

## Cuticular waxes in alpine meadow plants: climate effect inferred from latitude gradient in Qinghai-Tibetan Plateau

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### Keywords

Adaptation, average chain length, ecosystem, herbs, *n*-alkane, soil, temperature.

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### Abstract

Alpine meadow ecosystems are susceptible to climate changes. Still, climate impact on cuticular wax in alpine meadow plants is poorly understood. Assessing the variations of cuticular wax in alpine meadow plants across different latitudes might be useful for predicting how they may respond to climate change. We studied nine alpine meadows in a climate gradient in the east side of Qinghai-Tibetan Plateau, with mean annual temperature ranging from  $-7.7$  to  $3.2^{\circ}\text{C}$ . In total, 42 plant species were analyzed for cuticular wax, averaged 16 plant species in each meadow. Only four plant species could be observed in all sampling meadows, including *Kobresia humilis*, *Potentilla nivea*, *Anaphalis lac-teal*, and *Leontopodium nanum*. The amounts of wax compositions and total cuticular wax in the four plant species varied among sampling meadows, but no significant correlation could be observed between them and temperature, precipitation, and aridity index based on plant species level. To analyze the variations of cuticular wax on community level, we averaged the amounts of *n*-alkanes, aliphatic acids, primary alcohols, and total cuticular wax across all investigated plant species in each sampling site. The mean annual temperature, mean temperature in July, and aridity index were significantly correlated with the averaged amounts of wax compositions and total cuticular wax. The average chain length of *n*-alkanes in both plant and soil linearly increased with increased temperature, whereas reduced with increased aridity index. No significant correlation could be observed between mean annual precipitation and mean precipitation from June to August and the cuticular wax amounts and average chain length. Our results suggest that the survival of some alpine plants in specific environments might be depended on their abilities in adjusting wax deposition on plant leaves, and the alpine meadow plants as a whole respond to climate change, benefiting the stability of alpine meadow ecosystem.

### Introduction

Alpine meadows are widely distributed in Qinghai-Tibetan Plateau, and they are considered to be sensitive to climate change. As the outmost surface of plant, cuticular wax directly contacts with the environment and also shows great sensitivity to climate change (Shepherd and Griffiths 2006). Cuticular wax plays pivotal physiological and ecological roles in the interactions between plants and their abiotic and biotic environments (Bouzoubaa et al. 2006). It inhibits the loss of water from leaf surface, limits water transportation into leaf, increases disease resistance, provides ultraviolet light protection to leaf tissue, and protects plants from air pollution (Pannell and Fields 2014). The responses of cuticular wax under different environmental

stresses have been shown to be an adaptive mechanism of plants (Whitfield et al. 1996; Dunbar-Co et al. 2009; Gonzalez and Ayerbe 2010). Therefore, assessing the variations of cuticular wax in alpine plant species across different latitudes might be useful for predicting how they may respond to changing climate.

Controlled experiments have shown that the wax production at lower temperature ( $15^{\circ}\text{C}$ ) was relatively higher than that at higher temperature ( $25^{\circ}\text{C}$ ; Baker 1974; Wolff and Morgan-Richards 1998, Kartini & Azminah, 2012). In another study with wild-type *Arabidopsis thaliana* and wax mutants, Ni et al. (2014) found that the amounts of wax accumulated significantly under cold stress ( $4^{\circ}\text{C}$ ), mainly attributing to significant increase in alkanes and secondary alcohols. Under the conditions of lower tem-

peratures, plants of *Brassica oleracea*, *B. kale*, and *B. napus* had more of the decarbonylation products (alkanes, secondary alcohols, and ketones) and less reductive products (free fatty acids, aldehydes, primary alcohols, and esters) than at higher temperature (Baker 1974; Ohsawa and Ide 2008; Kim and Donohue 2013). All these studies indicate that wax compositions differ in their responses to temperatures and the roles of different wax compositions might be also different. The plants in alpine meadow have short growing period and suffer periodic stresses from low temperatures in growing season. Subtle changes in mean annual temperature in these areas might shift phenological phases and alter plant reproduction and even community (Sala et al. 2000; Zubair et al. 2012). However, how cuticular wax production in alpine plants responses to climate change is still uncertainty.

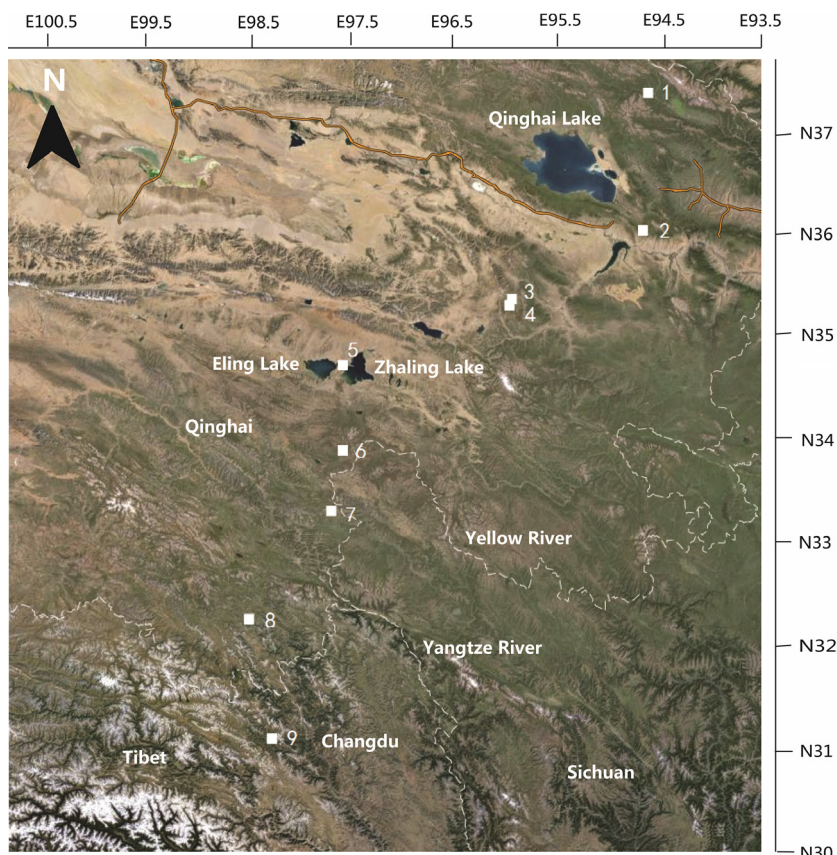
Plant cuticular waxes are typically mixtures of primary *n*-alcohols, *n*-aldehydes, and fatty acids as well as *n*-alkanes, each of these compound classes comprising a homologous series of chemicals with chain lengths ranging from 20 to almost 40 carbons (Jetter et al. 2006). Among them, long-chain *n*-alkanes in plants are widely used as biomarkers to track terrestrial climates and ecosystems in the geologic past to further our understanding of ancient environmental and climate change (Mosle et al. 1998; Ronsted et al. 2000; Nikolic et al. 2013). Duan and He (2011) reported that carbon preference index (CPI) and average chain length (ACL) of *n*-alkanes in plants from low latitudes were higher than those from high latitudes. Dodd and Poveda (2003) reported that greater ACL of *n*-alkanes in *Juniperus communis* at low and at high elevations was possibly a result of adaptation to minimize cuticular permeability due to high summer temperatures at low elevation and freezing causing physiological drought at high elevations. On the one hand, the difference in chain length distribution might be attributed to illumination difference at different sites (Ohsawa and Ide 2008; Kim and Donohue 2013). On the other hand, temperature might affect the decarbonylation pathways in charge of alkane synthesis. Zhang et al. (2004) reported that the concentrations of C<sub>27</sub>, C<sub>29</sub>, and C<sub>35</sub> in leaves of *Miscanthus sinensis* and that of C<sub>31</sub> in leaves of *Pleiblastus chino* were positively correlated with growing season temperature. In another study with *Austrocedrus chilensis*, Bush and McInerney (2013) reported that mean annual temperature was most significant for synthesis of the longer chain hydrocarbons. However, greater *n*-alkane ACL at both low and high elevation in the study of Dodd and Poveda (2003) indicated that the effects of temperature on chain length distribution might be complicated. Thus, variation in the abundances of long-chain-length *n*-alkane calls for a more directed examination of the effects of temperature on plant *n*-alkane distributions in natural environments (Duan and He 2011).

