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Morphometric distinction between bilobate phytoliths from *Panicum miliaceum* and *Setaria italica* leaves

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Abstract The development of identification criteria for crop plants based on phytoliths is of high relevance for archaeology, palaeoecology and plant systematics. While identification criteria are available for major food crops, these are mostly based on phytoliths from inflorescences, while other plant parts remain undetected. This paper focuses on bilobate phytoliths from leaves of *Panicum miliaceum* L. (common millet) and *Setaria italica* (L.) P. Beauv. (foxtail millet), two taxa that co-occur in regions of Asia and Europe since prehistory and regularly occur at archaeological sites in Eurasia. Leaves of the investigated taxa were systematically sampled to explore the variation of short cells and to collect 27 morphometric variables of bilobate phytoliths with newly developed open-source software. The data was analysed by discriminant analysis, analysis of variance and multiple comparison tests. The resulting morphometric data from five populations per species enables a distinction between the bilobate phytoliths of *P. miliaceum* and *S. italica*. Observed differences between populations within species affect only few parameters. This possibility to classify populations of bilobate phytoliths from *P. miliaceum* and *S. italica* leaves offers a new method for the detection and identification of these taxa in archaeology, amongst others.

Keywords Prehistory · Archaeobotany · *Panicum miliaceum* · *Setaria italica* · broomtail millet · proso millet · foxtail millet · Phytolith morphometry · Bilobates · Leaf anatomy · Identification criteria

Introduction

Phytolith systematics, of major relevance for plant systematic, taxonomy, palaeoecology and archaeology, is a strongly developing field, and it is frequently mentioned as a critical topic for research development (Mulholland and Rapp 1992, 10; Piperno 2006, 79; Ball et al. 2009; Shillito 2013). Phytoliths, consisting of biogenic opal, offer valuable applications such as the identification of plant taxa from sedimentary archives, the detection of plant material from anthropogenic deposits and the understanding of past plant use through the analysis of food products, construction material and temper and crop processing residues. Indeed, the identification of plant parts (culm/leaves/inflorescences) and the understanding of their use are fundamental to unravel social organization (e.g. Anderson 2003; Harvey and Fuller 2005). The ongoing need for further development of phytolith systematics partly relates to redundancy and multiplicity of these particles: Many plants can produce the same type of phytolith, and various phytolith types are produced in the different parts of a single plant. There are, nevertheless, multiple examples showing strong potentials for taxonomic identification on the family, subfamily, genus and sometimes the species level.

Concerning Old World taxa, phytolith systematics has focused on the domesticated cereals *Avena sativa* (oat), *Triticum* sp. (wheat), *Hordeum* sp. (barley), *Panicum miliaceum* (broomcorn millet), *Setaria italica* (foxtail millet) and *Oryza* sp. (rice), while identification criteria are additionally available for *Musa* spp. (banana) and some major palms (Rosen 1992; Ball et al. 1993, 2009; Pearsall et al. 1995; Zhao et al.

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1998; Mbida et al. 2000; Portillo et al. 2006; Lu et al. 2009a; Fenwick et al. 2011; Gu et al. 2013; Weisskopf and Lee 2014). The identification criteria for these taxa are all partly based on morphometry, and much work is still ahead to expand the plants investigated. In archaeology for instance, there is the need to extend the research to other crops but also their wild relatives. Millets, a group of grasses that belong to the Panicoideae and Chloridoideae subfamilies and that are used as crops in various parts of the world, received until recently little attention. Radomski and Neumann (2011), Zhang et al. (2011), Madella et al. (2014a, b) and Weisskopf and Lee (2014), amongst others, made a start with the study of variation within millets. Second, most morphometric studies on taxonomic identification of economic plants are primarily based on phytoliths from inflorescences with the exception of rice (Gu et al. 2013) and einkorn (Ball et al. 1993). Apart from inflorescences, however, grass leaves are also well-known for their taxonomic diagnostic value (Ellis 1987; Metcalfe 1960). Leaves, although often overlooked in the archaeological record, are a non-dietary by-product of cereal harvests that is of substantial economic importance, as known from ethnography and archaeology (Grubben and Partohardjono 1996; Lancelotti and Madella 2012; Ryan 2011). Ethnographic studies show the use of stalks and leaves of various millets for animal fodder, hay as well as silage, plaiting, building, fencing, thatching, brooms and fuel (Grubben and Partohardjono 1996). The development of taxonomic identification of phytoliths from leaves is therefore highly relevant.

In order to further work towards the development of plant identification criteria based on phytoliths, the aim of this study is to investigate phytolith morphometry from leaves of *Panicum miliaceum* L. (common millet) and *Setaria italica* (L.) P. Beauv. (foxtail millet), both members of the Poaceae, subfamily Panicoideae, tribe Paniceae. The two crops were selected because they have been of high economic importance, in particular in Asia and Europe, since prehistory. Moreover, the geographical distribution of *P. miliaceum* and *S. italica* considerably overlaps, which makes distinction relevant. Genetic and archaeobotanical evidence points to China as the origin of both taxa; *S. italica* was probably also domesticated elsewhere (De Wet et al. 1979; Li et al. 1995; Fukunaga et al. 2002, 2006; Lu 2002; Hunt et al. 2008, 2011; Lu et al. 2009b; Zhao 2011; Motuzaite-Matuzeviciute et al. 2013b; Bestel et al. 2014).

There are various studies on plant systematics, anatomy, physiology, taxonomy, carbon isotopes and phytolith extraction methods that discuss silicification and phytoliths of *P. miliaceum* and/or *S. italica* (for late 19th and early 20th century bibliography see: Formanek, Neubauer and Netolitzky in Powers 1992; more recent bibliography: Clark and Gould 1975; Hodson et al. 1982; Hodson and Parry 1982; Parry and Hodson 1982; Pearsall et al. 1995; Zuo and Lü 2011; Rajendiran et al. 2012; Sivasubramanian et al. 2013; Parr and Sullivan 2014; Wang et al. 2014). Especially for archaeology, those studies presenting identification criteria are most relevant. Until recently, identification of Panicoideae was primarily based on the general morphology of phytoliths, and on comparisons between reference material and archaeological material (Madella 2001, 2007; Rosen 2001; Li et al. 2007; Itzstein-Davey et al. 2007; Atahan et al. 2008). After the development of partial morphometric identification criteria for *P. miliaceum* and/or *S. italica* (Lu et al. 2009a; Zhang et al. 2011; Weisskopf and Lee 2014), their application in archaeology has quickly gained terrain, and it is applied to fields such as the detection of crop plants and food products, domestication and crop dispersal, agricultural practices, human impact and related social developments (Lu et al. 2005; Zhang et al. 2010, 2012; Gong et al. 2011; Chen et al. 2012; Weisskopf et al. 2014; Weisskopf and Lee 2014; Dal Corso 2014). Most of these studies are based on identifications from inflorescence phytoliths, while there are a few exceptional studies on Panicoideae stem/leaf phytoliths from archaeological tools or material used for basketry (Di Lernia et al. 2012; Ma et al. 2014).

