

Phytolith analysis for the identification of barnyard millet (*Echinochloa* sp.) and its implications

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Abstract *Echinochloa* was an important prehistoric food crop of early agriculture in Asia. Macro-remains can be used to identify *Echinochloa*. However, when few macro-remains are available, phytolith analysis can be performed. In this study, we examined the phytolith morphology of the glumes, lemmas, and paleas from the inflorescence bracts of nine *Echinochloa* species from different regions of China and obtained diagnostic, morphological, and morphometric characteristics for *Echinochloa*. Phytoliths in *Echinochloa* are different from those in most known crops except those in *Setaria italica* and *Panicum miliaceum*. We found the following two diagnostic features within an epidermal silica layer that can be used to distinguish *Echinochloa* sp. from *S. italica* and *P. miliaceum*: (1) the β -type undulated patterns with constricted top of the undulation amplitude and (2) the discriminant functions based on the morphometric data of the β -type undulated patterns, which suggested that 94.9 % of the cross-validated data were correctly classified into *Echinochloa*, *S. italica*, and *P. miliaceum*. Thus, we established the phytolith identification criteria for *Echinochloa*; this could have important implications in plant taxonomy, archaeobotany, and plant domestication.

Keywords Phytolith · *Echinochloa* · Barnyard millet · Domestication · Archeology

Introduction

Phytoliths are silica bodies mostly deposited in the intercellular or intracellular structures of plants; they are particularly abundant in plants belonging to *Poaceae* (Piperno 2006; Wang and Lu 1993) and are useful in archeological studies (Madella and Db, 2007; Pearsall 1989; Piperno 2006; Wang and Lu 1993). Although numerous plants belong to the family *Poaceae*, phytolith morphology has been mainly analyzed in food crops such as rice (Pearsall et al. 1995; Sato et al. 1990; Zhao et al. 1998), wheat and barley (Ball et al. 1999; Ball et al. 2001; Parry and Smithson 1966; Tubb et al. 1993), millet (Lu et al. 2009b; Madella et al. 2013; Weisskopf and Lee 2014; Zhang et al. 2011), and maize (Holst et al. 2007; Mulholland et al. 1988; Piperno and Pearsall 1993) due to their importance; considering the diagnostic morphology of phytoliths, they have been successfully used to elucidate the origin and domestication of these crops (Chen et al. 2012; Fuller et al. 2007; Horrocks et al. 2009; Lu et al. 2002; Lu et al. 2009a; Madella et al. 2014; Piperno et al. 2009; Piperno and Stothert 2003). However, other plants in this group, which might have been crops in the past but are treated as weeds at present, have been rarely studied, and phytolith analysis in such plants might provide important information on the use of these traditional plants.

Echinochloa is a genus belonging to the subfamily *Panicoideae* of *Poaceae*; species belonging to this genus are found worldwide and are commonly known as barnyard millet (or barnyard grass). The following two cultivars of this genus were formerly considered to be important crops in East Asia (Yabuno 1987): *Echinochloa esculenta* (also known as

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Japanese barnyard millet) and *Echinochloa frumentacea* (also known as Indian barnyard millet or sawa millet). Only these two barnyard millets are still cultivated and used as food (Gupta et al. 2009; Harlan 1989; Roshevits 1980; Yabuno 1987), and most other barnyard grasses are presently considered harmful weeds to crops (particularly rice) (Holm et al. 1977; Pandey et al. 2014). Archeological studies suggested that *Echinochloa* has been cultivated for centuries in Asia, particularly in Japan, where barnyard millet cultivation might have commenced 4000 years ago, and the oldest caryopsis of this genus found was more than 9000 years old (Crawford 1983; Watanabe 1970). In Japan, previous studies conducted scanning electron microscopy to examine the surface characteristics of the caryopsis and husks (Tsubakisaka 1988; Tsubakisaka 1993), as the preservation condition in Japan was generally good. However, in China, archeological studies performed using floatation analysis have suggested that seeds of *Echinochloa* were found at the sites across Paleolithic to Neolithic periods (Bestel et al. 2014; Jin et al. 2014; Lee et al. 2007; Wu et al. 2010; Zhang 2012), but their density was very low. Even in northeast China, where it has been suggested that *Echinochloa* may have been farmed, the remains of *Echinochloa* were also very low (Yang et al. 2010), and it was hard to find evidence of *Echinochloa* as a crop or wild resource. When macro-remains are not applicable, phytolith analysis could be employed, since phytoliths can be preserved in most cases. In recent studies (Madella et al. 2013; Weisskopf and Lee 2014), the phytoliths of *Echinochloa* inflorescence were observed and contrasted with those from other plants, but these studies provided an overall view. Hence, an in-depth study was needed to better understand the phytolith morphology in the inflorescence of *Echinochloa*.

In this study, samples were obtained from nine species belonging to *Echinochloa* from different regions of China (Fig. 1); six samples were obtained from *Echinochloa crus-galli* and its varieties, and three samples were obtained from

three different species (Table 1 and Fig. 2). A systematic and detailed analysis of the phytoliths produced in the spikelet of *Echinochloa* sp. was conducted, and diagnostic morphological characteristics of phytoliths were used to identify distinct features of different *Echinochloa* sp. Furthermore, useful parameters to assist in the discrimination of other millets were revealed. Our findings might assist in the archeological identification of *Echinochloa* sp. and provide taxonomic information.

Materials and methods

Sample preparation

The samples used in this study were collected during field trips; no necessary permits for the described field investigations were needed, since all field trips were carried out in non-restricted wild areas and no endangered or protected species were included. Some of the species were identified by Prof. Zhijian Feng from the East China Normal University and Prof. Shuzhi Cheng from the Institute of Botany Chinese Academy of Sciences.