Unlike cultivated plants or trees as we stated above, the plants in alpine meadow are annual or perennial grasses and exist in a mixture of different populations. Climate warming alters plant diversity and species richness of alpine meadow (Chawla et al. 2008; Shimono et al. 2010; Chen et al. 2013), and different plant species differ in their responses to climate warming (Jonas and Geber 1999; Frei et al. 2014). Plant species also show difference in cuticular wax characteristics (Barthlott et al. 1998; Jetter et al. 2006); thus, their responses to climate change might also differ among plant species. So far, no studies exist, which address climate impacts on the responses of cuticular wax in alpine meadow plants. Therefore, in this study, we investigated how cuticular waxes in leaves of alpine meadow grasses respond to climate change along latitude gradients (N31–N37) in the east side of Qinghai-Tibetan Plateau. The plant species varied greatly along latitude gradients, and thus, we analyzed species diversity and sampled most of the plant species averaged 16 species in each meadow. Surface soil samples were also analyzed for *n*-alkane distribution to investigate the long-term response of cuticular wax in alpine meadow plants to climate change. Soil *n*-alkanes come from plants and are widely used in palaeoenvironmental research due to their relatively high preservation potential (Rommerskirchen et al. 2003; Shimono et al. 2010). Wang et al. (2008) collected 26 modern soil samples from various climate conditions and reported that the distributions of lipids molecules in modern soils in China recorded well signals of climates from quite different climatic regions. Specifically, we addressed the following two aspects: (1) How does cuticular wax in alpine meadow plants respond to climate change on plant species level? (2) Can *n*-alkane distribution in soils and plant community reflect the responses of alpine meadow plants to climate change?

## Materials and Methods

### Study area

The study was conducted on alpine meadows in the east side of the Qinghai-Tibetan Plateau (Fig. 1). In total, nine sampling areas were set on a transect from northeast (N37.43, E101.16) to southwest (N31.12, E96.35), with altitude ranging from 3447 to 4845 m (Table 1). In each sampling area, there were three sampling sites (100 × 200 m) with similar altitude as three replicates. The distance between sampling sites was beyond 1000 m. The region has a typical plateau continental climate, and the growing period of the plants only lasts 2–3 months from June to August. The mean annual temperature and the mean annual precipitation of the sampling regions range from −7.7 to 3.2°C and 303 to 566 mm, respec-



**Figure 1.** Map of the study area. White rectangles indicate sampling areas (1–9).

**Table 1.** Basic description of the sampling areas and the numbers of plant species sampled for wax extraction in each area.

Area	Latitude (N)	Longitude (E)	Altitude (m)	Annual temperature (°C)	Annual precipitation (mm)	No. of species sampled <sup>1</sup>
A1	37°43'	101°16'	3482	−2.0	560	18
A2	36°16'	101°12'	3809	−2.8	324	15
A3	35°52'	099°56'	3447	1.8	379	18
A4	35°50'	099°55'	3759	0.0	379	14
A5	34°54'	099°31'	4589	−5.9	303	18
A6	34°07'	097°39'	4845	−7.7	303	13
A7	33°51'	096°55'	4015	−4.3	517	13
A8	32°37'	096°33'	3929	3.2	542	22
A9	31°12'	096°35'	3871	2.6	566	19

<sup>1</sup>The number of species sampled was the number of plant species qualified for leaf wax extraction in each sampling site.

tively. About 60–70% of the precipitation was in plant growing period. The alpine meadows were owned by local farmers and used mainly for grazing yak and Tibetan sheep. Parts of the meadows were overgrazed and degraded severely (Mazurek and Simoneit 1997). In this study, only meadows with good conditions in fenced pasture were sampled. Plant compositions in alpine meadow varied greatly at different habitats (Wang et al. 2008); therefore, we only investigated the meadows with *Kobresia humilis* as one of the predominant plant species. The vegetation coverage of the nine sampling areas was all above 90%, and the dominant plant species included *K. humilis*,

*K. parva*, *K. graminifolia*, *Leontopodium nanum*, *Potentilla nivea*, *Anaphalis lacteal*, *Taraxacum mongolicum*, *Polygonum viviparum*, *P. macrophyllum*, *Lancea tibetica*, *Carex atrofusca*, and *Thalictrum alpinum*. The soil type was alpine meadow soil (Cambisol, FAO).

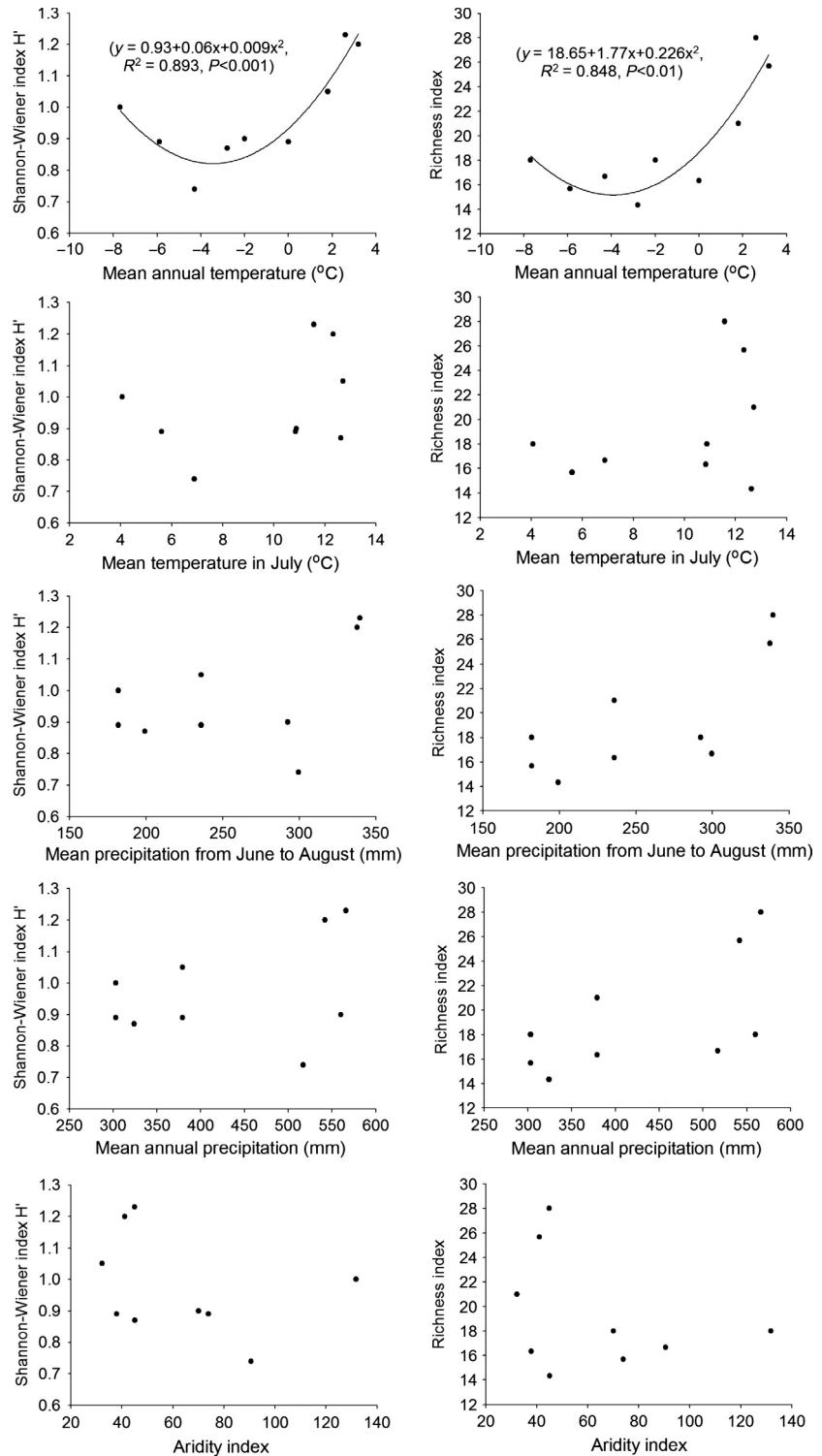
### Species richness and diversity

In each sampling site, the numbers of plant species and plant density of each species were counted in five quadrats (1 × 1 m) randomly distributed to calculate the Shannon–Wiener index ( $H'$ ) using the following equation:

$$H' = - \sum P_i \ln P_i$$

Species richness is the number of different species represented in 1-m<sup>2</sup> area.

where  $P_i$  is the relative plant density of the  $i$  species.