Concerning the systematics of millets based on phytoliths from leaves, Renvoize (1987) explored the variation of short cell morphotypes in 101 genera of Paniceae, and Lu and Liu (2003) and Fahmy (2008) demonstrated the potential of morphometric analysis of bilobates, diagnostic of Panicoideae, for taxonomic classification. In addition, there are various regional ecological studies that compare phytoliths from leaves of multiple taxa, including local *Panicum* and/or *Setaria* species (e.g. Ellis 1988; Zucol 1998; Krishnan et al. 2000), but these studies often focus on wild taxa and exclude major subsistence crops. Interestingly, leaf anatomical studies by Shaheen et al. (2011, 2012) commenced with a taxonomic identification of *Panicum* and *Setaria* species from Pakistan, including *P. miliaceum* and *S. italica* by means of phytolith morphotypology, amongst others, but the wider validity of the results is unclear since information on the sample size is lacking.

Within the above-presented framework of archaeobotany and the study of past societies, this investigation aims to examine whether bilobate and cross-shaped phytoliths allow for taxonomic identification of *P. miliaceum* and *S. italica* by leaf phytolith morphometry. The focus is on bilobates since these are short cells that silicify frequently and in large numbers, independent of environmental conditions, and thus can also be expected to occur frequently in archaeological assemblages. Bilobates and crosses were studied together since they can be considered as variations of the same morphotype (cf. Ball and Brotherson 1992) and since the large variation and subtle differences hamper the sharp separation between two separate groups.

The analysis of bilobate phytoliths was conducted by semi-automatic morphometric analysis (Out et al. 2014). The study included populations grown in various parts of the world to

increase the applicability of the observations and conclusions and to explore the variation within single taxa. The main questions are as follows:

- Can bilobate phytoliths from *P. miliaceum* and *S. italica* be distinguished from each other by morphometry, i.e. is there a difference in bilobate morphometry between the species?
- Is there a significant difference in bilobate phytolith morphometry between the various investigated populations of either *P. miliaceum* and *S. italica*, i.e. is there a difference within species?

Materials and methods

Table 1 shows the studied plant material and the experimental design. The study included five populations of both *P. miliaceum* and *S. italica*. Plants grown in Barcelona and Kyoto were grown from seeds obtained from the National Small Grains Collection, the North Central Regional Plant Introduction Station and the Plant Genetic Resources Conservation Unit, all part of the National Plant Germplasm System of the United States Department of Agriculture. Populations provided by the Botanical Institute of Barcelona were grown in the Botanical Garden of Barcelona. Further, plant material was kindly made available by G. Thijsse (Naturalis Biodiversity Centre, the Netherlands), M.K. Jones (University of Cambridge, UK) and D.Q. Fuller (University College London, UK).

The sampling strategy aimed to include two samples from two leaf blades from two plants per population. The leaves included the leaf below the highest leaf and the third leaf from the plant base, the latter representing a random leaf. The lowest and highest leaves were avoided since those are thought to have the highest risk of differences in leaf development and silicification. Leaf blades rather than sheaths were selected since blades are taxonomically more relevant (Metcalfe 1960, xviii) and since they may show more silica deposition due to higher evapo-transpiration (Prychid et al. 2004, 383; Chauhan et al. 2011, 842). Spodograms showing surface views of the in situ phytoliths were prepared according to the following protocol:

1. After maturation and decease of a plant, leaf fragments of 1–2 cm wide were collected from the middle of the lower and upper part of a leaf.
2. Samples were soaked in distilled water overnight to soften them (this shortens step 4).
3. Samples were cleaned 10 min in an ultrasonic bath to remove any dust or contaminants.
4. Samples were fragmented and soaked in household bleach until they became transparent. The length of this process depended on the individual samples.
5. Samples were rinsed by first soaking them in distilled water overnight and then briefly soaking them in ethanol (90 %).
6. Samples were mounted on a microscope slide with the abaxial or adaxial side randomly facing up. The ethanol was left to evaporate and the samples were mounted with the permanent mounting liquid Entellan™.

For the analysis, the following procedure was carried out:

1. The variation of morphotypes was explored non-quantitatively.
2. Microphotographs of bilobate short cells in the costal zone (veins) were taken at ×630 magnifications with a Leica DM2500 microscope equipped with a Leica DFC490 camera. Photographs of up to 10 clearly visible short cells per vein were taken to assure random sampling within leaves. Nodular (notched) bilobates were excluded.
3. The outline of at least 50 phytoliths per sample was drawn using a Bamboo Fun drawing pen (Wacom) and a FIJI macro (Schindelin et al. 2012) especially developed for this purpose (Out et al. 2014).
4. A total of 27 meaningful variables of size and shape (see Table 2) were measured by another newly developed FIJI macro (ibid.).
5. Descriptive statistics, including the mean, minima, maxima and standard deviation, were calculated for each of the variables at the level of species, population, plant, leaf and sample. The data is available from the corresponding author. Minimum sample sizes were calculated following the formula of Ball et al. (2006), assuring a 90 % confidence level that the sample means are within 5 % of the actual population means on the level of leaves, plants and populations:

$$N_{\min} = Z^2{}_{\mu/2} X S^2 / (\mathrm{ME})^2$$

where $N_{\min}$ is the minimum sample size, $Z^2{}_{\propto/2}=1.64$, which is the square of the two-tailed value at $\propto=0.10$, S^2=the variance, and $(\mathrm{ME})^2$=the square of the desired margin of error, which is here 0.05 x the sample mean.
6. To test whether the measurements allow for differentiation between the two taxa, a discriminant analysis was applied using the statistical software SPSS v.21 (IBM 2012).
7. To further test whether there is a taxon effect on the measured morphometrics and, moreover, to test whether there is an effect of population on the measured values, the statistical analysis also included the definition of an

Table 1 The studied plant material and experimental design

Species	Subspecies/ variety	Pop. nr.	Accession	Reference	Growth location and year	Location of origin	Plants	Leaves/ plant	Samples/ leaf	Total samples	Total phytoliths	Sampling
Panicum miliaceum (L.)	miliaceum	1	IMF-CSIC 1	PI 578074	Barcelona, Spain (2011)	Nebraska, USA	2	2	2	8	400	Standard
	miliaceum	2	IMF-CSIC 2	PI 463490	Barcelona, Spain (2011)	India	2	2	2	8	400	Standard
	miliaceum	3	Botanical Garden Barcelona	PI 170593	Barcelona, Spain (2013)	Turkey	2	2	2	8	400	Standard
	var. album	4	George Pitt Rivers Laboratory, University of Cambridge	GPR (P 455 261 Mil 83)	Cambridge, UK	Russia	2	2	2	8	400	1 x plant unknown, presumably standard
		5	George Pitt Rivers Laboratory and Institucion Mila y Fontanals	GPR/IMF	Korea	Korea	2	2	2	8	400	Random leaves
Setaria italica (L.) P. Beauv.	italica	6	IMF-CSIC 1	PI 614815	Barcelona, Spain (2011)	Nebraska, USA	2	2	2	8	400	Standard
	italica	7	IMF-CSIC 2	PI 173805	Barcelona, Spain (2011)	Turkey	2	2	2	8	400	Standard
		8	National Herbarium of the Netherlands	NHNL (L 0580035)	Anambas islands, Indonesia (1928)	Indonesia	2	2	2	8	400	Samples close to each other (6 cm)
		9	Institute of Archaeology, University College London	UCL 1 (234)	Cambridge, England (1999)	Sanganakallu, India	2	2	2	8	400	Random leaves
	italica	10	Botanical Garden Barcelona	PI 464176	Barcelona, Spain (2013)	Andhra Pradesh, India	2	2	2	8	400	Standard
N total							20	40	80		4000	