The spikelet of *Echinochloa* sp. were dissected into four major parts (Fig. 3): (1) two glumes, (2) lemma and palea of sterile floret, (3) lemma and palea of fertile floret, and (4) seed. Since the lemma of sterile florets is usually similar to the glume, and the palea of sterile florets is thin and transparent, the lemma and palea are considerably different from those of fertile florets (which are thick and hard); thus, for simplicity, in the present study, the words “lemma” and “palea” refer to the lemma and palea of fertile florets. Every part of the samples was cleaned with distilled water in an ultrasonic cleaner, and then prepared for wet oxidation, as described by previous study (Lu et al. 2009b). The description of phytoliths in *Echinochloa* follows the studies on *Setaria* and *Panicum*

Fig. 1 Sample distribution in different regions of China. The code on the map refers to Table 1

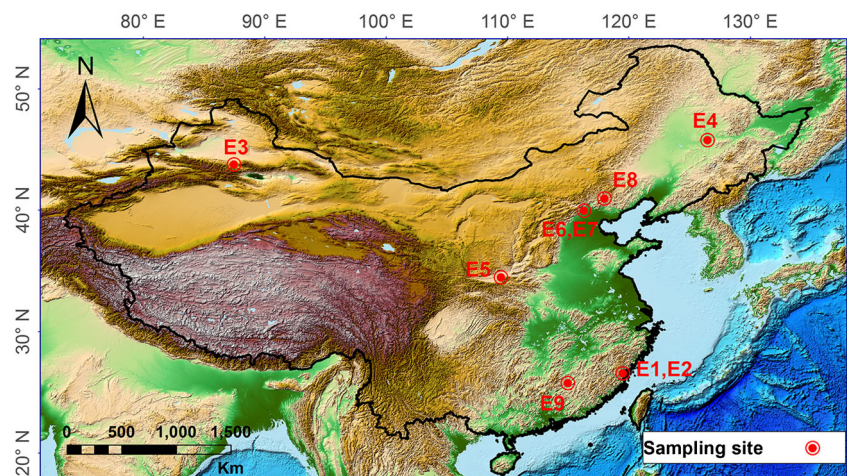


Table 1 Information on the plants studied

| Sample code | Breed name | Section | Species | Province | Precipitation | Sample date |
|-------------|--------------|---------------------|--|--------------|-----------------|-------------|
| E1 | Changmangbai | Section Hispidula | <i>Echinochloa crusgavonis</i> (H. B. K.) Schult. | Fujian | >800 mm/year | 1989 |
| E2 | Guangtoubai | Section Echinochloa | <i>Echinochloa colona</i> (L.) Link | Fujian | >800 mm/year | 1989 |
| E3* | Baicao | Section Echinochloa | <i>Echinochloa crus-galli</i> (L.) Beauv. | Xinjiang | <200 mm/year | 2014 |
| E4* | Etoubai | Section Echinochloa | <i>Echinochloa crus-galli</i> (L.) Beauv. | Heilongjiang | 400~800 mm/year | 2014 |
| E5 | Xiaohanbai | Section Echinochloa | <i>Echinochloa crus-galli</i> (L.) Beauv. var. <i>austro-japonensis</i> Ohwi | Shaanxi | 400~800 mm/year | 1993 |
| E6 | Wumangbai | Section Echinochloa | <i>Echinochloa crus-galli</i> (L.) Beauv. var. <i>mitis</i> (Pursh) Peterm.Fl. | Beijing | 400~800 mm/year | 1999 |
| E7 | Xiyehanbai | Section Echinochloa | <i>Echinochloa crus-galli</i> (L.) Beauv. var. <i>pratricula</i> Ohwi | Beijing | 400~800 mm/year | 2007 |
| E8 | Xiyehanbai | Section Echinochloa | <i>Echinochloa crus-galli</i> (L.) Beauv. var. <i>pratricula</i> Ohwi | Hebei | 400~800 mm/year | 2014 |
| E9 | Yingfubai | Section Phyllopogon | <i>Echinochloa glabrescens</i> Munro ex Hook. f. | Jiangxi | >800 mm/year | 2014 |

E3 is immature which have no seeds but only green husks, but the lemma and palea reach the similar size with other species; E4 is a cultivar which is mostly used as forage

(Lu et al. 2009b; Zhang et al. 2011) and the ICPN rules (Madella et al. 2005).

Data measurement and analysis

The parameters of the phytoliths were measured using a Leica DM 750 microscope by using software LAZ v4.5.

The Kruskal-Wallis test, a non-parametric test, was used to determine the differences in the distribution of parameters between the different sample groups since the data were not normally distributed. The significance level was set at $p < 0.05$.

In the discriminant function analysis, the numbers of parameters for *Echinochloa* sp., *P. miliaceum*, and *S. italica* were 36, 36, and 27, respectively. All data used were the mean

value of each parameter; morphology of phytolith and data for *P. miliaceum* and *S. italica* were adapted from previous study (Lu et al. 2009b).

Results

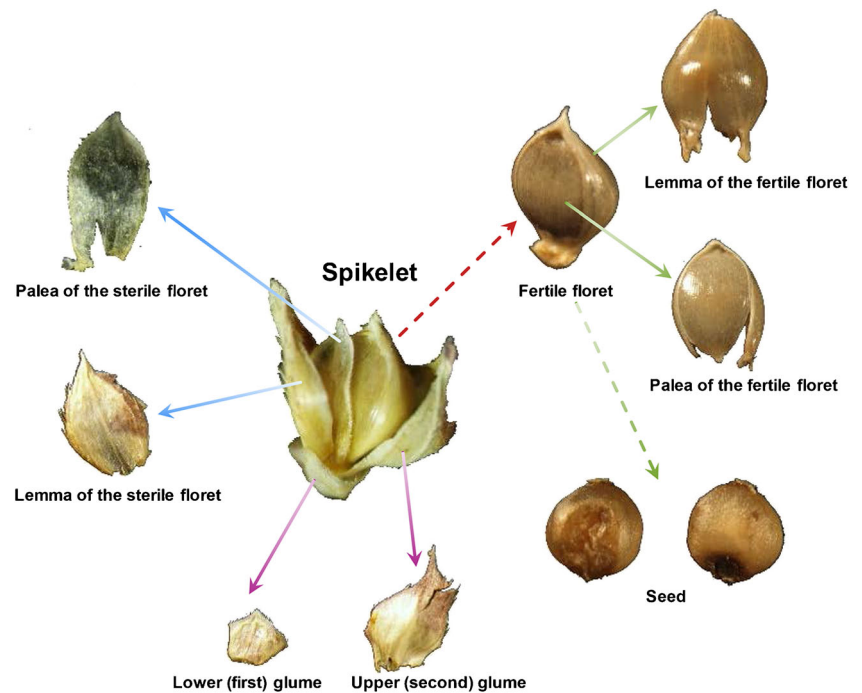
Phytolith morphology in the glumes

All the nine species had similar glume morphology and produced the same types of phytoliths (Fig. 4a). The most abundant phytolith type was bilobate phytoliths (silicified short cells); silicification also occurred in the long cells to form elongate phytoliths, in hair cells to form acicular or unciform phytoliths, in the hair base to form small tabular phytoliths,

