

1 **Phylogenetic, ecological and intraindividual variability patterns in grass phytolith shape**

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27 **Summary**

- 28 • Grass silica short cell (GSSC) phytoliths appear to be the most reliable source of fossil
29 evidence for tracking the evolutionary history and paleoecology of grasses. In recent
30 years, modern techniques have been used to quantitatively assess phytolith shape vari-
31 ation. This progress has widened opportunities with respect to the classification of
32 grass fossil phytoliths. However, phylogenetic, ecological and intraindividual variabil-
33 ity patterns in phytolith shape remain largely unexplored.
- 34
- 35 • The full range of intraindividual phytolith shape variation (3650 2D outlines) from 73
36 extant grass species, 48 genera, 18 tribes, and 8 subfamilies (with special attention
37 paid to Pooideae) was analysed using the geometric morphometric analysis based on
38 the semilandmarks spanning phytolith outlines.
- 39
- 40 • Although we showed that 2D phytolith shape is mainly driven by deep-time diversifi-
41 cation of grass subfamilies, a closer look uncovered distinct phytolith shape variation
42 in early-diverging lineages of Pooideae.
- 43
- 44 • The phylogenetic pattern in phytolith shape was successfully revealed by applying ge-
45 ometric morphometrics to 2D phytolith shape outlines. This finding strengthens the
46 potential of phytoliths to track the evolutionary history and paleoecology of grasses.
47 Moreover, geometric morphometrics of 2D phytolith shape proved to be an excellent
48 tool for analysis requiring large sums of phytolith outlines, making it useful for quanti-
49 tative palaeoecological reconstruction.

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58 **Key words:**

59 paleoecology, grass phylogeny, phytoliths, landmark-based geometric morphometrics,
60 Pooideae

61 **Introduction**

62 As the plant silica microfossils, phytoliths have the great potential to track the evolutionary
63 history and paleoecology of grasses (Poaceae) (Strömberg, 2005; Prasad *et al.*, 2011;
64 Strömberg, 2011; Strömberg, 2013). Due to their composition of biogenic opaline silica
65 ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$), phytoliths are preserved in various sedimentary environments, even when other
66 grass fossils are not (Piperno, 2006; Strömberg *et al.*, 2018). Grass phytoliths, and grass silica
67 short cell (GSSC) phytoliths, in particular, are known to vary in shape depending on multiple
68 taxonomic levels (subfamilies, tribes, sometimes even genera; Metcalfe, 1960; Mulholland &
69 Rapp, 1992; Piperno & Pearsall, 1998; Rudall *et al.*, 2014), whereas other grass fossil remains
70 such as pollen, leaves, or seeds are either rare or not taxonomically informative below the
71 family level (Jacobs *et al.*, 1999; but for the case of pollen see Mander *et al.*, 2013). The
72 recent work of Gallaher *et al.* (2020) quantitatively documented that GSSC phytolith 3D
73 shape carries a strong phylogenetic signal, one which can distinguish grass subfamilies and
74 tribes. They investigated 70 species of early-diverging grasses, plus Oryzoideae and
75 Bambusoideae. However, phylogenetic, ecological, and intraindividual variability patterns in
76 phytolith shape in other grass taxonomic groups remain unexplored.

77 The greatest amount of grass diversity falls in either of two clades, the BOP (Bambusoideae,
78 Oryzoideae, Pooideae) and PACMAD clade (Panicoideae, Arundinoideae, Chloridoideae,
79 Micrairoideae, Aristidoideae, and Danthoioideae) (Strömberg, 2011; Soreng *et al.*, 2015,
80 2017). Twiss *et al.* (1969) proposed three major divisions of GSSC phytoliths corresponding
81 to the three dominant grass subfamilies native to the Great Plains of the United States: bi-
82 /poly-lobate/cross in Panicoideae, saddle in Chloridoideae, and circular/oblong/rectangular in
83 Pooideae. These divisions are mainly useful for phytolith research in regions where grasses
84 are abundant and diverse on this (sub-familiar) taxonomic level [(for example, in grasslands
85 of the North American Great Plains (Fredlund & Tiezsen, 1994) and Sub-Saharan Africa
86 (Barboni *et al.*, 2007; Bremond *et al.*, 2005)]. However, they are less informative in regions
87 where only one subfamily dominates (and has dominated throughout long-term history), as,
88 for example, in large areas of temperate and boreal regions of Eurasia, where subfamily
89 Pooideae prevails (Gibson, 2009).

90 Pooideae is the largest Poaceae subfamily, with almost 4000 species, most of them adapted to
91 open and cold environments (Bouchenak-Khelladdi *et al.* 2010; Edwards & Smith, 2010;
92 Soreng *et al.*, 2017; Schubert *et al.*, 2019a, b). While considerable effort has been made to
93 refine the categorization of phytolith shape variation within subfamilies like Panicoideae,

94 Chloridoideae, Bambusoideae and Oryzoideae (Fahmy, 2008; Novello *et al.*, 2012; Lu & Liu
95 2003; Neumann *et al.*, 2017; Cai & Ge 2017; Gallaher *et al.*, 2020), phytolith shape variation
96 in Pooideae is largely unexplored, with only a handful of studies touching this area (for
97 example in studies where *Stipa* type phytoliths are recognised, Gallego & Distel 2004;
98 Silantyeva *et al.*, 2018; Mullholand, 1989).

99 Individual grass subfamilies are mostly adapted to certain environmental conditions and tend
100 to prevail in specific vegetation zones (Gibson, 2009). In phytolith analysis, this association is
101 used to define indices that can be applied as proxies of past environments. For example, the
102 so-called aridity index (Iph; Diester-Haass *et al.* 1973; Alexandre *et al.*, 1997) presents the
103 proportion of Chloridoideae prevailing in arid conditions (saddle-shaped morphotypes) and
104 Panicoideae prevailing in more humid conditions (bilobates, crosses), and can be used as a
105 proxy for aridity in past ecosystems. Similarly, the climatic index (Ic; Twiss, 1992) can be
106 used to reconstruct past climates on the basis of the proportion of morphotypes characteristic
107 of Pooideae adapted to open and cold environments (rondels) versus the proportion of
108 morphotypes characteristic of Chloridoideae and Panicoideae, which are adapted to higher
109 temperatures. However, it is untenable to use phytolith spectra *per se* for the indication of past
110 habitat conditions without a knowledge of the distribution of phytolith variation across the
111 phylogenetic tree, including the proportion of intraspecific variation.

112 Our recent study (Hošková *et al.*, 2021) observed very low GSSC phytolith shape plasticity
113 between populations of the same species, compared to interspecific variation, according to a
114 hierarchically designed study of two grass species with restricted ecological niches
115 (*Brachypodium pinnatum*, *B. sylvaticum*). Thus, we considered phytolith shape variation due
116 to environmental conditions to be negligible. However, significantly high residual variation
117 (51% of the total variation, which was not captured by the defined levels: species,
118 populations, individuals, leaves and parts of leaves) was related to the intraindividual
119 variation of phytolith shape within the individual sample. This type of variation (which is
120 intrinsic to phytoliths and demarcates it from variation in other fossils) causes species to carry
121 more than one phytolith morphotype, resulting in an overlap of phytolith shapes between taxa
122 (e. g. Rovner & Russ, 1992). Piperno & Pearsall (1998), in their seminal work, proposed that
123 intraindividual variation in phytolith shape may be lineage-specific; thus, species could vary
124 in the amount of intraindividual phytolith shape variation on the basis of their position in the
125 phylogenetic tree. Thus, knowledge of the full range of intraindividual variation could
126 complete our image of the phylogenetic pattern in phytolith shape.

127 In order to quantify the proportion of intraindividual variation in relation to the phylogenetic
128 pattern in phytolith shape, we used methods of geometric morphometrics (Hošková *et al.*,
129 2021). We explored whether GSSC phytolith shapes changed in response to diversification
130 during grass evolution and how closely phytolith shapes reflect phylogenetic relationships in
131 the Poaceae family. As the degree of intraindividual variation in phytolith shape in most
132 grasses has never been explored, we aimed to separate the intraindividual variation from the
133 variation due to phylogeny. We paid particular attention to the understudied grass subfamily
134 Pooideae, adapted to open and cold conditions, focusing, on the potential affinity of
135 ecological adaptation of species to phytolith shape variation within this group.