**Figure 2.** Relationship between climate factors and plant species richness index and Shannon–Wiener index ( $H'$ ).

### Collecting leaf samples

Based on the results of the vegetation investigation, the plant compositions between sampling areas were not exactly the same. Therefore, most of the plant species in each sampling area were collected from three sampling sites separately. A total of 450 leaf samples were collected from 27 sampling sites. Leaf samples from four plant species could be obtained in all sampling areas, including *K. humilis*, *P. nivea*, *A. lacteal*, and *L. nanum*. In each site, at least thirty expanded healthy leaves were sampled randomly from 20 to 30 plants. For plants with small leaves, such as species from *Kobresia* and *T. alpinum*, the numbers of the leaves were beyond 100. The leaves were collected within 14 days in July 2013. To avoid the difference of wax deposition in different plant development stages, we sampled from vegetative plants. The leaves were washed gently in water to exclude dusts on leaf surface and stored in absorbent papers. The absorbent papers were changed every other day until the leaves were fully dried without going moldy.

### Collecting soil samples

Soils were sampled in 0–20 cm soil layer from the quadrat used for vegetation investigation. Before soil sampling, 0–5 cm soil layer was removed to reduce plant litters. In total, 10 subsamples (two in each quadrat) were composited together in each sampling site, approximately 700 g. The soils free of plant residues and roots were air-dried and stored at <4°C.

### Cuticular wax extraction

Dried leaves were used to extract cuticular waxes. In a pre-experiment study, the dried leaves were extracted in chloroform for 30s, 50s, 60s, and 80s. The results showed that 60s was sufficient to extract most of the cuticular waxes on plant leaves. Therefore, in this study, approximately 10–20 leaves (depending on leaf size) were extracted in 50 mL chloroform containing 25 µg hexadecane as internal standard at room temperature for 1 min. The extracts were dried in a nitrogen stream at 40°C and derivated using 50 µL of BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) for 20 min at 100°C. The surplus BSTFA was evaporated under nitrogen, and the sample was redissolved in 1 mL *n*-hexane for GC and GC/MS analysis for wax compositions.

### Soil lipid extraction

Approximately 20 g dried soil samples were sieved (<250 µm) and subject to Soxhlet extraction using

dichloromethane/methanol (DCM/MeOH, 9:1 v/v) for 24 h, as reported by Naafs et al. (2004). The extract was then rotary evaporated and purified over a Na<sub>2</sub>SO<sub>4</sub> column. The dry extract was dissolved in DCM/isopropanol (2:1 v/v) and filtered using a Pasteur pipette packed with defatted wool, 0.5 cm Na<sub>2</sub>SO<sub>4</sub>, and 2 cm SiO<sub>2</sub> and dried in nitrogen stream. All samples were dissolved in 0.5 mL *n*-hexane for GC analysis.

### GC analysis

The GC analysis was carried out with 9790 II gas chromatograph (Zhejiang Fuli Analytic Instruments Co., Wenlin, China). The GC column was DM-5 30 m × 0.32 mm × 0.25 µm capillary column (Dikma Technologies Inc. Beijing, China). Nitrogen was served as the carrier gas. The injector and FID detector temperatures were set at 300 and 320°C, respectively. The oven temperature of GC was programmed with an original temperature of 80°C and increased at 15°C/min to 260°C, where the temperature remained 10 min. The temperature was then increased at 2°C/min to 290°C and further increased at 5°C/min to 320°C, where the temperature remained 10 min.

Quantification was based on FID peak areas. Amounts of *n*-alkanes, acids, alcohols, and other wax compositions per unit leaf area were calculated by the hexadecane. After wax extraction, the surface areas of leaves were measured with a WinFOLIA professional leaf image analysis system (Regent Instrument Inc, Quebec, Canada) and digitizing scanner (EPSON V750, Magano-ken, Japan). The amount of wax was expressed in µg/cm<sup>2</sup>.

To assure the authenticity of aliphatic acids, primary alcohols, and *n*-alkanes, samples were also run in GC/MS-GP2010 (Shimadzu Technologies Co., Kyoto, Japan), and commercialized standards were used as outer standard.

### Calculation of CPI and ACL values

Various indices, derived from the concentration data, were used to characterize the wax composition distributions. The CPI of *n*-alkanes was calculated using the Mazurek and Simoneit (1997) equation as follows:

$$\text{CPI}_{\text{total}} = \frac{1}{2} \left( \frac{\sum \text{odd } C_{n(17-31)}}{\sum \text{even } C_{n(18-32)}} + \frac{\sum \text{odd } C_{n(19-33)}}{\sum \text{even } C_{n(18-32)}} \right)$$

where *C* is the relative content of *n*-alkanes and *n* is the carbon number.

The total ACL was calculated using the Poynter and Eglinton (1990) equation.

$$\text{ACL}_{\text{total}} = \frac{\sum C_n \times n}{\sum C_n}$$

## Statistical analysis

The average annual temperature for each sampling meadow was calculated from 20 years measured air temperature data from the nearest climate stations (situated 5–70 km away) using a lapse rate of 0.6°C per 100 m elevation (Poynter and Eglinton 1990). The data were obtained from the mean values of three replicates. The relative abundance of *n*-alkanes (acids or primary alcohols) was calculated by the weight percentages of every *n*-alkane (acid or primary alcohol) homolog in all *n*-alkanes (acids

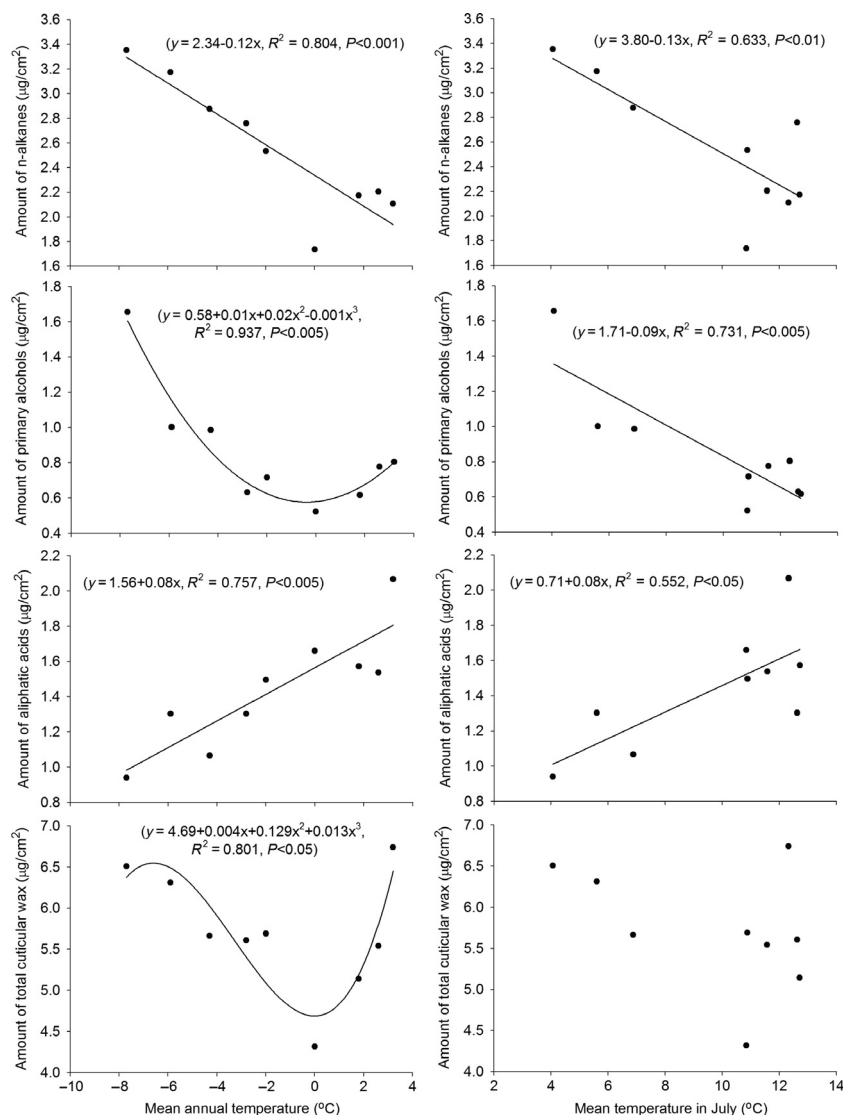
or primary alcohols). Regression analysis was conducted between climate factors (estimated mean annual temperature, mean temperature in July, mean annual precipitation, mean rainfall from June to August, and aridity index) and the amounts of total cuticular wax and wax compositions, *n*-alkanes ACL and CPI in soils and leaf cuticular wax, and plant species richness and Shannon–Wiener index (SigmaPlot 10.0; SPSS Inc. Chicago, USA). An aridity index was calculated as  $I = P/(T + 10)$ , where *P* is the annual precipitation in mm and *T* is the mean annual temperature in degrees centigrade (Dodd and Poveda 2003).