Fig. 2 Photographs of the spikelet of the nine samples. The code on top left refers to Table 1



Fig. 3 Illustration of the structure of the spikelet of *Echinochloa* sp. studied



and rarely in the stomata to form silicified stomata and silicified stomata guard cells. The glumes could contain a silica layer (which could also be named as silica skeleton or multi-cell phytoliths), as has been reported by previous researchers (Madella et al. 2013; Weisskopf and Lee 2014), but the silica layer in the glumes was usually very weakly silicified, and it was rarely observed in our samples.

Phytolith morphologies in the lemma and palea of sterile florets

The morphology and phytolith types of the lemma of sterile florets were mostly the same as those of the glumes; the palea of the sterile florets of all samples was thin and transparent and produced no phytoliths. The morphologies of bilobate phytoliths produced in the lemma of the sterile florets were found to be the same as the bilobate phytoliths produced in the glumes (Fig. 4a). However, the bilobate phytoliths produced in the lemma and palea of the fertile florets were different (Fig. 4b). On the basis of this information, we divided the

bilobate phytoliths into the following two types: the glume-type bilobate phytoliths (Fig. 4a) that had cavate ends and the lemma-type bilobate phytoliths (Fig. 4b) that had semicircular ends. In the lemma and palea of fertile florets, the lemma-type bilobate phytoliths could be observed only around the top margin; however, in the lemma of sterile florets and glumes, the glume-type bilobate phytoliths could be observed from the base to top. The glume-type bilobate phytoliths were observed in the lemma of sterile florets in most samples; however, in the lemma of sterile florets of sample E9, the lemma-type bilobate phytoliths were observed, and other phytolith types in the sterile florets of sample E9 were also the same as those in the lemma of fertile florets.

Phytolith morphologies in the lemma and palea of fertile florets

Being the most important parts of the spikelet, the lemma and palea of fertile florets are thick and glossy; the lemma usually has a bulging center, whereas the palea is relatively flat. They

Fig. 4 Comparison of phytolith types based on glume type and lemma type. **a** Glume-type phytoliths mostly occur in glumes and sterile florets. **b** Lemma-type phytoliths mostly occur in fertile florets

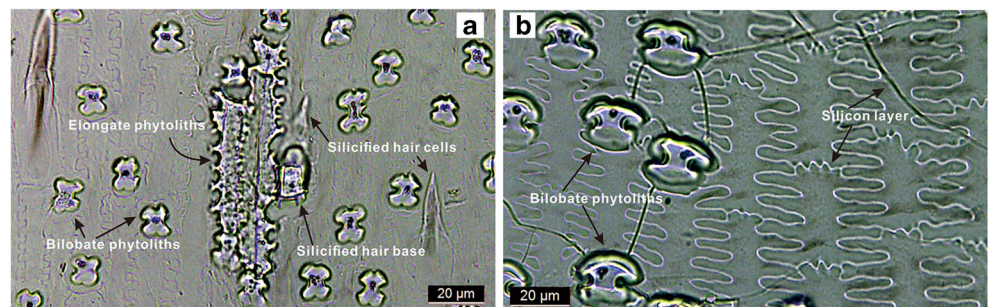
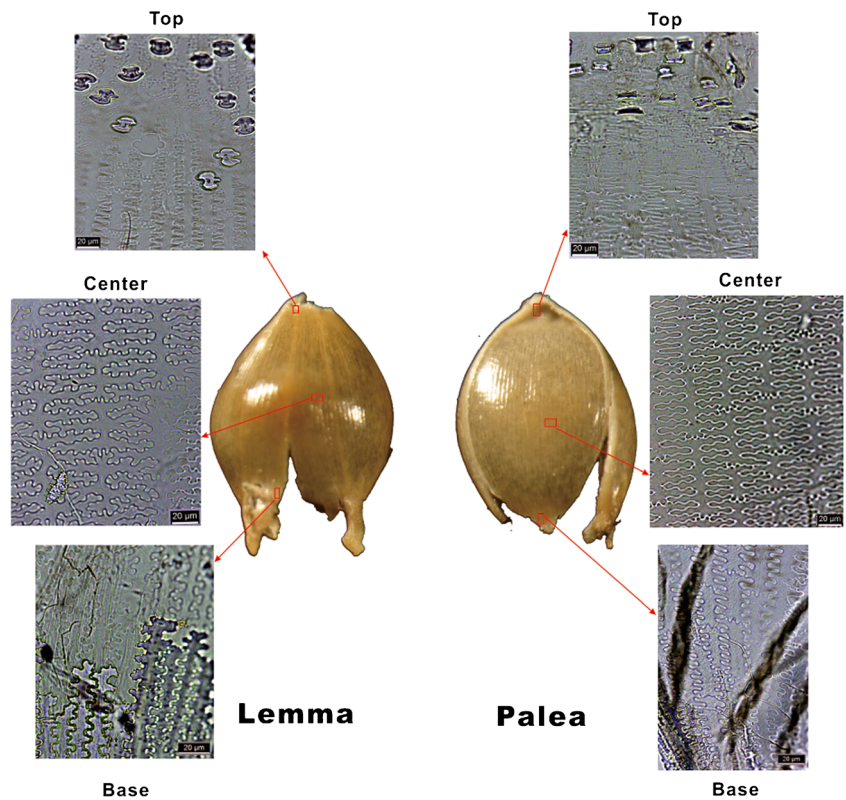