136 Geometric morphometrics is undoubtedly one of the most frequently applied techniques of the
137 biological shape analysis today (Polly & Motz, 2016). It enables size to be effectively
138 removed and thus focuses purely on the analysis of shape, allowing the simple collection of
139 coordinate data and easy visualisation of results as transformations of shapes themselves
140 rather than as tables of numbers (Bookstein, 1989; Klingenberg, 2013). Hence, unlike the
141 traditional description of predefined phytolith morphotypes {(International Code for Phytolith
142 Nomenclature 2.0 [International Committee for Phytolith Taxonomy (ICPT), 2019]},
143 geometric morphometrics allows the quantification and visualisation of a continuous phytolith
144 shape variation. Therefore, different phytolith morphotypes are analysed together within a
145 single multivariate space (morphospace), and, as a result, the major trends in shape variation
146 are explored. The Procrustes superimposition method, which is the core of the geometric
147 morphometric analysis, relies on the point-to-point correspondence of individual landmarks
148 among the analysed specimens. In case of phytolith outlines, these points are represented by
149 series of equidistant semilandmarks. In those phytoliths having symmetric 2D shapes, there
150 may be two or more fixed points delimiting individual symmetric curves. These points are
151 typically derived from the orientation of phytoliths within plant tissue (Hošková *et al.*, 2021).

152 **Materials and Methods**

153 *Plant material processing*

154 3650 modern grass phytoliths from 73 species, 48 genera, 18 tribes, 8 subfamilies were
155 analysed (Table S1).

156 Plant material was processed following the *in situ* charring method of Kumar *et al.* (2017).
157 This method preserves the original phytolith position within the plant epidermis. One leave
158 per plant per species was sampled. Leaves were cleaned in an ultrasonic cleaner (Digital

159 Ultrasonic Cleaner CE-7200A). The segment of leave was laid on a glass slide. Small pieces
160 of folded aluminium foil were placed near the two shorter sides of the slide. Another glass
161 slide was placed on top of the slide, holding the sample in place. The slides were put into a
162 muffle furnace at 550 °C for 5 h. The aluminium foil between the slides prevented the slides
163 from sticking together. The slides containing burnt material were washed with 1 N HCl and
164 distilled water (using a pipette). After the slides dried, plant material placed on the bottom
165 slide was covered with one drop of a 15 % solution of glycerol and the cover slide. The slides
166 were then analysed using transmission light microscopy (Leica DM 1000 LED).

167 *Data acquisition*

168 Sequential microphotographs of rows of GSSC phytoliths in the charred epidermis were
169 acquired under ×400 magnification (Leica camera ICC50 W). The planar view of GSSC
170 costal (over veins) phytolith morphotypes with a long axis parallel with the long axis of the
171 leaf was chosen for the analysis. First, two fixed landmarks were placed at the phytolith edges
172 perpendicular to the longest axis of the leaf (Fig. 1; Methods S1). Then, 48 equidistant points
173 were placed along both outline halves, resulting in 96 points which were treated as
174 semilandmarks in the subsequent geometric morphometric analysis. For each individual
175 phytolith image (3650 in total), 98 two-dimensional points were digitised. This approach was
176 only applied to phytolith morphotypes observed from the planar view called bilobates,
177 polylobates, saddles, crenates and trapezoids {(International Code for Phytolith Nomenclature
178 2.0 [International Committee for Phytolith Taxonomy (ICPT), 2019]}, whereas rondels,
179 positioned from the planar view in leaf epidermis, had no identifiable landmarks and were not
180 analysed. During the plant material processing, segments of leaves were laid on a glass slide
181 with random orientation regarding abaxial–adaxial leaf sides; however, the outer periclinal
182 surface of phytoliths was chosen for phytolith outline analysis since under light microscopy it
183 has clear, distinct edges that are well defined. Digitisation was carried out using the semi-
184 automated background curves tool in TpsDig, ver. 2.31 (Rohlf, 2015). Equidistant positions of
185 semilandmarks along the outlines relative to the positions of the fixed landmarks were
186 obtained using the ‘*digit.curves*’ function in GEOMORPH, v. 3.3.2 (Dryen & Mardia, 2016;
187 Adams *et al.*, 2021), in R, v. 3.6.3 (R Core Team, 2020) (for summarized methodological
188 workflow see Supplemental files Methods S2).

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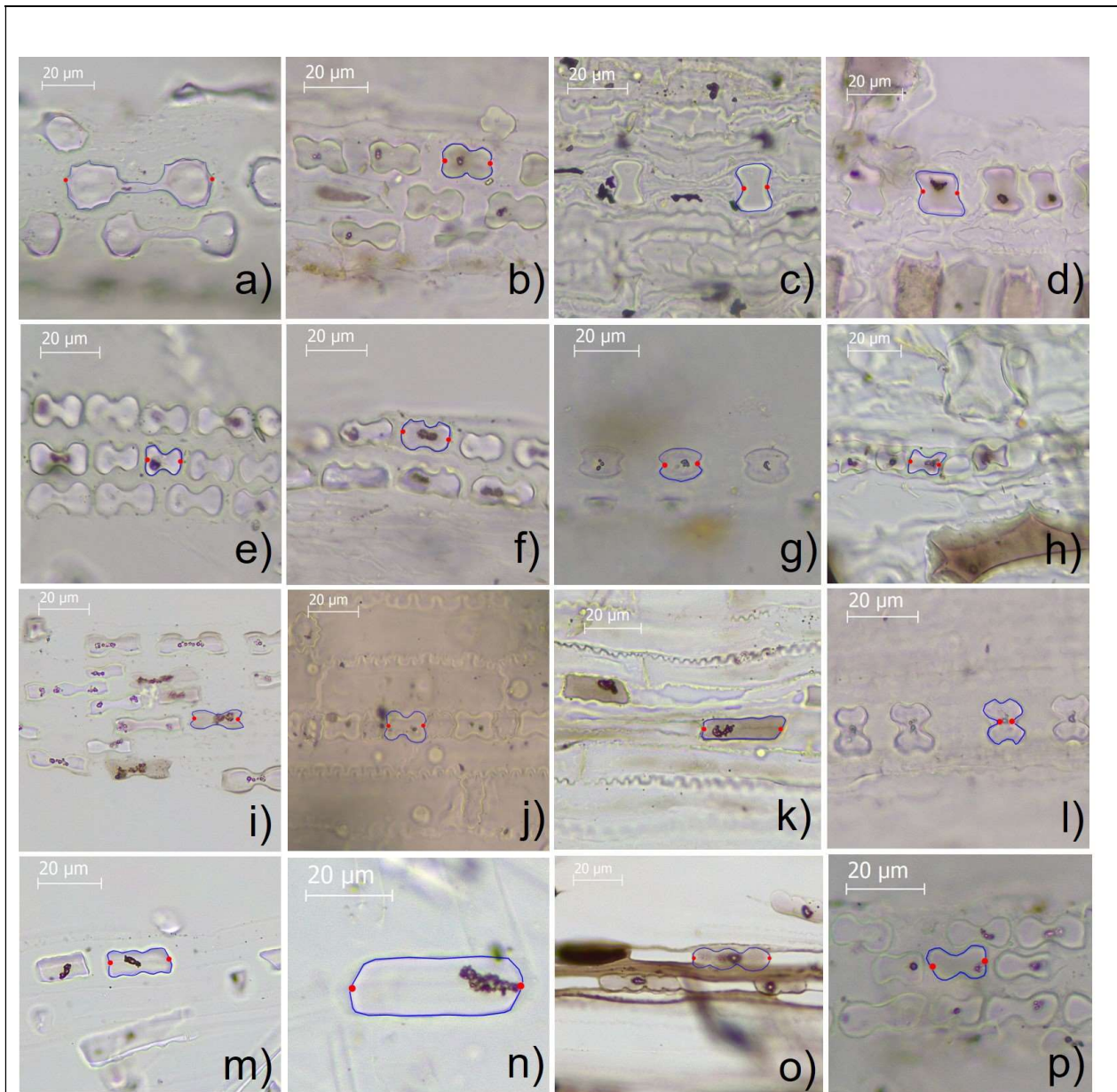


