



An orthogonal experimental study of phytolith size of *Phragmites communis* in northeast China

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Based on the widespread distribution and abundant phytoliths produced by *Phragmites communis*, phytoliths of *P. communis* in northeast China were investigated to identify the primary influencing factors on phytolith size using an orthogonal experimental method. Temperature and precipitation were found to be the prominent factors influencing the sizes of short-cell phytoliths including the saddle and the rondel morphotypes, whereas there were no obvious variations in the width-to-length ratios of short-cell phytoliths in response to environmental factors. These results indicate that short-cell phytoliths are genetically stable, confirming that they can be used for the classification of grasses. Moreover, precipitation was the main factor influencing the sizes of the lanceolate phytoliths and silicified stomata, and their width-to-length ratios changed significantly in response to precipitation and habitat differences. These findings reveal that lanceolate and silicified stomata phytoliths are sensitive to environmental changes, indicating their usefulness for palaeoenvironment reconstruction. Moreover, variations in their width-to-length ratios and their sizes may provide some information for plant classification, even at species level. Finally, the results of a principal component analysis of the sizes of *P. communis* phytoliths further verify the reliability of the orthogonal experiment results, in that temperature and precipitation were the prominent factors influencing the size of all *P. communis* phytoliths except for the length of saddle and width of the saddle morphotypes. This research into the primary environmental significance of the sizes of *P. communis* phytoliths may contribute to improving the precision of plant classification and the model for quantitative reconstruction of the palaeoenvironment to aid in reconstructions of palaeovegetation and palaeoclimate.

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As phytoliths precipitate in or amongst the cells of living plant tissues, their morphology is controlled by the plant tissues themselves. Plant tissues are influenced, in turn, by the environment and the physiological mechanisms of the plant; therefore, phytoliths are closely linked to environmental factors and plant physiological mechanisms (Lu & Liu 2003; Lu *et al.* 2007; Jie *et al.* 2010a; Liu *et al.* 2013). It has been reported that under different temperature and humidity conditions, the numbers of different phytolith types follow different statistical patterns (Prebble & Shulmeister 2002; Piperno 2006). Recent studies of changes in phytolith content and size under the influence of elevated atmospheric CO₂ concentrations or simulated warming have further revealed that the contents and sizes of phytoliths change with environmental factors (Ge *et al.* 2010; Jie *et al.* 2010b). However, it is not yet known what environmental factors are favour phytolith formation and whether the environmental factors influencing different phytoliths are consistent or not. There is also still no consensus on the environmental implications of phytoliths. For instance, there is still some uncertainty about the environmental significance of bulliform phytoliths. Messenger *et al.* (2010) showed that the content of bulliform phytoliths could be used to indicate the water conditions as recorded by grasses,

whereas Wang *et al.* (2003) showed that bulliform phytoliths in topsoil were representative of a warm climate. Additionally, elongated phytoliths are widely distributed in topsoil in the arid region of northwest China (Wang & Lu 1992). They are also representative of a cold climate when calculating the warm index of the phytolith assemblage, which is the ratio of warm-group phytoliths (i.e. panicoid, bulliform and long saddle phytoliths) vs. the sum of warm-group phytoliths and cold-group phytoliths (i.e. festucoid, elongate and lanceolate phytoliths) (Wang *et al.* 2003). This uncertainty about the environmental implications of phytoliths can lead to difficulties in the quantitative reconstruction of palaeoenvironments based on phytolith data. Thus, it is important to investigate the primary factors influencing phytolith characteristics to further understand the role of phytoliths in environmental reconstructions and interpret phytolith assemblages in sediments.

Phragmites communis is a plant of the Arundoideae and contains abundant silicon. This species is widely distributed in wetlands amongst different temperature zones and humidity areas. The aim of this study was to investigate the relationships amongst temperature, precipitation, habitat differences and phytolith size in *P. communis* in northeast China.

Moreover, the primary factors influencing phytolith size were evaluated by an orthogonal experimental method to reveal the climatic implications of *P. communis* phytoliths. The data presented herein may help improve the precision of plant classification and of quantitative reconstructions of palaeoenvironments.

Study area

The study site in northeast China is located at latitude 39°40'–53°30'N, longitude 115°05'–135°02'E (Ma *et al.* 2007). The region can be divided into a cold temperate zone, a temperate zone and a warm temperate zone from north to south, and a humid area, a semi-humid area and a semi-arid area from east to west. Northeast China has four distinct seasons, with a long winter and a short summer. The annual average temperature ranges from –4 to +11 °C. The annual average precipitation ranges from 1000 mm in the east to 350 mm in the west (Zhao *et al.* 2011). *Larix gmelinii* predominates in the cold temperate zone; needled and broad-leaved mixed forest covers the eastern part of the temperate zone; and *Pinus tabulaeformis* Carr is found in the Liaoning Hills in the warm temperate zone. The influence of the monsoon is obvious at 115–135°E because of the presence of needled and broad-leaved

mixed forest, meadow steppe and steppe. The main soil types in northeast China are brown coniferous forest soils in the cold temperate zone, dark brown mixed forest soil in the temperate zone, and forest steppe chernozem and meadow steppe chernozem in the temperate zone (Sun *et al.* 2006).