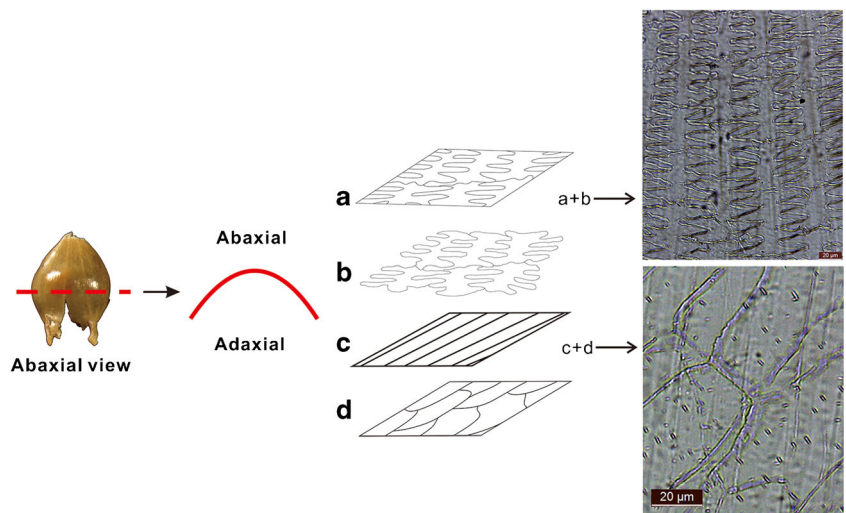
Fig. 5 Phytolith types in the lemma and palea of fertile florets



both wrap the seed tightly and, in general, produce similar types of phytoliths (Fig. 5), which mainly include bilobate phytoliths (lemma-type), silicified long cells, and the silica layer. The surface of the lemma and palea is covered by a silica layer with undulated patterns. The lemma-type bilobate phytoliths only occur around the top of the lemma and palea, and the silicified long cells are mostly found around their base. The undulated patterns grow longer and the branches have more protuberance in the center of both the lemma and palea compared with the ones around the margin.

To understand the position of phytolith occurrence, we investigated the location of phytolith production. In our study, four layers were observed (Fig. 6). From the abaxial (outside) to adaxial (inside) sides, the layers included the following: (a) silica layer, this layer covers the surface of the lemma and palea and has undulated characteristic patterns that can be used as a diagnostic tool; this layer was found in all the samples. (b) Epidermal long cells, this layer consists of epidermal long cells that are usually strongly attached to the silica layer; these cells are occasionally silicified and are mostly found at the base (Fig. 5). (c) Hypoderm fibers, this layer mostly

Fig. 6 Layers of the lemma and palea observed from abaxial to adaxial sides. **a** Silica layer. **b** Epidermal long cells. **c** Hypoderm fibers and vascular tissues. **d** Inner epidermis



consists of fibers and vascular tissues; silicification seldom occurs in this layer. (d) Inner epidermis, this layer consists of straight-walled long cells; silicification was not observed in this part in any of the samples. Silicification (the silica layer) was stronger in the outer side of both the lemma and palea and thus could be assumed to help protect the seeds.

The undulated patterns of the surface silica layer were characteristic and diagnostic; such a pattern was named β type (Fig. 7). The β -type undulated patterns could be divided into four levels. Depending on the growth of this pattern (from β -I to β -IV), the undulation amplitude grows longer and has more protuberance. The basic type of undulated patterns only occurs around the margin of the lemma and palea; from the margin to center, the undulated patterns become β type and grow from β -I to β -IV. The lemma and palea produce almost similar β -type undulated patterns, but the protuberance of the undulation amplitude in the palea is often smaller than that in the lemma, forming a smoother pattern in the palea than in the lemma. However, the β -IV undulated patterns are observed in the lemma of all samples but are absent in the palea of some samples.

Morphometric analysis of the β -type undulated patterns in the lemma and palea

According to the study of *S. italica* and *P. miliaceum* (Lu et al. 2009b) and our observation, three important parameters (Fig. 8) were used to characterize the morphological variations of the structures of the β -type undulated patterns: (1) W = width of ending interdigitation of the β -type undulated patterns, (2) H = length of undulation amplitude of the β -type undulated patterns, and (3) R = ratio of the width of ending interdigitation to undulation amplitude [$R = W/((H_1 + H_2)/2)$]. These three parameters were relatively stable among the different *Echinochloa* samples (Table 2). In the nine samples ($n = 1395$), W , H , and R values ranged from 1.1 to 14.4 μm (average, $4.86 \pm 1.82 \mu\text{m}$), 6.50 to 61.00 μm ($27.42 \pm 9.84 \mu\text{m}$), and 0.06 to 0.89 (0.19 ± 0.09), respectively. The H value varied extensively since the β -type undulated patterns have four levels, and the higher β level usually has higher H value.

Fig. 7 Illustration of the β -type undulated patterns and their distribution

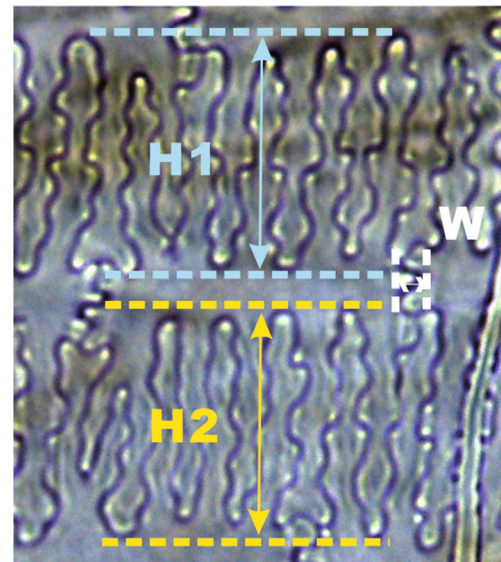
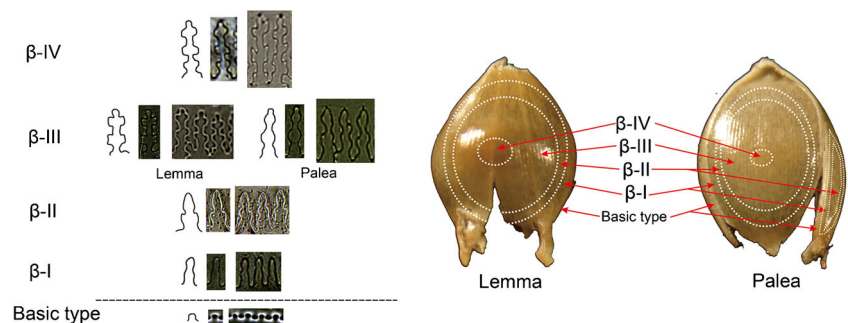