Fig. 1 Microphotographs of charred grass epidermises with GSSC phytoliths. Red dots indicate two fixed landmarks; blue lines indicate phytolith outline with 96 equidistant semilandmarks. (a) *Aristida rhiniochloa*, Aristidoideae; (b) *Hakonechloa macra*, Arundinoideae; (c) *Bambusa tuldoidea*, Bambusoideae; (d) *Arundinaria gigantea*, Bambusoideae; (e) *Danthonia alpina*, Danthonioideae; (f) *Schismus arabicus*, Danthonioideae; (g) *Cynodon dactylon*; (h) *Brachyelytrum erectum*, Pooideae; (i) *Bothriochloa ischaemum*, Panicoideae; (j) *Coix lacrymajobi*, Panicoideae; (k) *Alopecurus pratensis*, Pooideae; (l) *Zizania latifolia*, Oryzoideae; (m) *Bromus erectus*, Pooideae; (n) *Holcus lanatus*, Pooideae; (o) *Melica picta*, Pooideae; (p) *Stipa sibirica*, Pooideae.

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194 *Data analysis*

195 Generalised Procrustes Analysis of phytoliths with biradial symmetry

196 Geometric morphometrics was performed on a data set of 3650 phytolith configurations, each
197 consisting of 98 landmark coordinates (following Hošková *et al.*, 2021). The 2D shape of
198 phytoliths from planar view (long axis of phytolith parallel with the long axis of the leaf) is
199 typical by its biradial symmetry, meaning that left-right and upper-bottom parts of the 2D
200 outline are not differentiated. Thus, to achieve correspondence of all phytolith configurations
201 under study, we applied geometric morphometrics for the analyses of biradial symmetry
202 (Savriama *et al.*, 2010; Savriama & Klingenberg, 2011; Savriama *et al.* 2012; Neustupa,
203 2013) (Methods S1). A Generalised Procrustes Analysis, which minimises the sum of squared
204 distances between corresponding landmarks to extract shape data by removing the extraneous
205 information of size, location and orientation, was applied (e. g. Zelditch *et al.*, 2012; Dryden
206 & Mardia, 2016). The semilandmark position was optimised by their iterative sliding along
207 the curve tangents to achieve the lowest bending energy yielding the smoothest possible
208 deformation between each configuration and the mean shape (Bookstein, 1997; Pérez *et al.*,
209 2006; Gunz & Mitteroecker, 2013). Original phytolith configurations were transformed and
210 re-labelled and then subjected to a Generalised Procrustes Analysis. The resulting multiplied
211 dataset consisted of Procrustes coordinates of original configurations and transformed and re-
212 labelled copies (a reflected copy about the horizontal adaxial–abaxial axis; a reflected copy
213 about the vertical left-right axis; a copy reflected about both axes) (Savriama & Klingenberg,
214 2011; Klingenberg, 2015; Savriama, 2018; Hošková *et al.* 2021). By averaging the original
215 configuration and transformed copies of each specimen, symmetrised phytolith configurations
216 were obtained. These are symmetric and thus invariant under all transformations. A
217 Generalised Procrustes Analysis was conducted using the ‘*procGPA*’ function in *SHAPES*, v.
218 1.2.5 in R, v. 3.6.3.

219 Quantification of symmetric and asymmetric components of shape variation

220 Principal component analysis (PCA) was conducted with the superimposed Procrustes
221 coordinates consisting of all the original configurations and their transformed copies. This
222 PCA separated components of symmetric shape variation (variation between symmetrised
223 configurations) from three components of asymmetry (asymmetry under reflection in the
224 adaxial–abaxial direction, asymmetry in the left-right direction and asymmetry regarding both
225 these axes) (Savriama *et al.*, 2010; Klingenberg, 2015). The proportion of variation in the sub-

226 spaces of biradial symmetry and three asymmetric patterns were quantified by summing up
227 the percentages of variance explained by PCs belonging to a given subspace using scores
228 from PCs obtained by *'procGPA'* function in *SHAPES*, v. 1.2.5 in R, v. 3.6.3.

229 Quantification of different sources of shape variation

230 Different sources of the shape variation among phytoliths were quantified by multivariate
231 Procrustes analysis of variance (ANOVA) of the symmetrised configurations of individual
232 phytoliths (e. g. Klingenberg, 2015). Data were analysed in a nested structure that was
233 reflected by the Procrustes ANOVA models. The analysis decomposed the matrix of
234 Procrustes distances among individual configurations into different sources specified by the
235 independent factors. Besides quantifying the Procrustes sum of squares (SS) spanned by each
236 factor and their proportion on the total variation (η^2), the significance of the effects were
237 evaluated by comparing their original Procrustes SS values with their random distribution
238 yielded by 999 permutations (Schaefer *et al.*, 2006; Neustupa & Woodard, 2021). The
239 randomisation design reflected the nested structure of the independent factors. The main
240 effect evaluating the differentiation between the phytoliths of the BOP and PACMAD
241 lineages was tested against the random distribution based on the repeated reshuffling of
242 individual subfamilies between BOP and PACMAD. Likewise, the SS spanned by the
243 'subfamily' effect nested within 'BOP vs. PACMAD' were evaluated by comparison with the
244 random distribution yielded by reshuffling of tribes among subfamilies within the BOP and
245 PACMAD groups. Then, the effect of tribes was tested against the random SS distribution
246 based on the reshuffling of genera among different tribes, and the effect of genus identity on
247 phytolith shapes was tested against the random SS distribution based on a reshuffling of
248 species among genera. Finally, the differentiation of phytoliths among the species was
249 evaluated by randomisation of individual specimens. The function *'procD.lm'* in *GEOMORPH*, v.
250 3.3.2 (Dryen & Mardia, 2016; Adams et al., 2021), in R, v. 3.6.3 (R Core Team, 2020) was
251 used for the decomposition of sources of phytolith shape variation. Pairwise randomised
252 residual permutation procedure *posthoc* tests were performed using *'pairwise'* function in *RRPP*
253 v. 0.5.2 in R, v. 3.6.3.

254 To visualise the discrimination of grass subfamilies by their phytolith shape, canonical
255 variates analysis (CVA) was performed on symmetrised phytolith shape configurations in
256 MorphoJ (Klingenberg, 2011).

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258 Amount of intraindividual variation in phytolith shape in individual grass species

259 The amount of intraindividual variation in phytolith shape in individual grass species,
260 represented by average Procrustes distances of individual phytoliths to species centroids, was
261 compared using the function ‘*betadisper*’ of in VEGAN (Oksanen *et al.* 2019) in R v. 3.6.3.

262 Phytolith shape and grass phylogeny

263 Grass phylogeny was generated (using S3 scenario) with function ‘*phylo.maker*’ in
264 V.PHYLOMAKER, v.0.1.0 in R, v. 3.6.3 (Jin & Qian 2019) using ‘backbone’ tree based on
265 molecular data from seed plant phylogeny (mega-tree ‘GBOTB.extended.tre’; Smith &
266 Brown, 2018). Out of the 73 species examined, 54 species were in the Smith & Brown (2018)
267 backbone tree with the rest added using the S3 scenario of Jin & Qian (2019).

268 To visualise the phylogenetic history of phytolith shape change, grass species positions along
269 PC1 were mapped on the grass phylogeny tree. Likewise, PCs spanning different components
270 of asymmetric variation were mapped on this tree. Intraindividual shape variation, represented
271 by averaged distances from species group centroid in multivariate space, was also mapped on
272 the grass phylogeny. We used function ‘*phylosig*’ in R/PHYTOOLS v. 0.7.47 (Revell, 2012) to
273 determine Pagel's lambda as a measure of phylogenetic signal in individual components of the
274 shape data (individual PCA axes, amount of intraindividual variation). We used function
275 ‘*pgls*’ in R/CAPER, v. 1.0.1 to calculate confidence intervals (Orme *et al.*, 2018). We used
276 function ‘*contMap*’ to visualise the phylogenetic history of individual components of the
277 shape data, using the function ‘*fastAnc*’ in R/PHYTOOLS v. 0.7.47 to reconstruct maximum
278 likelihood values at tree nodes (Revell, 2012).

