



## Large-scale patterns of stomatal traits in Tibetan and Mongolian grassland species

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### Abstract

We aimed to disentangle the influence of environmental variables on the spatial patterns in stomatal occurrence and stomatal traits. We surveyed the stomatal occurrence and the stomatal length (SL), density (SD) and index (SI) of 99 species in 150 grassland sites on the Tibetan and Inner Mongolian Plateau to explore their spatial patterns and evaluate the influence of climate. Of all species studied, two thirds were amphistomatous. The species from the Tibetan Plateau had larger but fewer stomata than those from Inner Mongolia. Among the climate factors examined, temperature and insolation affected SD, SI and SL, whereas the CO<sub>2</sub> partial pressure and the relative humidity affected SD. The climatic variables showed similar effects on the spatial variation of the adaxial and abaxial stomatal traits. We conclude that the low temperature and high insolation at high altitudes may be responsible for the larger and fewer stomata in plants on the Tibetan Plateau. The stomatal parameters that are presented here can be used to model the gas exchanges at the ecosystem scale.

### Zusammenfassung

Unser Ziel war es, den Einfluss von Umweltfaktoren auf die räumlichen Muster des Auftretens der Eigenschaften von Stomata zu entschlüsseln. Wir untersuchten das Auftreten von Stomata, ihre Länge (SL), ihre Dichte (SD) und den Verteilungsindex (SI) von 99 Arten aus 150 Grasländern auf dem Hochland von Tibet und in der Inneren Mongolei, um ihre räumlichen Muster zu erkunden und den Einfluss des Klimas zu bewerten. Von allen untersuchten Arten, waren zwei Drittel amphistomatisch. Die Arten vom tibetischen Hochland besaßen größere aber weniger Stomata als die aus der Inneren Mongolei. Von den untersuchten Klimafaktoren beeinflussten Temperatur und Sonneneinstrahlung SD, SI und SL, während der CO<sub>2</sub>-Partialdruck und die relative Luftfeuchte SD beeinflussten. Die Klimafaktoren zeigten ähnliche Effekte auf die räumliche Variation von adaxialen und

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abaxialen Stomamerkmale. Wir schließen, dass die geringe Temperatur und starke Sonneneinstrahlung in großen Höhen für die größeren und weniger zahlreichen Stomata auf dem Hochland von Tibet ursächlich sein könnte. Die Stomaparameter, die hier vorgestellt wurden, können benutzt werden, um den Gasaustausch auf Ökosystemebene zu modellieren.

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## Introduction

Stomata are the essential connections between the internal air spaces of plants and the external atmosphere. Because they regulate plant water loss and carbon gain, they exert control over ecosystem water and carbon cycles (Hetherington & Woodward 2003). The evolution of stomata is considered a key event in the overall evolution of advanced terrestrial plants (Raven 2002). Stomata are present on both surfaces of many leaves (amphistomatous) or on only one surface, i.e., either the adaxial (hyperstomatous) or the abaxial (hypostomatous) surface. Even for amphistomatous species, some studies have found that stomata are frequently present in different numbers on both leaf surfaces (Taylor et al. 2012). Moreover, stomata vary in number and size within and among species (Hetherington & Woodward 2003). By addressing the relationship between the stomata occurrence (adaxial, abaxial or both leaf surfaces) and stomatal traits and the environmental variables, we can understand the adaptive mechanisms and strategies of plants at the scale of ecosystems.

Numerous studies have reported the patterns of stomatal structure and development (Bergmann & Sack 2007; Casson & Gray 2008; Casson & Hetherington 2010) and the responses of the stomatal traits such as stomatal size, aperture, density and index to atmospheric CO<sub>2</sub> concentrations (Woodward 1987; Franks & Beerling 2009; Mott 2009), light intensity (Hovenden & Vander Schoor 2006; Wang, Noguchi, & Terashima 2008; Mott 2009), water regimes (Lake & Woodward 2008; Xu & Zhou 2008) and temperature variation (Hovenden 2001; Luomala, Laitinen, Sutinen, & Vapaavuori 2005; Fraser, Greenall, Carlyle, Turkington, & Friedman 2009). A few studies have also focused on the spatial variation in the stomatal traits within specific species or groups of species along environmental gradients. For example, a study that investigated *Leymus chinensis* along a large-scale longitudinal gradient in Northeast China showed that both stomatal density and stomatal index were positively correlated with the mean annual precipitation (MAP) and the growing season precipitation (GSP; Wang et al. 2011). In a study of sedges in the Eurasian arctic, the stomatal density increased with temperature and precipitation, whereas the stomatal size showed the opposite response (Stenström, Jónsdóttir, & Augner 2002).

Among the studies of stomatal traits along the environmental gradients, the altitudinal variation is relatively well documented. The stomatal density of all 12 species of three life forms (trees, shrubs and herbaceous plants) declined with altitude in the Southern Alps in New Zealand (Körner,

Bannister, & Mark 1986). Pteridophytes from the Bolivian Andes showed an increase in stomatal density with altitude for six species, and no correlation for another five species (Kessler, Siorak, Wunderlich, & Wegner 2007). Other studies suggest that stomatal density decreases (Gou et al. 2005) or increases (Kofidis, Bosabalidis, & Moustakas 2003; Kouwenberg, Kurschner, & McElwain 2007) with altitude or is independent of it (Li, Zhang, Liu, Luukkanen, & Berninger 2006). The variation in stomata occurrence and stomatal index has been rarely investigated.

