Bilobate phytolith size matters for taxonomical and ecological identification of Chad grasses: A case study on 15 species

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A B S T R A C T

The Poaceae family produces diagnostic phytoliths commonly called Grass Silica Short Cell Phytoliths (GSSCP), of which shapes and sizes are very distinguishable from those produced by other plant families. Grass Silica Short Cell Phytoliths are usually classified into four main categories: the rondels, bilobates, crosses, and saddles. Among them, the bilobates have in common a general "dumbbell" shape consisting of two lobes inter-connected by a shank. They represent the dominant morphotypes observed in the leaf tissues of many Panicoideae grass species. Besides, they tend to often be related to this subfamily in the fossil record, especially when they occur in large amounts. Several studies based on modern African grasses have however demonstrated that bilobates were also abundantly produced by a few members of the Aristidoideae, Chloridoideae, and Oryzoideae grass subfamilies which, like Panicoideae, are today mainly distributed in tropical low-altitude areas. The relationship between bilobates and Panicoideae is thus not so direct in the tropics. As a result, it can lead to significant misinterpretations when applied to paleoenvironmental and archaeological surveys.

This study investigates variations in the size of bilobate phytoliths in 15 African grass species, including eight Panicoideae, three Aristidoideae, two Chloridoideae, and two Oryzoideae, with the intention to explore new alternatives for refining their identification in the past deposits of tropical Africa. Different statistical analyses (Principal Component Analysis, Mann–Whitney tests, decisional trees) applied to the previous dataset allowed testing whether: (i) bilobate length and width significantly vary between grass species; (ii) these variations (if so) have the potential to be used as taxonomical and/or ecological proxies.

Our analyses confirm that bilobate length is significant in discriminating dry-adapted Aristidoideae and Chloridoideae species from the other sub-families. In particular, a bilobate base longer than 25 μm is almost exclusively observed for Aristidoideae and Chloridoideae, which is in agreement with previous studies. Although subject to less variations, the length/width of bilobate shanks and the average length of their lobes appear significantly useful to refine grass taxonomical identification and discriminate <120 cm-high grasses from taller grasses. Yet, it seems that the key towards a robust identification of bilobate phytoliths involves considering the size parameters.

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1. Introduction

Phytoliths are microscopic amorphous hydrated silica (SiO_2) particles produced by plants. They are shaped in and between plant cells by precipitation and accumulation of the groundwater soluble silica taken up to tissues (Kumar et al., 2017; Piperno, 2006; Sangster and Parry, 1969). After plants die and decay, phytoliths are incorporated in modern surface soils or sediments where they eventually get preserved through time, including across large geological time scales (Piperno and Sues, 2005). This ability makes them suitable tools for reconstructing past vegetation and climate of the Earth (Harris et al., 2017; Novello et al., 2017; Dunn et al., 2015; Barboni et al., 2010), as for instance documenting plant evolution (Prasad et al., 2011), fossil plant ecology (Novello et al., 2015) and physiology (e.g., grass photosynthesis) (Strömberg and McInerney, 2011). In archaeological contexts, phytoliths have largely been employed to document the history of plant domestication (Madella et al., 2014) and fire technology (Albert and Cabanes, 2007), and also food uptake habits to a lesser extent (Power et al., 2016).

Phytoliths consist of a variety of morphologies depending on the cells, tissues, and plants (taxonomy) they are forming in. The Poaceae family (grasses) accumulates more significant amounts of silica in its tissues than most of the other plant families, and especially than many of the eudicotyledoneous lineages (Katz, 2014). Among the phytoliths produced by grasses, the Grass Silica Short Cell Phytoliths (so-called

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GSSCPs), are diagnostic to the family. Their overall aspect, as well as their broadly defined shape and size ranges are common features that have been used to identify them in modern and past deposits (Piperno, 2006).

In Africa, GSSCPs have been largely employed to document grass-dominated vegetation assembly and dynamics during the Plio-Pleistocene (Novello et al., 2017; Rossouw and Scott, 2011; Barboni et al., 1999), where savanna and grassland ecosystems today represent about half of the African continental surface (Gibson, 2009). In order to refine the taxonomical and/or environmental values of the GSSCPs pre-served in paleontological and archaeological deposits of Africa, phyto-lith analysts have developed tools and calibration lines based on both modern plant and soil reference material (Esteban et al., 2017; Neumann et al., 2017; Liu et al., 2016; Aleman et al., 2014; Cordova, 2013; Novello et al., 2012; Cordova and Scott, 2010; Mercader et al., 2010; Barboni and Bremond, 2009; Rossouw, 2009; Fahmy, 2008). Studying modern African grass material has for instance permtted to de-fine trapeziform polylobate GSSCPs as reliable indicators for the C$_3$-Pooideae subfamily in the East African regions (Barboni and Bremond, 2009; Rossouw, 2009; Bremond et al., 2008), and squat saddles as indic-ative of the Chloridoideae subfamily (Neumann et al., 2017; Mercader et al., 2010) and/or of African dry-adapted grasses (Novello et al., 2012; Barboni and Bremond, 2009).

Among the main GSSCP morphological categories, the bilobate ones possess a general "dumbbell" (Metcalfe, 1960) shape consisting of two lobes inter-connected by a shank (Fahmy, 2008; Lu and Liu, 2003). They are observed in large amounts in the leaf epidermis of the Panicoideae grass subfamily, but also of several species among the Aristidoideae, Chloridoideae, and Oryzoideae subfamilies (Neumann et al., 2017; Novello et al., 2015; Mercader et al., 2010; Rossouw, 2009), which all occur today in low altitude regions of tropical Africa (Schmidt et al., 2011; Poilecot, 1999). The shape and size of bilobates are varying from a taxon to another, and these variations have been ap-preciated differently according to authors, most likely depending on their goals and on the descriptive criteria they used for their analyses. Barboni and Bremond (2009), for instance, inventoried and described 23 distinct bilobate morphotypes after scanning the leaf epidermis pho-tographs of 184 East African grass species (Palmer and Gerbeth-Jones, 1986; Palmer et al., 1985; Palmer and Tucker, 1981, 1983), whereas Rossouw (2009) and Mercader et al. (2010) recognized only three and nine distinct morphotypes from the analysis of 309 and 60 southern African grass species/specimens, respectively. As another example, Novello et al. (2012) described 15 distinct bilobate morphotypes from analyzing 37 grass species from Central Africa. At first sight different, all these studies present nonetheless the similarity to have (mostly) taken into account qualitative criteria for describing bilobate phytoliths. These qualitative criteria include the shape of the base, the shape of the top, and how they differ or not from each other, and finally the shape of the outer margins (lobes). Still, these qualitative descriptive criteria ap-plied to grass phytolith identification have been proved to provide ei-ther very little or inexistent information about their taxonomy, ecology or physiology. Indeed, while a single study suggested that bilobates with concave and convex lobes can be conventionally associ-ated with the Panicoideae subfamily in the context of Africa (Mercader et al., 2010), other authors conversely pointed out significant redundancy in the bilobates produced across the entire grass family as a barrier to produce refined grass taxonomical identification (Barboni and Bremond, 2009). But what if the size could help pushing further the identification of the bilobate phytoliths? In that vein, Novello et al. (2012, 2015) considered bilobate total length and section height as quantitative criteria to add to qualitative criteria; this allowed isolating several bilobate morphotypes informative of grass ecology to use for paleoenvironmental studies. These author's analyses especially showed that tabular <25 μm bilobates with concave lobes, as well as trapeziform (≥5 μm section) bilobates (including the *Stipa*-type) (Fredlund and Tieszen, 1994) were preferentially observed in aquatic grass species,