279 The phylogenetic tree of the studied taxa was projected onto the shape tangent space by
280 squared-change parsimony in MorphoJ (Klingenberg, 2011). The resulting tree was plotted in
281 the plane of the PC1 vs PC2 ordination plot of the species-level averaged shapes.

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287 **Results**

288 *Decomposition of symmetric and asymmetric variation in phytolith shape*

289 The first group of PCs, associated with entirely symmetric shape variation, accounted for
290 89.5 % of the total variation of the data set. The second group of PCs, associated with three
291 subspaces of asymmetric shape variation, accounted for 11.0 % of the total variation. Thus,
292 the PCA results indicated that phytolith shape variation consisted mainly of symmetric
293 variation between individual phytoliths and relatively little asymmetric shape variation within
294 phytoliths (e. g. between their left and right sides). Therefore, in the following analyses of
295 phytolith shape, we considered only the data set of the symmetrised phytolith configurations.

296 *Sources of variation in phytolith shape*

297 Individual taxonomic levels accounted for 81.9 % of the total variation in symmetric phytolith
298 shapes (Fig. 2, Table 1). Residual variation consisting of phytolith shape variation within
299 individuals was considerably lower (18.2 %). Variation between subfamilies was the most
300 pronounced (42.7 %, $P = 0.001$), followed by variation between tribes (14.0 %, $P=0.001$),
301 species (13.3 %, $P=0.001$), and genera (10.4 %, $P=0.9$). The phytolith shape variation
302 between PACMAD and BOP clades accounted for only 1.4 % of the total variation ($P=0.7$).

303 Significant differences in phytolith shape were found for all the 28 subfamily pairs in the
304 *posthoc* pairwise tests (at the significance level of 0.01) (Fig. 3; Table S2).

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317 **Table 1** Results of Procrustes ANOVA evaluating different sources of variation in
318 symmetrised phytolith shape. df: degrees of freedom; SS: sum of squares; MS: means
319 squares; η^2 : proportion on the total variation; P: probability of the null hypothesis. ***, P <
320 0.001

Source	df	SS	MS	η^2	P
BOPxPACMAD	1	0.742	0.742	0.014	0.7
Subfamily (BOPxPACMAD)	6	22.861	3.81	0.427	0.001***
Tribe (subfamily)	10	7.465	0.747	0.14	0.001***
Genus (tribe)	30	5.589	0.186	0.204	0.9
Species (genus)	26	7.135	0.274	0.133	0.001***
Residuals	3576	9.713	0.003	0.182	
Total	3649	53.506			

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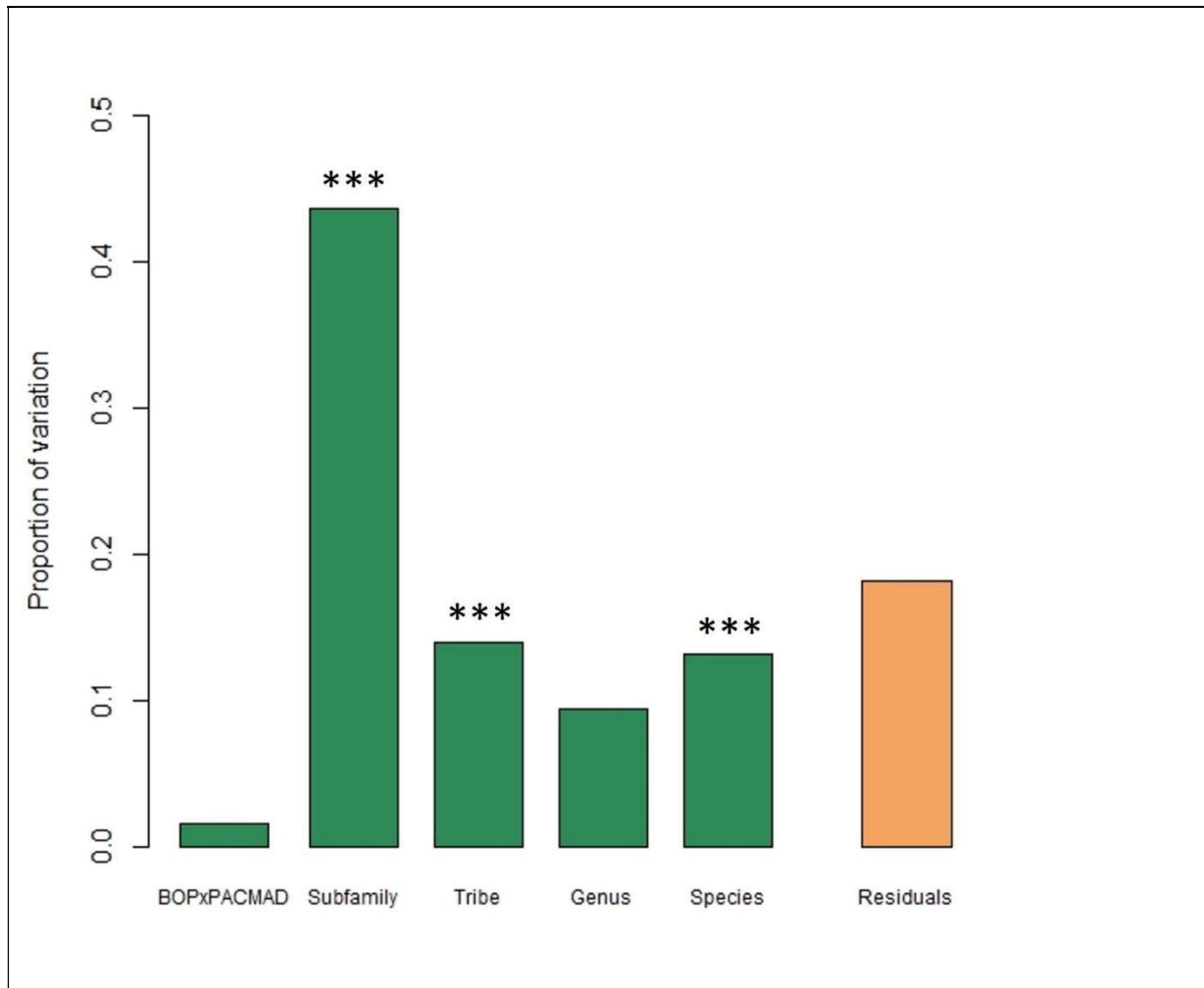


Fig. 2 Coloured bars represent the proportion of variation in phytolith shape attributed to the various taxonomic levels and residual intraindividual variation. Stars indicate statistically significant differences in phytolith shape between the subfamilies, the tribes and the species. ***, $P < 0.001$