Stomatal control of the exchange of CO<sub>2</sub> and water has been evaluated using stomatal conductance, which is directly influenced by the stomatal size and density (Willmer & Fricker 1996). In addition, the stomatal conductance of the adaxial and abaxial surfaces responds differently to environmental conditions such as light (Wang et al. 2008). Therefore, more comprehensive information of stomatal occurrences on leaves and their variation among species and functional groups may help improving our understanding of the stomatal regulation of gas exchange from the leaf scale to the ecosystem scale.

Here, we present a large-scale survey across the Chinese grassland biomes: from the temperate grasslands on the Inner Mongolian Plateau to the alpine grasslands on the Tibetan Plateau. Chinese grasslands provide an ideal opportunity for such studies because they have a large number of species and large gradients of atmospheric CO<sub>2</sub> concentration, insolation, temperature and precipitation (see Appendix A: Table 1). Our objectives were to: (1) document the patterns of stomatal occurrence and stomatal traits in different functional groups and biogeographic regions, and (2) determine the effects of climatic variables on the stomatal traits across Chinese grassland species.

## Materials and methods

### Study sites and sampling strategies

The study area covers the temperate grasslands on the Inner Mongolian Plateau and the alpine grasslands on the Tibetan Plateau (see Appendix A: Fig. 1). The climate of these regions is notably seasonal, with substantial annual variation in temperature, precipitation, relative humidity and insolation. Sample collection was conducted in late July and early August of 2003, 2004 and 2005. Sampling sites with flat topography were selected by visual inspection of the vegetation with a focus on sites that are subject to

minimal grazing and other anthropogenic disturbances. At each site (10 m × 10 m), the dominant species were selected for measurements in five plots (1 m × 1 m). However, at sites in species-poor grasslands, fewer species were sampled, to as few as one. For each species, healthy mature leaves of identical or similar position and developmental stage were collected from three to five plants and fixed immediately in FAA solution (formaldehyde-acetic acid-alcohol solution) in the field. In total, we investigated 99 species from 48 genera and 21 families over the 150 research sites; 51 species were measured at more than one site (see Appendix A: Table 2). We further distinguished three functional groups: graminoids, herbs and shrubs.

### Measurement of stomatal traits

Epidermal images were taken from the leaves using either the nail varnish imprint method (Hilu & Randall 1984) or the sodium hypochlorite solution method (Pan, Lu, & Wen 1990) in the laboratory. The epidermis that was used was generally obtained from the distal 1/3 of the leaf, with a length of 0.5 cm and avoiding major veins. The stomatal measurements were conducted using the Motic Microscopic Image System (Motic BA200, Motic, Inc., Xiamen, China). For each imprint/epidermis, five random views were selected and photographed under a microscope (10 × 40). Up to seven stomata were randomly chosen in each view, and the following measurements were made:

Stomatal length (SL, in  $\mu\text{m}$ ): the length between the junctions of two guard cells at each end of the stoma. The SL of each view was calculated as the mean value of all stomata measured.

Stomatal density (SD, in  $\text{mm}^{-2}$ ): the number of stomata per unit leaf surface area, which is calculated as the number of stomata in the view divided by the area viewed.

Stomatal index (SI, %): the number of stomata as a proportion to the number of epidermal cells, which is calculated by the number of stomata divided by the number of all epidermal cells in the view.

Abaxial ratio (ABR, %): for amphistomatous species, this ratio is the ratio of the stomata number on the abaxial surface to the total stomata number on both surfaces of a leaf.

For each individual, the stomatal traits were calculated as the mean values of all 3–5 leaves measured. At each site, the stomatal traits of each species were represented by the mean values of 3–5 individuals.

### Climate data

The climatic variables in this study, including the mean annual temperature (MAT) and the mean annual precipitation (MAP) of each sampling site, were extracted from the “Global Climate Data” (1951–2000) based on latitude, longitude and altitude (<http://www.worldclim.org/current>). The relative humidity and insolation of the sampling sites were

obtained from the “NASA Surface Meteorology and Solar Energy” dataset (22-year average) based on latitude and longitude (<http://eosweb.larc.nasa.gov/sse/>). Insolation is a measure of the solar radiation energy that is received on a given surface area and recorded during a given time, and it is used to represent light intensity in the present study. The growing season temperature (GST, from May to September), growing season precipitation (GSP) and growing season relative humidity (GSRH) were used in the final analysis to more accurately capture the climatic variation that is important to plant growth. The partial  $\text{CO}_2$  ( $P_{\text{CO}_2}$ ) of the atmosphere was calculated based on the altitude as:

$$P_{\text{CO}_2} = \frac{P_{\text{air},z}}{101,325} \times P_{\text{CO}_2,\text{sea level}}$$

where  $P_{\text{air},z}$  is the atmospheric pressure at altitude  $z$  in m, and  $P_{\text{CO}_2,\text{sea level}}$  is the  $\text{CO}_2$  partial pressure at sea level, which is equal to 37.5 Pa (Kouwenberg et al. 2007).

### Statistical analysis

All statistical analyses were performed using R version 2.14.1 (R Development Core Team 2011) or SPSS 16.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA with Tukey’s *post hoc* test for differences among the means was used to test the effects of region and functional group, and Pearson correlation was used to detect the correlations of stomatal traits at the species level. Considering the unbalanced data, we used Type III-sums in the one-way ANOVA.