whereas ≥25 μm bilobates were mostly produced by xerophytic grass species, confirming Barboni and Bremond (2009)'s first hypothesis on bilobate size increasing for dry-adapted grasses. Fahmy (2008) also combined detailed size and shape criteria to describe bilobate phytoliths in African grasses. His study includes 66 Paniceae species (a tribe among the Panicoideae subfamily) of West Africa and allowed de-fining a total of 22 bilobate morphotypes. In addition to outer margin features, Fahmy also took in account four distinct measurements: the length (divided up into four categories: >26; 18–26; 10–18; <10 μm) and the width (three categories: >16; 9–16; <9 μm) of the complete bilobate bodies, as well as the length (three categories: >7; 4–7; <4 μm) and the width (three categories: >7; 4–7; <4 μm) of their shank. According to this classification scheme, Fahmy concluded that bilobates with flattened, convex, and notched outer margins were the ones mainly produced by Paniceae, and that bilobate width seemed to corre-late with bilobate shank thickness. In line and as a continuity of Fahmy's work, Neumann et al. (2017) studied the bilobates produced by 51 grass species from West Africa, but this time including common representa-tives of the Aristidoideae, Chloridoideae, Oryzoideae, and Panicoideae subfamilies. Their final GSSCP classification, which is the most detailed produced for African grasses so far, includes a total of 93 bilobate morphotypes defined from the combination of both descriptive (shape, 3D morphology) and measured criteria (total length of the bod-ies, length of their shank). Statistical analyses applied to this dataset pointed out nine bilobate morphotypes as being of interest for documenting grass taxonomy and/or ecology. Among them, the scooped bilobates were for instance pointed out as indicative of the Oryzoideae subfamily. Their analyses also indicate that very long bilobates with convex ends and very long shanks are most commonly produced in the Aristidoideae subfamily and, more broadly, by xero-phytic grass species, which remains in agreement with Barboni and Bremond (2009)'s original hypothesis, and also with Novello et al. (2012)'s analyses.

The present study presents a dataset of five distinct size criteria mea-sured on bilobate phytoliths observed from a preliminary sample of 15 different grass species from Central Africa. The ability of this quantita-tive dataset to predict grass taxonomy (subfamily), as well as several grass ecological (water-requirement) and physiological variables (pho-tosynthesis) and traits (grass height) was tested through a series of sta-tistical analyses. Two main questions are addressed in this paper: (i) how bilobates vary in dimensions between grass species according to their taxonomy, ecology, physiology, and traits; (ii) whether the var-iations recorded (if so) could be potentially used to interpret their pres-ence in past deposits of tropical Central Africa; (iii) and finally, if these new results are in agreement with previous studies carried out in trop-ical areas of Africa, but also out of this continent.

2. Material

The 15 grass species selected for this study consist of eight Panicoideae including *Andropogon gayanus* Kunth *(*var. *gayanus), Andropogon pseudapricus* Stapf, *Anthephora nigritana* Stapf & C.E. Hubb., *Brachiaria xantholeuca* (Schinz) Stapf, *Cymbopogon giganteus* Chiov., *Loudetia annua* (Stapf) Hubb.*, Loudetia simplex* (Nees) Hubb.*, and *Pennisetum pedicellatum* Trin.; three Aristidoideae: *Aristida funiculata* Trin. & Rupr, *Aristida mutabilis* Trin. & Rupr., and *Aristida stipoides* Lam.; two Chloridoideae: *Ctenium elegans* Kunth and *Sporobolus helvolus* (Trin.) T. Durand & Schinz; and two Oryzoideae: *Oryza barthii* A. Chev. and *Oryza longistaminata* A. Chev. & Roehr. (Table 1). They all produce significant amounts of bilobate phytoliths in their leaf tissues (44–100%; Novello and Barboni, 2015), which jus-tifies their selection for this study. Besides, these 15 species altogether also represent a large array of diversity by including distinct photosyn-thetic pathways (limited to C$_3$-*Oryza*, C$_4$ in this study), life cycles (an-nual or perennial), water-requirements (helophytic, mesophytic, and xerophytic), and physiognomies (average height ranging from 22.5 to

Table 1
Taxonomy, water-requirement, life cycle, photosynthetic pathway, average height (cm), and preferred environment of the 15 Poaceae species selected for this study (Clayton et al., 2017; Poilecot, 1999; Schmidt et al., 2011; Watson and Dallwitz, 1992). The species (specimens) collected in Chad were identified by P. Poilecot (CIRAD).

Species	Subfamily (Tribe)	Water-requirement	Life cycle	Photosynth. pathway	Average height (cm)	Preferred vegetation type(s) and/or substrat	Herbarium specimen ID/Coordinates
Andropogon gayanus Kunth (var. *gayanus*)	Panicoideae (Andropogoneae)	Helophytic	Perennial	C_4, NADP ME	225	Stream banks, alluvial plain, and swamp margins	Herb. spe. no. 6764
Andropogon pseudapricus Stapf	Panicoideae (Andropogoneae)	Mesophytic	Annual	C_4, NADP ME	100	Savanna grassland to woodland; occasionally tropical montane forest	Herb. spe. no. 7890
Anthephora nigritana Stapf & C.E. Hubb.	Panicoideae (Paniceae)	Mesophytic	Perennial	C_4, NADP ME	100	Stream banks	11°43'7"N, 19°06'34"E, 568m
Aristida funiculata Trin. & Rupr	Aristidoideae (Aristideae)	Xerophytic	Annual	C_4, NADP ME	22.5	Sandy areas	Herb. spe. no. 8003
Aristida mutabilis Trin. & Rupr.	Aristidoideae (Aristideae)	Xerophytic	Annual	C_4, NADP ME	40	Sandy areas	Herb. spe. no. 7323
Aristida stipoides Lam.	Aristidoideae (Aristideae)	Xerophytic	Annual	C_4, NADP ME	120	Sandy areas	Herb. spe. no. 3958
Brachiaria xantholeuca (Schinz) Stapf	Panicoideae (Paniceae)	Xerophytic	Annual	C_4, PCK	40	Dry forest and savanna woodland	Herb. spe. no. 7307
Ctenium elegans Kunth	Chloridoideae (Cynodonteae)	Xerophytic	Annual	C_4, NAD ME	105	Savanna grassland to woodland	Herb. spe. no. 3961
Cymbopogon giganteus Chiov.	Panicoideae (Andropogoneae)	Mesophytic	Perennial	C_4, NADP ME	200	Savannas; sandy terraces	Herb. spe. no. 4890
Loudetia annua (Stapf) Hubb.	Panicoideae (Arundinelleae)	Mesophytic	Annual	C_4, NADP ME	75	Savanna woodland	Herb. spe. no. 6410
Loudetia simplex (Nees) Hubb.	Panicoideae (Arundinelleae)	Xerophytic	Perennial	C_4, NADP ME	90	Savanna woodland	Herb. spe. no. 6757
Oryza barthii A. Chev.	Oryzoideae (Oryzeae)	Helophytic	Perennia or annual	C_3	90	Low-energy stream banks and ponds	Herb. spe. no. 10411
Oryza longistaminata A. Chev. & Roehr.	Oryzoideae (Oryzeae)	Helophytic	Perennial	C_3	95	Stream banks and wetlands	Herb. spe. no. 6732
Pennisetum pedicellatum Trin.	Panicoideae (Paniceae)	Mesophytic	Annual	C_4, NADP ME	90	Dry forest and savanna grassland to woodland	Herb. spe. no. 6533
Sporobolus helvolus (Trin.) T. Durand & Schinz	Chloridoideae (Zoysieae)	Mesophytic	Perennial	C_4, NAD ME	37.5	Large inland distribution; occasionnaly coastal	Herb. spe. no. 7355