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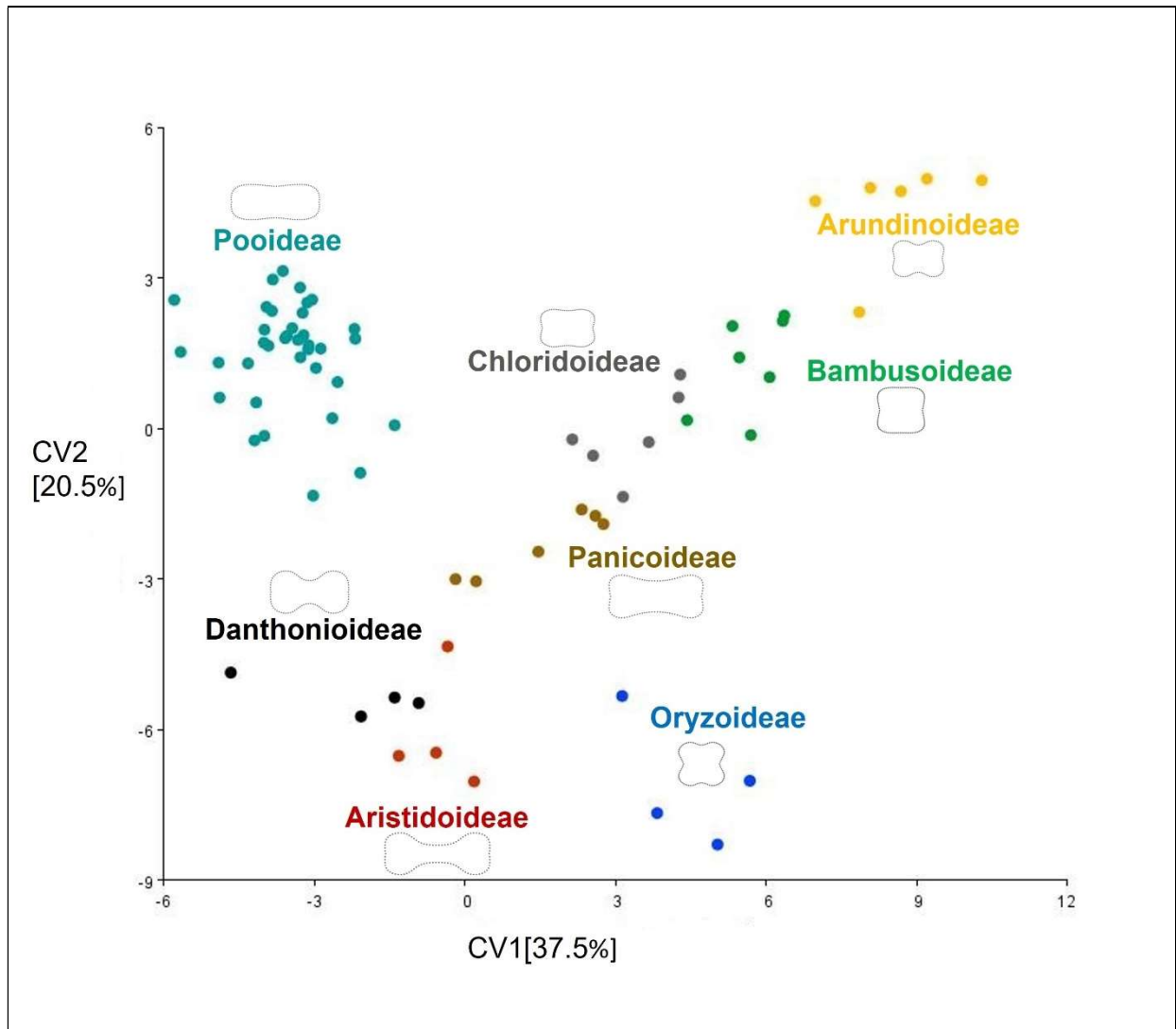


Fig. 3 Canonical variates analysis used to discriminate between grass subfamilies by symmetrised grass silica short cell phytoliths shape. Dots represent averaged phytolith shape of individual grass species. Colours of dots indicate grass subfamilies. Phylogenetic signal in phytolith shape is apparent from the distinctive position of individual grass subfamilies in the morphospace.

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351 *Intraindividual phytolith shape variation across species*

352 The amount of intraindividual GSSC phytolith shape variation differed across species (Fig. 4;
353 Notes S1). The phytolith shape of some species varied to such an extent that two different
354 morphotypes, as traditionally defined, occurred – we reported variation from bilobate- to
355 saddle-shaped phytoliths in *Eragrostis minor*, and variation from bilobate- to polylobate-
356 shaped phytoliths in *Brachypodium dystachion*, *B. pinnatum*, *Bromus inermis*, *Calamagrostis*
357 *epigejos*, *Bothriochloa ischaemum*, *Coix lacryma-jobi*, and *Digitaria sanguinalis*. Other
358 species, like *Bromus erectus* (tribe Bromeae), *Melica picta* (tribe Meliceae), *Festuca*
359 *gigantea*, *Festuca arundinacea*, *Helictotrichon pubescens*, *Milium effusum*, *Phleum pratense*,
360 *Poa nemoralis*, and *Trisetum flavescens* (tribe Poeae) significantly varied intraindividually in
361 the length and width of GSSC phytoliths (the so-called crenate morphotype). *Ehrharta erecta*
362 (tribe Ehrharteae) varied intraindividually in the length of the polylobate phytolith shape,
363 whereas *Brachyelytrum erectum* (tribe Brachyelytreae), *Dichanthium annulatum* (tribe
364 Andropogoneae), and *Echinochloa crus-galli* (tribe Paniceae) varied intraindividually in the
365 length of the bilobate shape.). In contrast, some species were very conservative in their
366 phytolith shape, like, for example, *Bambusa tuldoides*, *Phragmites australis*, and *Aristida*
367 *rhinochloa*.

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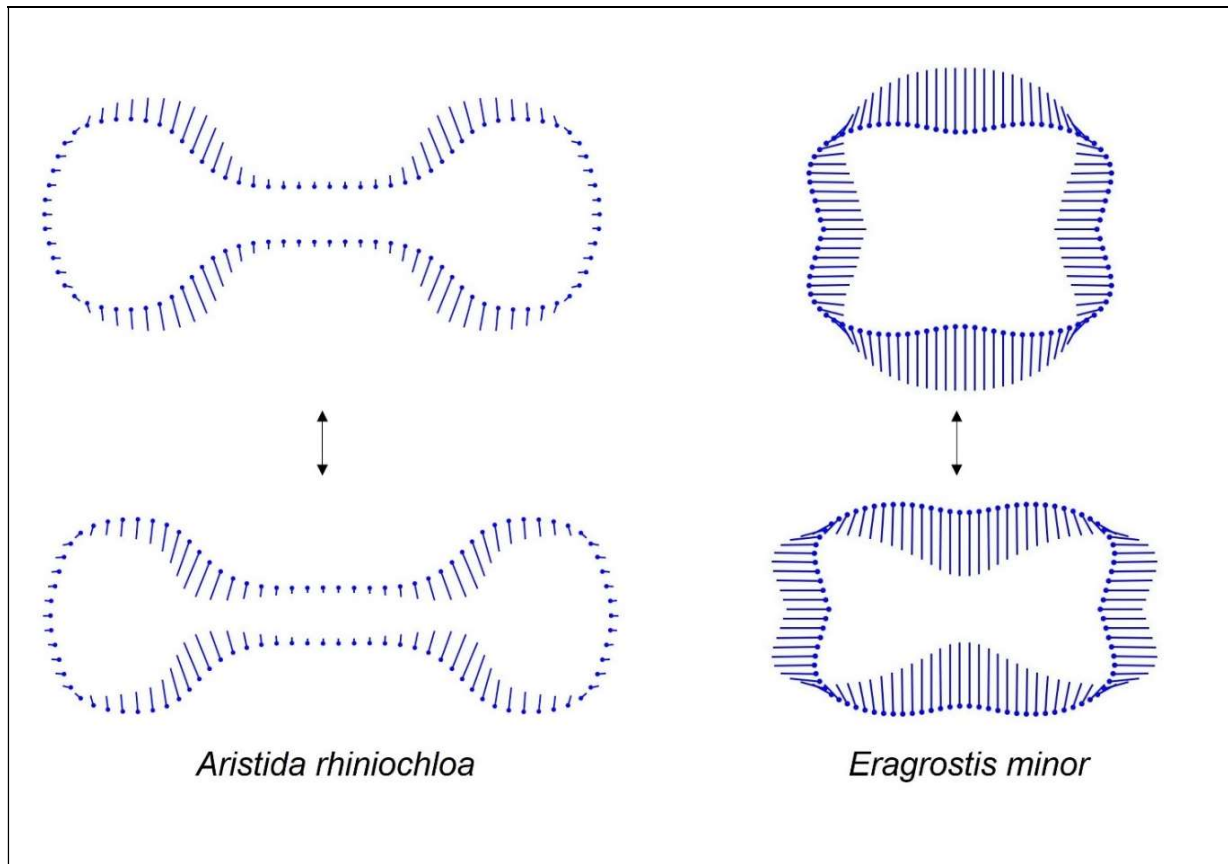
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Aristida rhinochloa

Eragrostis minor

Fig. 4 The amount of intraindividual variation in phytolith shape (represented by the average distance of individual phytolith to species centroid) is visualised by 'lollipop' graphs where the stick of the lollipop indicates the direction of the variation, whereas the lollipops (filled dots) represent together mean phytolith shape outline. *Aristida rhinochloa* represent species with less variable phytolith shape, whereas *Eragrostis minor* has highly variable phytolith shape. (For the phytolith outlines of more species see Supplemental files Notes S1)

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389 *Mapping phytolith shape data onto the phylogeny*

390 According to the PCA performed on the symmetrised phytolith configurations, the first two
391 PCs explained >96 % of the total variation (Fig. 5). Along PC1 (explaining 89.5 % of the total
392 variation), phytoliths varied between elongated shapes with two deeply incised lobes and
393 shorter and taller shapes on opposite sides. Along PC2 (explaining 7.0 % of the total
394 variation), phytolith shape varied between shorter outlines with two deeply incised lobes and
395 elongated shapes with a pronounced middle part on the opposite sides.