To account for the potentially confounding effect of phylogenetic relatedness on trait correlations, the phylogenetically independent contrasts (PIC; Felsenstein 1985) were calculated for the stomatal traits. First, we constructed a supertree of all species (see Appendix A: Fig. 2) based on the phylogeny of the Angiosperm Phylogeny Group (Chase et al. 2009). A phylogeny was generated using the online tool Phylomatic (<http://phylodiversity.net/phyloomatic/>). Independent contrasts were obtained using the *aotf* module of the software Phylocom v. 4.2 (Webb, Ackerly, & Kembel 2008). Then, the Pearson correlations among the stomatal traits were obtained.

To evaluate the effect of the environmental variables on the stomatal traits, redundancy analysis (RDA) was performed to explore the variables that significantly affected stomatal traits. The environmental variables that significantly correlated with stomatal traits were further tested for direct versus indirect effects using structural equation modeling (SEM; Grace & Pugsek 1997; Geng, Wang, Yang et al. 2012). SEM accounts for the roles of multiple variables in a single analysis, providing the mechanisms behind the overall patterns by partitioning the direct effects from the indirect effects that act through other components. Here, we used SEM to partition the total effect of the variables (screened out) on the stomatal traits. The R package “*sem*” was used to construct and test the SEMs for the stomatal length, stomatal density and stomatal index. We examined how well

**Table 1.** Number of amphistomatous, hyperstomatous and hypostomatous species for two regions and three plant functional groups, with relative proportions in each group shown in parentheses.

	No. of species	Amphistomatous	Hyperstomatous	Hypostomatous
Overall	99	62 (62.3%)	12 (12.1%)	24 (24.2%)
<b>Region</b>				
Inner Mongolia	42	31 (73.8%)	5 (11.9%)	5 (11.9%)
Tibetan Plateau	60	33 (55.0%)	8 (13.3%)	19 (31.7%)
<b>Functional groups</b>				
Graminoids	37	12 (32.4%)	10 (27.0%)	15 (40.5%)
Herbs	43	39 (90.7%)	0	3 (7.0%)
Shrubs	19	11 (57.9%)	2 (10.5%)	6 (31.6%)

A herb species with leaves in half-terete shape from Inner Mongolia, *Allium polyrhizum* is excluded from the summary.

the model fitted the data using the root-mean-square error approximation (RMSEA), the Bentler–Bonett normed fit index (NFI) and Bentler’s comparative fit index (CFI).

Some species were frequently sampled, whereas others only occurred at one or two sites in our dataset. To account for this variation in sample size, the data were analyzed at two levels: (1) the species level, with measurements of the stomatal traits averaged within species to produce a dataset of species means, and (2) the species-by-site level, with measurements of stomatal traits of each species at each site to produce a species-by-site dataset (He et al. 2006; Geng, Wang, Liang et al. 2012). The species level data were used to analyze the statistical description and trait correlations. The data on the species-by-site level were used to evaluate the effect of the environmental variables on the spatial patterns of the stomatal traits. Both datasets were used to analyze the comparisons among the regions and functional groups, and the results were generally similar, therefore only results from the species level are presented here for brevity.

## Results

### General patterns of stomata occurrence and stomatal traits

Among the 99 species, 62 are amphistomatous, 12 are hyperstomatous and 24 are hypostomatous (Table 1). Most of the hyperstomatous species (10 out of 12 species) are from the genus *Stipa*. Nine out of 24 hypostomatous species belong to the genus *Kobresia* (see Appendix A: Table 2). For the amphistomatous species, ABR ranged from 14.0 to 99.5% with a mean of 49.1% (Table 2).

Large variation in stomatal traits was observed. The traits varied by one (SL and SI) to three (SD) orders of magnitude across all types of stomatal occurrence (Fig. 1 and Table 2). SD showed larger variation (CV: 64.4%) than SL (CV: 34.5%) and SI (CV: 43.0%, Fig. 1). The mean values of SL, SD and SI for all species were 25.2  $\mu\text{m}$ , 267  $\text{mm}^{-2}$  and 20.7%, respectively (Fig. 1), and SL, SD and SI ranged between 9.9–47.2  $\mu\text{m}$ , 10–1190  $\text{mm}^{-2}$  and 1.7–46.6%, respectively (Table 2). Across all species, SL

negatively correlated with both SD ( $r = -0.561$ ,  $P < 0.001$ ) and SI ( $r = -0.505$ ,  $P < 0.001$ ), but only the negative correlation between SL and SD persisted ( $r = -0.578$ ,  $P < 0.001$ ) by PIC (Fig. 2).

### Regional and functional group differences in stomatal traits

The proportion of amphistomatous species was 73.8% in the studied species in the Inner Mongolian Plateau and 55.0% in the Tibetan Plateau (Table 1). The amphistomatous species from the Inner Mongolian Plateau showed a larger fraction of abaxial stomata than those from the Tibetan Plateau (ABR: 52.7 vs. 45.6%). The species from the Inner Mongolian Plateau generally have smaller SL but higher SD and SI than those from the Tibetan Plateau (SL: 19.5 vs. 29.1  $\mu\text{m}$ , SD: 357 vs. 205  $\text{mm}^{-2}$ , SI: 23.9 vs. 18.2%, Table 2).