200 cm) (Clayton et al., 2017; Schmidt et al., 2011; Poilecot, 1999; Watson and Dallwitz, 1992) (Table 1). Strict water-requirement assignments were given according to the most common habitats recorded in the distribution of each species. Each species was studied through a single specimen. Specimen samples were collected at the CIRAD herbarium in Montpellier (France) in 2009 or in southern Chad during the September–October 2010 time period following the rainy season (Table 1). In this case, P. Poilecot (CIRAD, Montpellier) did the identification of the different specimens directly collected. Either from the herbarium or the 2010's fieldwork season, each specimen possesses mature inflorescences, suggesting that the grasses were all at well-advanced growth stage during their collect. This increases the probability to work on complete grass silica short cell (here bilobate) silicification.

3. Methods

3.1. Data acquisition

Phytoliths were extracted from grass leaves/stems using alternating baths of nitric/perchloric acids (HNO_3/$HClO_4$) and hydrogen peroxide (H_2O_2 at 33%) at high temperatures (80–90 °C), and then mounted on slides using Canada basalm. Canada basalm (refraction index: 1.55) gives a better contrast than glycerin (refraction index: 1.47) to photograph phytoliths (Novello et al., 2015). A total of 49–55 of bilobate bodies were photographed for each specimen/species using a microscope-camera device set up at ×500 magnification. Each body was then measured using the imagery software PEGASE PRO. Five distinct measurements were performed: length of the base, length of the shank, average length of the two opposite lobes, width of the shank, and average width of the two opposite lobes (Fig. 1a). As bilobates consist of two parts, a top and a base (often the largest part), all measurements were systematically performed on the same part, here so the

base according to our definition (Fig. 1b). Moreover, the point where the phytolith outline switches from convex to concave was defined as being the limit between the bilobate shank and its lobes (Lu and Liu, 2003). All bilobates were measured independently from the shape of their outer margins (lobes). Finally, "nodular" bilobates (Neumann et al., 2017; Fahmy, 2008) were not taken into account in this study because they have also been alternatively classified in the polylobate morphotype category by other authors (Novello and Barboni, 2015).

3.2. Robustness and limitation of the dataset

The size robustness of the total number of bilobates used per species was first tested in order to ensure their suitability to be processed through statistical analyses. Size robustness was estimated through the margin of error, so-called E, calculated at a confidence interval of 90% (Ball et al., 2016) and applied to different samples (portions) of the measurement dataset (base length, shank length, shank width, etc.) according to the distinct grass trait variables considered for this study (subfamily, photosynthesis, life cycle, etc.): $E = [sqrt ((Z_{\alpha/2}^2 \times S^2)/N)]/M$, where $Z_{\alpha/2}^2 = 1.64$, which is the square of the two-tailed test (Z value at $\alpha = 0.10$); S^2: variance; N: sample group size; M: sample group mean. E (margin of error) values estimated for measurements (length of the base, length of the shank, average length of the two opposite lobes, width of the shank, and average width of the two opposite lobes) are summarized in SOM, Table S1. E values are <0.06 for all the samples, which suggests that the size of the dataset produced is large enough to ensure robust statistical analyses.

3.3. Are grass variables dependent from each other?

Correlation values between the different grass trait variables and their associated *p*-values (Pearson test) are presented as Supplementary

a.

b.

— — — top

.... base

■ shank
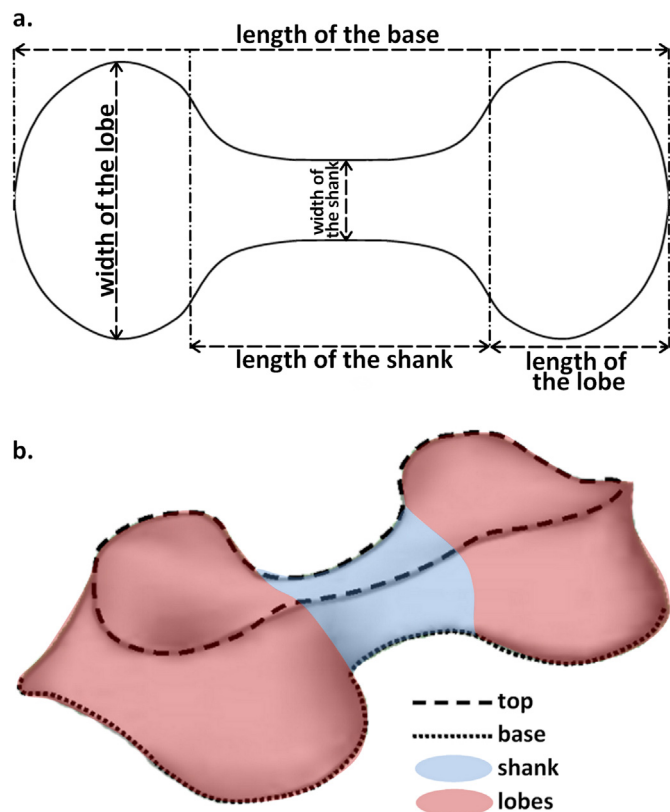
■ lobes

Fig. 1. a. A bilobate showing the five parameters measured for this study on a 2D projection; b. Top, base, shank, and lobes of bilobate on a 3D projection (after Madella et al., 2005).

online material (SOM, Table S2a and b). *P*-values > 0.05 support non-significant correlation values between grass traits, which is observed in most cases, whereas significant *p*-values indicate significant correlation values between traits. For significant *p*-values, the Oryzoideae subfamily is (100%) correlated to the C_3-*Oryza* photosynthetic pathway and (79%) to the helophytic type, while the Aristidoideae subfamily is (61%) correlated to the xerophytic type. Likewise, the helophytic type is (79%) correlated to the C_3 photosynthetic pathway and (-79%) to the C_4 photosynthetic pathways. The C_3-*Oryza*/C_4 (-100%), mesophtytic/xerophytic (-67%) and annual/perennial grasses (-87%) are also correlated. Finally, significant *p*-values is also observed between 20;40 cm-high/ 70;120 cm-high and perennial grasses/helophytic type but the correlation score is low (respectively −48% and 53%) (SOM, Table S2).