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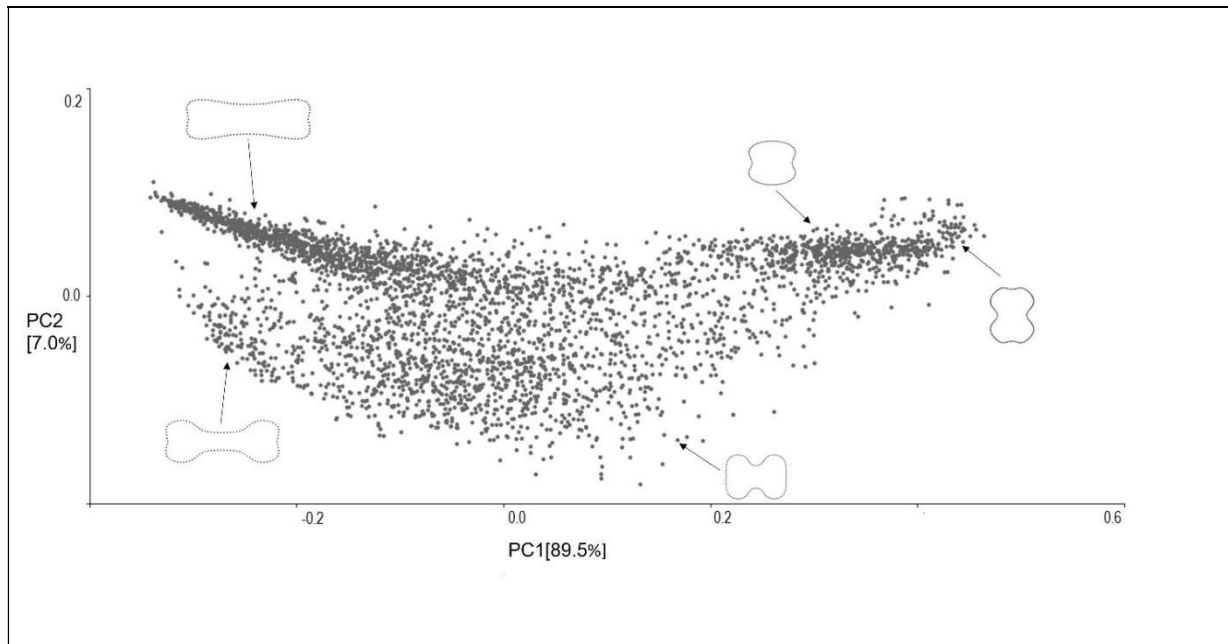


Fig. 5 Principal component analysis of 3650 symmetrised grass silica short cell phytolith shape configurations. Dots indicate individual phytoliths. The first two PCs are associated with the most pronounced shape variation within the dataset. Phytolith outlines represent shape variation along both axes.

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429 To visualise the phylogenetic history of symmetric phytolith shape change, we mapped grass
430 species positions along PC1 on the grass phylogeny tree (Fig. 6). The most prominent shape
431 variation in the data — an elongated shape with two deeply incised lobes vs shorter and taller
432 shapes — was strongly phylogenetically conserved at the subfamily level. Thus, individual
433 monophyletic subfamilies substantially differed in their phytolith shapes. On the other hand,
434 similar phytolith shapes occurred in two major grass subclades: BOP and PACMAD. The
435 measure of phylogenetic conservatism for individual PCs describing symmetrised shape
436 variation, along with the measure of phylogenetic conservatism in asymmetric components of
437 shape variation and in the amount of intraindividual variation (Fig. S1), are summarised in
438 Table 2.

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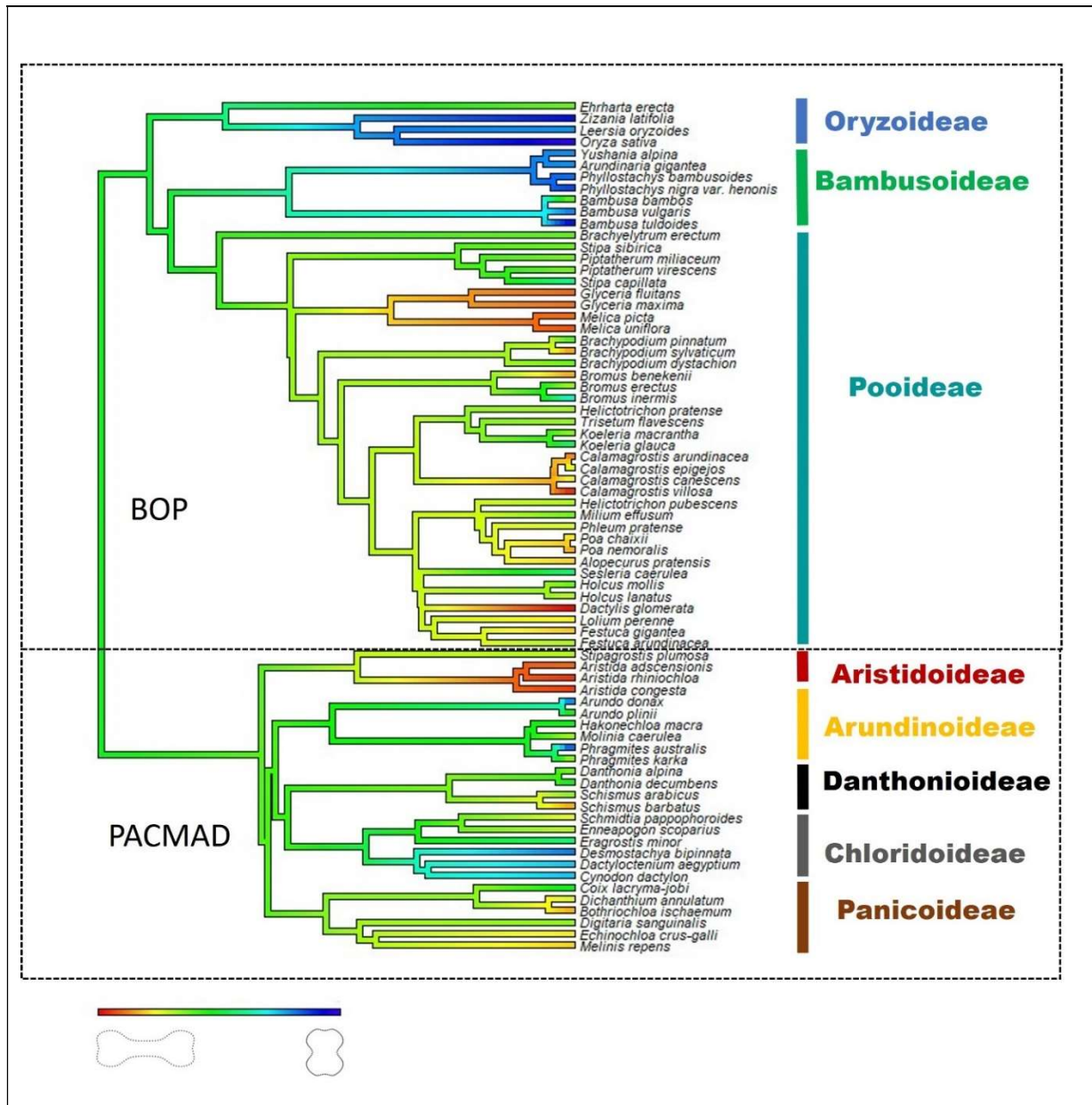


Fig. 6 Phytolith shape variation pattern across grass phylogeny. Grass phylogeny was constructed with V.PhyloMaker package in R (Jin & Qian, 2019), ‘backbone’ tree based on molecular data from seed plant phylogeny (mega-tree ‘GBOTB.extended.tr’; Smith & Brown, 2018). Grass species positions along the first principal component axis (PC1) were mapped on the tree. Colour scale indicates grass silica short cell phytolith shape variation along PC1.