Fig. 8 Parameters of the undulated patterns used in the study

Discriminating *Echinochloa* sp. from *Setaria italica* and *Panicum miliaceum*

Whether the β -type undulated patterns in *Echinochloa* sp. are distinct from those in other species is not yet known. The widely used criteria for species identification by using inflorescence phytoliths are mainly based on the morphology of inflorescence phytoliths in rice (Pearsall et al. 1995; Zhao et al. 1998), maize (Holst et al. 2007; Piperno and Pearsall 1993), wheat and barley (Ball et al. 1999; Ball et al. 2001; Parry and Smithson 1966; Tubb et al. 1993), foxtail millet, and common millet (Lu et al. 2009b; Zhang et al. 2011); the phytolith morphologies in rice, corn, wheat, and barley are apparently different from that in *Echinochloa* sp. However, the morphology of inflorescence phytoliths in *S. italica* and *P. miliaceum* is more similar to that in *Echinochloa* sp. Therefore, we mainly focused on these two species.

In our previous study (Lu et al. 2009b), we found that the differences in the morphology of the undulated patterns among Ω type (*S. italica*), η type (*P. miliaceum*), and β type (*Echinochloa* sp.) is mainly due to the differences in the undulation amplitude (Fig. 9). The basic morphologies of the Ω -type, η -type, and β -type undulated patterns were not

Table 2 Measured data of the parameters of the β -type undulated patterns

| Sample code | β level | W (μm) | | $(H_1 + H_2)/2$ (μm) | | R | | Count |
|-------------|---------------|-----------------------|------|-----------------------------------|------|---------|------|-------|
| | | Average | SD | Average | SD | Average | SD | |
| E1 | 1 | 4.21 | 1.71 | 13.4 | 3.32 | 0.32 | 0.11 | 45 |
| E1 | 2 | 4.62 | 0.83 | 23.94 | 4.25 | 0.20 | 0.05 | 44 |
| E1 | 3 | 5.91 | 1.19 | 29.90 | 3.73 | 0.20 | 0.04 | 46 |
| E1 | 4 | 6.51 | 1.87 | 33.81 | 4.18 | 0.20 | 0.48 | 44 |
| E2 | 1 | 3.88 | 1.91 | 14.86 | 3.88 | 0.26 | 0.10 | 42 |
| E2 | 2 | 4.18 | 1.41 | 23.06 | 3.79 | 0.18 | 0.06 | 42 |
| E2 | 3 | 4.34 | 0.99 | 33.53 | 4.17 | 0.13 | 0.03 | 42 |
| E2 | 4 | 5.11 | 1.25 | 41.83 | 2.17 | 0.12 | 0.03 | 21 |
| E3 | 1 | 5.64 | 2.48 | 17.35 | 3.84 | 0.34 | 0.18 | 42 |
| E3 | 2 | 6.82 | 2.13 | 25.67 | 3.5 | 0.27 | 0.08 | 43 |
| E3 | 3 | 7.11 | 1.87 | 35.71 | 2.84 | 0.20 | 0.05 | 42 |
| E3 | 4 | 8.15 | 2.01 | 43.68 | 3.98 | 0.19 | 0.04 | 21 |
| E4 | 1 | 5.24 | 1.61 | 18.32 | 4.16 | 0.30 | 0.13 | 42 |
| E4 | 2 | 4.87 | 1.56 | 25.84 | 4.9 | 0.20 | 0.08 | 41 |
| E4 | 3 | 4.35 | 1.28 | 34.87 | 5.17 | 0.13 | 0.04 | 42 |
| E4 | 4 | 4.92 | 1.25 | 42.91 | 3.14 | 0.11 | 0.03 | 42 |
| E5 | 1 | 5.03 | 1.45 | 18.55 | 5.4 | 0.28 | 0.10 | 42 |
| E5 | 2 | 5.46 | 1.58 | 24.35 | 2.85 | 0.23 | 0.07 | 41 |
| E5 | 3 | 5.58 | 2.53 | 34.88 | 3.32 | 0.16 | 0.07 | 42 |
| E5 | 4 | 5.56 | 1.38 | 41.21 | 4.17 | 0.13 | 0.03 | 42 |
| E6 | 1 | 3.69 | 1.21 | 14.90 | 3.06 | 0.26 | 0.09 | 42 |
| E6 | 2 | 3.73 | 1.17 | 21.94 | 2.17 | 0.17 | 0.05 | 42 |
| E6 | 3 | 4.55 | 0.71 | 31.72 | 2.12 | 0.14 | 0.02 | 42 |
| E6 | 4 | 5.78 | 1.26 | 40.22 | 2.45 | 0.14 | 0.03 | 21 |
| E7 | 1 | 3.42 | 1.53 | 13.89 | 2.63 | 0.25 | 0.10 | 42 |
| E7 | 2 | 3.96 | 0.98 | 20.69 | 2.62 | 0.19 | 0.05 | 42 |
| E7 | 3 | 3.51 | 0.83 | 28.63 | 4.28 | 0.13 | 0.04 | 42 |
| E7 | 4 | 3.81 | 1.00 | 36.40 | 3.57 | 0.11 | 0.03 | 21 |
| E8 | 1 | 3.61 | 1.22 | 16.87 | 2.97 | 0.22 | 0.07 | 42 |
| E8 | 2 | 3.94 | 0.87 | 23.22 | 2.3 | 0.17 | 0.04 | 42 |
| E8 | 3 | 5.20 | 1.38 | 31.77 | 2.87 | 0.16 | 0.04 | 42 |
| E8 | 4 | 5.79 | 1.39 | 48.41 | 6.18 | 0.12 | 0.03 | 21 |
| E9 | 1 | 4.09 | 1.39 | 20.67 | 3.22 | 0.20 | 0.06 | 42 |
| E9 | 2 | 3.58 | 1.04 | 25.93 | 2.63 | 0.14 | 0.04 | 42 |
| E9 | 3 | 5.27 | 1.22 | 36.20 | 3.24 | 0.15 | 0.03 | 41 |
| E9 | 4 | 6.50 | 1.55 | 43.65 | 3.47 | 0.15 | 0.03 | 21 |