Table 2 The applied morphometric variables of size and shape

Type	Label	Description	Unit
Size	ArBBox	Feret*Breadth, area of the bounding box along the Feret diameter, which is not necessarily the minimal bounding box.	μm^2
Size	Area	The area inside the polygon defined by the Perimeter.	μm^2
Size	Area equivalent diameter	Area Equivalent Diameter=sqrt ((4/π)*Area).	μm
Size	Breadth	The largest axis perpendicular to the Feret (not necessarily colinear).	μm
Size	Convex hull	Convex hull or convex polygon calculated from pixel centres. Perimeter calculated in a different way.	μm
Size	Concavity	Convex area-area.	μm^2
	Convex area	Area of the convex hull polygon (= Area/Solidity). Area calculated in a different way.	μm^2
Size	Curve length	The arc length of the centerline curve between the points with the largest separation.	μm
	Curve width	Maximum width perpendicular to medial axis.	μm
Size	Equivalent ellipse area	(π*Feret*Breadth)/4, this is the area of an ellipse with the same long and short axes as the particle.	μm^2
Size	Feret	Largest axis length=the longest distance between 2 points in the perimeter.	μm
Size	MaxR	Radius of the enclosing circle centred at the centre of mass. Centre of mass: the brightness-weighted average of the x and y coordinates of all pixels in the image or selection.	μm
Size	MBCRadius	Radius of the minimal bounding circle.	μm
Size	MinR	Radius of the inscribed circle centred at the centre of mass.	μm
Size	Perimeter	The length of the outside boundary of the selection, calculated from the centres of the boundary pixels.	μm
Size	Perimeter equivalent diameter	Area/π.	
Shape	Aspect ratio	Feret/Breadth.	
Shape	Circularity	$4*\pi*Area/Perimeter^2$, sometimes called Form Factor, distinguishes beteen perfect round circles and dentated circles.	
Shape	Compactness	Sqrt ((4/π)*Area)/Feret or alternatively ArEquivD/Feret.	
Shape	Convexity	Convex Hull/Perimeter, it is 1 for a perfectly convex shape, diminishes if there are surface indentations.	
Shape	Modification ratio	(2*MinR)/Feret.	
Shape	Rectangularity	Area/ArBBox, this approaches 0 for cross-like objects, 0.5 for squares, π/4=0.79 for circles and approaches 1 for long rectangles.	
Shape	RFactor	Convex hull/(Feret*π).	
Shape	Roundness	$4*Area/(\pi*Feret^2)$, it is 1 for a perfect circle and diminishes with elongation of the feature.	
Shape	Shape	$Perimeter^2/Area$.	
Shape	Solidity	Area/convex area, it is 1 for a perfectly convex shape, diminishes if there are surface indentations.	
Shape	Sphericity	MinR/MaxR.	

appropriate statistical mixed model (Laird and Ware 1982; Verbeke and Molenberghs 2000) and an ANOVA. Concerning the model, the data was assumed to be approximately normally distributed and to be heteroscedastic due to the different taxa, populations, plants and leafs. These assumptions are based on a graphical residual analysis (residual plots of each variable for each taxon). The statistical model included the taxa *P. miliaceum* and *S. italica*, and additionally the populations 1-5, the latter nested within the factor taxon as fixed factors. The factors plant, leaf and sample were regarded as random factors with sample being nested in leaf, leaf nested in plant and plant nested in population. Based on this model, an ANOVA was conducted to answer the questions of the trial.

8. To test which populations differed from each other (if relevant), multiple contrast tests (e.g. Bretz et al. 2011) were conducted to compare the mean values of the several levels of the influence factors per taxon, i.e. mean values of pairs of populations were compared. To do so, a corresponding cell means model was applied (Schaarschmidt and Vaas 2009). Steps 7 and 8 were carried out with assistance from M. Hasler (Kiel University) using the statistical software R (2013).

Results

Minimum sample size

Results of the test for the minimum adequate sample size of bilobates for species, population, plants, leaves and samples are summarized in Table 3. The outcome of the required minimum sample size depends on the level for which the sample size is calculated. While for *S. italica*, the minimum sample size calculated per sample is mostly the highest number, the minimum sample size for *P. miliaceum* is regularly higher when calculated per plant or population, suggesting that variation within plants and population is larger than variation within samples. On the sample level, the applied sample size of $N=50$ phytoliths per sample is sufficient to cover the variation for 18 of the 27 variables, while for some measurements, even smaller sample sizes can be used. The $N=50$ sample size does not meet the required minimum per sample for the nine variables area bounding box (ArBBox), area, concavity, convex area (CArea), equivalent ellipse area (EqEllAr), radius of the inscribed circle (MinR), perimeter equivalent diameter (PerEqD), modification ratio and sphericity. The required minimum sample size has nevertheless been reached due to the duplication in the experimental design, indicating that a representative data set has been investigated.

Phytolith morphotypes

The phytolith morphotypes in the prepared samples of *P. miliaceum* and *S. italica* leaf blades include long cells, bulliforms, short cells including bilobates, cross-like bilobates and crosses, nodular (notched) bilobates, trilobates and polylobates, cork cells, stomata, interstomatal cells, trichomes, including prickles and microhairs, and silicified fragments of vascular bundles. Although neither of the two species yield unique phytolith morphotypes, trilobates and particularly polylobates are rare in the investigated *S. italica* samples. Figures 1 and 2 show a selection of long cells and short cells. The shape of bilobates of both species shows great variation concerning the short ends of the lobes and the length of the shank between the lobes (see Figs. 1 and 2 and Supplementary Information Figs. 1 and 2). The long cells show straight and wavy edges and occasionally spiny ornamentation. Silicification is generally the highest along the leaf edge and in short cells.

Morphometric analysis

Descriptive statistics of the bilobates of altogether 4000 phytoliths from 10 populations, 20 plants, 40 leaves and 80 samples of *P. miliaceum* ($N=2000$) and *S. italica* ($N=2000$) are presented in Table 4 and in the Supplementary Information Tables 1, 2 and 3. Figure 3 shows boxplots of values per population for a representative selection of variables. The ranges of all variables overlap.

A stepwise discriminant analysis has been conducted to test if the morphometric data can be used to predict whether the bilobates represent *P. miliaceum* or *S. italica*. Significant mean differences are observed for all variables except the following: area, area equivalent diameter and perimeter equivalent diameter. Box's M indicates that the assumption of equality of covariance matrices is violated, which is, however, not regarded as problematic in the case of large sample sizes (Burns and Burns 2009). The developed discriminant function DF=1.061*Aspect Ratio + 2.014*Circularity + 0.844 * Roundness + 0.583*Solidity + 1.118*Shape – 0.298*Rectangularity – 1.352*ModRatio reveals a significant association ($p=0.000$) between the two taxa and the predictors, accounting for 56.6 % of the between-species variability. The cross-validated classification shows that 88 % of all phytoliths together are identified correctly, with 82.9 % of *Panicum* phytoliths and 93.2 % of the *Setaria* phytoliths being identified correctly. Thus, classification is possible when based on measurements of phytolith populations. To further test the validity of our results, the developed discriminant function has been applied to 200 phytoliths of *S. italica* taken from four samples from two leaves from a single plant grown in London (UCL collection ref. nr. 237). Of these phytoliths, 83.5 % are classified correctly as *S. italica*.

