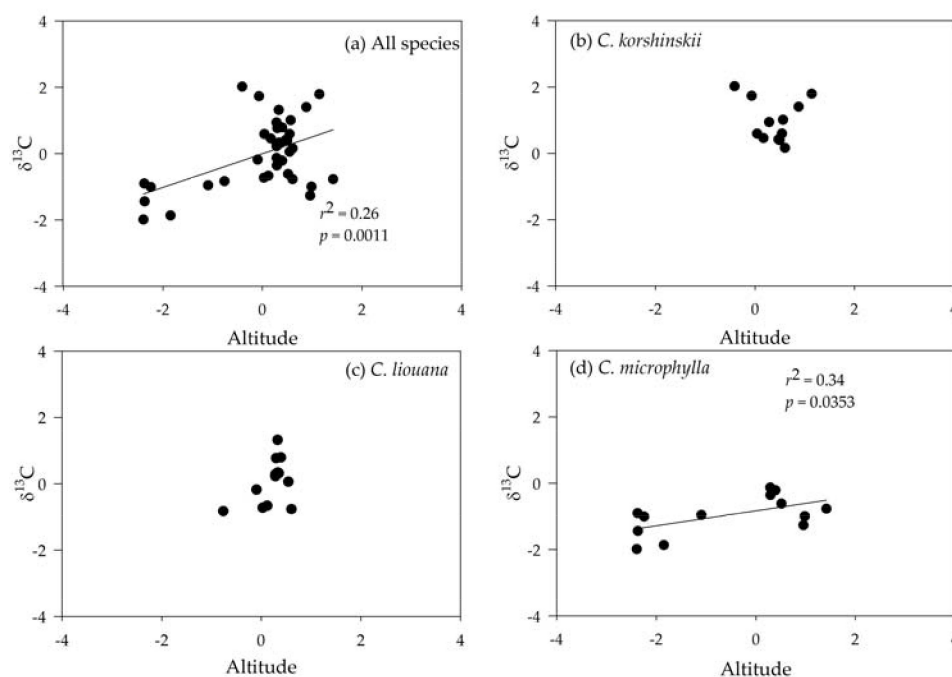
**Figure 5.** Relationship between leaf  $\delta^{13}\text{C}$  and relative humidity (RH) for all species (a), *C. korshinskii* (b), *C. liouana* (c), and *C. microphylla* (d). Linear fits, correlation coefficients, and  $p$ -values are shown.



**Figure 6.** Relationship between leaf  $\delta^{13}\text{C}$  and mean annual temperature (MAT) for all species (a), *C. korshinskii* (b), *C. liouana* (c), and *C. microphylla* (d). Linear fits, correlation coefficients, and  $p$ -values are shown.

### 3.4. Correlating Leaf $\delta^{13}\text{C}$ with Altitude

For all leaf samples, leaf  $\delta^{13}\text{C}$  was positively correlated with altitude (Figure 7). However, the altitudinal patterns of leaf  $\delta^{13}\text{C}$  varied with species and the relationship was only significant in *C. microphylla* (Figure 7d). Altitude was negatively correlated with RH ( $p < 0.001$ ) for all species, and was significantly negatively correlated with both MAT and RH for *C. microphylla* ( $p < 0.001$ ) (Table 2).



**Figure 7.** Correlation between leaf  $\delta^{13}\text{C}$  and altitude for all species (a), *C. korshinskii* (b), *C. liouana* (c), and *C. microphylla* (d). Linear fits, correlation coefficients, and  $p$ -values are shown.

**Table 2.** Correlating altitude with mean annual precipitation (MAP), mean annual temperature (MAT), relative humidity (RH), and leaf N and P concentrations for *C. korshinskii*, *C. liouana*, and *C. microphylla*. The symbol \*\*\* indicates statistical significance at  $p < 0.001$ .

Species	MAP	MAT	RH	N	P
All species	−0.274	−0.193	−0.780 ***	−0.099	−0.267
<i>C. korshinskii</i>	0.239	0.048	0.291	0.344	0.388
<i>C. liouana</i>	−0.510	−0.090	0.400	−0.017	0.294
<i>C. microphylla</i>	0.020	−0.890 ***	−0.947 ***	0.400	−0.344

### 3.5. Correlating Leaf $\delta^{13}\text{C}$ with Leaf Nutrient Concentrations

In all leaf samples, both leaf N and P concentrations were significantly negatively correlated with leaf  $\delta^{13}\text{C}$  (Table 3). However, nutrient patterns did not differ among species as the leaf  $\delta^{13}\text{C}$  showed no significant relationship between leaf N and P concentrations for each species (Table 3).

**Table 3.** Regression equations for leaf  $\delta^{13}\text{C}$  values against leaf N and P concentrations for *C. korshinskii*, *C. liouana*, and *C. microphylla*.