The sample code corresponds to the sample code of Table 1, and the β level corresponds to β -I, β -II, β -III, and β -IV, respectively, in Fig. 7

W width of ending interdigitation of the β -type undulated patterns, H undulation amplitude of the β -type undulated patterns, R ratio of width of ending interdigitation to undulation amplitude

substantially different. The morphology of level I undulation amplitude was similar among these three types of patterns, but they have different diagnostic characteristics: the Ω type is usually inflated on the top and symmetrical to the vertical axis, the η type is flat on the top and usually not symmetrical to the vertical axis, and the β type is constricted on the top and symmetrical to the vertical axis. The morphologies of level

II and III undulation amplitudes of the Ω , η , and β types have the same diagnostic characteristics as that of level I; further, the diagnostic characteristics of level II and III undulation amplitude were less variant and the characteristics were clearer and more stable than those of level I undulation amplitude. Hence, level I undulation amplitude is not suggested to be used for the discrimination of these three types of patterns,

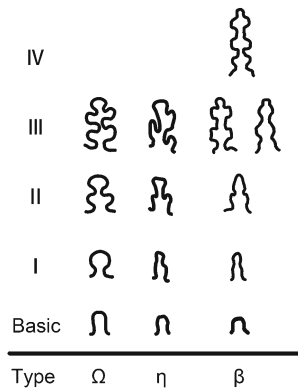


Fig. 9 The comparison of undulated patterns of *Setaria italica*, *Panicum miliaceum*, and *Echinochloa*

unless these diagnostic characteristics are distinctly visible. To our knowledge, level IV undulation amplitude is only observed in the β type; therefore, these three types could be

Table 3 Structure matrix and canonical discriminant function coefficients

| | | <i>W</i> | <i>H</i> | <i>R</i> | <i>L_v</i> |
|---------------------------|------------|----------|----------|----------|----------------------|
| Structure matrix | Function 1 | 0.338 | -0.238 | 0.760 | -0.049 |
| | Function 2 | 0.528 | 0.581 | 0.090 | 0.183 |
| Standardized coefficients | Function 1 | 0.015 | -0.591 | 1.205 | 1.240 |
| | Function 2 | 0.403 | 1.743 | 0.278 | -1.367 |

discriminated using the diagnostic characteristics of the undulation amplitude.

Although morphological differences are the criteria for direct identification and are easy to use, morphometric analysis provides statistical differences among patterns and assists in accurate identification. The Ω type and η type could be discriminated using the *W* and *R* values (Lu et al. 2009b); however, when the data for β -type pattern are included, many

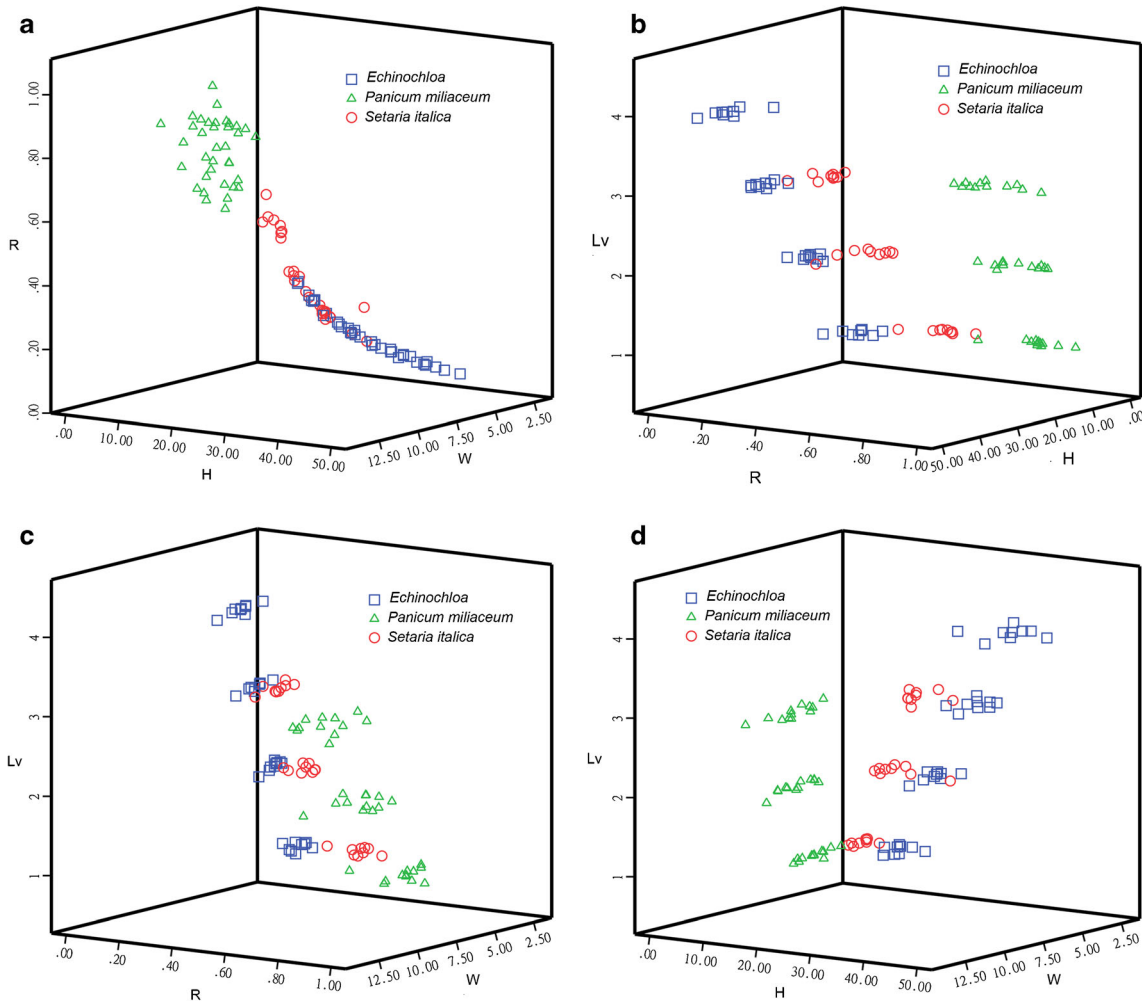


Fig. 10 Scatter plot of parameters of the Ω type, η type, and β type. Data of the Ω type and η type are adapted from Lu et al. [12]. *W* = width of ending interdigitation of the β -type undulated patterns, *H* = length of

undulation amplitude of the β -type undulated patterns, *R* = ratio of width of ending interdigitation to undulation amplitude, *L_v* = level of the undulation amplitude