In other words, compared with *S. italica*, the average leaf bilobates from *P. miliaceum* are characterised by lower values of aspect ratio, circularity, rectangularity and solidity, and higher values of modification ratio, roundness and shape (see Table 4). This means that on average, *P. miliaceum* bilobates are less elongated (aspect ratio and roundness), less roundish (circularity and shape) and more irregular of shape (modification ratio, rectangularity and solidity) then *S. italica* bilobates. The diagnostic variables are visualised in Fig. 4. Simplifying the results further, comparisons of the bilobates of the two species in Figs. 1 and 2 show that the short sides of the *P. miliaceum* bilobates are relatively concave while the short sides of the *S. italica* bilobates are more often relatively flat or convex. The complexity of the variables, the mostly subtle differences between the two species and the fact that seven variables are taken into consideration in the discriminant function implies that image analysis-assisted morphometry is required to distinguish between the two studied taxa.

Table 5 shows the results of the ANOVA, testing the effect of taxon as well as population within taxon on the measured values, and the main result of the multiple contrast tests. Supplementary Information Table 4 shows the detailed results of the multiple contrast tests. The ANOVA confirms the outcome of the discriminant analysis, showing that taxon

Table 3 The minimum required sample size for the different sampling levels, based on calculations for each sample, leaf, plant and population separately

P. miliaceum		Species	Population		Plant		Leaf		Sample		All values		
			Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Difference
≤50	ArEqD	15	10	25	10	30	5	15	5	20	5	30	25
	Breadth	20	15	25	15	30	10	25	10	25	10	30	20
	CHull	20	15	25	10	30	10	15	5	20	5	30	25
	Curve_length	20	20	25	15	30	10	20	10	25	10	30	20
	Curve_width	15	10	25	10	25	5	15	5	15	5	25	20
	Feret	20	15	25	15	35	10	20	10	20	10	35	25
	MaxR	20	15	25	15	35	10	20	10	20	10	35	25
	MBCRadius	20	15	25	15	35	10	20	10	20	10	35	25
	Perimeter	20	15	25	15	30	10	20	10	25	10	30	20
	AspRatio	15	10	20	10	25	5	25	5	30	5	30	25
	Circularity	10	10	10	10	15	10	15	5	15	5	15	10
	Compactness	5	5	5	5	5	5	5	5	5	5	5	0
	Convexity	5	5	5	5	5	5	5	5	5	5	5	0
	Rectangularity	5	5	10	5	10	5	10	5	10	5	10	5
	RFactor	5	5	5	5	5	5	5	5	5	5	5	0
	Roundness	15	10	15	10	15	10	15	10	20	10	20	10
	Shape	10	10	15	10	15	10	15	5	15	5	15	10
	Solidity	5	5	5	5	5	5	5	5	5	5	5	0
>50	ArBBox	80	45	105	40	120	25	70	20	80	20	120	100
	Area	70	35	95	30	115	20	60	15	70	15	115	100
	Concavity	135	100	140	85	155	60	150	40	165	40	165	125
	CArea	75	40	100	35	115	25	65	20	80	20	115	95
	EqEllAr	80	45	105	40	120	25	70	20	80	20	120	100
	MinR	60	45	65	35	65	30	75	20	95	20	95	75
	PerEqD	70	35	95	30	115	20	60	15	70	15	115	100
	ModRatio	75	65	80	50	80	50	105	30	135	30	135	105
	Sphericity	75	65	80	50	80	50	105	30	135	30	135	105
S. italica		Species	Population		Plant		Leaf		Sample		All		
			Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Difference
≤50	ArEqD	15	10	15	10	15	5	15	5	15	5	15	10
	Breadth	20	15	25	15	25	10	25	10	25	10	25	15
	CHull	15	15	15	10	20	10	20	10	20	10	20	10
	Curve_Length	20	15	20	10	20	10	20	10	20	10	20	10
	Curve_Width	15	10	15	10	15	10	15	5	15	5	15	10
	Feret	20	15	20	15	25	15	25	10	25	10	25	15
	MaxR	20	15	20	15	25	15	25	10	25	10	25	15
	MBCRadius	20	15	20	15	25	15	25	10	25	10	25	15
	Perimeter	20	15	20	10	20	10	20	10	25	10	25	15
	AspRatio	30	20	35	20	50	15	50	5	40	5	50	45
	Circularity	10	5	10	5	10	5	15	5	15	5	15	10
	Compactness	5	5	10	5	10	5	10	5	10	5	10	5
	Convexity	5	5	5	5	5	5	5	5	5	5	5	0
	Rectangularity	5	5	5	5	10	5	10	5	10	5	10	5
	RFactor	5	5	5	5	5	5	5	5	5	5	5	0
	Roundness	20	15	25	15	30	10	35	10	30	10	35	25
	Shape	10	5	10	5	10	5	15	5	15	5	15	10

Table 3 (continued)

P. miliaceum		Species	Population		Plant		Leaf		Sample		All values		
			Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Difference
	Solidity	5	5	5	5	5	5	5	5	5	5	5	0
>50	ArBBox	55	35	60	35	60	25	55	15	60	15	60	45
	Area	55	30	55	30	55	20	50	15	60	15	60	45
	Concavity	100	65	120	60	125	50	125	35	155	35	155	120
	CArea	55	35	60	30	60	25	55	20	65	20	65	45
	EqEllAr	55	35	60	35	60	25	55	15	60	15	60	45
	MinR	80	55	85	50	85	35	95	20	95	20	95	75
	PerEqD	55	30	55	30	55	20	50	15	60	15	60	45
	ModRatio	110	80	120	70	125	55	145	45	145	45	145	100
	Sphericity	105	80	115	70	125	55	145	45	140	45	145	100

Min. and Max. provide the lowest and highest minimum sample size within the relevant sampling level. Calculations for various levels are the same in the rare cases of restricted sampling, when, e.g. a population included one plant only (see Table 1)

significantly influences the measured variable values for 16 of the 27 variables of size and shape, i.e. that there is a difference between the measured values of *P. miliaceum* and *S. italica*. The ANOVA further demonstrates that there is a significant effect of population within taxon on the measured values: A few variables show differences between *P. miliaceum* and/or *S. italica* populations within single species. This concerns the five variables CHull,