**Table 2.** Amounts of cuticular waxes in leaves of *Kobresia humilis*, *Potentilla nivea*, *Anaphalis lacteal*, and *Leontopodium nanum* along latitude gradients.

	<i>n</i> -Alkanes	Aliphatic acids	Primary alcohol	Others <sup>1</sup>	Total
<i>Kobresia humilis</i>					
A1	1.28 ± 0.07 <sup>a,b</sup>	0.33 ± 0.12 <sup>a</sup>	0.85 ± 0.04 <sup>a,b</sup>	1.93 ± 0.16 <sup>a,b</sup>	4.40 ± 0.21 <sup>a</sup>
A2	1.81 ± 0.42 <sup>a</sup>	0.29 ± 0.05 <sup>a</sup>	0.82 ± 0.04 <sup>a,b</sup>	2.30 ± 0.50 <sup>a</sup>	5.22 ± 1.13 <sup>a</sup>
A3	0.94 ± 0.03 <sup>b</sup>	0.35 ± 0.02 <sup>a</sup>	0.59 ± 0.01 <sup>b</sup>	1.28 ± 0.13 <sup>a,b</sup>	3.15 ± 0.08 <sup>a</sup>
A4	1.41 ± 0.35 <sup>a,b</sup>	0.16 ± 0.02 <sup>a</sup>	0.50 ± 0.04 <sup>b</sup>	1.88 ± 0.51 <sup>a,b</sup>	3.94 ± 1.05 <sup>a</sup>
A5	0.86 ± 0.08 <sup>b</sup>	0.33 ± 0.11 <sup>a</sup>	0.67 ± 0.02 <sup>b</sup>	0.93 ± 0.29 <sup>b</sup>	2.78 ± 0.13 <sup>a</sup>
A6	0.82 ± 0.23 <sup>b</sup>	0.40 ± 0.18 <sup>a</sup>	1.30 ± 0.02 <sup>a</sup>	1.97 ± 0.63 <sup>a,b</sup>	4.49 ± 1.16 <sup>a</sup>
A7	1.90 ± 0.38 <sup>a</sup>	0.55 ± 0.34 <sup>a</sup>	0.28 ± 0.05 <sup>b</sup>	0.88 ± 0.08 <sup>b</sup>	3.61 ± 0.60 <sup>a</sup>
A8	1.57 ± 0.11 <sup>a,b</sup>	0.62 ± 0.28 <sup>a</sup>	0.54 ± 0.02 <sup>b</sup>	1.29 ± 0.45 <sup>a,b</sup>	4.03 ± 0.83 <sup>a</sup>
A9	1.37 ± 0.31 <sup>a,b</sup>	0.20 ± 0.02 <sup>a</sup>	0.46 ± 0.13 <sup>b</sup>	1.68 ± 0.20 <sup>a,b</sup>	4.40 ± 0.21 <sup>a</sup>
<i>Potentilla nivea</i>					
A1	1.90 ± 0.36 <sup>b</sup>	0.65 ± 0.17 <sup>b</sup>	0.27 ± 0.02 <sup>c</sup>	0.87 ± 0.08 <sup>c</sup>	3.68 ± 0.16 <sup>b</sup>
A2	4.95 ± 0.20 <sup>a</sup>	2.24 ± 0.62 <sup>a</sup>	0.59 ± 0.04 <sup>b,c</sup>	3.64 ± 0.35 <sup>a</sup>	11.41 ± 0.72 <sup>a</sup>
A3	1.46 ± 0.18 <sup>b</sup>	0.47 ± 0.15 <sup>b</sup>	0.19 ± 0.06 <sup>b,c</sup>	0.62 ± 0.01 <sup>c</sup>	2.74 ± 0.01 <sup>b</sup>
A4	1.64 ± 0.24 <sup>b</sup>	1.08 ± 0.12 <sup>b</sup>	0.32 ± 0.02 <sup>b,c</sup>	0.84 ± 0.07 <sup>c</sup>	3.89 ± 0.28 <sup>b</sup>
A5	2.44 ± 0.48 <sup>b</sup>	0.33 ± 0.12 <sup>b</sup>	0.38 ± 0.05 <sup>b,c</sup>	1.09 ± 0.39 <sup>c</sup>	4.24 ± 0.96 <sup>b</sup>
A6	6.01 ± 1.26 <sup>a</sup>	0.97 ± 0.09 <sup>b</sup>	1.43 ± 0.29 <sup>a</sup>	2.77 ± 0.17 <sup>b</sup>	11.18 ± 1.54 <sup>a</sup>
A7	2.22 ± 0.64 <sup>b</sup>	0.43 ± 0.09 <sup>b</sup>	0.76 ± 0.25 <sup>b,c</sup>	1.37 ± 0.26 <sup>c</sup>	4.78 ± 1.22 <sup>b</sup>
A8	1.36 ± 0.16 <sup>b</sup>	0.58 ± 0.20 <sup>b</sup>	0.32 ± 0.08 <sup>b,c</sup>	0.75 ± 0.12 <sup>c</sup>	3.01 ± 0.28 <sup>b</sup>
A9	2.17 ± 0.52 <sup>b</sup>	0.81 ± 0.35 <sup>b</sup>	0.67 ± 0.23 <sup>b,c</sup>	1.39 ± 0.33 <sup>c</sup>	5.04 ± 1.31 <sup>b</sup>
<i>Anaphalis lacteal</i>					
A1	5.43 ± 0.71 <sup>c</sup>	0.46 ± 0.06 <sup>b,c,d</sup>	0.52 ± 0.09 <sup>c,d</sup>	5.84 ± 0.82 <sup>c</sup>	12.25 ± 1.69 <sup>b</sup>
A2	3.40 ± 0.28 <sup>d,e</sup>	0.65 ± 0.21 <sup>b,c</sup>	0.75 ± 0.05 <sup>c</sup>	2.31 ± 0.01 <sup>d,e</sup>	7.12 ± 0.45 <sup>c,d</sup>
A3	0.69 ± 0.22 <sup>g</sup>	0.07 ± 0.02 <sup>e</sup>	0.05 ± 0.02 <sup>e</sup>	0.31 ± 0.10 <sup>f</sup>	1.12 ± 0.36 <sup>f</sup>
A4	3.90 ± 0.47 <sup>d</sup>	0.51 ± 0.12 <sup>b,c,d</sup>	0.43 ± 0.12 <sup>d</sup>	1.79 ± 0.48 <sup>d,e,f</sup>	6.63 ± 1.08 <sup>c,d</sup>
A5	1.95 ± 0.21 <sup>f</sup>	0.29 ± 0.11 <sup>d,e</sup>	0.33 ± 0.02 <sup>d</sup>	0.74 ± 0.06 <sup>e,f</sup>	3.31 ± 0.41 <sup>e,f</sup>
A6	5.21 ± 0.22 <sup>c</sup>	0.36 ± 0.05 <sup>c,d,e</sup>	0.53 ± 0.01 <sup>c,d</sup>	2.86 ± 0.67 <sup>d</sup>	8.97 ± 0.91 <sup>c</sup>
A7	2.26 ± 0.43 <sup>e,f</sup>	0.69 ± 0.11 <sup>b</sup>	0.52 ± 0.11 <sup>c,d</sup>	1.85 ± 0.27 <sup>d,e,f</sup>	5.33 ± 0.92 <sup>d,e</sup>
A8	7.99 ± 0.14 <sup>b</sup>	1.63 ± 0.03 <sup>a</sup>	1.93 ± 0.07 <sup>a</sup>	12.64 ± 0.96 <sup>a</sup>	24.21 ± 0.73 <sup>a</sup>
A9	12.79 ± 0.42 <sup>a</sup>	1.33 ± 0.03 <sup>a</sup>	1.31 ± 0.09 <sup>b</sup>	7.93 ± 0.16 <sup>b</sup>	23.37 ± 0.33 <sup>a</sup>
<i>Leontopodium nanum</i>					
A1	8.62 ± 1.23 <sup>a</sup>	0.67 ± 0.22 <sup>b</sup>	0.18 ± 0.02 <sup>b</sup>	2.29 ± 0.55 <sup>a,b</sup>	11.81 ± 1.87 <sup>a,b</sup>
A2	4.08 ± 0.52 <sup>d</sup>	0.45 ± 0.17 <sup>b</sup>	0.15 ± 0.04 <sup>b</sup>	1.31 ± 0.32 <sup>c,d</sup>	6.02 ± 1.03 <sup>d</sup>
A3	8.40 ± 1.16 <sup>a</sup>	0.71 ± 0.33 <sup>a,b</sup>	0.19 ± 0.15 <sup>b</sup>	2.72 ± 0.26 <sup>a,b</sup>	12.36 ± 1.67 <sup>a</sup>
A4	4.71 ± 0.25 <sup>c,d</sup>	0.39 ± 0.02 <sup>b</sup>	0.06 ± 0.00 <sup>b</sup>	1.22 ± 0.16 <sup>d</sup>	6.40 ± 0.42 <sup>c,d</sup>
A5	6.82 ± 0.80 <sup>a,b,c</sup>	0.54 ± 0.01 <sup>b</sup>	0.07 ± 0.04 <sup>b</sup>	1.39 ± 0.15 <sup>c,d</sup>	8.89 ± 0.97 <sup>b,c,d</sup>
A6	5.87 ± 0.04 <sup>b,c,d</sup>	0.32 ± 0.14 <sup>b</sup>	0.20 ± 0.03 <sup>b</sup>	1.35 ± 0.02 <sup>c,d</sup>	7.76 ± 0.12 <sup>c,d</sup>
A7	6.46 ± 0.11 <sup>a,b,c</sup>	1.17 ± 0.09 <sup>a</sup>	0.41 ± 0.02 <sup>a</sup>	2.86 ± 0.21 <sup>a,b</sup>	11.03 ± 0.39 <sup>a,b</sup>
A8	6.63 ± 0.37 <sup>a,b,c</sup>	0.47 ± 0.02 <sup>b</sup>	0.14 ± 0.01 <sup>b</sup>	2.18 ± 0.06 <sup>b,c</sup>	9.46 ± 0.42 <sup>a,b,c</sup>
A9	7.39 ± 0.48 <sup>a,b</sup>	0.23 ± 0.09 <sup>b</sup>	0.15 ± 0.01 <sup>b</sup>	3.15 ± 0.32 <sup>a</sup>	11.01 ± 0.32 <sup>a,b</sup>