3.4. Analytical approach

Measurement data were processed using the R statistics software (version 3.3.1). A series of analyses including a Principal Component Analysis, so-called PCA (package Ade4), box-plots, Wilcoxon–Mann–Whitney tests, and decision trees (packages Rpart, and Rpart.plot) was performed. In each analysis, data were independently grouped following taxonomy (sub-family level), photosynthetic pathway (C_3-*Oryza* and C_4), life cycle (annual or perennial), water-requirement (helophytic, mesophytic, or xerophytic), and height (20;40 cm-high, 70;120 cm-high, or 200;225 cm-high) (Table 1). These three height classes were defined after appreciating the range of the height values recorded from the literature for the 15 grass species selected for this study (SOM, Fig. S1). The PCA was performed to appreciate which size parameters had the most weight in the distribution of the data (correlation circle) and whether this distribution reflected any trend related to the different grass traits considered for this study. The analysis was carried out on standardardized (centered and scaled) data in order to

gauge dispersion from original variables expressed with different units. Box-plots of certain size parameters (pointed out by the PCA) were also represented. Differences between groups were tested using Wilcoxon–Mann–Whitney non-parametrical tests (at the level 0.01) applied to the original dataset (non-standardized). Non-parametrical tests were preferred since the distribution/variance of the original data does not display normality/are not homogeneous. Used as a bivariate test, Wilcoxon–Mann–Whitney allows testing whether bilobate dimensions vary significantly between two groups according to one parameter, while used as an univariate test it allows testing whether bilobate dimensions are significantly higher (or lower) in a group than in another according to one parameter. Finally, optimal decision trees were performed to characterize the threshold measurement values, below or above which, a bilobate can be associated with a certain grass subfamily, photosynthesis pathway, life cycle, water-requirement, and height category. A correlation matrix of the different grass attributes was simultaneously performed to discuss the results obtained. It is noteworthy that the species *Oryza barthii* was not included in the analyses dealing with life cycle for the reason that the life cycle of this species is triggered by its geographical setting and so varies between annual and perennial (Schmidt et al., 2011; Clayton et al, 2017; Poilecot, 1999).

4. Results

4.1. Dataset distribution

The PCA indicates that 83.7% of the variance included in the dataset is explained by the first two axes, and that these axes relate to the bilobate length and width size parameters, respectively (Fig. 2). PCA-axis 1 (which explains 56.6% of the total variance) distinguishes the bilobate individuals according to the length of their base, shank, and to the average length of their two opposite lobes, which all increase from the negative end to the positive end of axis 1 (Fig. 2a). These three variables, which are all related to the length, are correlated to each other (values: 0.86–0.89, SOM Table S3a). PCA-axis 2 (27.1% of the total variance) is mainly defined by the width of the shank and the average width of the two opposite lobes, which both increase from the negative end to the positive end of the axis 2 (Fig. 2). These two variables are independent from each other (value: 0.35, SOM Table S3a).

According to the PCA and box-plot graphs, there is a trend towards longer bilobates from Oryzoideae and Panicoideae grass species to Aristidoideae and Chloridoideae grass species (Figs. 2b and 3a). Alternatively, there is also a trend towards longer bilobates between humidity-loving grass species and xerophytic grass species (Figs. 2c and 3b). Finally, perennial grass species, C_3-*Oryza* grass species, and 200;225 cm-high grass species seem to produce shorter bilobates than the other grass species categories (Figs. 2d–f and 3c–e). These observed trends are being statistically tested in Section 4.2.

4.2. Is bilobate size displaying significant differences according to grass trait variables?

4.2.1. Subfamily

Mann–Whitney tests indicate that Oryzoideae bilobates are statistically shorter than Panicoideae, Aristidoideae, and Chloridoideae bilobates (SOM Table S4a). Indeed, pairwise comparisons between the length of their bases, shanks, and between the average length of their two opposite lobes are all significant at the 0.01 level. Aristidoideae bilobates are not significantly different from Chloridoideae bilobates for none of the length parameters measured (*p*-values >> 0.01) but still do appear significantly longer than Panicoideae bilobates (*p*-values < 0.01). Regarding shank width, Panicoideae and Chloridoideae bilobates on the one hand, and Aristidoideae and Oryzoideae bilobates on the second hand, are not statistically different from each other
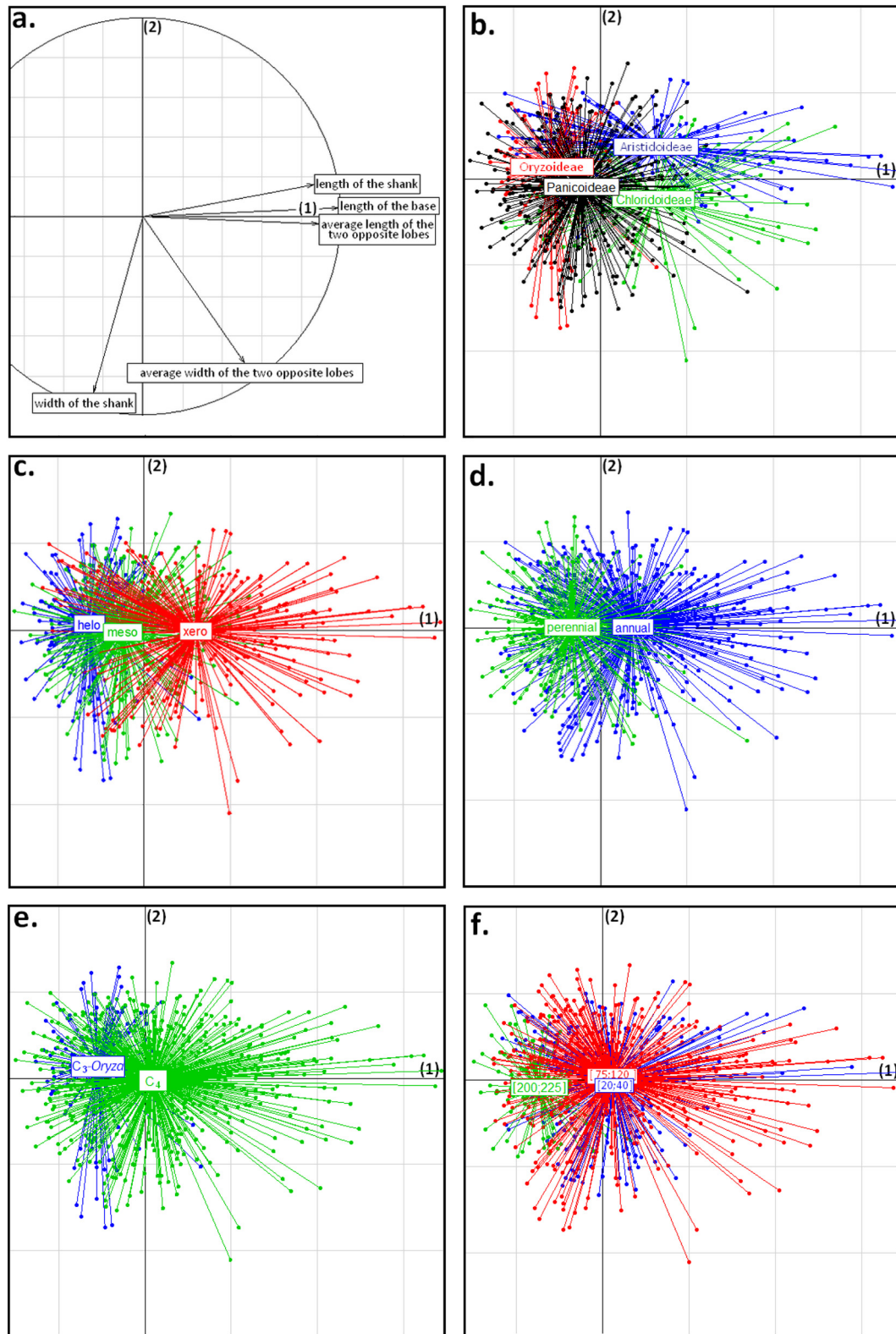
Fig. 2. a. Variable distribution according to the Principal Component Analysis. Each variable corresponds to a size parameter measured on bilobates (five in total). Axis 1 represents 56.6% of the total variance and axis 2 27.1% and scatter plots of bilobate individuals on axes 1 and 2 of the PCA with bilobate individuals are colored according to: b. Grass taxonomy (Aristidoideae, Chloridoideae, Oryzoideae or Panicoideae); c. Grass water-requirement (helophytic, mesophytic or xerophytic); d. Grass life cycle (annual or perennial); e. Grass photosynthetic pathway (C_3-*Oryza* or C_4); f. height of grass species (in cm). Segments connect each individual coordinate to a centroide estimated using s.class of R package Ade4.