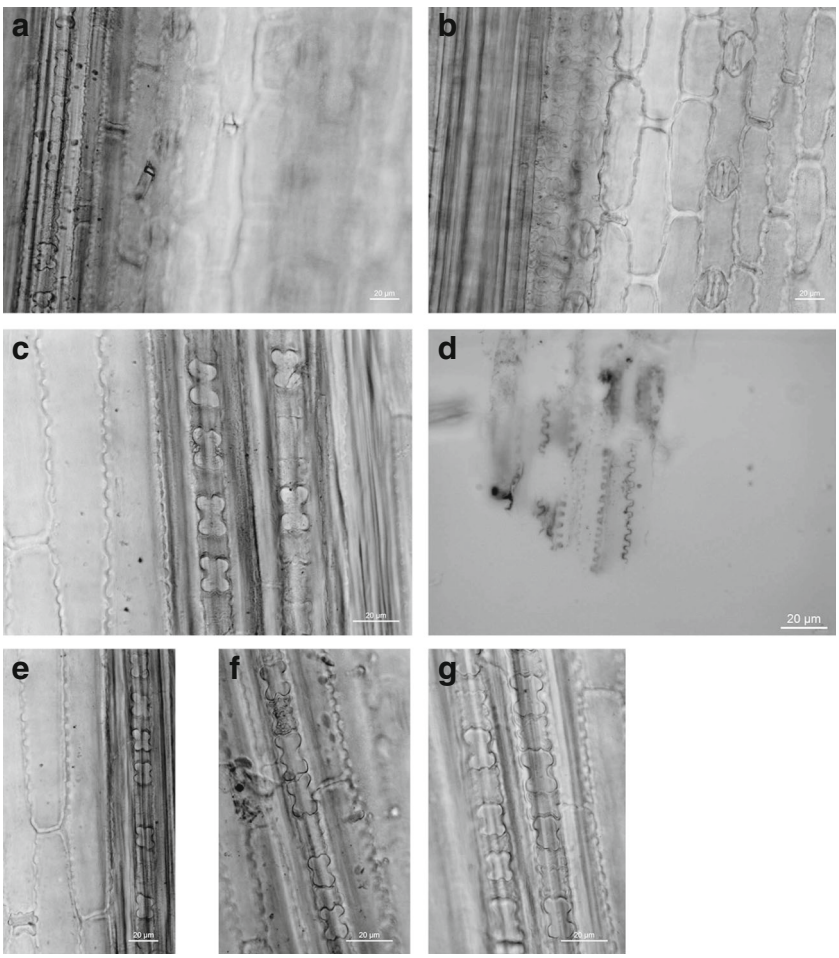


Fig. 1 *Panicum miliaceum*, leaf spodograms, mounted in water (all except **d**), and ashed material (**d**). **a**, **b** Position of investigated bilobate phytoliths in the leaf (two times the same tissue sample). **c**, **d** Shape of long cells. **e**, **f** Variation of short cells. **g** Silicified morphotypes in between the short cells. **a**, **b**, **f**, **g** Population 2, PI 463490; **c**, **e** population 1, PI 578074; **d** population 4, GPR (P 455 261 Mil 83)

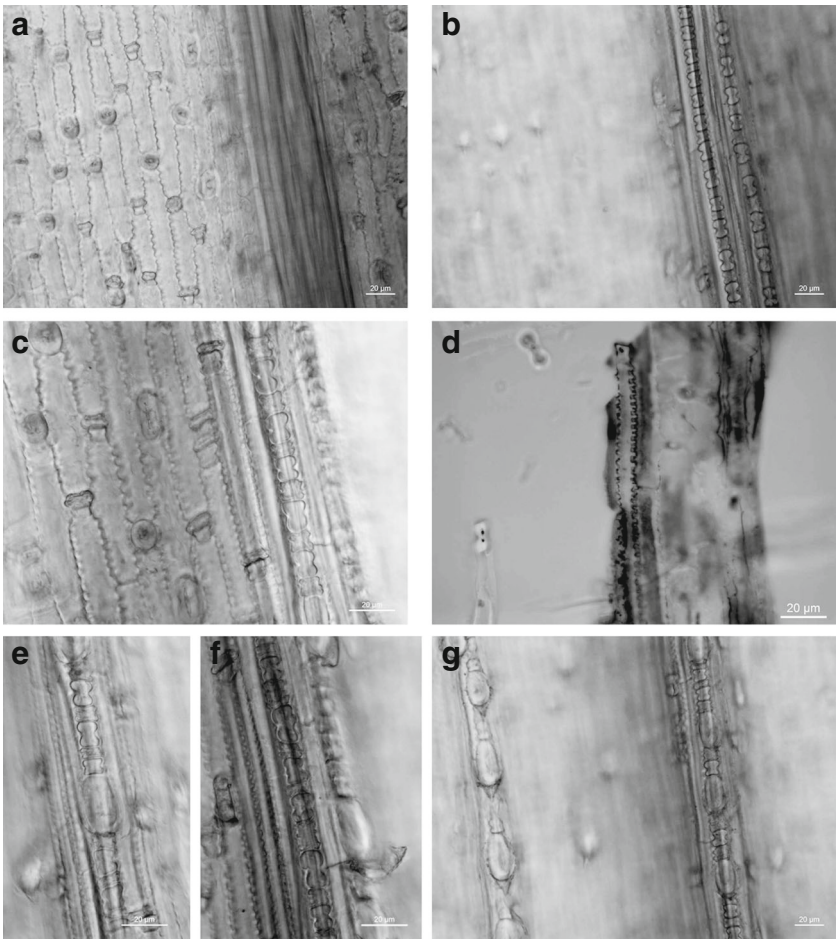


Fig. 2 *Setaria italica*, leaf spodograms, mounted in water (all except **d**), and ashed material (**d**). **a**, **b** Position of investigated bilobate phytoliths in the leaf (two times the same tissue sample). **c**, **d** Shape of long cells (**e**) and (**f**) variation of short cells. **g** Frequent occurrence of prickles; also note the silicified morphotypes in between the short cells (all except **d**): population 10, PI 464176; **d** population 9, UCL 1 (234)

circularity, solidity, convexity shape and concavity that are mostly measurements of shape.

The multiple comparison tests mostly confirm the ANOVA results, showing that the values of most variables (N=21 out of 27) do not statistically differ between populations within a single species. The multiple comparison tests show differences between populations for six variables: circularity, solidity, convexity, shape, ModRatio and sphericity. At the level of four individual variables, there are minor differences between the outcomes of the ANOVA and the multiple comparison tests; the multiple comparison tests, for example, indicate that there are differences between sphericity measurements in two *S. italica* populations, while the ANOVA states that population does not significantly influence these measurements. These minor differences are inherent to the use of a complex, mixed model (see "Materials and methods").

Based on the multiple comparison tests, differences between *P. miliaceum* populations are only observed for convexity measurements of two population pairs that both include population 5, grown in Korea (see Fig. 3c). This population differs from the other population because of a non-European location and by the fact that random leaves were sampled since the reference material available concerned plant fragments instead of a full plant. The differences between *S. italica* populations are observed for the variables circularity, modification ration, shape, solidity and sphericity (see Fig. 3b, d). Only circularity and shape show differences between more than one pair of populations (for these two variables, the sample size of measured phytoliths was sufficiently large). The significant differences between the *S. italica* populations all relate to population 4 (population 9 in Table 1), grown indoors in England with seeds from India, from which random leaves were used since the reference material concerned plant fragments only.

Discussion

Minimum sample size

Table 3 shows that while for many variables, the sample means were within 5 % of the actual population means on

Table 4 *P. miliaceum* and *S. italica*, descriptive statistics for morphometric variables of the bilobate phytoliths per species