With respect to the functional groups, most of the herbs are amphistomatous (90.7%), whereas almost half of the graminoids and shrubs are hyper or hypostomatous (Table 1). The shrubs had smaller SL but higher SD than the herbs and the graminoids, and the graminoids had similar SD compared with the herbs. The shrubs and herbs had similar SI, which was higher than that of the graminoids (Table 2).

### Effects of climate on the variation in stomatal traits

Altitude and the climatic variables that closely correlate with altitude, including  $P_{\text{CO}_2}$ , GST, insolation and GSRH (see Appendix A: Table 5), were significantly correlated with the variation in stomatal traits for all amphistomatous and hyper and hypostomatous species. However, GSP only significantly correlated with the variation in stomatal traits in hyper and hypostomatous species (Fig. 3, see Appendix A: Table 6). Furthermore,  $P_{\text{CO}_2}$ , GST, insolation, GSRH and altitude also showed significant correlation with the variation in the stomatal traits of adaxial and abaxial surface of amphistomatous species (see Appendix A: Table 7 and Fig. 3). Because these six variables were inter-correlated, the apparent relationships combined both direct and indirect effects. Therefore, we used



**Table 2.** Stomatal traits of amphistomatous, hyperstomatous and hypostomatous species from the two regions and three plant functional groups.

		Overall	Region		Functional groups		
			Inner Mongolia	Tibetan Plateau	Graminoids	Herbs	Shrubs
<b>Amphistomatous</b>							
SL ( $\mu\text{m}$ )	Mean	23.3	17.9 <sup>a</sup>	28.4 <sup>b</sup>	28.0 <sup>b</sup>	24.1 <sup>b</sup>	14.7 <sup>a</sup>
	Range	9.9–47.2	9.9–41.5	13.2–47.2	11.9–47.2	9.9–41.0	10.2–20.3
SD ( $\text{mm}^{-2}$ )	Mean	261	366 <sup>b</sup>	164 <sup>a</sup>	247 <sup>a</sup>	212 <sup>a</sup>	455 <sup>b</sup>
	Range	62–1190	120–1190	62–311	62–749	64–614	158–1190
SI (%)	Mean	23.2	24.5 <sup>b</sup>	22.1 <sup>a</sup>	18.3 <sup>a</sup>	23.6 <sup>b</sup>	25.4 <sup>b</sup>
	Range	7.5–44.6	11.6–46.6	7.5–35.7	7.5–26.5	7.5–46.6	12.0–45.0
ABR (%)	Mean	49.1	52.7 <sup>b</sup>	45.6 <sup>a</sup>	42.9 <sup>a</sup>	50.8 <sup>b</sup>	50.6 <sup>b</sup>
	Range	14.0–99.5	14.5–99.5	14.0–74.7	14.0–68.9	15.8–99.5	38.4–74.6
<b>Hyperstomatous</b>							
SL ( $\mu\text{m}$ )	Mean	26.9	27.4 <sup>a</sup>	26.6 <sup>a</sup>	28.9 <sup>b</sup>		17.0 <sup>a</sup>
	Range	16.1–33.1	25.7–29.1	16.1–33.1	25.1–33.1		16.1–17.9
SD ( $\text{mm}^{-2}$ )	Mean	293	339 <sup>a</sup>	264 <sup>a</sup>	307 <sup>a</sup>		212 <sup>a</sup>
	Range	10–435	261–435	10–413	250–435		10–413
SI (%)	Mean	18.8	22.3 <sup>b</sup>	10.1 <sup>a</sup>	22.3 <sup>a</sup>		10.1 <sup>a</sup>
	Range	1.7–25.6	14.2–25.6	1.7–18.4	14.6–25.6		1.7–18.4
<b>Hypostomatous</b>							
SL ( $\mu\text{m}$ )	Mean	28.7	18.4 <sup>a</sup>	31.4 <sup>b</sup>	33.2 <sup>b</sup>	22.8 <sup>a</sup>	20.4 <sup>a</sup>
	Range	11.0–39.4	14.8–23.5	11.0–39.4	15.5–39.4	15.3–26.9	11.0–28.3
SD ( $\text{mm}^{-2}$ )	Mean	276	370 <sup>a</sup>	252 <sup>a</sup>	226 <sup>a</sup>	159 <sup>a</sup>	460 <sup>b</sup>
	Range	24–590	221–590	24–539	159–359	24–306	325–590
SI (%)	Mean	15.0	21.6 <sup>b</sup>	13.4 <sup>a</sup>	13.7 <sup>a</sup>	15.4 <sup>ab</sup>	19.5 <sup>b</sup>
	Range	10.3–26.5	13.8–26.5	10.3–18.3	10.3–16.6	11.4–19.4	13.8–26.5

Means with different superscript letters are significantly different at the 5% significant level within the same group. SL, SD, SI are abbreviations for stomatal length, density and index. ABR denotes the proportion of abaxial stomata on a leaf.

SEM to evaluate the casual relationships among altitude,  $P_{\text{CO}_2}$ , GST, insolation and GSRH.