(p-values: 0.06 and 0.91, respectively). Besides, both Aristidoideae and Oryzoideae bilobates possess thinner shank than Panicoideae and Chloridoideae bilobates and these differences are significant (p-values $\ll$ 0.01). Finally, the average width of the two opposite lobes measured

in Oryzoideae bilobates is not significantly different from the one measured in Aristidoideae (p-value: 0.76) whereas in Chloridoideae, this size parameter is statistically higher than in all the other subfamilies (p-values $\ll$ 0.01). Threshold values of 25 μm for the base length and
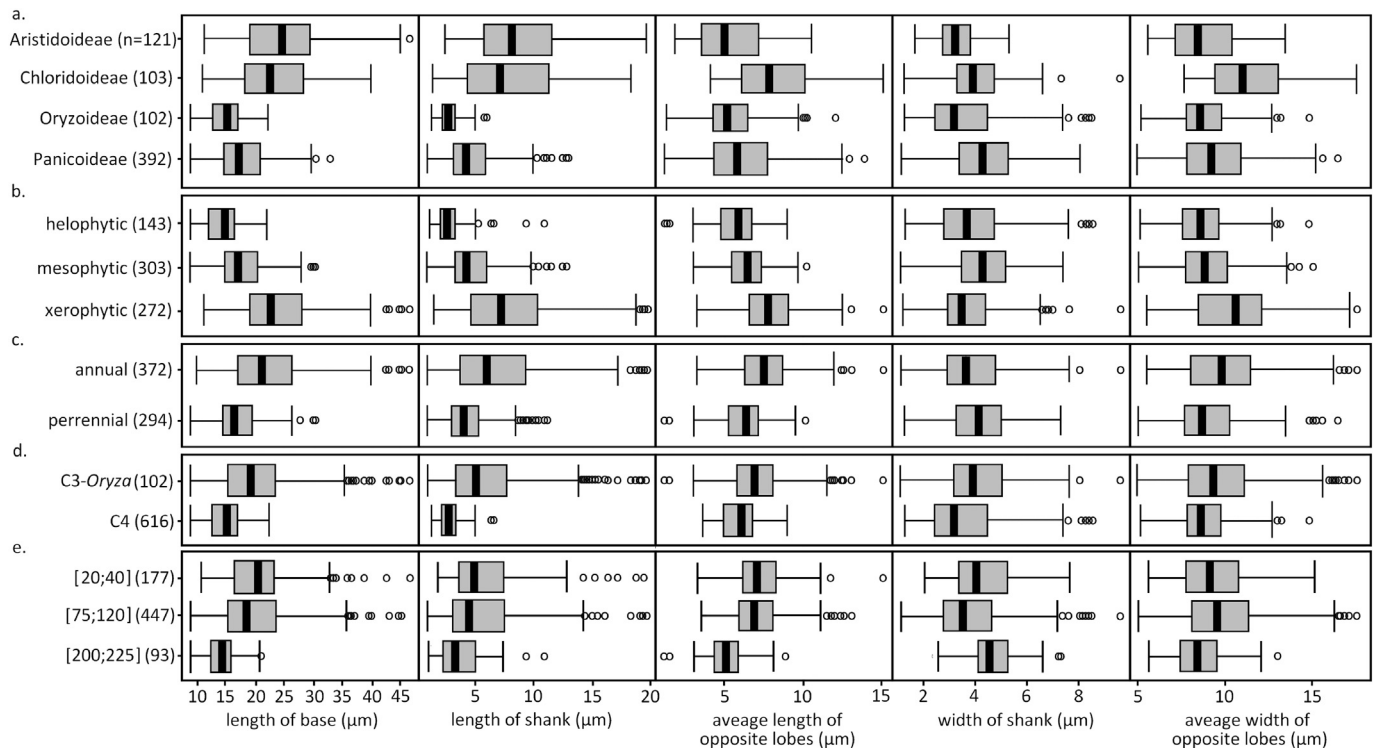
Fig. 3. Box-plots (min, first quartile, median, third quartile, and max values, plus outliers) on each of the five bilobate size parameters grouped according to: Grass taxonomy (Aristidoideae, Chloridoideae, Oryzoideae or Panicoideae); b. Grass water-requirement (helophytic, mesophytic or xerophytic); c. Grass life cycle (annual or perennial); d. Grass photosynthetic pathway (C_3-Oryza or C_4); e. height of grass species (in cm). Values in parentheses represent the number of bilobates included in each group.

of 12 μm for the average width of the two opposite lobes allow discriminating the Aristidoideae and Chloridoideae bilobates from the other grass bilobates with 65% and 83% of accuracy, respectively (Fig. 4a). It means that a bilobate base length ≥25 μm has higher probability to be observed in both Aristidoideae and Chloridoideae than in other subfamilies. Among the ≥25 μm-long bilobates, an average width of the two opposite lobes ≥12 μm is more frequently observed in Chloridoideae than in Aristidoideae. In comparison, both Panicoideae and Oryzoideae have a higher probability to produce bilobates with a base length <25 μm. Also, threshold values of 3.4 and 3.3 μm for the length and width of bilobate shank, respectively, allow discriminating Panicoideae from Oryzoideae with 70% accuracy.