Variable	*P. miliaceum*				*S. italica*			
	Mean	Min	Max	STD	Mean	Min	Max	STD
ArBBox	2920.28	959.00	8357.00	998.95	2538.54	972.00	5891.00	718.85
Area	1577.81	659.00	4700.00	499.22	1551.39	679.50	3925.00	433.63
ArEqD	44.33	28.97	77.36	6.59	44.03	29.41	70.69	6.08
AspRatio	*1.28*	*1.01*	*2.53*	*0.17*	*1.53*	*1.01*	*2.99*	*0.33*
Breadth	47.46	26.87	83.72	8.24	40.92	22.99	63.77	7.03
CArea	1917.38	723.50	5717.50	631.47	1800.91	755.00	4549.50	509.85
CHull	163.87	99.44	283.72	26.29	162.09	101.47	276.86	24.12
Circularity	*0.52*	*0.35*	*0.74*	*0.06*	*0.57*	*0.39*	*0.80*	*0.06*
Compactness	0.74	0.58	0.87	0.05	0.72	0.52	0.89	0.06
Concavity	339.57	64.50	1143.00	152.56	249.52	52.00	771.00	97.54
Convexity	0.84	0.71	0.93	0.03	0.88	0.76	0.95	0.03
Curve_Length	24.65	14.60	41.60	4.26	23.76	12.80	38.30	3.68
Curve_Width	10.18	6.70	17.30	1.53	9.68	6.40	15.60	1.37
EqEllAr	2293.58	753.20	6563.57	784.57	1993.77	763.41	4626.78	564.59
Feret	60.19	35.69	103.35	10.30	61.45	34.54	111.80	10.56
MaxR	30.84	18.04	53.34	5.30	31.37	17.82	57.24	5.39
MBCRadius	30.12	17.85	51.94	5.15	30.75	17.40	55.91	5.28
MinR	8.16	0.43	18.35	2.45	7.07	0.41	18.33	2.42
ModRatio	*0.28*	*0.02*	*0.59*	*0.09*	*0.24*	*0.02*	*0.70*	*0.10*
PerEqD	502.23	209.77	1496.06	158.91	493.82	216.29	1249.37	138.03
Perimeter	194.72	112.23	346.19	33.27	184.04	109.74	304.59	28.33
Rectangularity	*0.55*	*0.43*	*0.76*	*0.04*	*0.61*	*0.47*	*0.80*	*0.05*
RFactor	0.87	0.76	0.95	0.03	0.84	0.73	0.95	0.04
Roundness	*0.55*	*0.34*	*0.75*	*0.07*	*0.53*	*0.27*	*0.80*	*0.09*
Shape	*24.56*	*17.02*	*36.29*	*2.97*	*22.26*	*15.65*	*32.68*	*2.31*
Solidity	*0.83*	*0.71*	*0.93*	*0.04*	*0.86*	*0.73*	*0.96*	*0.03*
Sphericity	0.27	0.02	0.59	0.09	0.23	0.02	0.69	0.09

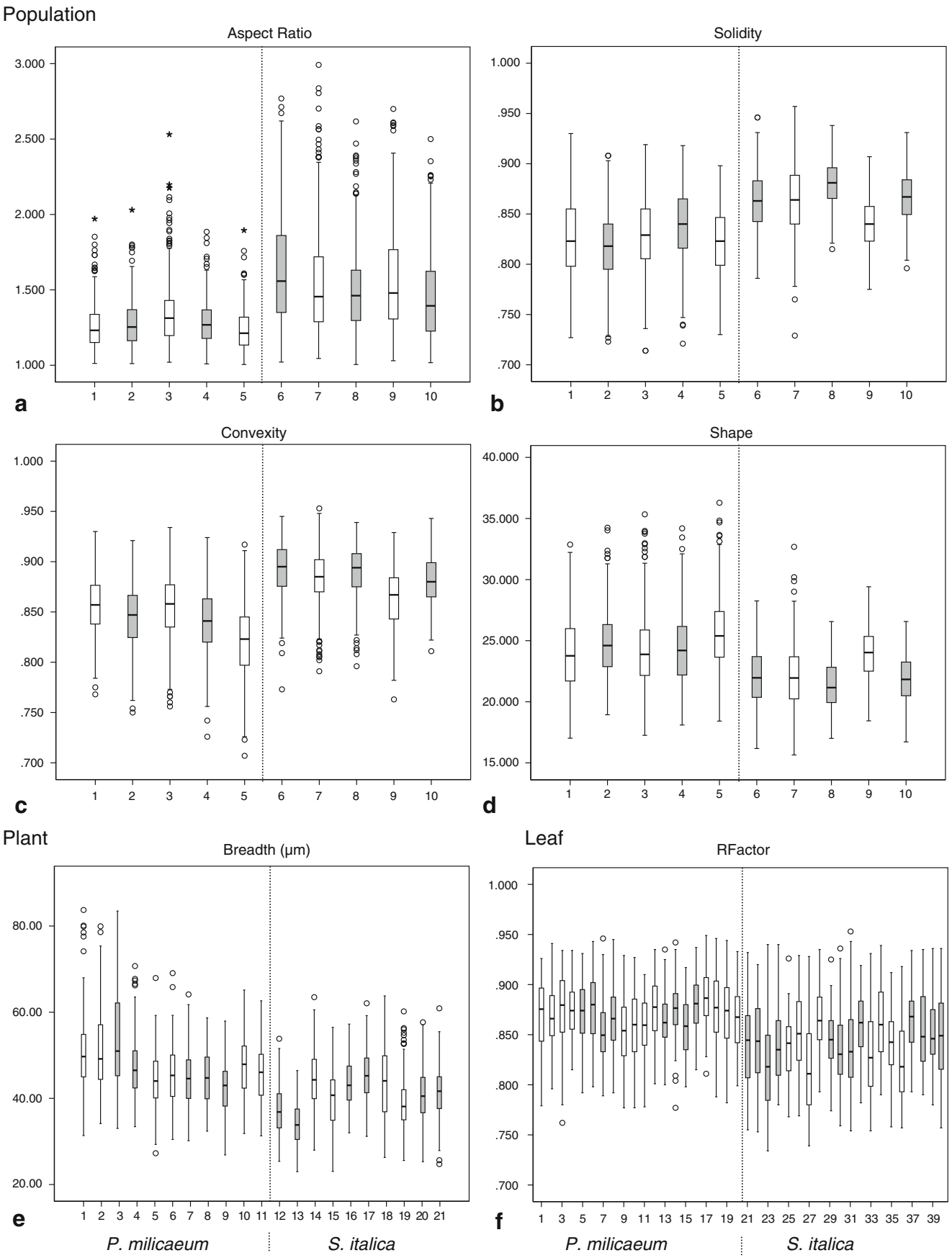
In italic: the parameters included in the discriminant analysis. For the units of measurement, see Table 2

STD standard deviation

all analysis levels, for some variables, particularly variables of size, this is not the case. While it could be argued that ideally, more phytoliths should have been measured per sample, the experimental duplication and the fact that minimum sample sizes per population are easily met makes the collection of further measurements of reduced relevance.

The sample size data moreover shows the diversity in variation within the studied material. While some samples, leaves, plants and populations have small variation (resulting in a small minimum required sample size), others have larger variation. In addition, particularly in the case of *P. miliaceum*, the minimum sample size is larger for the analysis levels of plant and population, pointing to an important level of variation within plants and populations. These results indicate that calculating a minimum required sample size based on morphometric data from single samples or from a single leaf may result in a too small sample size. This has consequences for studies in phytolith morphometry that use the above-provided formula to calculate a minimum sample size for studies in modern-day and archaeological material. This is of relevance for

Fig. 3 *P. miliaceum* and *S. italica*, a selection of representative bilobate morpometrics shown in *boxplots per population* (aspect ratio and solidity), plant (convexity and shape) and leaf (breath and RFactor), based on 2000 measurements of each species. **e** Population 4 is presented as three plants due to uncertainties in the sample strategy on plant level. The three plants probably represent two plants. The order of the populations, plants and leaves corresponds with Table 1. *White bars* uneven population numbers, *black bars* even population numbers. O= outlier: >1,5 and <3× the interquartile range from a quartile, *=extreme outlier: >3× interquartile range from a quartile)


Population
Aspect Ratio
Solidity
a
b
Convexity
Shape
c
d
Plant
Leaf
Breadth (μm)
RFactor
e
f
P. milicaeum
S. italica
P. milicaeum
S. italica