Data followed with different small letters showed significance at  $P < 0.05$  according to least significant difference (LSD) test.

<sup>1</sup>Others were the sum of secondary alcohols, ketones, aldehydes, esters, and unidentified.



**Figure 3.** Relationship between temperature and the amounts of *n*-alkanes, aliphatic acids, primary alcohols, and total cuticular wax across plants in alpine meadows along latitude gradients.

## Results

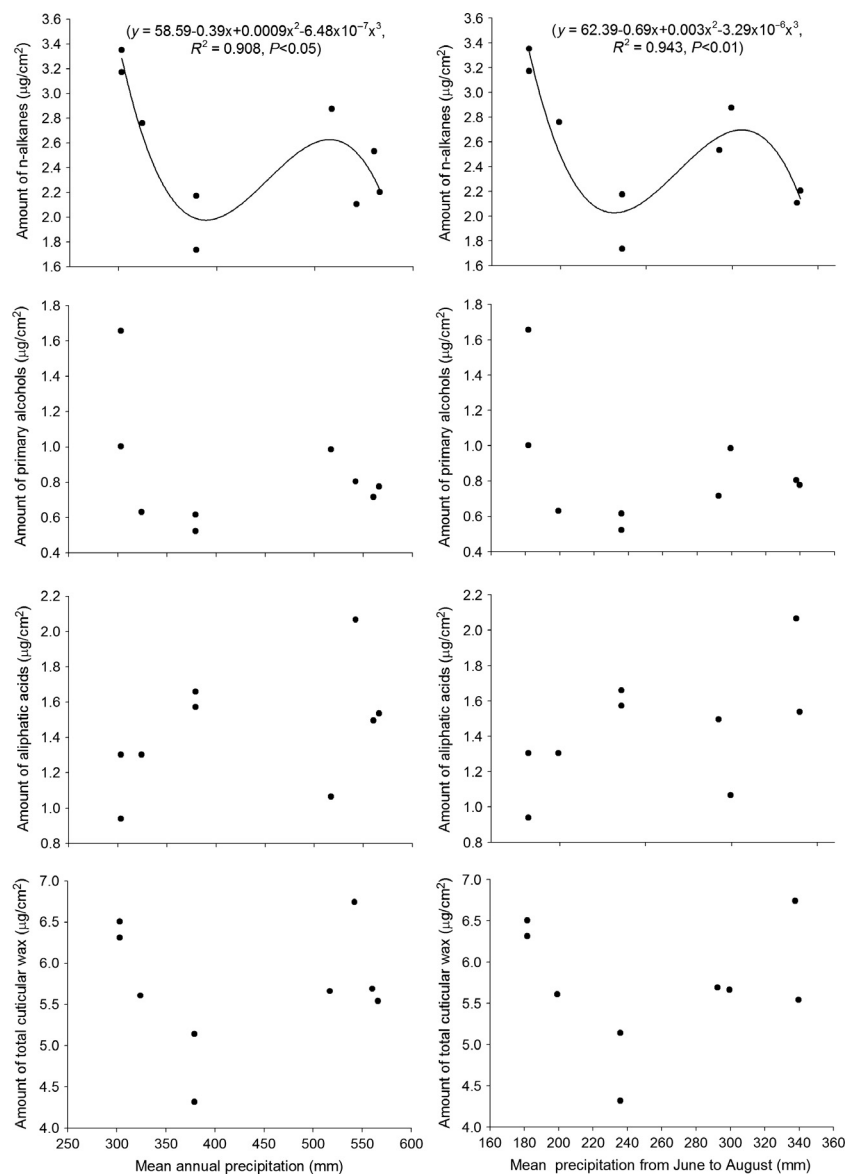
### Plant species richness and diversity

Although alpine meadows were sampled according to latitude gradients from N37.43 to N31.12, the values of the environmental factors of the sampling meadows depended upon both latitude and altitude (Table 1). The estimated mean annual temperature ranged from  $-7.7^{\circ}\text{C}$  (4845 m, N34) to  $3.2^{\circ}\text{C}$  (3929 m, N32), whereas the mean annual precipitations ranged from 303 (4854 m, N34) to 566 mm (3871 m, N31). The plant community composition differed among sampling areas with species richness ranging from 28 (3871 m, N31) to 16 (3809 m, N36). Only four plant species could be observed in all sampling areas, including *K. humilis*, *P. nivea*, *A. lacteal*, and *L. nanum*. *K. humilis* was one of the most dominant plant species in

all sampling areas. The estimated mean annual temperature was positively correlated with the Shannon–Wiener index ( $H'$ ) ( $R^2 = 0.893$ ,  $P < 0.001$ ) and species richness ( $R^2 = 0.848$ ,  $P < 0.01$ ) (Fig. 2). No significant correlation was observed between plant diversity and mean annual precipitation, mean precipitation from June to August, mean temperature in July, and aridity index.