Material and methods

Experimental design

Twelve sampling sites were selected along the precipitation gradient between 115 and 135°E and the temperature gradient between 39 and 53°N (Fig. 1). The 12 sampling sites were divided into four sections from south to north along the temperature gradient: Dandong–Panjin–Tongliao, Longwan–Changchun–Changling, Mudanjiang–Harbin–Daqing and Tongjiang–Beian–Nehe. In each section, there are large precipitation differences amongst the sites (Fig. 1). As the sampling sites at Nehe were damaged, *P. communis* samples were collected from the other 11 sites. The sampling sites represent three levels of precipitation and four levels of temperature. Five *P. communis* samples were collected from aquatic habitat (W) and another five from xerophytic habitat (T) at each sampling site in September 2012.



Fig. 1. Location of sampling sites in northeast China.

Extraction methods

The phytoliths were isolated using wet ashing (Wang & Lu 1992) as follows.

- Cleaning: third or fourth leaf of each plant sample was selected to reduce experimental error. Each leaf was added to a test tube, to which distilled water was added. The leaves were then cleaned in an ultrasonic shaking instrument to remove any soil contamination.
- Oxidization: the five dry clean leaves from five *P. communis* samples in each site were cut into small pieces and mixed. A 0.20 g sample of the dry clean leaves was added to a test tube and mixed with concentrated nitric acid until the organic matter was fully oxidized.
- Centrifugation and cleaning: to dilute the acid, distilled water was added and the mixture was centrifuged three times at 2000 rpm for 20 min. Absolute ethanol was then added to the test tube and the mixture was centrifuged at 2000 rpm for 20 min.
- Slide preparation: the liquid was shaken well and one to three drops of it was placed on a glass microscope slide, which was then heated over a spirit lamp until all the ethanol had evaporated. Canada balsam oil (one to two drops) was then added and a cover slip was placed on top.
- Identification: the samples were examined under a MOTIC biomicroscope (DMBA 300, MOTIC China Group Co., Ltd, Xiamen, China) at a magnification of 900 \times . More than 300 phytolith grains were counted for each slide. Only phytoliths with a diameter >10.00 μm and that could be taxonomically identified were counted.

Selection of experimental method

The orthogonal experimental method is a highly efficient method for dealing with multifactor experiments and screening optimum levels by using the orthogonal design table. Therefore, we employed an orthogonal experimental design to examine the primary factors influencing *P. communis* phytolith size. A mixed-level orthogonal experimental design of three factors was chosen according to the research requirements, and it takes into account that any two factors interacted with each other. The three representative factors considered were temperature, precipitation and habitat differences. Three corresponding levels (high, middle and low) were used for temperature or precipitation, whereas two (xerophytic and aquatic habitats) were included for habitat differences. An orthogonal design table was also drawn (Table 1). The three or two ordered degree values of each factor in the same level were summed, and the corresponding average value M_i and range ($R = M_{\max} - M_{\min}$) were calculated. R represents the influence of the factors on *P. communis* phytolith size, with a higher R indicating a stronger impact upon the size of *P. communis* phytoliths. Range analysis shows the order of the variables influencing the target qualitatively. However, it could not distinguish the undulation of each factor level quantitatively. Moreover, it could not distinguish whether the influence of a variable is noticeable. To overcome these limitations, analysis of variance (ANOVA) was also used to determine how much of the variation was contributed by each factor and to identify significant effects to clarify the differences (Wu & Leung 2011). Statistical analyses were conducted using SPSS18.0 with the level of significance set at $P < 0.05$.

Table 1. Design matrix based on $L_{18}(2 \times 2^7)$ orthogonal array.

Experiment number	Factor						
	Habitat	Temperature	Temperature \times habitat	Precipitation	Precipitation \times habitat	Temperature \times precipitation	
1	1	1	1	1	1	1	1 1
2	1	1	2	2	2	2	2 2
3	1	1	3	3	3	3	3 3
4	1	2	1	1	2	2	3 3
5	1	2	2	2	3	3	1 1
6	1	2	3	3	1	1	2 2
7	1	3	1	2	1	3	2 3
8	1	3	2	3	2	1	3 1
9	1	3	3	1	3	2	1 2
10	2	1	1	3	3	2	2 1
11	2	1	2	1	1	3	3 2
12	2	1	3	2	2	1	1 3
13	2	2	1	2	3	1	3 2
14	2	2	2	3	1	2	1 3
15	2	2	3	1	2	3	2 1
16	2	3	1	3	2	3	1 2
17	2	3	2	1	3	1	2 3
18	2	3	3	2	1	2	3 1

Selection of phytolith types

Based on the classification criteria of Wang & Lu (1992) and the International Code for Phytolith Nomenclature (ICPN) 1.0 protocol, the *P. communis* phytoliths are mainly short-cell phytoliths (including the saddle and rondel morphotypes) and non-short-cell phytoliths (including the lanceolate, elongate and bulliform morphotypes). Lanceolate phytoliths originate from hair cells and the latter two from long cells and bulliform cells, respectively. Considering the relatively large proportion of saddle and rondel phytoliths present in *P. communis*, and that hair cell phytoliths are sensitive to environmental changes (Jie *et al.* 2010b), these types were examined in this study.