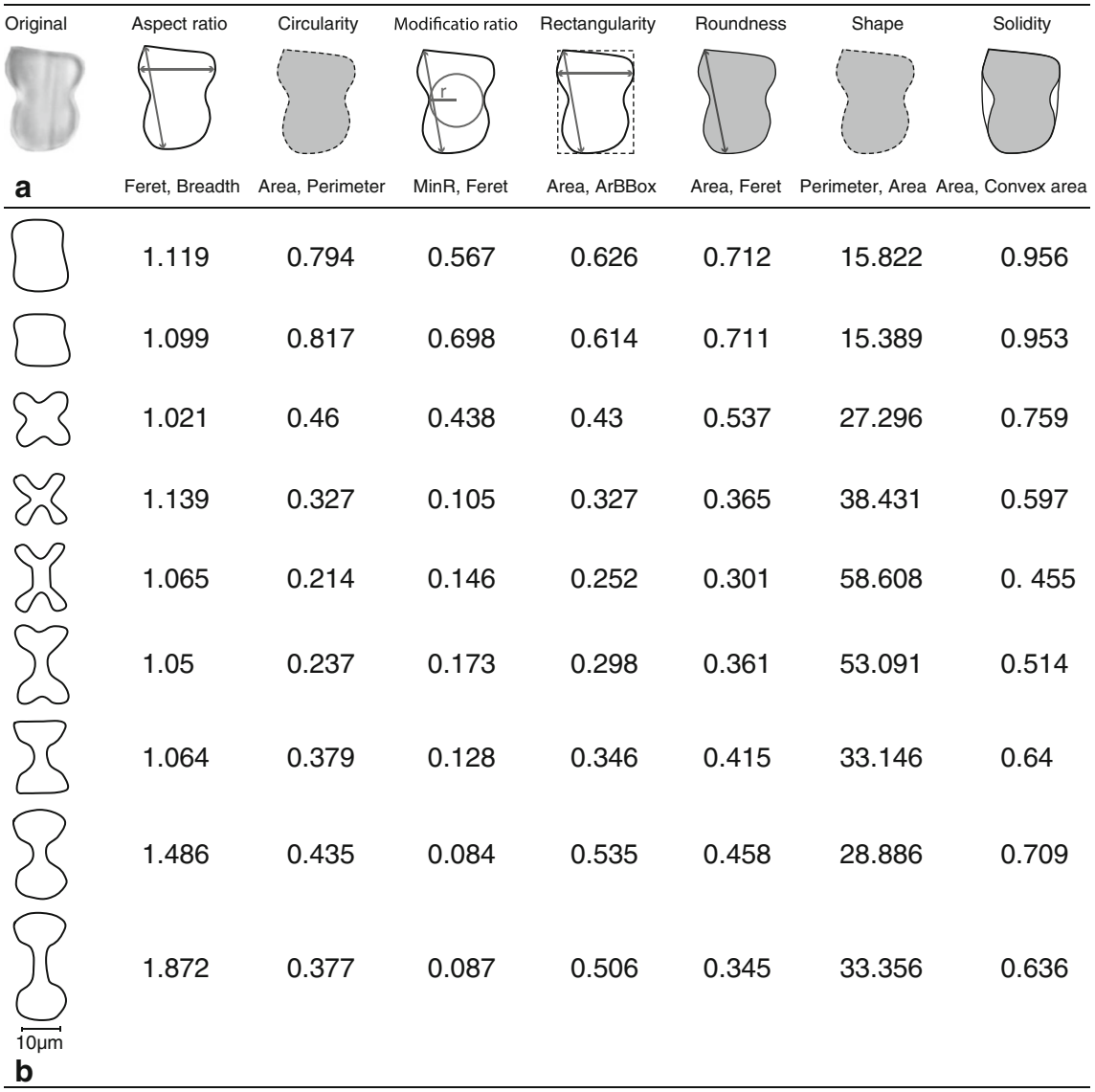


Fig. 4 Visualisation of the morphometric parameters included in the discriminant function. **a** Visualisation of concepts, after Ball et al. (2015). *1* Bilobate from a *S. italica leaf vein. 2* Aspect ratio: Feret/Breadth. *3* Circularity: $4\times\pi\times$Area/Perimeter^2, sometimes called form factor. It is 1 for a perfect circle and diminishes for irregular shapes. *4* Modification ratio (2×MinR)/Feret. *5* Rectangularity: Area/ArBBox. This approaches 0 for cross-like objects, 0.5 for squares, $\pi/4=0.79$ for circles and approaches 1 for long rectangles. *6* Roundness: $4\times$Area/($\pi\times$Feret^2). It is 1 for a perfect circle and diminishes with elongation of the feature. *7* Shape: Perimeter^2/Area. *8* Solidity: Area/Convex area. It is 1 for a perfectly convex shape, diminishes if there are surface indentations. **b** Measurements of the morphometric parameters included in the discriminant function of various phytolith models to show how shape influences the parameters

taxonomic identification by phytolith morphometry considering the tendency to develop identification criteria based on small sample sizes of material and often from few populations, and the regular lack of information on the experimental design and the quantities of reference material investigated.

Concerning the analysis of archaeological samples that are expected to represent either *P. miliaceum* or *S. italica*, the results indicate that a minimum of 35 phytoliths per sample can be sufficient for the analysis of data of many variables. Future reference studies should at best include material from more than one sample, leaf and plant, as indicated by our results for the minimal required sample size on the plant and population levels.

Classification by morphometry: differences between *P. miliaceum* and *S. italica*

This study, based on five populations per species, all of which are of a different genetic origin and some were grown under distinct environmental conditions, shows that taxonomic distinction between bilobates of leaves from *P. miliaceum* and

Table 5 The *p* values of the ANOVA testing the effect of taxon and population within taxon on the measured values of *P. miliaceum* and *S. italica*, as well as a general result showing whether there are differences between populations

Variable	ANOVA		Mult contrast tests	
	Taxon	Pop. in taxon	Pop. in taxon	
ArBBox	0.0368	0.1378	n.s.	
Area	0.7976	0.1617	n.s.	
ArEqD	0.8152	0.1406	n.s.	
AspRatio	.0001	0.3546	n.s.	
Breadth	0.0005	0.0958	n.s.	
CArea	0.3064	0.1628	n.s.	
CHull	0.6423	0.0384	n.s.	
Circularity	<.0001	0.011	Sign.	4–1:S_it 0.04109 *
				4–2:S_it 0.04900 *
				4–3:S_it 0.00582 **
				5–4:S_it 0.03369 *
Compactness	0.0172	0.3066	n.s.	
Concavity	0.0001	0.03	n.s.	
Convexity	<.0001	0.0088	Sign.	5–1:P_mi 0.0170 *
				5–3:P_mi 0.0188 *
Curve_length	0.1559	0.1090	n.s.	
Curve_width	0.1022	0.1216	n.s.	
EqEllAr	0.0368	0.1378	n.s.	
Feret	0.4507	0.1567	n.s.	
MaxR	0.5296	0.1465	n.s.	
MBCRadius	0.4527	0.1580	n.s.	
MinR	0.0107	0.1097	n.s.	
ModRatio	0.0012	0.0736	Sign.	4–3:S_it 0.0418 *
PerEqD	0.7976	0.1617	n.s.	
Perimeter	0.0586	0.1520	n.s.	
Rectangularity	<.0001	0.153	n.s.	
RFactor	0.0001	0.4349	n.s.	
Roundness	0.0210	0.3086	n.s.	
Shape	<.0001	0.0095	Sign.	4–1:S_it 0.04772 *
				4–3:S_it 0.00642 **
				5–4:S_it 0.03154 *
Solidity	<.0001	0.0328	Sign.	4–3:S_it 0.0128 *
Sphericity	0.0012	0.0673	Sign.	4–3:S_it 0.0368 *

N.s. no significant difference, *sign.* significant difference, significance codes: 0.001 '**' 0.01 '*' 0.05. For further details of the multiple contrast tests, see the text and Supplementary Information Table 3

S. italica by phytolith morphometry is achievable. The 88 % chance of correct identification with discriminant analysis is reasonably comparable with other, routinely accepted morphometric phytolith identification criteria (Ball et al. 1999, 2006; Piperno 2009). The exploration of the phytolith morphotypes suggests that frequency analysis of short cells from leaves, and particularly short cells other than bilobates may be a relevant additional tool to support the morphometric identification. This remains a topic for future research.