Nutrient Variables	Species	Statistic		
		Slope	$r^2$	$p$ -Value
Leaf N	All species	−0.11	0.26	0.0010
	<i>C. korshinskii</i>	−0.10	0.03	0.6026
	<i>C. liouana</i>	−0.25	0.03	0.6061
	<i>C. microphylla</i>	0.46	0.23	0.0975
Leaf P	All species	−0.44	0.20	0.0051
	<i>C. korshinskii</i>	−0.24	0.12	0.2760
	<i>C. liouana</i>	0.14	0.02	0.6625
	<i>C. microphylla</i>	−0.03	0.002	0.8936

### 3.6. Relationship between Leaf $\delta^{13}\text{C}$ and Climatic Variables, Altitude, and Leaf Nutrients

Multiple regressions indicated that MAP and RH both contributed to the variation of leaf  $\delta^{13}\text{C}$  across all populations, while MAP and RH were dominant in influencing the variation of leaf  $\delta^{13}\text{C}$  for *C. korshinskii*, MAP and MAT contributed to variation in leaf  $\delta^{13}\text{C}$  of *C. liouana*, and MAT mainly controlled leaf  $\delta^{13}\text{C}$  variation of *C. microphylla* (Table 4).

**Table 4.** Results from multiple stepwise regressions testing relationships between leaf  $\delta^{13}\text{C}$  and mean annual precipitation (MAP), mean annual temperature (MAT), relative humidity (RH), and leaf N concentration for *C. korshinskii*, *C. liouana*, and *C. microphylla*. The most parsimonious model was found by selecting variables using a stepwise selection procedure based on Akaike information criteria (AIC).  $F$ - and  $p$ -values are shown for each species, along with standardized coefficients and  $r^2$  for the regressions.

Species	a (Constant)	bx1 (MAP)	Standardized Coefficients	cx2 (MAT)	Standardized Coefficients	dx3 (RH)	Standardized Coefficients	ex4 (N)	Standardized Coefficients	F	p Regression	$r^2$
All species	−16.37	−0.01	−0.63 $p < 0.001$			−0.14	−0.41 $p < 0.001$			49.78	<0.001	0.74
<i>C. korshinskii</i>	−26.22	−0.01	−1.15 $p < 0.001$					0.08	0.56 $p = 0.013$	21.08	<0.001	0.82
<i>C. liouana</i>	−21.00	−0.01	−0.91 $p = 0.003$	−0.39	−0.55 $p = 0.045$					7.29	0.011	0.59
<i>C. microphylla</i>	−26.84			−0.18	−0.72 $p = 0.005$					12.03	0.005	0.52

The regression models are  $y = a + bx1 + cx2 + dx3 + ex4$ . x1, x2, x3, and x4 are MAP, MAT, RH, and leaf N concentration, respectively.



## 4. Discussion

### 4.1. Variation in Leaf $\delta^{13}\text{C}$ of Three *Caragana* Species along the Transect

It has been reported that foliar  $\delta^{13}\text{C}$  varies in C3 plants from  $-20\text{‰}$  to  $-35\text{‰}$  (mean  $-26\text{‰}$ ) [53], and that foliar  $\delta^{13}\text{C}$  in C3 desert plants ranges from  $-21\text{‰}$  to  $-29\text{‰}$  [54,55]. Consistent with these studies, we found here that leaf  $\delta^{13}\text{C}$  of the three *Caragana* species varied widely along the arid and semi-arid transect, ranging from  $-28.65\text{‰}$  to  $-24.77\text{‰}$  (mean  $-26.73\text{‰}$ ). In addition, populations of each species differed significantly in leaf  $\delta^{13}\text{C}$ , which is consistent with many other studies [6–10,25]. Generally, leaf  $\delta^{13}\text{C}$  is related to the ratio of  $\text{CO}_2$  partial pressure inside the leaf and ambient air ( $c_i/c_a$ ) and has been found to be strongly affected by many factors, such as precipitation [16], temperature [19], irradiance [56], and leaf intrinsic traits such as leaf N and P concentrations [33–35,40–42]. In our study, there were substantial differences in these factors across sampling sites for each species (Table 1, Table S1), which might account for the large variation in leaf  $\delta^{13}\text{C}$  among populations of each species.