Gas exchange in higher plants mainly occurs through actively regulated pores situated on the leaf epidermis, called stomata (Aasamaa *et al.* 2001). Stomata are composed of two specialized epidermal cells called guard cells that face each other (Sangster 1970). The cross-sectional area, where gas fluxes take place, is determined by both stomatal opening (pore aperture) and stomatal anatomical features (size and density) (Taylor *et al.* 2012). Previous studies of environmental factors affecting the anatomical characters of stomata showed that they were sensitive to both temperature and precipitation (Pandey *et al.* 2007; Giday *et al.* 2013). However, other studies reported that temperature did not have a significant impact on stomata (Reynolds-Henne *et al.* 2010). Consequently, there is currently no unified understanding of what environmental factors have the greatest influence on stomata.

Additionally, the silicified stomata is the amorphous hydrated silica ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$) in the stomata (Motomura *et al.* 2006). Its morphology is largely related to the stomata themselves. Therefore, the main factors influencing the silicified stomata were also evaluated by an orthogonal experimental method in the present study.

Selection and measurement of phytolith morphological parameters

Phytolith size was measured under a microscope (DMBA 300, MOTIC China Group Co., Ltd) at a magnification of 900 \times . Measurements included the length, width and height of various parameters. The overall length, length of saddle, overall width, and width of saddle were used for the saddle phytoliths; the upper base, etue and height for the rondel phytoliths; and the overall length and width for the lanceolate phytoliths and the silicified stomata (Fig. 2). Overall, 4560 phytolith particles were measured and 10 320 original data values of phytolith size were obtained. The detailed original data values for the different phytolith types are shown in Tables 2 and 3. All data are expressed as the mean values \pm SD.

Results

The foremost factor affecting the size of short-cell phytoliths

The results of the range analysis and ANOVA of the saddle phytolith sizes are shown in Table 4 and Figs 3, 4.

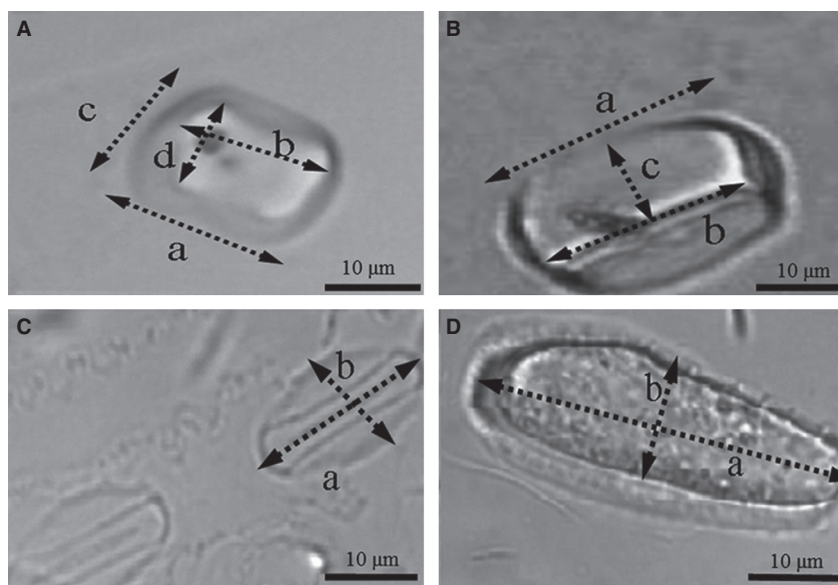


Fig. 2. Sketch of *Phragmites communis* phytoliths. A. Saddle phytoliths: a = overall length; b = length of saddle; c = overall width; d = width of saddle. B. Rondel phytoliths: a = etue; b = upper base; c = height. C. The lanceolate: a = length; b = width. D. The silicified stomata: a = length; b = width. E. Silicified stomata: a = overall length; b = overall width. F. Lanceolate phytoliths: a = overall length; b = overall width.

Table 2. Parameters of short-cell phytoliths. T = xerophytic habitats; W = aquatic habitats.