4.2.2. Water-requirement

Our analyses indicate a positive gradient in the length of bilobates from moisture-adapted grass species to dry-adapted grass species (Figs 2c, 3b). Besides, it turns out that the bilobates produced by xerophytic species possess bases, shanks, and lobes that are on average significantly longer than those of bilobates produced by mesophytic and helophytic species (p-value < 0.01) (SOM Table S4b). Besides, a similar significant trend is observed from helophytic to mesophytic species, the latter producing longer bilobates than helophytic grasses. Regarding the average width of the two opposite lobes, it is significantly larger for the bilobates produced by xerophytic grass species than for those produced by mesophytic and helophytic grass species (p-values < 0.01). Finally, the width of bilobate shanks is statistically larger in xerophytic species than in mesophytic and helophytic species (p-value < 0.01). Decision trees indicate that a bilobate base length ≥27 μm allow discriminating part of the bilobates produced by xerophytic species from those produced by moisture-adapted species (94% of accuracy), while a bilobate base length ranging in between 20 and 27 μm combined with an average width of the two opposite lobes ≥10 μm allow discriminating the rest of the bilobates produced by xerophytic species (74% of accuracy) (Fig. 4b). Best chances to discriminate bilobates produced by helophytic grasses from bilobates produced by other grasses

would be obtained by combining the three following size parameter threshold values: a bilobate base length <20 μm, a shank length <3.4 μm, and a shank width <4 μm. In this study, this combination discriminates the two groups with 76% of accuracy. Finally, bilobates produced by mesophytic grasses get discriminated in this study with accuracies ranging from 50 to 70% after combining four size parameter threshold values: a length base <20 μm and length shank <3.4 μm, completed by an average width of the two opposite lobes <11 μm or a shank width ≥4 μm.

4.2.3. Life cycle

Our analyses indicate that bilobates produced by annual grasses have statistically longer bases and shanks, as well as larger lobes than those produced by perennial grasses (p-values << 0.01) (Extended Data Table Sc). Conversely, bilobate shanks for perennial grasses appear significantly larger than for annual grasses. According to decision trees (Fig. 4c), base lengths ≥21 μm are mostly recorded for annual grasses and allow discriminating the bilobates produced by this group of grasses with 84% of accuracy. Conversely, bilobates possessing a base length <21 μm and an average width of the two opposite lobes ≥11 μm are mostly recorded in annual grasses. These size parameter features allow discriminating annual grass bilobates from perennial grass bilobates with 67% accuracy.

4.2.4. Photosynthetic pathway

Bilobates produced by C_3-Oryza grasses possess larger shanks and lobes, as well as longer bases, shanks, and lobes than the bilobates produced by C_4 grasses (p-values << 0.01) (SOM Table Sd). According to decision trees, threshold values of 3.4 and 3.3 μm for the length and width of shanks, respectively, are enough to discriminate C_3-Oryza grasses from C_4 grasses with 80–84% accuracy (Fig. 4d).

4.2.5. Grass height

Bilobates produced by 200;225 cm-high grasses possess shorter bases, shanks, and lobes, as well as thinner lobes than the bilobates
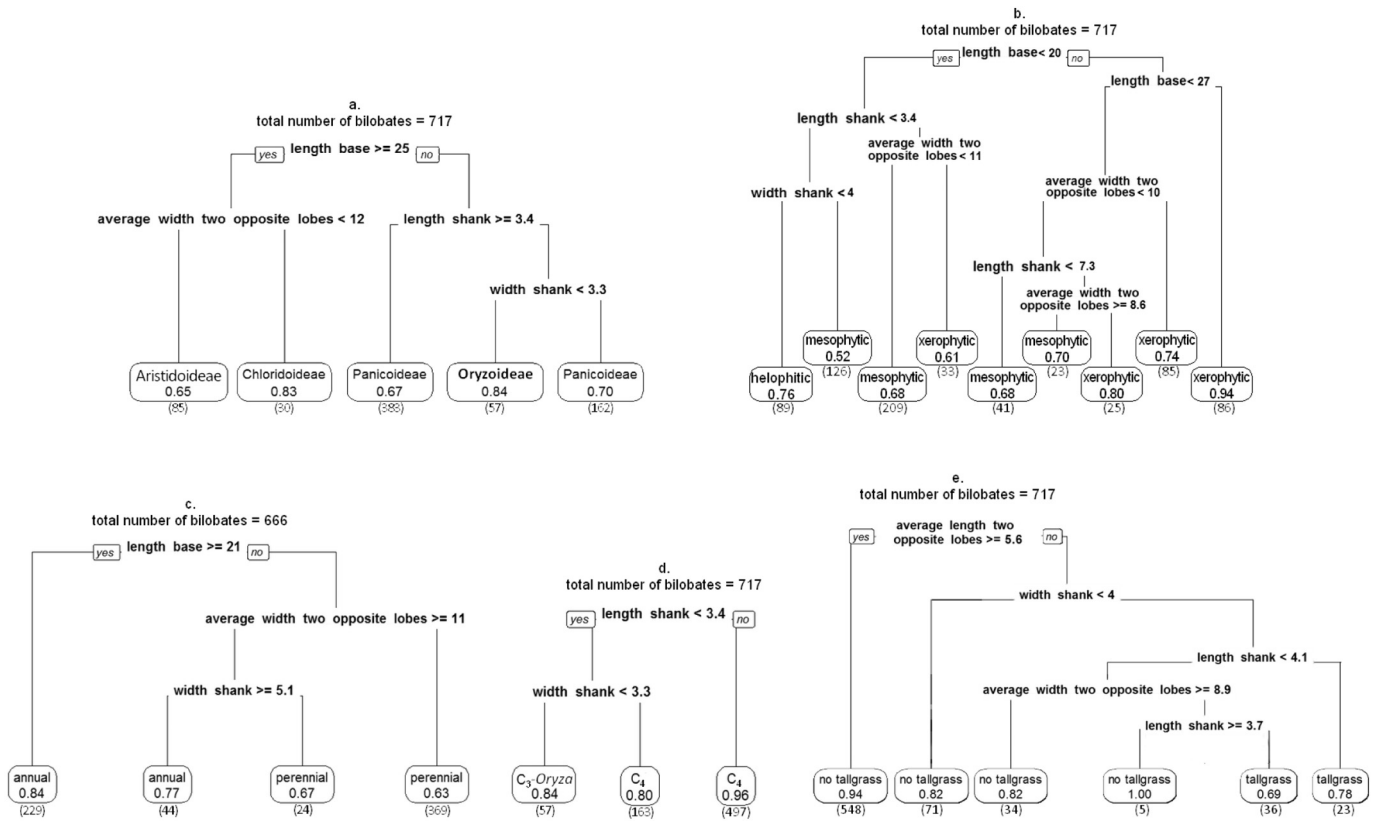
Fig. 4. Optimal decision trees compiling the five bilobate size parameters to document: Grass taxonomy (Aristidoideae, Chloridoideae, Oryzoideae or Panicoideae); b. Grass water-requirement (helophytic, mesophytic or xerophytic); c. Grass life cycle (annual or perennial); d. Grass photosynthetic pathway (C_3-*Oryza* or C_4); e. height of grass species (in cm). Probabilities of assignment to a group are represented by values ranging from 0 to 1. Numbers in parentheses represent the number of bilobates supporting each tree partitioning.