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461 **Table 2** Measure of phylogenetic conservatism in averaged phytolith shape represented
 462 by principal components of symmetric (PC1-PC4) and asymmetric shape variation (vertical,
 463 left-right, transversal). Phylogenetic conservatism of the amount of intraindividual variation
 464 in symmetrised phytolith shape was also measured (see also Fig. S1). The amount of
 465 intraindividual variation in phytolith shape in individual grass species is represented by
 466 average distance of individual phytolith to species centroid. N=73

Type of shape variation	Proportion of variation (PCA)	(%)	Pagel's lambda (λ)	Lower 95% CI	Upper 95% CI
<i>Symmetric</i>	PC1	89.5	0.686	0.453	0.843
	PC2	7.0	0.517	0.208	0.753
	PC3	1.5	0.523	0.236	0.75
	PC4	1.0	0.486	0.083	0.764
<i>Intraindividual Symmetric</i>	-	-	0.428	0.053	0.744
<i>Asymmetric</i>	Vertical	1.9	0.188	NA	0.627
	Left-right	2.4	6.10^{-5}	NA	0.219
	Transversal	6.7	6.10^{-5}	NA	0.455

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481 The phylogenetic tree of the subfamily Pooideae was also projected onto the shape tangent
482 space by squared-change parsimony. The resulting tree was plotted in the plane of the PC1 vs
483 PC2 ordination plot of the species-level mean shapes (Fig. 7). The phylogenetically closely-
484 related taxa were not necessarily close to each other in the morphospace, so there are long
485 branches that criss-cross the plot. On the other hand, a number of other closely-related taxa,
486 especially *Stipa capillata*, *S. sibirica*, *Piptatherum miliaceum*, *P. virescens* in the tribe Stipeae
487 and *Melica picta*, *M. uniflora*, *Glyceria fluitans*, *G. maxima* in the tribe Meliceae showed
488 close correspondence between phylogeny and phytolith shape variation described by the first
489 two PCs.

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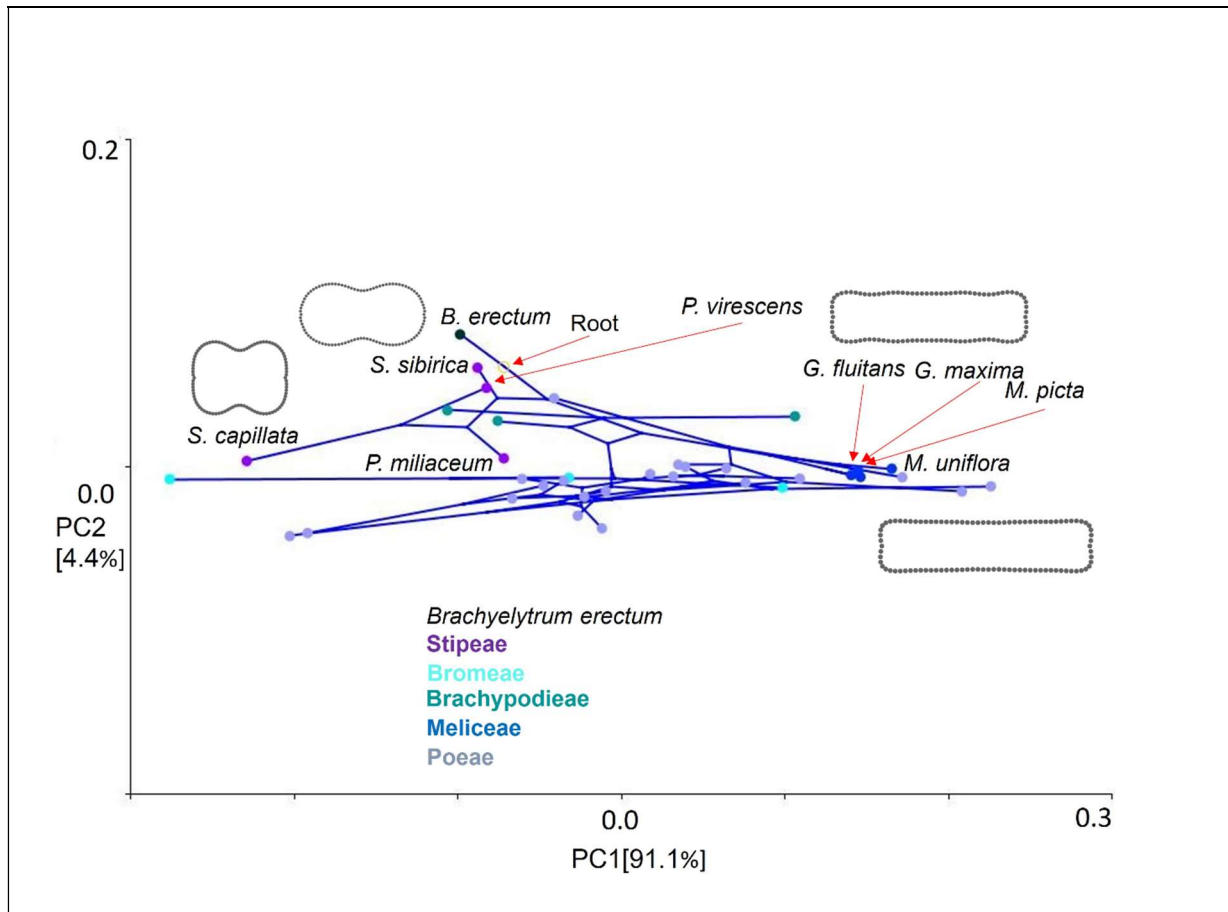


Fig. 7 Grass phylogeny mapped onto morphospace represented by the PC1 vs PC2 of the subfamily Pooideae. Coloured dots indicate analysed species from the various tribes; lines represent phylogenetic tree diversification. Closely related species (*Stipa capillata*, *Stipa sibirica*, *Piptatherum miliaceum*, *Piptatherum virescens*, *Melica picta*, *Melica uniflora*, *Glyceria fluitans*, *Glyceria maxima*) with phytolith shape variation well correlated with relationships in phylogenetic trees and their phytolith shape outlines are indicated.

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517 **Discussion**

518 *The observed evolutionary pattern in phytolith shape*

519 In general, the results of this study concur with previous reports that GSSC phytolith shapes
520 closely reflect phylogenetic relationships between taxa (Piperno & Pearsall, 1998; Gallaher *et*
521 *al.*, 2020). Thus, in the case of Poaceae, this means that phytolith shape changed along with
522 other anatomical and micromorphological leaf traits in response to their diversification in the
523 course of evolutionary history (Thomasson, 1978; Romaschenko, 2011; Rudall *et al.*, 2014).
524 The deep-time diversification of grass subfamilies mainly drives the basic pattern of phytolith
525 shape variation. The ancestors of recent grass species probably carried phytoliths of similar
526 shapes to those we see in extant species occurring in modern ecosystems (Thomasson, 1987;
527 Strömberg, 2005; Prasad *et al.*, 2005, 2011).