Site		Saddle phytoliths (μm , mean value \pm SD)				Rondel phytoliths (μm , mean value \pm SD)		
		Length of saddle	Width of saddle	Overall length	Overall width	Upper base	Etue	Height
Dandong	T	7.92 \pm 2.10	6.02 \pm 2.74	12.71 \pm 2.73	9.85 \pm 3.83	5.86 \pm 0.95	9.72 \pm 1.32	4.77 \pm 0.91
	W	7.85 \pm 1.68	5.23 \pm 1.85	11.78 \pm 0.65	9.01 \pm 2.86	5.68 \pm 0.81	9.74 \pm 0.98	4.87 \pm 0.98
Panjin	T	7.13 \pm 1.83	5.10 \pm 1.82	11.66 \pm 2.18	9.13 \pm 2.87	5.39 \pm 1.17	9.44 \pm 1.50	4.07 \pm 0.83
	W	7.95 \pm 2.08	5.92 \pm 1.71	12.46 \pm 2.33	10.15 \pm 2.51	5.88 \pm 1.51	10.13 \pm 2.07	4.07 \pm 1.01
Longwan	T	10.09 \pm 2.41	7.17 \pm 2.59	15.26 \pm 8.25	11.43 \pm 3.29	6.93 \pm 1.26	11.12 \pm 1.80	5.37 \pm 1.17
	W	10.90 \pm 2.79	8.48 \pm 3.36	16.94 \pm 2.95	13.56 \pm 4.06	7.85 \pm 1.36	13.28 \pm 1.45	6.00 \pm 1.15
Tongliao	T	8.38 \pm 1.85	5.70 \pm 2.28	12.18 \pm 2.04	9.32 \pm 2.69	6.48 \pm 1.24	9.78 \pm 1.70	4.40 \pm 1.08
	W	8.75 \pm 2.13	6.65 \pm 2.07	12.78 \pm 2.28	10.48 \pm 3.10	6.59 \pm 1.37	9.95 \pm 1.34	4.77 \pm 1.05
Changchun	T	9.84 \pm 2.15	6.60 \pm 2.58	13.01 \pm 2.36	8.02 \pm 1.71	10.91 \pm 1.54	6.03 \pm 2.00	6.85 \pm 1.28
	W	10.78 \pm 2.43	6.80 \pm 2.37	13.88 \pm 2.58	8.89 \pm 3.40	12.92 \pm 1.67	7.97 \pm 1.64	7.85 \pm 1.35
Mudanjiang	T	10.34 \pm 2.58	7.26 \pm 2.86	15.89 \pm 2.61	11.69 \pm 4.01	7.70 \pm 1.36	12.35 \pm 1.60	5.45 \pm 0.99
	W	10.88 \pm 2.45	8.10 \pm 3.37	16.43 \pm 2.43	12.00 \pm 4.07	7.75 \pm 1.30	12.26 \pm 2.25	5.74 \pm 1.21
Changling	T	10.15 \pm 1.93	7.02 \pm 2.29	14.39 \pm 1.99	11.26 \pm 2.85	7.11 \pm 1.76	11.09 \pm 1.96	5.04 \pm 1.19
	W	8.10 \pm 2.09	6.36 \pm 2.08	12.99 \pm 2.23	10.32 \pm 3.19	5.84 \pm 1.13	9.95 \pm 1.68	4.84 \pm 0.93
Harbin	T	7.35 \pm 1.55	4.79 \pm 1.86	9.99 \pm 1.69	7.55 \pm 2.29	6.16 \pm 1.12	9.03 \pm 1.34	3.68 \pm 1.01
	W	6.96 \pm 1.32	4.69 \pm 1.35	9.67 \pm 1.40	7.67 \pm 2.05	5.45 \pm 1.03	8.34 \pm 1.26	3.65 \pm 0.78
Daqing	T	7.78 \pm 1.58	5.26 \pm 1.40	12.08 \pm 1.77	9.10 \pm 2.73	5.79 \pm 1.14	9.47 \pm 1.56	4.58 \pm 0.91
	W	7.79 \pm 1.88	5.35 \pm 1.64	12.49 \pm 2.27	9.08 \pm 2.81	5.59 \pm 1.23	9.42 \pm 1.55	4.5 \pm 0.95
Tongjiang	T	10.89 \pm 1.74	7.42 \pm 2.19	15.03 \pm 2.06	12.22 \pm 2.59	6.80 \pm 1.63	10.53 \pm 2.08	5.16 \pm 2.18
	W	10.67 \pm 2.76	7.66 \pm 2.94	15.67 \pm 2.83	11.96 \pm 2.78	6.36 \pm 1.23	10.12 \pm 1.58	6.01 \pm 1.88
Beian	T	9.03 \pm 2.11	5.43 \pm 1.88	12.54 \pm 2.12	9.37 \pm 2.51	6.25 \pm 1.30	9.55 \pm 1.73	4.14 \pm 0.96
	W	8.62 \pm 1.77	5.19 \pm 1.82	11.84 \pm 1.77	8.67 \pm 2.56	5.59 \pm 1.03	8.96 \pm 1.60	3.83 \pm 0.55

Table 3. Parameters of lanceolate phytoliths and silicified stomata. T = xerophytic habitats; W = aquatic habitats.

Site		Lanceolate phytoliths (μm , mean value \pm SD)		Silicified stomata (μm , mean value \pm SD)	
		Overall length	Overall width	Overall length	Overall width
Dandong	T	30.99 \pm 8.81	11.71 \pm 2.71	18.32 \pm 1.62	8.85 \pm 0.94
	W	39.41 \pm 23.66	13.00 \pm 5.75	19.11 \pm 1.82	9.54 \pm 1.20
Panjin	T	19.44 \pm 5.44	9.08 \pm 1.69	16.17 \pm 1.51	8.02 \pm 1.04
	W	35.52 \pm 11.89	13.65 \pm 5.03	20.32 \pm 3.43	9.89 \pm 1.74
Longwan	T	34.52 \pm 18.98	13.97 \pm 4.02	20.25 \pm 1.51	10.24 \pm 0.92
	W	36.21 \pm 14.47	15.23 \pm 5.43	20.04 \pm 1.41	9.64 \pm 0.92
Tongliao	T	21.79 \pm 9.76	8.89 \pm 3.26	16.89 \pm 2.00	8.24 \pm 1.67
	W	35.26 \pm 18.13	14.87 \pm 3.54	17.73 \pm 1.53	8.36 \pm 0.94
Changchun	T	11.02 \pm 2.17	8.50 \pm 1.64	19.02 \pm 1.34	9.44 \pm 0.87
	W	9.86 \pm 11.17	8.46 \pm 10.64	23.22 \pm 1.64	10.73 \pm 1.17
Mudanjiang	T	34.66 \pm 17.88	15.34 \pm 5.01	20.25 \pm 2.04	10.55 \pm 1.39
	W	35.86 \pm 12.13	15.16 \pm 3.82	21.31 \pm 1.67	10.84 \pm 1.83
Changling	T	26.72 \pm 10.85	12.94 \pm 4.94	17.94 \pm 1.60	8.21 \pm 0.85
	W	24.03 \pm 8.60	11.41 \pm 3.26	17.60 \pm 1.81	7.96 \pm 1.46
Harbin	T	18.95 \pm 4.33	7.95 \pm 1.02	16.49 \pm 1.80	8.01 \pm 1.16
	W	19.36 \pm 4.47	7.97 \pm 1.34	16.40 \pm 1.56	7.47 \pm 0.76
Daqing	T	22.42 \pm 8.40	9.46 \pm 2.55	16.90 \pm 1.37	7.96 \pm 0.95
	W	24.03 \pm 10.53	10.19 \pm 3.69	17.02 \pm 1.23	7.77 \pm 0.80
Tongjiang	T	36.04 \pm 13.25	16.35 \pm 4.08	18.42 \pm 1.65	9.01 \pm 1.28
	W	35.57 \pm 8.52	16.73 \pm 3.80	21.53 \pm 1.30	10.63 \pm 0.89
Beian	T	26.17 \pm 11.60	10.97 \pm 2.96	17.64 \pm 1.84	8.25 \pm 1.06
	W	22.33 \pm 7.95	9.41 \pm 2.41	17.14 \pm 1.07	8.30 \pm 0.91