The final SEM model (Fig. 4) for all species fitted the data well (RMSEA: 0.078, NFI: 0.997, CFI: 0.998, P value: 0.082) and explained the variations of 23.6%, 13.9% and 10.6% in SL, SD and SI, respectively. Insolation ( $\lambda = 0.26$ ) and GST ( $\lambda = -0.20$ ) had strong direct effects on the variation of SL.  $P_{\text{CO}_2}$  had the strongest effect ( $\lambda = -0.59$ ) on the variation of SD, followed by insolation ( $\lambda = -0.53$ ), GST ( $\lambda = 0.43$ ) and GSRH ( $\lambda = -0.18$ ). GST had a stronger effect ( $\lambda = 0.30$ ) on the variation of SI than insolation ( $\lambda = -0.16$ ) and  $P_{\text{CO}_2}$  ( $\lambda = -0.11$ ). Although there were significant bivariate relationships between altitude and the stomatal traits, altitude only had significant indirect effects through its direct effects on  $P_{\text{CO}_2}$ , GST and insolation. Overall, GST strongly affects SL and SI, insolation strongly affects SL and  $P_{\text{CO}_2}$  strongly affects SD.

The final SEM model (see Appendix A: Fig. 4) for the amphistomatous species showed similar effects of the climatic variables on the variation of stomatal traits to the overall model. However, we could not construct a model for only the hyper and hypostomatous species because of the small sample size.

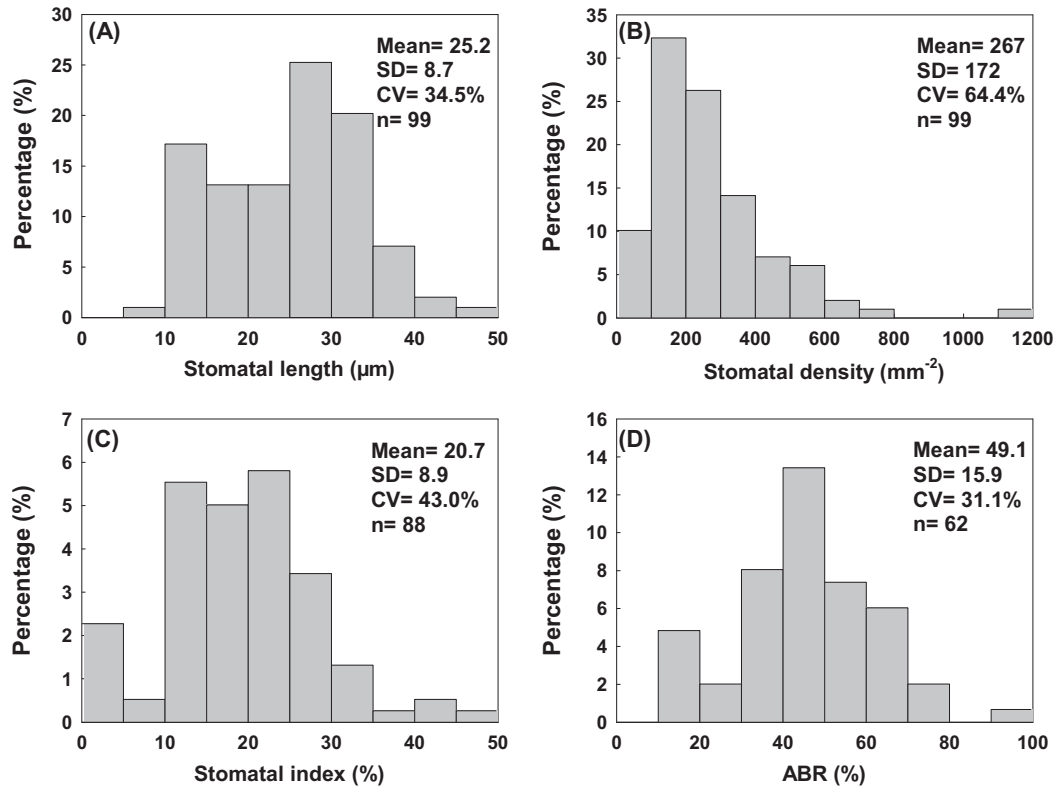
## Discussion

To the best of our knowledge, the relationships between the environment and the stomatal traits have not been previously explored at a large spatial scale. Our study explored the macroecological variation in stomatal traits and their relationships to the environmental gradients over a large biogeographical range from the Inner Mongolian Plateau to the Tibetan Plateau.

### Effect of climatic variables on the spatial variation of the stomatal traits

The present study showed that the species from the Inner Mongolian Plateau produce smaller but more stomata than those from the Tibetan Plateau. These differences are partly because of the different environmental conditions. On average, the climatic variables accounted for 16.0% of the variation in stomatal traits.

Consistent with previous reports from altitude transects elsewhere, altitude is the most notable variable that affects the stomatal traits (Körner 2003; Hovenden & Vander Schoor



**Fig. 1.** Frequency distributions of stomatal length (A), stomatal density (B), stomatal index (C) and ABR (the proportion of the abaxial stomata on a leaf) (D) across all species.

2006). However, altitude is actually a composite term that encompasses many factors that may change with elevation. Thus, the altitude can affect the stomatal traits through one or a combination of multiple factors that change with elevation (Beerling & Kelly 1996). Our analysis showed that altitude correlated with stomatal traits through its effects on  $P_{\text{CO}_2}$ , GST, insolation and GSRH.