Leaf  $\delta^{13}\text{C}$  for all *Caragana* species was negatively correlated with MAP (Figure 4a), which is consistent with findings from many previous studies [9,18,19,26]. This occurs because water limitation caused by low precipitation can lead to stomatal closure and lowered discrimination against the heavy isotope of  $\text{CO}_2$  ( $^{13}\text{C}$ ), thus leading to an increase in  $\delta^{13}\text{C}$  [57]. However, among the three species, *C. korshinskii* and *C. liouana* showed significant negative correlations with MAP while no significant correlation was found for *C. microphylla* (Figure 4b–d). One study has shown that leaf  $\delta^{13}\text{C}$  decreases with MAP, but only below a certain value. Above this value,  $\delta^{13}\text{C}$  shows no significant change [58]. However, the MAP ranges in regions where *C. microphylla* was observed in our study (from 325 to 434 mm) were much lower than the above-reported threshold point [58]. We thus speculate from this narrow precipitation range that water availability was not the key factor influencing WUE and plant growth for *C. microphylla*. This conclusion was further supported by the stepwise regression, which indicated that MAP was the main determinant for the variation of leaf  $\delta^{13}\text{C}$  for *C. korshinskii* and *C. liouana*, but not for *C. microphylla* (Table 4).

Temperature is another important climatic factor influencing plant photosynthesis, and thus leaf  $\delta^{13}\text{C}$  [14]. Previous studies have found either negative, positive, or inconclusive relationships between leaf  $\delta^{13}\text{C}$  and temperature [19,59,60]. In our present study, we found that leaf  $\delta^{13}\text{C}$  increased with MAT along the arid and semi-arid transect across all populations. In these regions, we found that MAT showed a significant negative correlation with MAP ( $r = -0.52$ ;  $p = 0.0056$ ). The increase in temperature, decrease in precipitation, and their interaction effect profoundly influenced leaf  $\delta^{13}\text{C}$ , resulting in reduced  $c_i$ , and thus increasing leaf  $\delta^{13}\text{C}$  [61]. Considering each species separately, only the leaf  $\delta^{13}\text{C}$  of *C. microphylla* showed a significant negative correlation with MAT (Figure 6). This result was inconsistent with findings from one previous study [19]. One explanation for such a negative correlation might be that the increased leaf thickness found in cold environments increases internal mesophyll resistance and reduces  $c_i/c_a$ , thus increasing  $\delta^{13}\text{C}$  values [14]. Also, half of the sites had MATs below  $5\text{ °C}$  and low temperatures increase the probability of cold-induced droughts that cause stomatal closure and increase  $\delta^{13}\text{C}$  [62]. *C. korshinskii* and *C. liouana* mainly occurred in regions with a narrow range (4 to  $8\text{ °C}$ ) of MAT, so their photosynthetic rates were probably less affected by temperature [19]. Furthermore, results from the stepwise regression confirmed that MAT was the main contributor to the variation of leaf  $\delta^{13}\text{C}$  in *C. microphylla*, but not for *C. korshinskii* or *C. liouana* (Table 4).

Leaf N and P concentrations have been previously reported to correlate positively with leaf  $\delta^{13}\text{C}$  because of their vital role in plant photosynthesis [29–34]. Nevertheless, our results indicated that leaf  $\delta^{13}\text{C}$  showed a negative relationship with N and P concentrations for all species, which has also been observed in several previous studies [28,40,41,63–65]. Moreover, the negative correlation between leaf  $\delta^{13}\text{C}$  and leaf N concentration is reported to be independent of functional group, vegetation type, and altitude, suggesting that this is a general trend for plants [66,67]. Other studies have interpreted the negative correlation between leaf  $\delta^{13}\text{C}$  and leaf N concentration as an autocorrelation due to low water availability in semi-arid environments [63,67]. The negative correlation between leaf  $\delta^{13}\text{C}$  and P

was likely linked to the movement of P from soil to root surfaces, which partially depends on the mass flow of the soil solution that is caused by plant transpiration [67–69]. However, we found that leaf  $\delta^{13}\text{C}$  showed no significant relationship with leaf N and P across sampling sites for either species (Table 3), which might imply that the variation in leaf  $\delta^{13}\text{C}$  values was more likely caused by stomatal limitations within each species, rather than N- and P-related changes in photosynthetic efficiency [67–70].

Altitudinal patterns of leaf  $\delta^{13}\text{C}$  are complicated because many interrelated abiotic factors vary with altitude, including temperature, precipitation, relative humidity, and atmospheric  $\text{CO}_2$  pressure, as well as with leaf intrinsic traits, like leaf N and P concentrations [8,12,14,71]. In agreement with many previous results [14,42,71], the leaf  $\delta^{13}\text{C}$  of the combined data and that of *C. microphylla* were positively correlated with altitude, but the leaf  $\delta^{13}\text{C}$  of *C. korshinskii* and *C. liouana* were unrelated to altitude (Figure 7), possibly because the growth range of *C. microphylla* covers a wide altitudinal gradient (227–1789 m asl) compared to the other two species (1039–1456 m asl for *C. korshinskii*; 893–1456 m asl for *C. liouana*). The leaf  $\delta^{13}\text{C}$  of all populations was positively correlated to elevation, which may have been because altitude was strongly negatively correlated with RH ( $r = -0.780$ ,  $p < 0.001$ ; Table 2). RH in turn showed a significant negative correlation with leaf  $\delta^{13}\text{C}$  (Figure 5). The positive correlation found between the leaf  $\delta^{13}\text{C}$  of *C. microphylla* and altitude was owed to altitude being significantly and negatively correlated with both MAT and RH (Table 2). Additionally, altitude showed no significant relationship with MAP, MAT, RH, and leaf N and P concentrations for *C. korshinskii* and *C. liouana*, likely accounting for the insignificant correlation between leaf  $\delta^{13}\text{C}$  and altitude (Table 2). Similar results were also found in a previous study [27].