Evaluation of the *R*-value of each factor revealed that temperature has the greatest influence on the length of saddle, whereas precipitation has the greatest influence on the width of saddle, overall length and overall width. ANOVA revealed that temperature, precipitation

and precipitation \times habitat had the greatest impacts on the width of saddle and length of saddle ($P<0.01$), with precipitation having the greatest effects on the overall width and length ($P<0.05$). Overall, the range analysis and ANOVA revealed that temperature has the greatest

Table 4. Analysis of variance (ANOVA) of the size and width-to-length ratio of *Phragmites communis* phytoliths.

Phytolith morphotype	Parameter	Factor				
		Habitat	Temperature	Temperature×habitat	Precipitation	Precipitation×habitat
Short-cell phytoliths	Saddle					
	Length of saddle	0.677	0.002	0.301	0.009	0.003
	Width of saddle	0.367	0.024	0.513	0.024	0.054
	Length	0.596	0.018	0.416	0.004	0.02
	Width	0.448	0.251	0.232	0.014	0.244
Rondel	Width of saddle/length of saddle	0.384	0.564	0.919	0.346	0.75
	Width/length	0.379	0.106	0.176	0.25	0.079
	Upper base	0.718	0.034	0.421	0.001	0.002
	Etue	0.620	0.902	0.230	0.027	0.538
	Height	0.367	0.004	0.365	0.143	0.007
Lanceolate	Height/upper base	0.131	0.168	0.026	0.001	0.009
	Height/etue	0.883	0.064	0.238	0.141	0.069
	Length	0.195	0.252	0.56	0.012	0.585
	Width	0.263	0.792	0.612	0.031	0.532
	Width/length	0.851	0.013	0.335	0.024	0.025
Silicified stomata	Length	0.042	0.043	0.693	0.011	0.025
	Width	0.342	0.245	0.993	0.006	0.024
	Width/length	0.041	0.333	0.448	0.012	0.115

effect on the length of saddle, whereas precipitation has strongest effects on the width of saddle, as well as the overall width and length. Moreover, the range analysis and ANOVA also revealed that the width-to-length ratios of *P. communis* saddle phytoliths, including the width of saddle/length of saddle and overall width/length did not change with changes in environmental factors (Table 4, Figs 3, 4), implying that saddle the shape of saddle phytoliths is stable.

The range analysis and ANOVA of the sizes of the rondel phytoliths (Table 4, Figs 3, 4) indicated that temperature, precipitation and precipitation×habitat had the greatest effects on the upper base, whereas temperature and precipitation x habitat are the key factors affecting the height. The *R*-value of precipitation to the etue was the largest amongst all these ranges (Fig. 3), implying that precipitation is the most significant factor influencing the etue. The ANOVA (Table 4) further indicated that variations in precipitation have significant effects on the etue (*P*<0.05). Based on these findings, precipitation is the strongest influencing factor for the etue of the rondel phytoliths. As shown in Table 4 and Fig. 4, precipitation, precipitation×habitat and temperature×habitat significantly affect the height/upper base, whereas the height/etue do not change in response to any factors.

Based on the above analysis, temperature and precipitation are the key factors influencing short-cell phytoliths (including the saddle and rondel types). Additionally, there are slight variations in the width-to-length ratios of short-cell phytoliths other than the height/upper base of the rondel types in response to environmental factors.

The foremost factor affecting the size of lanceolate phytoliths

Based on the magnitude of the *R*-values, the influence of the experimental factors on the size of lanceolate phytoliths is ranked as follows: precipitation>temperature>habitat>precipitation×habitat>temperature×habitat>temperature×precipitation for their overall length; precipitation>temperature×precipitation>habitat>temperature×habitat>precipitation×habitat>temperature for their overall width; temperature>precipitation>precipitation×habitat>temperature×habitat>temperature×precipitation>habitat for their overall width/length ratio (Figs 3, 4). The *R*-value of precipitation is the largest amongst all of these ranges, implying that precipitation is the most significant factor influencing the size of lanceolate phytoliths. The ANOVA (Table 4) indicated that variations in precipitation have significant effects on the overall length, width and width/length of the lanceolate phytoliths (*P*<0.05). Based on these findings, precipitation was the strongest influencing factor, which is consistent with the results of the range analysis (Figs 3, 4).