The taxonomic distinction of phytoliths from *P. miliaceum* and *S. italica* leaves is highly relevant for systematic and archaeobotany since:

- It offers a new classificatory criterion to distinguish between the two taxa;
- It furthers the level of taxonomic significance of different types of plant tissues; indeed, our results show that taxonomic identification by phytolith morphometry is possible using phytoliths from leaves (previously only tested in rice; Fujiwara 1993; Gu et al. 2013);
- The improved identification of leaves by phytolith analysis allows for the identification of by-products of millet harvests such as leaf fodder that are of substantial economic importance, as known from ethnography and archaeology (see "Introduction"), which may moreover benefit our understanding of producer and consumer sites and the social organisation of prehistoric societies (Fuller et al. 2014);
- The result can be directly applied to the archaeology of Asia and Europe to identify these crops of major economic importance since prehistory. Although most populations were grown in Europe and a further check for application in Asia may be useful, the large number of investigated populations, their large geographic and environmental variation and the attested restricted variation in the bilobates size and shape supports the criteria's wide applicability;
- The taxonomic characterisation at genus level (*Panicum* versus *Setaria*) may also be applicable in plant remains from archaeological sites in Africa.

For the proper application of the illustrated discriminant function as a taxonomic identification criterion for archaeological and palaeoecological assemblages, various aspects should be kept in mind. First, morphometric analysis of phytoliths from archaeological sites should always be based on statistically significant assemblages rather than individual phytoliths, since single cells have high variability. The minimum sample size recommended for morphometric analysis of bilobates from leaves from *P. miliaceum* and *S. italica* is discussed above ("Discussion", "Minimum sample size"). Second, differences between the reference material and archaeological assemblages may occur. For example, post-depositional factors, such as size-selective preservation and dissolution, may affect the composition of the assemblage (cf. Albert et al. 2009). Third, bilobate phytoliths are produced by many more Panicoideae taxa, including both wild and domesticated plants. The presented identification method can therefore best be applied to sites and/or regions where *P. miliaceum* and *S. italica* are expected based on the evidence from macroremains and/or inflorescence phytoliths. Further

research is needed to clarify the bilobate phytoliths production and significance in related taxa and to avoid false positive identification of wild relatives. This means that the current identification criteria should be preferably applied to closed contexts (e.g. pit linings, roof thatching and possibly dung) from fully fledged agricultural sites rather than early sites in which domestication processes are still in place. In fully agricultural sites, the highest input will be from domestic species, also for secondary products. Moreover, there is the need to compare the short cell bilobate phytolith morphometry of *P. miliaceum* and *S. italica* with that of other Panicoideae taxa that have been used as crops or famine food in East Asia and South Asia, such as *Panicum sumatrense* Roth (little millet), *Setaria pumila* (Poir.) Roem. and Schult. (yellow foxtail millet), *Setaria verticillata* (L.) P. Beauv. (bristley foxtail), *Urochloa ramosa* (L.) T.Q. Nguyen (browntop millet), *Echinochloa colona* (L.) Link ssp. *frumatenacea* (sawa millet) and *Paspalum scrobiculatum* L. (kodo millet). Although the taxonomic identification of *P. miliaceum* and *S. italica* bilobates for some sites with mixed assemblages thus requires further research on related taxa, the difference in bilobate size and shape of *P. miliaceum* and *S. italica* does nevertheless allow the recognition of the input of two different taxa.

Classification by morphometry: differences between populations within species

Comparison between the populations per species shows highly similar results within species, strengthening the wider applicability of differences between species and stressing the restricted role of within-species genetic variation on phytolith morphometry. The minor differences observed between the populations of *P. miliaceum* and *S. italica* affect only few variables and can be considered to reflect variation of biological objects. The minimal variation observed in *P. miliaceum* particularly relates to the population grown outside Europe and under substantially different climatic conditions (as it has been observed also for *Sorghum bicolor* (L.) Moench; Out and Madella unpublished results). In contrast to *P. miliaceum*, the difference between populations observed in *S. italica* is less likely to be explained directly by climatic/environmental conditions, since the slightly outstanding population of *S. italica* was like most other populations grown in Europe, while the population grown outside Europe (Indonesia) did not differ from the other populations. While both genetic and environmental factors may play a role, the precise cause of the small difference between the *S. italica* population from England and the others remains unknown.

Identification of plant parts

Besides differentiation between the bilobates of *P. miliaceum* and *S. italica* leaves also the differentiation by phytolith analysis between leaves and other plant parts is highly relevant for archaeology, thus aiming at a better detection and identification of non-dietary crop products. A distinction between leaves and inflorescences is firstly possible by means of morphotype comparison, since inflorescences are dominated by dendriform morphotypes, leaves by a combination of bulliforms, stomata, interstomatal cells, bilobates and smooth and wavy long cells (see also Figs. 1 and 2), and stems are dominated by smooth long cells. Since bilobates occur in both leaves, culms and inflorescences, a possible question for future research is whether there is a difference in size and shape in *P. miliaceum* and *S. italica* bilobates from different plant parts, and whether different short cell morphotypes are produced in the various plant parts. Interestingly, apart from phytoliths also starch is argued to be able to provide information about plant parts (detection of starch from *Panicum* culms on harvesting tools, Yang et al. 2013).

Conclusions

Analysis of 27 morphometric variables from five populations per species shows that it is possible to distinguish between bilobate phytoliths from leaves of *P. miliaceum* and *S. italica*. Differences within the species are observed, but they are little, and there is some overlap between the two taxa. This makes the objective method of morphometry based on image analysis highly suitable to apply for distinction between the two taxa. The new results are not only relevant for archaeology but also for plant systematics and palaeoecology amongst others. Detection and identification of *P. miliaceum* and *S. italica* in archaeological and palaeoecological records were already possible by means of caryopses (seeds), starch and phytoliths from inflorescences (phytoliths: see "Introduction"; starch: Yang et al. 2005; Yang et al. 2012; macroremains: e.g. Knörzer 1971; Kroll 1983, p. 43 ff.), while *P. miliaceum* can also be detected biochemically by means of miliacin (Motuzaite-Matuzeviciute et al. 2013a). The newly developed identification method for bilobate phytoliths from leaves strengthens this set of tools, now also allowing for detection of leaves that presumably have been of economic importance, e.g. as construction material and fodder, since prehistory. While this paper focuses on variation within species, the main suggestion for future research concerns the comparison with related taxa.

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