produced by 20;40 and 75;120 cm-high grasses, and these differences are significant at the 0.01 level (SOM Table S4e). Conversely, there are no significant differences in the size of bilobates produced by 20;40 cm-high and 75;120 cm-high grasses. Shanks are conversely significantly larger for 20;40 cm-high grasses than for 75;120 cm-high grasses, but thinner for 75;120 cm-high grasses than for 200;225 cm-high grasses (p-values $\ll$ 0.01). 20;40 and 75;120 cm-high grasses, which eventually differ from one another for only one single bilobate size parameter (shank width), were on purpose merged into one single category, the so-called "no tall" grass category, in direct opposition to the "tall" grass category including the 200;225 cm-high grasses, in order to simplify and improve the decision tree analysis. According to the analysis, an average bilobate lobe length ≥5.5 µm is mainly observed for the "no tall" species and allows discriminating them with 94% accuracy (Fig. 4e).

5. Discussion

5.1. A small dataset... but yet indicating encouraging preliminary results

Our dataset includes 15 grass species, which are well represented in grass-dominated vegetation of the sub-Saharan regions. Based on Novello et al.'s grass phytolith reference collection, this dataset represents 27% of the species in which bilobates where found dominant (>33% of the GSSCP assemblages) compared to other main GSSCP categories (rondels, crosses, polylobates, saddles). Whether this dataset remains limited by not including all the main bilobate grass producers, it nevertheless appears well-balanced for the population used after we tested its robustness through the E value calculation. Indeed, dependence between variables was observed only a few times and they remain totally unbreakable from the original ecology and physiology of the taxa involved: Oryzoideae species are all C_3 and water-dependent,

while the genus *Aristida* is dry-adapted. Still based on Novello et al.'s dataset, the Panicoideae sub-family is the less well represented by including only 18% of genera with bilobate-dominated assemblages. This low representativeness can be first thought to explain why the Panicoideae sub-family as a whole is more difficult to discriminate than the other subfamilies. Alternatively, it can simply be that because the Panicoideae subfamily is much more diverse than the other subfamilies, this cannot lead to a well-defined bilobate size signal. Working at a smaller taxonomical level (tribe, genus?) could therefore be a potential key to obtain a better discrimination in the case of an extended dataset.

5.2. Bilobate size matters for documenting grass taxonomy

Our study indicates that the base length in bilobates has the potential to be used for identifying grass subfamilies in the context of paleontological/archaeological surveys. In our dataset, both Chloridoideae and Aristidoideae produce on average longer bilobates than the other grass subfamilies do. The value of 25 µm for bilobate length is pointed out as a significant threshold to be used for segregating Chloridoideae and Aristidoideae from other subfamilies in past phytolith assemblages.

Likewise, Lu and Liu (2003) observe that Chloridoideae (four species) and Aristidoideae (one species) grasses produce longest bilobates than the other subfamilies (79 species). This includes *Aristida desmantha* (Aristidoideae), *Ctenium aromaticum, Eragrostis ferruginea, E. japonica,* and *Leptochloa chinensis* (Chloridoideae), which happen to mostly occur in warm and arid regions (Lu and Liu, 2003).

The threshold value of 25 µm for bilobate length was previously and arbitrary used in Novello et al. (2012)'s GSSCs classification after their analysis of the bilobate content of different sub-Saharan grass subfamilies. In fact, 25 µm is very close to the 26 µm threshold value first defined by Fahmy (2008) in his study about Paniceae grasses. The 26 µm threshold value for bilobate length got eventually used by Neumann

et al. (2017) to describe bilobate phytoliths in West African grasses, as an expansion of Fahmy's work. The Aristidoideae species studied by Neumann et al. (2017) consist of six species all related to the *Aristida* genus. Besides, 27 to 70% of the bilobates produced by these species are >26 μm-long. These authors also observed >26 μm-long bilobates in 16 non-Aristidoideae grass species (out of 46 grass species they studied and which produce bilobates); for more than half of these species (9/16), >26 μm-long bilobates represent less than 15% of their GSSCP assemblages; while in *Sporobolus festivus*, (Chloridoideae), *Pennisetum pedicellatum, Andropogon fastigiatus, and Echinochloa pyramidalis* (Panicoideae), >26 μm-long bilobates account for more than 19%, and up to 40–54% in *Loudetia togoensis, L. simplex* (Panicoideae), and *Pennisetum ramosum* (Panicoideae). Yet, long bilobates with convex ends (very abundant in the *Aristida* genus), or alternatively made of one convex end/one flattened end, and a in both cases with a very long shank, seem to be only produced by Aristidoideae. The ones with flattened ends and very long shank are conversely more widely produced across the grass family. Both shank and total bilobate length, in addition to lobe shape description seem significant to combine to identify Aristidoideae grasses from a past GSSCP assemblage.

The bilobate size pattern drawn in this study for the Panicoideae by the decision tree analysis is applicable to most of the Paniceae species studied by Fahmy (2008). This author reported that out of the 66 Paniceae grasses in his dataset, 52 exclusively produce <25 μm-long bilobates, while six dominantly produce <25 μm-long bilobates. The lack of <25 μm-long bilobates is only observed for eight species: *Cenchrus biflorus, C. ciliaris, Digitaria horizontalis, D. lecardii, D. ternata, Pennisetum purpureum, Setaria longiseta* and *S. pallida-fusca*. As a comparison, >25 μm-long bilobates account for significant amounts (43–50%) in the *Cenchrus biflorus* and *Setaria longiseta* specimens studied by Novello et al. (2012), but are not found exclusive. Only three species in Fahmy's dataset: *Beckeropsis laxior, B. uniseta* and *Paspalum orbiculare* produce bilobate whom shanks possess length and width <3.4 and <3.3, respectively, and are among the few not following the result of our decision tree analysis.

Regarding the Chloridoideae family in Neumann's dataset, >26 μm-long bilobates are only observed in the genus *Sporobolus*, and especially in *S. helvolus* (19%). In our dataset, they were also found abundant in *S. helvolus*, but also in *Ctenium elegans*, which does not appear in Neumann's dataset. Yet, the trend we observe for bilobate length in Chloridoideae seems to apply only for a few genera among the subfamily. This could be obviously tested through a broader set of species of *Sporobolus* and *Ctenium* African species, as well as in other Chloridoideae species producing bilobates like the species *Enneapogon desvauxii* (Novello and Barboni, 2015).

At the difference with previous studies, we pointed out in our study another size criteria than the length: the average bilobate lobe width, which when ≥12 μm, could be potentially used to discriminate the very long Chloridoideae bilobates from the very long Aristidoideae ones.