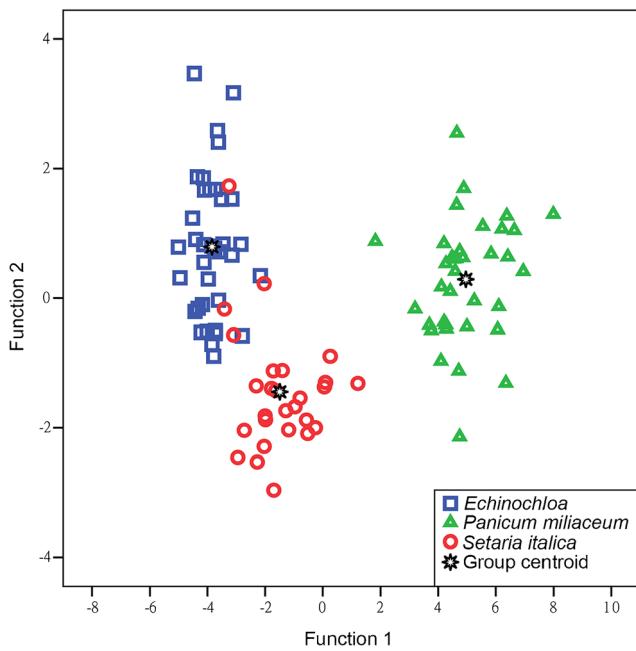


Fig. 11 Discriminant function analyses of *Echinochloa*, *P. miliaceum*, and *S. italica*

characteristics overlap between the Ω -type and β -type patterns. The discrimination becomes difficult even when parameter H is added (Fig. 10a). To address this problem, we used the level of undulation amplitude as a parameter and found that the Ω -type, η -type, and β -type patterns were clearly divided into different groups, although there was some overlap between the Ω -type and β -type patterns (Fig. 10b–d); these results suggest that the level of undulation amplitude is an important parameter to discriminate the three types of patterns.

Table 4 Classification results of the discriminant function analysis

| | | | Predicted membership | | | Total |
|-----------------|---------|---------------------|----------------------|---------------------|-------------------|-------|
| | | | <i>Echinochloa</i> | <i>P. miliaceum</i> | <i>S. italica</i> | |
| Original | Count | <i>Echinochloa</i> | 36 | 0 | 0 | 36 |
| | | <i>P. miliaceum</i> | 0 | 36 | 0 | 36 |
| | | <i>S. italica</i> | 4 | | 23 | 27 |
| | Percent | <i>Echinochloa</i> | 100 | 0 | 0 | 100 |
| | | <i>P. miliaceum</i> | 0 | 100 | 0 | 100 |
| | | <i>S. italica</i> | 14.8 | 0 | 85.2 | 100 |
| Cross validated | Count | <i>Echinochloa</i> | 35 | 0 | 1 | 36 |
| | | <i>P. miliaceum</i> | 0 | 36 | 0 | 36 |
| | | <i>S. italica</i> | 4 | 0 | 23 | 27 |
| | Percent | <i>Echinochloa</i> | 97.2 | 0 | 2.8 | 100 |
| | | <i>P. miliaceum</i> | 0 | 100 | 0 | 100 |
| | | <i>S. italica</i> | 14.8 | 0 | 85.2 | 100 |

Ninety-six percent of original grouped cases are correctly classified. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the function derived from all cases other than that case. Of cross-validated grouped cases, 94.9 % are correctly classified

Discriminant function analysis is useful for the identification of boundaries between groups of objects, and it has been successfully used to discriminate *S. italica* and *Setaria viridis* (Zhang et al. 2011); therefore, this method was used to determine the differences in Ω -type, η -type, and β -type patterns in this study. Parameters W , H , R , and L_v (level of undulation amplitude) were important and used in the discriminant function analysis. The results of this analysis are shown in Table 3. The following two canonical discriminate functions were used in the analysis: function 1 explained 94.7 % of the variance, and function 2 explained 5.3 % of the variance. Parameter R had the largest absolute correlation with function 1, indicating that it contributed most to function 1; parameters H , W , and L_v had the largest absolute correlation with function 2, indicating that they contributed more to function 2 (Table 3). These two functions were used to plot the data (Fig. 11). Three groups having distinct centroids were obtained; *P. miliaceum* had no intersection with *S. italica* and *Echinochloa*, whereas *S. italica* had slight intersection with *Echinochloa*. The accuracy of the classification was ascertained by cross validating the results (Table 4). Up to 96 % of the original data and 94.9 % of the cross-validated data were correctly classified, suggesting that the discriminant functions obtained using parameters W , H , R , and L_v could be reliably used to discriminate *Echinochloa*, *P. miliaceum*, and *S. italica*.

Discussion

In all, eight species and six varieties of the genus *Echinochloa* are grown in China; in our study, four species and three varieties from different locations were

Table 5 The result of non-parametric test for W , H , and R of β -type undulated patterns between different groups

| Category | Null hypothesis | Test | Significance | Decision |
|---------------|--|--|--------------|----------------------------|
| Section | The distribution of W is the same across different sections | Independent-sample Kruskal-Wallis test | 0.384 | Retain the null hypothesis |
| | The distribution of H is the same across different sections | Independent-sample Kruskal-Wallis test | 0.717 | Retain the null hypothesis |
| | The distribution of R is the same across different sections | Independent-sample Kruskal-Wallis test | 0.097 | Retain the null hypothesis |
| Precipitation | The distribution of W is the same across different precipitation | Independent-sample Kruskal-Wallis test | 0.002 | Reject the null hypothesis |
| | The distribution of H is the same across different precipitation | Independent-sample Kruskal-Wallis test | 0.949 | Retain the null hypothesis |
| | The distribution of R is the same across different precipitation | Independent-sample Kruskal-Wallis test | 0.065 | Retain the null hypothesis |

The significant level is 0.05

used to observe the morphology of phytoliths formed in the bracts of spikelet. Bilobate phytoliths and a silica layer with undulated patterns were the main phytolith types produced in the bracts of spikelet, and β -type undulated patterns of silica layer were the diagnostic feature of *Echinochloa* sp. Although we conducted an in-depth analysis, some aspects remained to be discussed.