#### 4.2. Difference in WUE between the Three Caragana Species: Implications for Their Geographical Distribution

Alternative distributions, a situation in which one species replaces another in an ecosystem geographically, is a common phenomenon in closely related plant species [45,72,73]. Still, a primary focus in ecology remains fixed on understanding the underlying mechanisms governing species' geographic distributions [74]. It is now generally accepted that the distribution patterns of plant species reflect an evolutionary response to long-term environmental change, which can be partially shaped by environmental gradients and partially by characteristics of a plant species [72–75]. Therefore, here we firstly determined the key environmental factors that control variation in the leaf  $\delta^{13}\text{C}$  of all species by relating leaf  $\delta^{13}\text{C}$  of all samples to MAP, MAT, RH, altitude, and leaf N and P, which are reported to be crucial factors influencing plant distribution [1,4,5,10,12,14,70]. The results indicated that, although leaf  $\delta^{13}\text{C}$  decreased significantly with MAP, RH, and leaf N and P concentrations and increased with MAT and altitude across all species, the multiple regressions suggest that MAP and RH are the primary determinant factors controlling the variation of leaf  $\delta^{13}\text{C}$  (Table 4). These results support previous conclusions that water availability, which is closely related to MAP and RH, appears to be a selective force playing an important role in shaping the distribution of *C. korshinskii*, *C. liouana*, and *C. microphylla* from east to west in northern China [53].

Secondly, to assess the impact of a species physiology on its spatial distribution, it is essential to compare tolerance in conditions of limited water availability between species. As previously reported, leaf  $\delta^{13}\text{C}$  is related to WUE and plants with higher  $\delta^{13}\text{C}$  or WUE tend to have a higher drought tolerance during periods of low water availability [10,16,19,23]. The present results indicated that the mean leaf  $\delta^{13}\text{C}$  was highest in *C. korshinskii*, intermediate in *C. liouana*, and lowest in *C. microphylla*, with the differences being significant (Figure 3), which is consistent with results from previous studies [51,52]. Although the growth range of *C. microphylla* occurs across a relatively wider altitudinal and temperature gradient, the pluviometric range is limited and tends to be higher than the growth ranges of the other two species, which probably results in lower values of  $\delta^{13}\text{C}$  for *C. microphylla* than in the other two species. The growth range of *C. korshinskii* appears to be characterized by lower precipitation than the area where *C. liouana* grows (Table 1, Table S1), which results in the highest values of leaf  $\delta^{13}\text{C}$  among the three species. In a previous study examining the ecophysiological responses of these three species to water stress [51], we also found that *C. korshinskii* exhibits lower

sensitivity of the photosynthetic rate and growth, a lower specific leaf area, higher biomass allocation to roots, and higher levels of water use efficiency to drought compared with the other two species. Taken together, these results support differentiation in drought tolerance among the three species because of long-term adaptation to distinct water availability conditions. *C. microphylla*, *C. liouana*, and *C. korshinskii* showed an increasing sequential drought tolerance, consistent with their distinct geographical distributions from east to west in northern China.

## 5. Conclusions

Across species, leaf  $\delta^{13}\text{C}$  significantly decreased with MAP, RH, and leaf N and P concentrations and increased with altitude and MAT. However, patterns and environmental controls of leaf  $\delta^{13}\text{C}$  varied with species; where *C. korshinskii* was mainly controlled by MAP and leaf N concentration, *C. liouana* was controlled by both MAT and MAP, and *C. microphylla* was mainly controlled by MAT. These findings are essential in improving predictions of how each species will respond to future climate change accompanied by increasing temperature and more frequent drought events in dry areas. Regardless of species, MAT and RH are important determinant factors influencing variation in leaf  $\delta^{13}\text{C}$  along the study transect. We further found that *C. microphylla*, *C. liouana*, and *C. korshinskii*, showed an increasing sequential drought tolerance, as reflected by their integrated WUE (leaf  $\delta^{13}\text{C}$ ) and consistent with their distinct geographical distributions from east to west in northern China.

**Supplementary Materials:** The following is available online at [www.mdpi.com/1999-4907/9/6/297/s1](http://www.mdpi.com/1999-4907/9/6/297/s1), Table S1: Spatial and climate data for each sampling site of the three *Caragana* species.

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