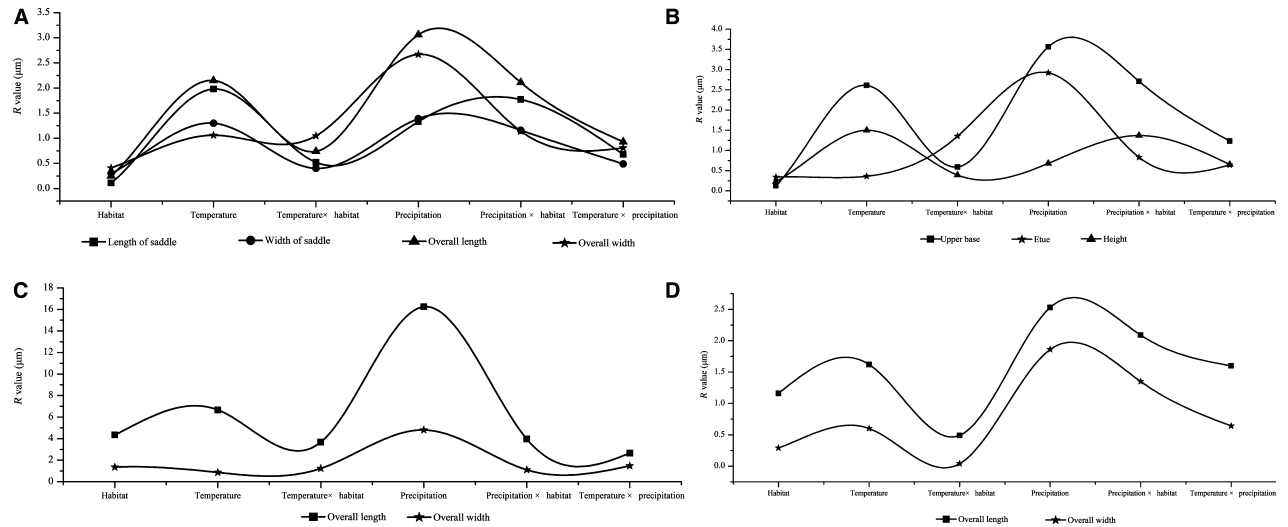


Fig. 3. Results of orthogonal experiments evaluating the sizes of *Phragmites communis* phytoliths. A. Saddle phytoliths. B. Rondel phytoliths. C. Lanceolate phytoliths. D. Silicified stomata.

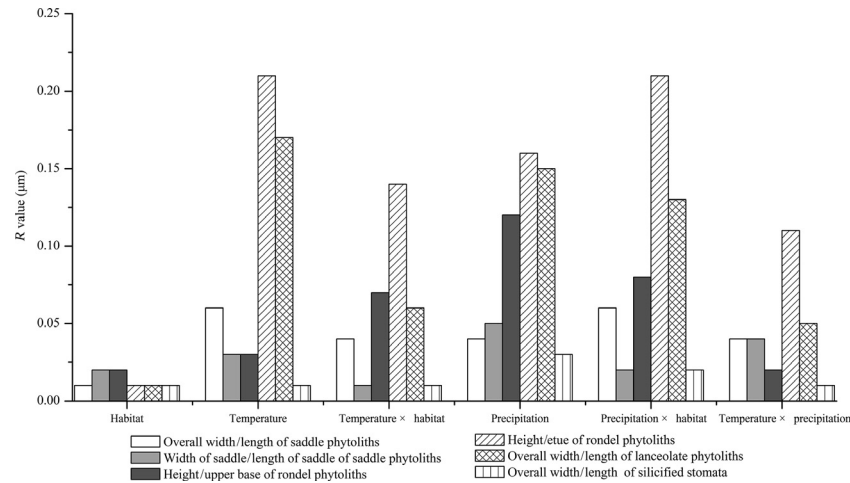


Fig. 4. Results of orthogonal experiments evaluating the width-to-length ratio of *Phragmites communis* phytoliths.

The foremost factor affecting the size of the silicified stomata

Table 3 summarizes the sizes of the silicified stomata in the present study. The size of the silicified stomata is affected by the six factors in different ways. The results of the ANOVA (Table 4) demonstrate that all factors significantly affect the overall length of the silicified stomata ($P < 0.05$). Additionally, precipitation and precipitation × habitat have strong effects on the overall width of the silicified stomata ($P < 0.05$), in comparison with dry conditions, precipitation conditions being better for the formation of silicified stomata ($P < 0.01$). Precipitation and habitat also have significant effects on the overall width/length of the silicified stomata ($P < 0.05$). According to the R -value of each factor, the influences of the experimental factors upon the silicified stomata are ranked as follows: precipitation > precipita-

tion > habitat > temperature > temperature × precipitation > habitat > temperature × habitat for their overall length; precipitation > precipitation × habitat > temperature × precipitation > temperature > habitat > temperature × habitat for their overall width; and precipitation > precipitation × habitat > temperature > temperature × precipitation > temperature × habitat > habitat for their overall width/length ratio (Figs 3, 4), which is consistent with the results of the ANOVA. Accordingly, we believe that the size and width-length ratios of the silicified stomata are sensitive to variations in water difference.