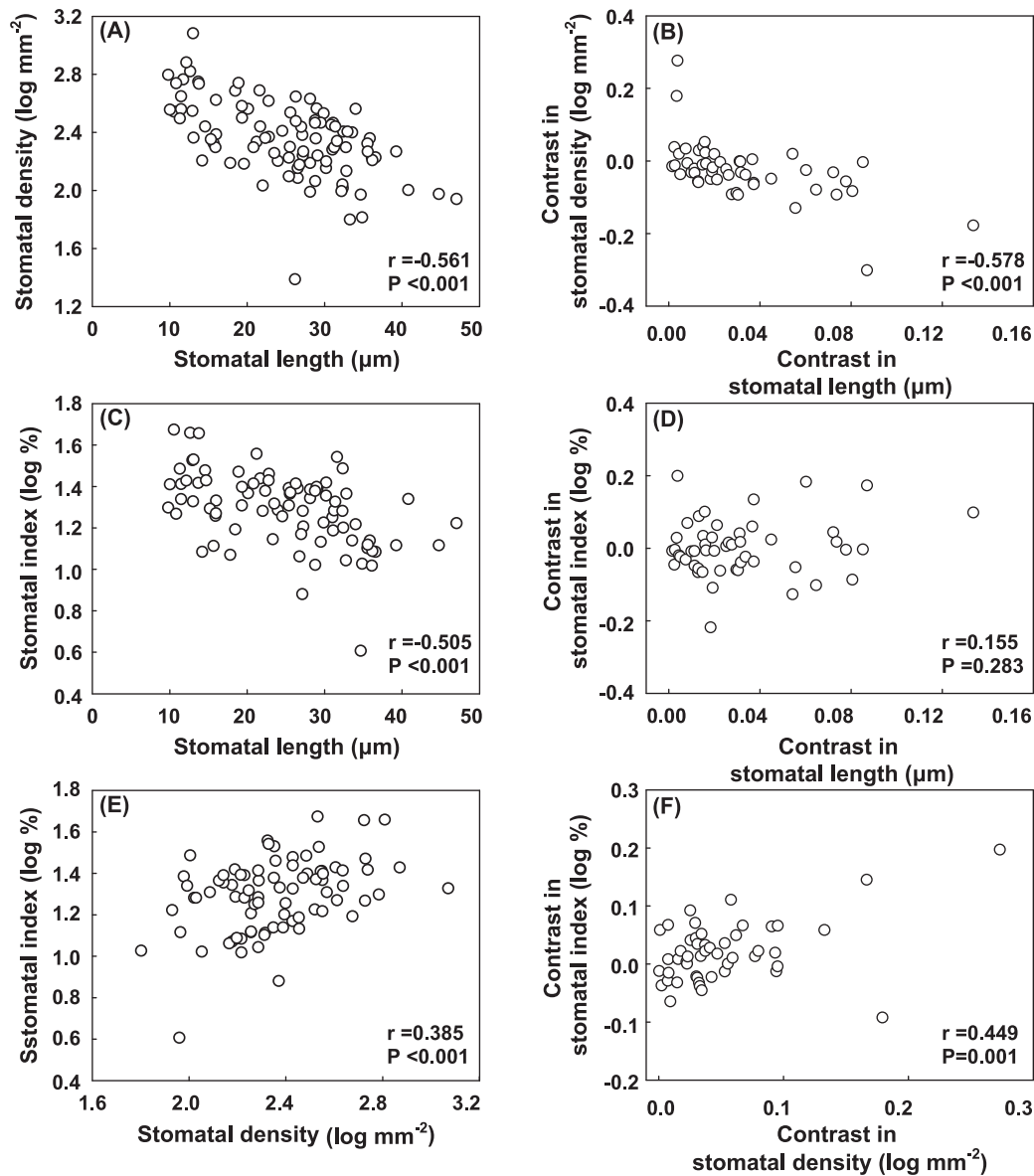
Temperature is believed to have no direct effect on stomatal traits (Willmer & Fricker 1996), but the field experiments showed that temperature indirectly affects the traits through other environmental factors (Hovenden 2001). Our study showed that temperature was an important factor for the differences in stomatal traits between two regions. Similarly, a study on leaf anatomy across the Chinese grassland also showed that the species from the Tibetan Plateau have larger mesophyll cells than the species from the Inner Mongolian Plateau because of the difference in temperature (Ma et al. 2012). As Körner et al. (1986) noted that low temperature at high altitude was responsible for the larger mesophyll cells of the plants, we suggest that the low temperature in the Tibetan Plateau may be responsible for the larger stomata, which leads to fewer stomata because of the trade-off between stomatal size and frequency (Hetherington & Woodward 2003).

Light intensity positively correlated with SD in previous studies (Hovenden & Vander Schoor 2006; Mott 2009), but insolation negatively affected SD in our study. It should

be noted that high insolation at high altitude is always associated with low temperatures (Körner 2003). Körner (2003) proposed that low temperature and strong insolation may co-determine the leaf structures at high altitude. This hypothesis is consistent with our result that low GST and high insolation on Tibetan Plateau may be responsible for larger SL, which results in lower SI and SD.

Apparently,  $P_{\text{CO}_2}$  had a positive effect on SD (Fig. 4), which was inconsistent with previous results from fossil records and manipulated experiments, which showed that plants produce more stomata under lower  $P_{\text{CO}_2}$  (Royer 2001; Franks & Beerling 2009). We suggest that the effect of  $P_{\text{CO}_2}$  on the stomatal traits was overridden by GST and insolation across the Chinese grassland. Despite the lower  $P_{\text{CO}_2}$ , the species on the Tibetan Plateau produce fewer stomata because of low temperature and strong insolation in this region, which may result in larger stomata as we discussed above. When the effects of GST and insolation are eliminated in the structural equation model (Fig. 4),  $P_{\text{CO}_2}$  maintains a negative effect on SD, which suggests that the effects of temperature and light intensity should be considered when applying the  $P_{\text{CO}_2}$ -SD/SI relationship (Royer 2001) to reconstruct pre-historical  $\text{CO}_2$  levels.