### Cuticular waxes based on species level

Plant species differed significantly in their amounts of cuticular waxes (Table 2). Among the four plant species in Table 2, weight proportion of *n*-alkanes in leaves of *L. nanum* was the highest (70.22%), followed by *P. nivea* (48.55%) and *A. lacteal* (51.25%), with *K. humilis* the least (34.86%). The average weight proportion of aliphatic acids and primary alcohols across four plant species



**Figure 4.** Relationship between precipitation and the amounts of *n*-alkanes, aliphatic acids, primary alcohols, and total cuticular wax across plants in alpine meadows along latitude gradients.

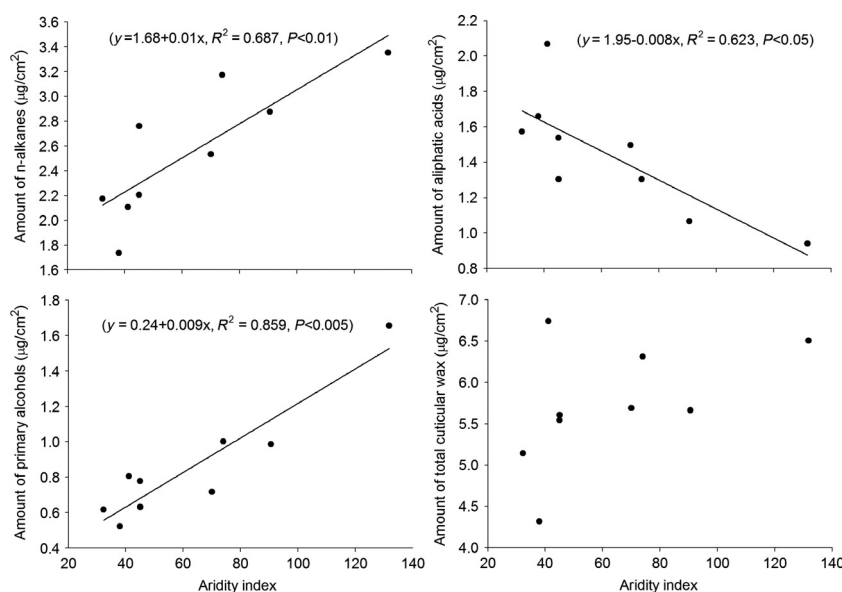
ranged from 15.70 to 5.79 and 16.95 to 1.85%, respectively. The amounts of wax compositions and total cuticular wax in the four plant species varied among sampling areas, but no significant correlation could be observed between them and the climate factors (Table 2, Figs S1–S4). The difference in total cuticular wax amount reached 21 times between the lowest and the highest for *A. lacteal*, four times for *P. nivea*, and two times for *L. nanum* and *K. humilis*, across nine sampling areas.

### Cuticular waxes based on community level

In total, 42 plant species were analyzed for cuticular wax, averaged 16 plant species in each sampling area

(Table S1). To analyze the variations of cuticular wax on community level, we averaged the amounts of *n*-alkanes, aliphatic acids, primary alcohols, and total cuticular wax across all investigated plant species in each sampling site. Regression analysis indicated that the estimated mean annual temperature and mean temperature in July were significantly correlated with the averaged amounts of *n*-alkanes, aliphatic acids, primary alcohols, and total cuticular wax, except for insignificant relationship between mean temperature in July and total cuticular wax amounts (Fig. 3). Overall, the amounts of *n*-alkanes and primary alcohols reduced, whereas the amount of aliphatic acids rose with increased temperatures. The amount of total cuticular wax reduced first and then





**Figure 5.** Relationship between aridity index and the amounts of *n*-alkanes, aliphatic acids, primary alcohols, and total cuticular wax across plants in alpine meadows along latitude gradients.

increased. There existed no significant relationship between mean annual precipitation and mean precipitation from June to August and the amounts of cuticular wax compositions except for *n*-alkane (Fig. 4). The amounts of *n*-alkanes and primary alcohols linearly increased, whereas the amount of acids decreased with increased aridity index (Fig. 5). No relationship was observed between total cuticular wax amounts and aridity index.

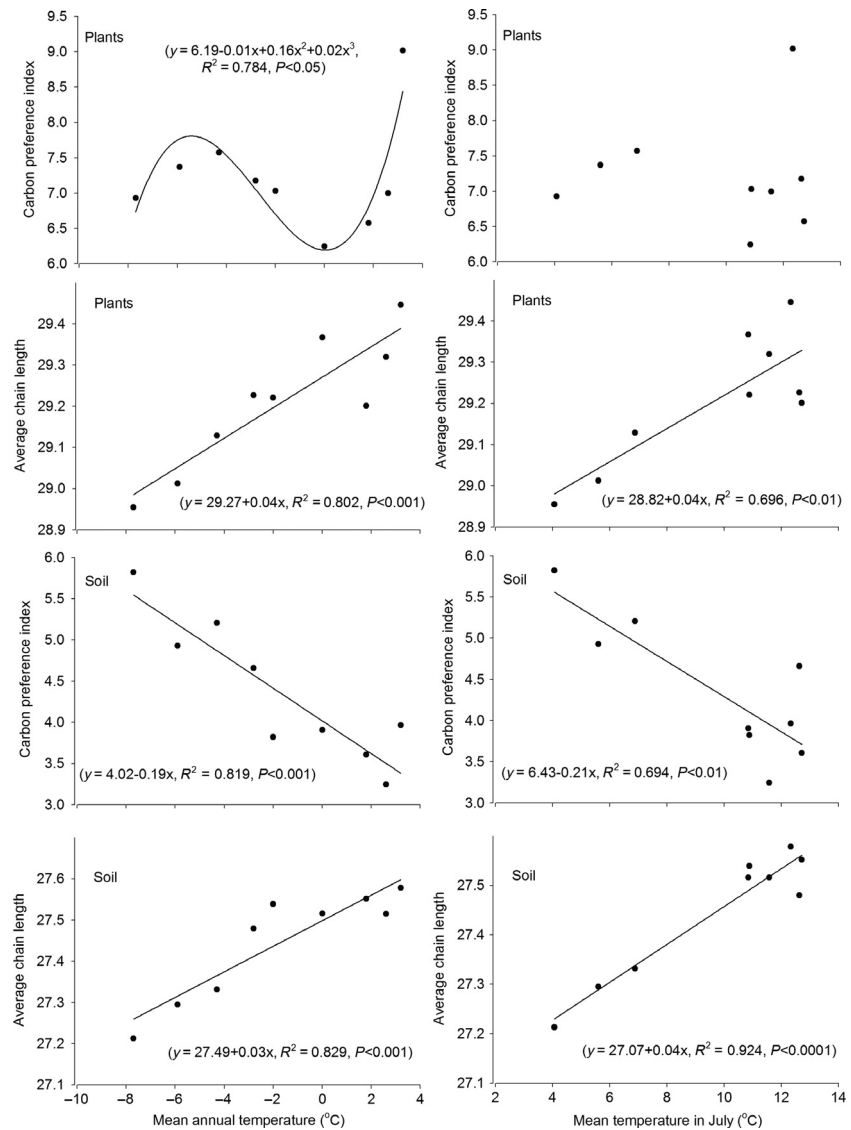
### Average chain length and CPI of *n*-alkanes in plants and soils

The ACL and CPI of *n*-alkane in plants were calculated across all investigated plant species in each sampling area. The ACL in plants ranged from 28.95 to 29.44, and strong positive linear correlation was observed between ACL and the estimated mean annual temperature and mean temperature in July (Fig. 6). The CPI in plants increased from 6.93 to 7.51 when mean annual temperatures increased from  $-7.7$  to  $-4.3^\circ\text{C}$ , then decreased first to 6.57 at  $0^\circ\text{C}$ , and followed by an increase to 9.53 at  $3.2^\circ\text{C}$ , showing significant cubic correlation ( $R^2 = 0.784$ ,  $P < 0.05$ ). The CPI and ACL in soil ranged from 3.25 to 5.82 and 27.21 to 27.58, respectively, relatively lower than in plants. The CPI in soil linearly decreased, whereas the ACL increased with increased temperatures. There existed no significant relationship between CPI and ACL in plants and soils and mean annual precipitation and mean precipitation from June to August (Fig. 7). The CPI linearly increased, whereas the ACL decreased with increased aridity index except for insignificant relationship between aridity and CPI in plants (Fig. 8).