Principal component analysis (PCA) of phytolith size

PCA is a method of reducing environmental variables. Therefore, PCA analysis of the sizes of *P. communis* phytoliths was conducted to further clarify the influence of temperature, precipitation and habitat

differences and to verify the results of the orthogonal experiment. The first and second PCA axes explained 100.00% of the variation (Table 5), indicating that the phytolith sizes are mainly affected by the environmental factors represented by these two axes, with the factor represented by the first axis being most important.

The PCA revealed that all parameters except the length of saddle and width of saddle phytoliths are distributed in the positive direction on the first axis and at the direction of their maximum (Table 5). Additionally, the range analysis and ANOVA revealed that precipitation has an important influence on *P. communis* phytolith sizes (Table 4, Figs 3, 4), and so the first axis could be considered as the axis of precipitation. For the second axis, the length of saddle and length of saddle phytoliths is distributed in the positive direction on the second axis, whereas the overall length and width of lanceolate phytoliths is distributed in the negative direction (Table 5). The second axis could be considered the axis of temperature; therefore, the results of the PCA show that *P. communis* phytolith size is influenced by precipitation and temperature, especially precipitation, further confirming the reliability of the orthogonal experiment results.

Discussion

The most influential factors influencing *P. communis* phytoliths

Evaluation of the uppermost factors influencing *P. communis* phytoliths reveals that temperature, precipitation and precipitation × habitat have great effects on phytoliths, with precipitation having the greatest effect. Hong *et al.* (2008) found that the anatomical structures of *P. communis* were sensitive to precipitation in northeast China, and that the diameter of vascular bundles increased with increas-

ing precipitation. Work on the morphological and anatomical changes of *Annona glabra* L. and *Lotus creticus* also showed similar trends in Brazil and Spain, that is, the anatomical changes of *Annona glabra* L. and *Lotus creticus* were largely related to precipitation (Bañona *et al.* 2004; Soami *et al.* 2008). As changes in the morphological and anatomical changes lead to variations in leaf cell morphological change, we speculate that the cells of plant leaves are also sensitive to precipitation in northeast China. Guo & Zhu (1994) conducted a correlation analysis of the relationship between *Aneurolepidium chinense* (Trin.) Keng community biomass and climatic factors from 1978 to 1990 in northeast China and found that precipitation was the main factor influencing community biomass. Overall, these findings indicate that increased precipitation may strengthen the assimilation of silica by *P. communis*, resulting in larger phytoliths. Furthermore, the height, biomass and spatial distribution of wetland plants are determined by the availability of water (Luan *et al.* 2006; Webb *et al.* 2012; Johns *et al.* 2015), which suggests that the sizes of *P. communis* phytoliths in northeast China may be closely related to precipitation. Lu *et al.* (2006) investigated 243 surface soil phytolith samples from China and the results of canonical correspondence analysis and detrended correspondence analysis suggested that the mean annual precipitation was the dominant variable controlling the spatial distribution of the phytolith assemblages. Zhang (2006) also found that the mean annual temperature and relative humidity were the key factors influencing the contents of different phytolith types. Consequently, as similar with different phytolith assemblages, phytolith size is also strongly related to environmental changes. This suggests that phytolith size may also be a valuable tool in palaeoenvironmental reconstructions, with potential for improving their precision.

Our experimental results also revealed that there are no obvious variations in the width-to-length ratios of short-cell phytoliths under the influence of environmental factors, whereas there are clear changes in lanceolate phytoliths and in silicified stomata. Lawton (1980) showed that short-cell phytoliths were mainly controlled by genetic factors, whereas bulliform cell phytoliths and silicified stomata were largely influenced by environmental factors associated with transpiration. Many investigations of the formation mechanisms of phytoliths have further reported that short-cell phytoliths are under a considerable degree of active genetic control (Hodson *et al.* 2005; Madella *et al.* 2009). Conversely, environmentally controlled solidification occurred in cells that did not have genetic control over silica deposition in their lumen, such as epidermal long cells (Piperno 1988), and the mechanism of deposition was related to water flow (Rich-

Table 5. Factor scores and cumulative contribution rate determined by PCA.

Phytolith morphotype		Parameter	Main composition factor	
			1	2
Short-cell phytoliths	Saddle phytoliths	Length of saddle	−0.377	0.926
		Width of saddle	0.908	0.419
		Overall length	0.412	0.911
		Overall width	−0.221	−0.975
	Rondel phytoliths	Upper base	0.987	0.163
		Etue	0.903	−0.430
		Height	0.997	−0.078
Lanceolate phytoliths		Overall length	0.710	−0.705
		Overall width	0.767	−0.641
Silicified stomata		Overall length	0.981	0.196
		Overall width	0.798	0.603
Cumulative contribution rate (%)			60.518	100.000

mond & Sussman 2003; Madella *et al.* 2009), which is consistent with our experimental results. Our findings verify the genetic stability of short-cell phytoliths and the reliability of short-cell phytoliths for the identification of grasses, further confirming the effectiveness of phytoliths as an indicator of palaeoclimate.