Considering the important role of water in regulating the stomatal development, structure and movement (Buckley 2005), we failed to detect a significant effect of GSP on



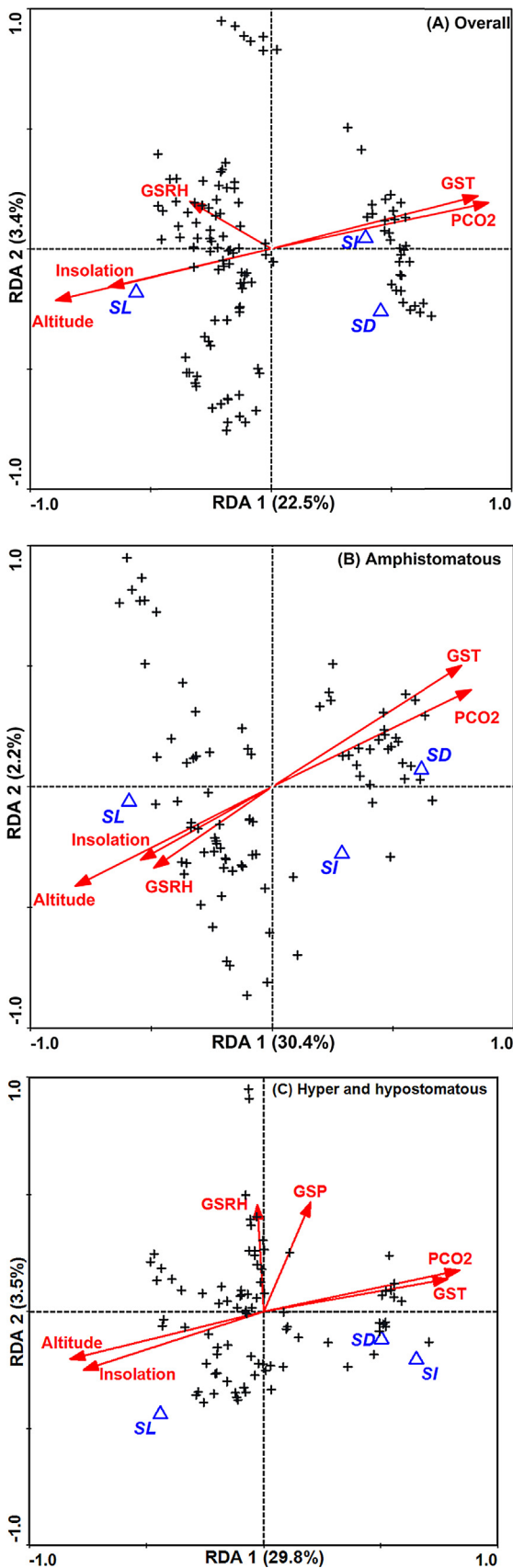
**Fig. 2.** Correlations of stomatal length and stomatal density, SL and stomatal index, SD and SI. Left half: inter-specific correlation (A, C, E); right half: phylogenetically independent contrasts (PIC) correlation (B, D, F).

the variation of stomatal traits. Although GSRH negatively correlates with SD, its effect was much weaker than GST, insolation and  $P_{CO_2}$ . We suggest that GSP and GSRH are not important factors in determining the distribution of stomatal traits in large spatial scale.

Combined with previous studies on leaf anatomy across the Chinese grassland (Ma et al. 2012) and the studies of alpine plants from the Central Alps (Körner 2003), our results suggest that the combination of low temperature and high light intensity at high altitudes may be responsible for the larger and fewer stomata on the Tibetan Plateau, whereas  $P_{CO_2}$  negatively correlates with the stomatal frequency even at low temperatures.

### Stomatal occurrence

Our results showed that two thirds of the species across the Chinese grassland are amphistomatous, and the amphistomatous species from the Tibetan Plateau produce more stomata on the adaxial surface than do those from the Inner Mongolian Plateau. Amphistomatous leaves are considered to have a higher maximum stomatal conductance than hyper or hypostomatous leaves (Beerling & Kelly 1996) because of their ability to reduce the intercellular diffusion resistance and increase the leaf boundary layer conductance (Parkhurst 1978; Mott & Michaelson 1991). Therefore, amphistomatous leaves are likely advantageous where the photosynthetic



rates are potentially limited by a low leaf conductance (e.g., low CO<sub>2</sub> concentration), and/or where the light intensity is high (Mott & Michaelson 1991). A pioneer survey of 469 British plant species shows that amphistomatous leaves are predominant in species that live in non-shaded environments, where CO<sub>2</sub> may limit photosynthesis, whereas hypostomatous leaves frequently occur in species that live in shaded habitats, where CO<sub>2</sub> is unlikely to limit photosynthesis (Peat & Fitter 1994). In addition, *Ambrosia cordifolia* was found to produce hypostomatous leaves in low-light-intensity condition and amphistomatous leaves in high-light-intensity condition (Mott & Michaelson 1991). The amphistomatous plants from high altitudes in the Central Alps also showed a higher fraction of adaxial stomata (Körner, Allison, & Hilscher 1983).