## Discussion

### Responses of plant leaf cuticular wax to climate factors on plant species level

Development of a cuticular wax layer was fundamental to the successful colonization of land by plants (Jetter et al. 2006). In this study, 450 plant samples were analyzed for cuticular wax, belonging to 42 plant species along a latitude gradient. The plants showed great difference in their amounts of wax compositions and total cuticular wax among species. For example, average amount of *n*-alkanes in leaves of *L. nanum* reached  $6.55 \mu\text{g}/\text{cm}^2$ , whereas that was only  $1.32 \mu\text{g}/\text{cm}^2$  in *K. humilis*. These plant species were widely distributed in alpine meadow and showed great adaptation to local environments (Sun et al. 2014); therefore, their adaptabilities to alpine environments could not be distinguished from the difference in cuticular wax amount. However, the cuticular wax amounts varied among sampling areas within plant populations. This suggested that plants might adapt to different environments by altering wax depositions. Leaf waxes of *Dryas octopetala* and *Saxifraga oppositifolia* from snow-free, wind-swept microsites had significantly higher abundances of *n*-alkanes than in those plants growing in adjacent, swale areas where snow accumulates in winter (Li et al. 2012). Under controlled environmental conditions, the variations of cuticular wax amount and compositions have been shown to be related to their adaptations (Whitecro and Armstrong 1972; Shepherd et al. 1995; Dodd et al. 1998; Gordon et al. 1998). In this study, based on individual plant population for the

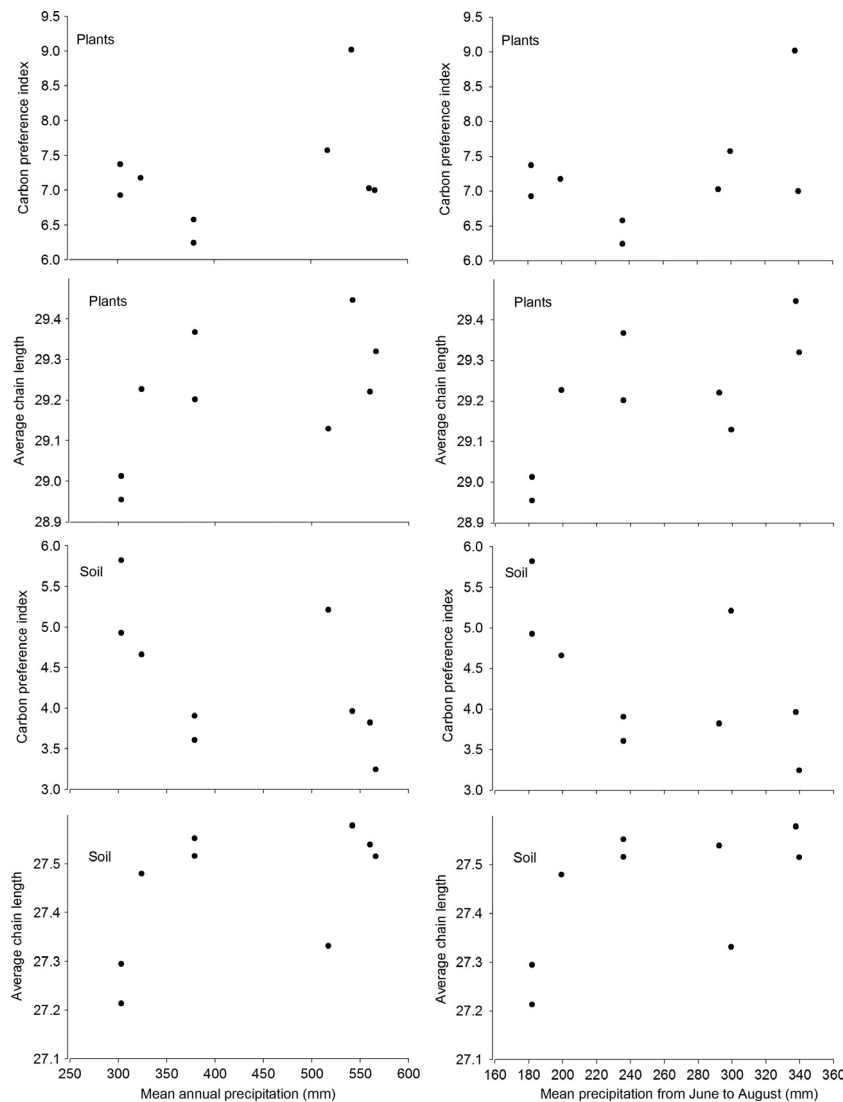


**Figure 6.** Relationship between temperature and carbon preference index and average chain length of *n*-alkane across plants and soils in alpine meadows along latitude gradients.

four plant species distributed in all sampling areas, no significant relationship could be observed between the amounts of wax compositions and the mean annual temperatures, mean temperature in July, mean annual precipitation, mean precipitation from June to August, and aridity index. Therefore, such variations could not simply be attributed to the variations of individual climate factor. On the one hand, the possible influence of one climate factor on wax deposition might be neutralized by the effects of other factors in field. As reviewed by Shepherd and Griffiths (2006), irradiation, temperature, humidity, water, and salinity would all influence wax depositions. This suggested that the wax deposition might be a comprehensive response of plant to changeable environmental conditions. On the other hand, these plant species might somewhat be tolerant to

the environmental changes under natural conditions, partially attributing to their relatively stable wax depositions, or the selection pressure caused by latitude and altitude differences might not be big enough to alter wax deposition on these plant species.

Our findings on alpine meadow grasses could not support the results from the tresses (shrubs) or cultivated plants. No significant increase in wax amount was produced at lower temperatures. Alpine meadow plants confront fluctuating weathers, and the temperature might be minus during growing season. Under such complicated environments, the variations of cuticular wax might not be adapted to changing mean annual temperature in alpine meadow. We concluded that, for the four investigated grasses, the climate change will not necessarily lead to a shift of wax deposition on leaves of alpine plants.

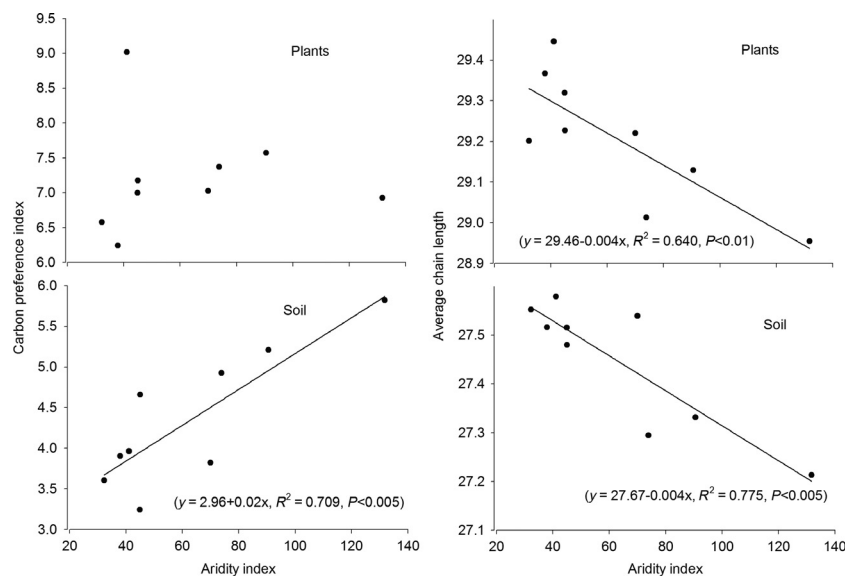


**Figure 7.** Relationship between precipitation and carbon preference index and average chain length of *n*-alkane across plants and soils in alpine meadows along latitude gradients.