Nevertheless, a number of the non-short-cell phytoliths, such as the bulliform cell phytoliths (Wang *et al.* 1997; Huang & Zhang 2000; Ma & Fang 2007; Xu 2010) and long cell phytoliths (Nasu *et al.* 2007; Zhang *et al.* 2011a) have been also conducted to distinguish plant species, even at the species level. These findings suggest that there may be still some identification information regarding morphological parameters of the non-short-cell phytoliths. Therefore, the variations in width-to-length ratios and size of *P. communis* lanceolate phytoliths recorded in this study may also provide some information for plant classification. However, further studies are needed to ascertain this conclusion. Accordingly, greater attention should be given to the sizes of non-short-cell phytoliths in future studies as this may provide information that would strengthen their role in plant classification, which would improve the accuracy of palaeovegetation reconstruction.

The most influential factors influencing P. communis silicified stomata

In our study, precipitation and precipitation \times habitat had strong effects on the sizes of the silicified stomata, and precipitation and habitat also significantly influenced the width-to-length ratios of the silicified stomata. In general, the sizes and the width-to-length ratios of the silicified stomata are sensitive to water variations. Recent studies of the environment factors affecting the stomata features showed that seasonal temperature and precipitation were significant environmental factors for stomata (Pandey *et al.* 2007; Shtein *et al.* 2011; Giday *et al.* 2013). However, prior to these works, light intensity rather than temperature was considered as the most important environmental factor influencing stomata (Dickison 2000), and temperature was thought to have little influence on stomata (Bañona *et al.* 2004). Therefore, it is clear that there are differences in the main environmental factors influencing stomata amongst studies. Accordingly, it is likely that an environment cask effect of distinct study area leads to the main environmental factors that caused the stomata size to differ. The *P. communis* in our experiment came from sampling sites in wetlands in northeast China, and the presence of wetland plants is determined by the availability of water. Peng *et al.* (2007) investigated variations in rice stomatal resistance in Qingan County of Heilongjiang Province, China (45°63'N, 127°3'E), and used factor analysis to determine the

main environmental variables that influenced this resistance. They suggested that moisture factors including relative humidity, vapour pressure deficit and soil water played the most important roles, whereas the effects of temperature and light were relatively small. Accordingly, *P. communis* silicified stomata may also be more sensitive to changes in moisture content that lead to significant differences in their size and shape under different precipitation conditions.

Therefore, our results reveal that silicified stomata are sensitive to environmental factors, especially precipitation. Many studies have used stomata as a supplementary source of palaeoecological data to study tree lines with greater precision than can be achieved with pollen alone (Hansen *et al.* 1996; Gervais & MacDonald 2000; Gervais *et al.* 2002). Combined stomatal and pollen results from Tianchi Lake in the Loess Plateau also revealed the recent forest history and confirmed the local presence of conifer trees (*Abies* and *Pinus*) in the vicinity of the lake to further successfully reconstruct the Holocene environment and vegetation in China (Zhang *et al.* 2011b). Overall, these findings indicate the potential of using silicified stomata analysis as a palaeoecological tool in palaeoenvironmental reconstructions.

Significant works on the morphological characters of stomata have shown that they can be used to classify different coniferous species. Conifer stomata are morphologically variable and can be identified at least to family and in some instances to species level (Hansen 1995; Yu 1997). Wan *et al.* (2007) successfully discriminated Pinaceae and Cupressaceae based on the sizes of stomata. Later, Álvarez *et al.* (2009) found that 10 morphological parameters of stomata could be used to differentiate *Pinus sylvestris* and *P. uncinata*. More recently, Zhang *et al.* (2011b) measured seven variables of stomata from eight conifer species and suggested that conifer stomata could be identified to at least genus level. They then used their reference stomata results for the identification of fossil stomata from the Tianchi Lake. These findings all suggest that stomata can also be used for the identification of other plant taxa, even to the species level. Therefore, the variations in the width-to-length ratios and sizes of the silicified stomata of *P. communis* observed in this study may also contain some information that could be used for identification. Studies on the silicified stomata of modern plants would be helpful to improve the precision of distinguishing the species represented in fossil materials to further aid the reconstruction of regional palaeovegetation.

Conclusions

Range analysis and ANOVA revealed that under the influence of temperature, precipitation, habitat differ-

ences and interactive effects between these factors, variations in temperature and precipitation have significant effects on the sizes of saddle and rondel phytoliths in *P. communis*. More specifically, temperature is the prominent factor influencing the length of saddle phytoliths and the height of rondel phytoliths, whereas precipitation has significant effects on the sizes of other parameters of saddle and rondel phytoliths. However, there are no obvious variations in the width-to-length ratios of short-cell phytoliths in response to environmental factors. These results indicate that short-cell phytoliths are genetically stable, confirming that they could be used for the classification of grasses.

Precipitation was found to be the main factor influencing the sizes of lanceolate phytoliths and silicified stomata, and their width-to-length ratios changed significantly in response to precipitation and habitat differences. These data reveal that lanceolate phytoliths and silicified stomata are sensitive to environmental changes, indicating their usefulness for palaeoenvironment reconstruction. Additionally, variations in width-to-length ratios and size may also provide information for plant classification, even to the species level. Accordingly, increasing the use of these characteristics in plant classification could contribute to improving its accuracy and that of palaeovegetation reconstruction.

The PCA of the size of *P. communis* phytoliths conducted here supported the results of our orthogonal experiment and confirmed that temperature and precipitation are the prominent factors influencing the size of *P. communis* phytoliths. The PCA also revealed the environmental implications of the size of *P. communis* phytoliths to further enrich their environmental context.

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