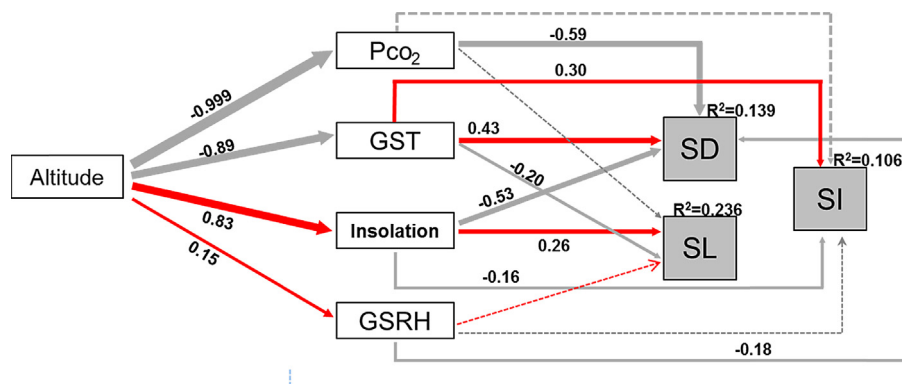
Many recent studies have focused on leaf functional traits, such as leaf lifespan, specific leaf area, leaf nitrogen and phosphorous contents, photosynthetic rates, and other leaf morphological and physiological traits. A global leaf economic spectrum, which consists of key chemical, structural and physiological properties across and within biomes, has been constructed (Wright et al. 2004). However, despite their great importance for understanding the mechanisms of plant adaption to environmental changes, the stomatal traits have received less attention in the large-scale studies. The macroecological patterns of stomatal traits are shaped by the adaptations of plants to the physical environments and the historical constraints. Understanding these adaptations is important to better understand the plant functional traits and to model the responses of vegetation to the change in climate and land use. The next step is to examine whether there is any link between the stomatal traits and the plant economic traits across large-scale geographic regions.

### Limitations of the current study

We attempt to explore the effects of environmental variables on the variation in stomatal traits in the present study. It is challenging for ecologists to link the local-scale stomatal traits to the large-scale climatic variables because the stomatal traits are influenced by both the large-scale climate and the local site conditions such as altitude, latitude, longitude, slope aspect, slope inclination and P<sub>CO<sub>2</sub></sub>. Although there are significant correlations between the longitude/latitude and the

**Fig. 3.** Redundancy analysis (RDA) of stomatal traits including stomatal length (SL), stomatal density (SD) and stomatal index (SI) for all observations (A,  $n = 244$ ), and for amphistomatous (B,  $n = 135$ ), and hyper and hypostomatous (C,  $n = 111$ ) species. Black crosses represent each observation, blue triangles indicate stomatal traits, and red arrows indicate significant environmental variables. GST denotes growing season temperature, GSP denotes growing season precipitation and GSRH denotes growing season relative humidity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)





**Fig. 4.** Final structural equation model of stomatal length (SL), stomatal density (SD) and stomatal index (SI) for all observations. Non-significantly correlated paths are showed in dashed lines. The thickness of the solid arrows reflects the magnitude of the standardized structure equation model (SEM) coefficients. The red lines represent positive correlations and gray lines represent negative correlations.  $R^2$  values represent the proportion of variance explained for each variable. GST denotes growing season temperature and GSRH denotes growing season relative humidity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

stomatal traits, only altitude and  $P_{CO_2}$  were used in the final analysis. The reasons are as follows. (1) The latitude and the longitude correlate highly with the altitude along the Inner Mongolian Plateau and the Tibetan Plateau (see Appendix A: Table 5). (2) Compared to latitude and/or longitude, the relationships between  $P_{CO_2}$ , GST and insolation with altitude are globally more consistent. The slope aspect and the inclination would influence the stomatal traits through their effects on climate such as the solar input (Chapin, Matson, & Vitousek 2011). However, the effect of slope on the variation in stomatal traits is notably small in the present study, due to the flat topography for most sampling sites on the plateau.

The main objective of the present study was to investigate the large-scale regional pattern of stomatal traits and explore the effects of the climatic variables on such spatial patterns. More studies are required to validate the reported patterns in more species. The integration of more site conditions and leaf traits into the model will be helpful in exploring the effects of the environmental variables on the variation of the stomatal traits in further studies.

## Conclusions

In the current study, it was found that two-thirds of the species surveyed across the Chinese grassland are amphistomatous; most of the herbs are amphistomatous, whereas almost half of the graminoids and shrubs are hyper or hyposomatous. The shrubs produce smaller but more stomata than the herbs and the graminoids. The amphistomatous species from the Inner Mongolian Plateau showed a larger fraction of abaxial stomata than those from the Tibetan Plateau. The plants on the Tibetan Plateau produce larger but fewer stomata than those on the Inner Mongolian Plateau. The climatic variables showed similar effects on the spatial variation of the adaxial and abaxial stomatal traits. We found that  $P_{CO_2}$ , GST, insolation and GSRH directly affect the stomatal traits,

whereas altitude indirectly affects the stomatal traits through these four variables. The low temperature and the high insolation at high altitude on the Tibetan Plateau may be responsible for the larger and fewer stomata.  $P_{CO_2}$  negatively correlates with the stomatal density, but its effect was overridden by temperature and insolation across the Chinese grassland.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.baae.2014.01.003>.

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