The silica layer in the lemma and palea of the fertile florets

Soluble silicon is taken up with water by plants and accumulated at sites from where water evaporates (Raven 1983). Although water mostly escapes from the stomata, some water diffuses out through the epidermal cells and cuticle (Soni et al. 1972); therefore, silicon might be translocated to and deposited at the epidermis after transpiration. In rice husk, a silica layer was found between the cuticle and epidermal cells (Yoshida et al. 1962b); the formation of this “cuticle-silica layer” could be the result of the deposition after transpiration, but it could also be caused by the

silicification of the cuticle layer. However, the exact mechanism of the formation of this layer has not yet been completely investigated. In our study, such silica layer was observed in the outermost part of the lemma and palea of our samples; it might have also formed due to the deposition of soluble silicon and mineralization of cuticle.

Silicon in crops has many benefits (Guntzer and Keller 2012), and phytoliths play an important role in the beneficial aspects of silicon (Piperno 2006; Wang and Lu 1993). Phytoliths in the rind of *Cucurbita* help resist insects and fungi (Piperno 2006; Piperno et al. 2002), and the silica layer in the rice husk also provides protection against fungi and parasites (Yoshida et al. 1962a) and limits water loss from the cuticle (Yoshida et al. 1962b). Since the silica layer in *Echinochloa* sp. covers the entire surface of lemma and palea, like in the rice husk, it might have similar protective functions to resist insects and fungi and control the transpiration rate. The silica layer on the surface of lemma and palea might play a similar role in other plant species such as *S. italica* and *P. miliaceum* and their relatives.

Table 6 Comparison of the phytolith in inflorescences bracts for *Echinochloa* sp., *P. miliaceum*, and *S. italica*

| Parts of spikelet | | <i>Echinochloa</i> | <i>P. miliaceum</i> | <i>S. italica</i> |
|-----------------------------------|---------------------------------------|--|--|---|
| Glumes | Main phytolith types | Bilobate with cavate ends | Bilobate with cavate ends | Bilobate with cavate ends |
| Lemma and palea of sterile floret | Main phytolith types | Bilobate with cavate ends | Bilobate with cavate ends | Bilobate with cavate ends |
| Lemma and palea of fertile floret | Main phytolith types | Bilobate with semicircular ends, silica layer | Silica layer | Silica layer |
| | Surface of the silica layer | Smooth | Scattered spots | Regularly arranged papillae |
| | The type of undulated patterns | β type (β -I, β -II, β -III, β -IV) | η type (η -I, η -II, η -III) | Ω type (Ω -I, Ω -II, Ω -III) |
| | The character of undulation amplitude | Constricted on top, symmetry to the vertical axis | Flat on top, not symmetry to the vertical axis | Inflated on top, symmetry to the vertical axis |
| | Parameters of the undulated patterns | $W = 4.86 \pm 1.82 \mu\text{m}$ $R = 0.19 \pm 0.09$ | $W = 8.95 \pm 2.02 \mu\text{m}$ $R = 0.79 \pm 0.12$ | $W = 4.37 \pm 0.89 \mu\text{m}$ $R = 0.33 \pm 0.11$ |

The stability of the β -type undulated patterns in *Echinochloa*

The morphology of phytoliths is known to vary within and across individuals owing to many factors such as maturity stage (Hodson et al. 1985; Sangster 1970), transpiration rate (Whang et al. 1998), tissue type (Ball et al. 1993; Mulholland et al. 1988), and growth environments (Mulholland et al. 1990; Mulholland et al. 1988). Determining whether the β -type undulated patterns differ between species and environments is necessary to use these patterns as diagnostic tools. Thus, assessing the stability of the β -type undulated patterns and the possible influence of different factors on these patterns is necessary. The information provided in Table 1 was used to divide the nine samples into different groups: (1) according to sections, i.e., Section Hispidula, Section *Echinochloa*, and Section Phyllopon, (2) and according to the precipitation information of the sampling sites, i.e., humid (precipitation, >800 mm/year), semihumid (precipitation, 400–800 mm/year), and arid (precipitation, <200 mm/year). The β -type undulated patterns between the groups were compared using the three parameters (*W*, *H*, and *R*). The Kruskal-Wallis test was used to determine whether there were differences in the distribution of parameters among the groups (Table 5). The three parameters were not significantly different across the different sections, indicating that the morphology of β -type undulated patterns is stable in *Echinochloa*. The width of ending interdigitation was significantly different across the different precipitation levels; however, the length of undulation amplitude was highly coherent, indicating that the width of ending interdigitation might be sensitive to water stress.

The samples used in this study were collected from different regions of China; factors that could affect the morphology of phytoliths were mostly included. Except parameter *W* that showed to be affected by water supply, regardless of immaturity, different sections, and growing environments, our results suggest that the morphology and morphometric parameters of the β -type undulated patterns have no significant differences among the different samples, suggesting that these patterns could be stable enough to be used as an identification criterion for *Echinochloa* sp.

Conclusions

Phytoliths are more durable than seeds in various conditions, and phytolith analysis could be helpful in the identification of *Echinochloa* when macro-remains are not found. Our study focused on the phytoliths in the inflorescence bracts of *Echinochloa*, which could be preserved in most cases, and provided diagnostic characteristics of the β -type undulated patterns for more precise identification and discrimination of

Echinochloa (Table 6) from archaeobotanical remains and assist in understanding their cultivation and domestication. To our knowledge, the β -type undulated patterns in the lemma and palea of *Echinochloa* are unlike those found in the phytolith patterns of other species of *Poaceae*, but further studies are needed to confirm our findings.

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Author contributions H.L. and Y.G. conceived the experiment. H.L., Y.G., C.W., K.H., and X.H. collected and prepared the samples. Y.G. and J.Z. performed the experiment. All authors contributed to data analysis. Y.G. and H.L. wrote the manuscript.

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