528 We found differences between subfamilies known for their distinct phytolith shape
529 (Chloridoideae, Oryzoideae, Bambusoideae, Panicoideae, Pooideae; Twiss *et al.*, 1969;
530 Gallaher *et al.*, 2020) but also between smaller subfamilies like Aristidoideae, Arundinoideae
531 and Danthonioideae. We also revealed diversification in phytolith shape in some tribes.
532 Axially-oriented bilobate-shaped phytoliths of *Ehrharta erecta* (Ehrharteae) differed from
533 those in Oryzoideae, while *Oryza sativa*, *Zizania latifolia*, and *Leersia oryzoides* (Oryzeae)
534 formed a separate cluster within this subfamily. Similarly, bilobate-shaped phytoliths of
535 *Eragrostis minor* and *Enneapogon scoparius* (Eragrostideae) differed from those in
536 Chloridoideae. Also, *Stipa* and *Piptatherum* (Stipeae) and *Melica* and *Glyceria* (Meliceae)
537 differed from Pooideae. From this, it is clear that further analysis of phytolith shape
538 employing a more comprehensive range of grass tribes would be useful.

539 *The intraindividual variation in relation to the phylogenetic pattern in phytolith shape*

540 The amount of intraindividual variation was variable across species. However, the
541 phylogenetic signal in intraindividual variation was generally low. Nevertheless, we detected
542 high intraindividual variation in some early-diverging lineages (as was previously shown by
543 Piperno & Pearsall, 1998). The phytolith shapes of *Eragrostis minor* were the most variable –
544 it carried saddle-shaped phytoliths characteristic of its subfamily Chloridoideae and bilobate-
545 shaped phytoliths generally attributed to Panicoideae. Similarly, polylobate-shaped phytoliths
546 occurred in *Ehrharta*, the lineage diverging early from Oryzoideae, itself characterised by tall,
547 transversally-oriented, bilobate-shaped phytoliths. In Pooideae, the early-diverging lineages
548 (Brachyelytrum, Brachypodieae, Bromaeae, and Meliceae) also significantly varied in

549 phytolith shape. However, we also found some highly variable phytolith shape in 'crown'
550 groups (e. g. Poeae, Andropogoneae) and very conservative phytolith shape in early-diverging
551 lineages (e. g. Aristideae). Further studies of phylogenetic signals in intraindividual phytolith
552 shape variation are needed to clarify these patterns observed in phytolith shape.

553 Whether there is a phylogenetic signal in intraindividual phytolith shape variation or not, we
554 still need to consider this variation while classifying fossil phytoliths. In other words, we must
555 consider whether to classify fossil taxa on the basis of comparison with averaged phytolith
556 shape (representing whole intraindividual shape variation by a single shape) or compare them
557 with whole intraindividual phytolith shape variation within the species of our reference
558 collection. According to the findings of this study, the second option seems to be the better
559 approach since the averaged phytolith shape of some species does not reflect the natural
560 variation in phytolith shape (as seen in the extreme case of *Eragrostis minor*). A reference
561 collection based on the whole intraindividual phytolith shape variation of the studied species
562 is necessary.

563 *The ecological component in phytolith shape variation*

564 Although we found a clear phylogenetic signal in phytolith shape, we consider the high
565 contrast in terms of the phytolith shape (namely the distinction between elongated and shorter
566 shapes) of some closely related taxa as striking. As shown before, grass long cell phytoliths,
567 like crenate and polylobate GSSC phytoliths in some species, are consistently bigger and
568 proportionally longer in reduced light conditions (Dunn *et al.*, 2015). It was suggested that the
569 addition of trichomes and stomata to the epidermis might shorten phytoliths, and that the
570 densities of both stomata and trichomes increase with enhanced light conditions in grasses
571 (Allard *et al.*, 1991; Knapp & Gilliam, 1985). Focusing on the Pooideae subfamily, we found
572 such a contrast between the Stipeae and Meliceae tribes. Stipeae comprises species that
573 generally occupy drier open grasslands and steppe communities (e. g. Romaschenko *et al.*,
574 2011, 2012). Meliceae is mostly found in shady woodlands or wet environments. More
575 studies focusing on phytolith shape variation along environmental gradients and on
576 comparison with other anatomical traits (e. g. stomata and trichome densities) would be
577 needed in these groups; however, on the basis of what we have learned from this dataset, we
578 expect this shape variation to be more an outcome of the long-term ecological adaptation of
579 species than a short-term plastic response of phytolith shape to the environment. Further
580 testing is crucial in this direction.

581 *Limitations of the current study*

582 Geometric morphometrics enables the quantitative assessment of phytolith shape variation
583 and the exploration of trends in phytolith shape variation within one morphospace. However,
584 the Procrustes superimposition of phytolith shapes may not be universally applicable to all
585 known morphotypes occurring in real samples. As shown in this study, in phytoliths with
586 symmetric 2D shapes, there may be two or more fixed points delimiting individual symmetric
587 curves. These points are typically derived from the orientation of phytoliths within plant
588 tissue. This applies to wide range of phytolith morphotypes; however, phytoliths that do not
589 bear any such corresponding points, such as those classified into rondel or spheroid groups,
590 would be unsuitable for a clear-cut morphometric analysis by the generalized Procrustes
591 analysis. This is the major limitation of our approach, because it prevents us from including
592 all grass species that carry only such morphotypes, such as the genera *Nardus*, which
593 represents an early-diverging lineage of Pooideae, or *Festuca* (in particular, species with
594 narrow leaves).

595 Landmark-based geometric morphometrics applied to a 3D phytolith surface model to
596 quantify the overall GSSC phytolith shape partially overcomes this problem (Gallaher *et al.*,
597 2020). 3D shape assessment helps to establish the positional homology of all GSSC phytolith
598 morphotypes, allowing them to be analysed within a common framework. However, the 3D
599 morphometrics of phytoliths is based on confocal microscopy (Evet & Cuthrell, 2016), which
600 makes it more expensive and time-consuming than analysis based on light microscopy. The
601 fact that 2D geometric morphometrics is more accessible allows robust studies based on large
602 datasets. This particularly applies to the use of geometric morphometrics in paleoecology,
603 where high numbers of phytoliths are typically used for the reconstruction of vegetation
604 dynamics (Piperno, 2006; Strömberg, 2009).

605 *Conclusions*

606 In this study, we demonstrated that the 2D shape of GSSC phytoliths is highly relevant for the
607 reconstruction of the evolution and paleoecology of Poaceae. Although we showed that
608 phytolith shape is mainly driven by the deep-time diversification of grass subfamilies, a closer
609 look also uncovered distinct phytolith shape variation in early-diverging lineages of the
610 subfamily Pooideae. Geometric morphometrics proved to be particularly helpful in this
611 regard. It enables the quantitative assessment of the entire phytolith shape and visualises its
612 variation. Moreover, the geometric morphometrics of 2D phytolith shape is cheap and

613 relatively non-laborious, making it an excellent tool for achieving goals requiring large sums
614 of phytolith outlines, such as in palaeoecological reconstruction.

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620 searched for us every grass species we wished for. We thank Matthew Nicholls for his
621 diligent proofreading of the final manuscript.

622 **Author contributions**

623 KH conceived the study, conducted imaging and data analysis. KH and AP collected plant
624 material in the field. JN developed the scripts for automated analysis of phytolith shape with
625 biradial symmetry and Procrustes ANOVA. KH wrote the text with contribution of JN, PP,
626 and AP. All authors approved the final version of the manuscript.

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877 **Supplemental files**

878 Table S1 List of grass species used for analysis of grass silica short cell phytoliths within *in*
879 *situ* charred epidermis.

880 Table S2 Tests of significant difference in phytoliths shape between the subfamilies.

881 Fig. S1 Pattern in the amount of intraindividual phytolith shape variation across grass
882 phylogeny.

883 Methods S1 Workflow sequence of landmark-based geometric morphometrics performed on
884 phytoliths with biradial symmetry.

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886 Methods S2 Workflow sequence of landmark-based geometric morphometrics and other
887 methods used in the current study.

888 Notes S1 Zip file of the 'lollipop' graphs visualising the amount of intraindividual variation in
889 phytolith shape for all grass species under study.

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