### Responses of plant leaf cuticular wax to climate factors on plant community level

As plant species differed among nine sampling sites, we averaged the amounts of cuticular waxes across all investigated plant species in each sampling site, aiming to analyze the variation of cuticular wax on community level along latitude and altitude gradients. Results from regression analysis indicated that the amounts of wax compositions and total cuticular wax were influenced more by temperature than precipitation. No significant relationship was observed between mean annual precipitation and mean precipitation from June to August and the amounts of cuticular wax compositions except for *n*-alkane. However, the amounts of *n*-alkanes and primary alcohols reduced, whereas the amount of aliphatic acids rose with increased temperatures, while the amount of total cuticular wax

reduced first and then increased. Such variations suggested that deposition of *n*-alkanes and primary alcohols might be sensitive to environmental temperatures on plant community level, and more deposition at severely lower temperature might attribute to plant adaptation. Such relationship as well as the insignificant relationship based on plant species level as we discussed above implied that appearance of specific plant species in certain habitat might be benefitted from their wax deposition for alpine meadow plants. These plants might be sensitive to environmental changes and their distribution might be influenced by the ability in adjusting wax deposition. Controlled experiments have shown that plants adjust their wax deposition to changing environments to improve their adaptation (Whitecro and Armstrong 1972; Shepherd and Griffiths 2006; Yeats and Rose 2013). The different responses of cuticular wax compositions to changing climates also suggested that the



**Figure 8.** Relationship between aridity index and carbon preference index and average chain length of *n*-alkane across plants and soils in alpine meadows along latitude gradients.

potential roles of the cuticular wax compositions might differ in improving plant adaptation. In a study with *Picea abies*, Gordon et al. (1998) reported that the proportion of nonacosane diols increased, whereas that of alkyl esters decreased with increasing UV-B dose. Controlled environment experiments showed that velvetleaf plants grown under drought stress or low-temperature treatments had greater leaf cuticular wax deposition compared to plants grown in soil with moisture at field capacity or a high-temperature regime (Hatterman-Valenti et al. 2011). In this study, we also observed that the amounts of *n*-alkanes and primary alcohols linearly increased, whereas the amount of acids decreased with increased aridity index. The increase in *n*-alkane amount was observed in many plants subjected to drought stresses, attributing to their adaptation (Kim et al. 2007; Kosma et al. 2009). This suggested that the aridity index also played important roles in governing wax depositions for alpine plants. Alpine plants have evolved to exist in fluctuating weathers which are rarely ideal for maintenance of normal physiology (Thomas et al. 2004), and thus, formation of specific adaptive mechanism is the prerequisite for alpine plants to survive (Hoerandl and Emadzade 2011; Kim and Donohue 2013; Ma et al. 2015). Based on community level, we concluded that the survival of some alpine plants in certain environment might be depended on their abilities in adjusting wax deposition on plant leaves.

Based on community level, we also found that the ACL in plants linearly rose with increased mean annual temperature and mean temperature in July, whereas CPI showed cubic relationship with mean annual temperature. On the one hand, higher ACL at higher temperature was consistent with the results of other studies (Duan and He 2011; Bush and McInerney 2013), mainly attributing to

the increase of long-chain-length *n*-alkanes at higher temperature. On the other hand, the variations of CPI indicated that the responses of odd and even number carbon alkanes depended upon the temperature. In this study, some plant species appeared in alpine meadows were rare, their leaves were difficult to be sampled for wax extraction, and thus, not all plant species were included in cuticular wax analysis. Considering that there exist deviations when using community level, we analyzed the ACL and CPI of *n*-alkanes in 0–20 cm soil layer, reflecting the *n*-alkane distribution as a whole. Like the ACL in plants, ACL in soil also linearly rose with increased temperatures. This further confirmed our finding that climate change will alter the distribution of *n*-alkane chain length. The CPI in soil reduced with increased temperature, suggesting that higher temperature relatively increased the proportion of even number *n*-alkane. Tipple and Pagani (2013) also reported that average *n*-alkane chain lengths recovered from soils, *Acer rubrum*, and *Juniperus virginiana* leaves showed significant correlations with mean annual temperature. ACL in both soils and plants decreased, whereas CPI in soils rose with increased aridity index. These results indicated that the *n*-alkane distribution in soils was consistent with the *n*-alkane distribution in plants for alpine meadow. The relatively lower ACL and CPI in soil than those in plants were attributed to the influence from soil microbes (Lichtfouse 1998).

### ***n*-Alkane distribution and plant diversity**

Leaf cuticular *n*-alkanes have been widely used as markers in the chemotaxonomy of plant populations under different growing conditions (Li et al. 2012, 2013; Bush and McInerney 2013). In this study, based on plant commu-

nity level, the variations of *n*-alkane ACL and CPI depended upon the temperatures and aridity index, implying that alpine plant communities under different growing conditions might also be different. The variation based on plant community level might be caused by the difference of plant species appeared in different alpine meadows. Despite the common plant species in all sampling areas, unique plant species could be observed in each sampling area. In a study at western Himalaya, Chawla et al. (2008) also reported that the plant diversity was less at higher altitudinal ranges, whereas the uniqueness was relatively high with high species replacement rates. During the past five decades, the temperature in Qinghai-Tibetan Plateau has increased by 0.2°C per decade since 1960 (Gomez-Estrada et al. 2011), and this temperature rise consequently resulted in an increase of plant diversity (Yang et al. 2004; Ma and Fang 2006; Xu and Xue 2013). In this study, higher plant species richness and Shannon–Wiener index were also observed at higher temperatures. Although no direct evidence proved that plant diversity was related to leaf cuticular *n*-alkanes, the sensitivity of both plant diversity and leaf cuticular *n*-alkanes to varying mean annual temperature implied that certain relationship might exist between them. As we stated above, the existence of specific plant species in certain sampling area somewhat was attributed to its wax deposition and *n*-alkane distribution. Therefore, it might be the difference of leaf cuticular wax in plants which resulted in the uniqueness of plant species in certain sampling area and thus causing the variations of plant diversity.

## Conclusion

This is one of the first studies that addressed the effects of climate changes on leaf cuticular waxes in alpine meadow plants. Our result demonstrates that the climate change will not necessarily lead to a shift of wax deposition on leaves of alpine plants based on plant species level. However, the significant relationship between the temperature and aridity index and the amounts of cuticular wax and the ACL and CPI of *n*-alkanes in both plants and soils based on plant community level suggested that the alpine meadow plants as a whole respond to climate change, benefiting their adaptabilities. In addition, our findings imply that the variations of plant leaf cuticular wax may be related to the variation of plant diversity, which needs further studies.

## Acknowledgments

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## Conflict of Interest

None declared.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Relationship between climate factors and the amounts of *n*-alkane in *Kobresia humilis*, *Potentilla nivea*, *Anaphalis lacteal*, and *Leontopodium nanum*.

**Figure S2.** Relationship between climate factors and the amounts of acids in *Kobresia humilis*, *Potentilla nivea*, *Anaphalis lacteal*, and *Leontopodium nanum*.

**Figure S3.** Relationship between climate factors and the amounts of primary alcohols in *Kobresia humilis*, *Potentilla nivea*, *Anaphalis lacteal*, and *Leontopodium nanum*.

**Figure S4.** Relationship between climate factors and the amounts of total cuticular wax in *Kobresia humilis*, *Potentilla nivea*, *Anaphalis lacteal*, and *Leontopodium nanum*.

**Table S1.** Plant species sampled in each alpine meadow.