Most of the Oryzoideae bilobates we photographed are defined by a base length <25 μm, as well as by short (<3.4 μm) and thin (<3.3 μm) shanks. Lu and Liu (2003) observed that about 70% of the bilobates produce by Oryzoideae (*Oryza sativa, Leersia hexandra, Zizaniopsis miliacea* and *Z. caduciflora*) possess a shank that appear much shorter than the 1/3 of their lobe length, which again suggests relatively short shank among the bilobates produced by this subfamily. However, this bilobate type is also produced in significant abundance by *Panicum amarum*, a typical helophytic species among the Panicoideae (Lu and Liu, 2003), As another comparison, the three *Oryza* species (Oryzoideae) studied by Neumann et al. (2017) only produce <26 μm-long bilobates, among which 65–88% possess a <4 μm shank or an absence of shank (defined as "constriction between the lobes"). The combination of <26 μm total length and <4 μm shank length for bilobates appears nonetheless totally common among the Panicoideae subfamily (Neumann et al., 2017) since it is observed for every single species, and represents between 6 and 94% of the bilobates produced. The Panicoideae subfamily, in fact,

was the most difficult to pull apart in our tree analysis. The size criteria used by Neumann et al. (<26 and <4 μm) are slightly broader than the ones pointed out in our tree analyses (<25 and 3.4 μm), in addition to not take into account shank width, which yet appears to play a role in discriminating Oryzoideae from Panicoideae in our analyses.

5.3. Bilobate size matters for documenting grass ecology

Our analyses indicate that xerophytic grass species produce on average longer and larger bilobates than humidity-loving species, the shank width being the only exception. Lu and Liu (2003) discussed on a similar relationship between bilobate length and grass water-requirement preference after looking at bilobate phytoliths in 250 grasses from tropical China and the USA. They observed that moisture-adapted species tend to yield lobate silica bodies with much shorter shanks than dry-adapted species. Barboni and Bremond (2009) had also hypothesized on a similar relationship based on the observation of grass epidermis photographs from 184 East African species. According to Neumann et al. (2017), any correlation observed between GSSCP morphotypes and grass water-requirement is at the first place linked to grass taxonomy, which seems to be confirmed in this study directly at the genus level, and not necessarily at the subfamily level. The genera *Aristida* (Aristidoideae) and *Ctenium* (Chloridoideae), for instance, are primarily found in dry areas (Poilecot, 1999; Watson and Dallwitz, 1992). They specifically produce bilobates that are significantly much longer than the helophytic (Poilecot, 1999) *Oryza* species (Oryzoideae) (this study).

A number of arguments, however, would suggest that bilobate size could also be triggered by water availability rather than being only related to grass taxonomy. Indeed, different arrangements and shapes of the grass leaf epidermis cells (phenotypic plasticity), and so phytoliths, have been observed in a couple of species under variable water stress and growing conditions (Honaine et al., 2017; Kumar et al., 2017; Markovich et al., 2015; Naskar et al., 2018; Rudall et al., 2014). In *Cortaderia selloana* (Danthonioideae) from the Pampean region (Argentina), the distribution of the silicified leaf epidermal cells looks different in specimens showing xerophytic-adapted modified leaves (Honaine et al., 2017). Naskar et al. (2018) saw a similar trend in the arrangement of phytoliths/leaf epidermal cells in Panicoideae species from India as a response to salinity increasing. Moreover, the Chinese Bambusoideae species *Dendrocalamus ronganensis* produces different phytolith assemblages under different environmental conditions (Li et al., 2017).

Decision tree analyses indicate that an average lobe length ≥5.5 μm is the best to discriminate "no tall" (<120 cm-high) grasses from "tall" grasses (>120 cm-high). In Sub-Saharan Africa, the ratio of "tall" versus "no tall" grasses is influenced by the water availability, the temperature, and the length of the growing season (Schmidt et al., 2011). Low rainfall pattern is correlated with "no tall" grasses for this area (Pasturel et al., 2016). An indirect relationship between bilobate length and grass water-requirement might therefore exist.

5.4. Implications for life cycle and photosynthetic pathways

Our results indicate that annual grasses produce longer bilobates than perennial grasses, as xerophytic species also do compare to humid-loving species. The annual life cycle in plants is an adaptation to avoid arid conditions under seasonal climates (Le Houérou, 1993). Besides, non-annual (perennial) species are positively correlated to the xerophytic water-requirement in our dataset. The significant variations we observed in bilobate length according to grass life cycle is therefore more likely to reflect grass water-requirement and/or grass preferred climate context.

This study shows that C_3 grasses produce shorter and thinner bilobates than C_4 grasses do, as Neumann et al. (2017) also observed and Barboni and Bremond (2009) had previously suggested. Helophytic species in our study are positively correlated to the C_3 photosynthetic

pathway, while variations in bilobate size in C_3-*Oryza* and C_4 appeared close to humidity-loving and xerophytic species, respectively. Variations in bilobate size between the C_3-*Oryza* and C_4 photosynthetic pathways is therefore more likely an indirect reflection of grasses adaptation to wet conditions (Lu and Liu, 2003; Barboni and Bremond, 2009).

6. Conclusion

In this study, we measured the bilobate silica short cell phytolith produced by 15 sub-Saharan grass species and analyzed these data through statistical methods to see whether size showed a potential for documenting grass taxonomy, water-requirement, life cycle, physiology, and habitat. Our analyses demonstrated the existence of a correlation between bilobate phytolith length (base, lobes, shank), width (base, lobes) and water-requirement. This finding is in line with a couple of previous studies performed in tropical Africa (Barboni and Bremond, 2009; Neumann et al., 2017), as well as in tropical Asia and North America (Lu and Liu, 2003).

Yet, it is still not clear whether this correlation is indirect and so primarily taxonomy-related (Neumann et al., 2017), and/or whether the environment itself plays a significant role in determining the size of the bilobates produced by grasses as suggested by a few studies (Barboni and Bremond, 2009; Lu and Liu, 2003; Novello, 2012; Novello et al., 2015). It is however noteworthy that the Aristidoideae (genus *Aristida*), Chloridoideae (*Ctenium elegans*), and Panicoideae (*Brachiaria xantholeuca* and *Loudetia simplex*) associated with dry areas in our dataset produce bilobates that are significantly much longer than the helophytic Oryzoideae (genus *Oryza*) and Panicoideae (*Andropogon gayanus*). The variations observed in bilobate size according to the grass life cycle and photosynthetic pathway appear not relevant in this study since they more likely reflect differences in grass water-requirement. A larger dataset, especially including other grass subfamilies like the C_3 early grass lineages, Bambusoideae, and Pooideae could improve the discrimination of C_3 grasses.

The measurements performed in this study consist of simple distances between two points, which at the end is still an incomplete estimate of bilobate phytolith variation. A study combining conventional and more complex measurements could permit going further in the taxonomic and ecological identification of grass bilobate phytoliths. By using different measurements like the circumference and area parameters on bilobates, Liu et al. (2016) managed, for instance, to discriminate Panicoideae grass species from Pooideae and Arundinoideae grasses with 73% of accuracy from a set of 12 Chinese Poaceae species. In the same line, morphometric analyses like the ones developed by Ball et al. (2016) and Portillo et al. (2006) on cereals could be also used for the identification of wild grasses. At this stage, catching the more detailed variations in bilobates seem the key to further refine their taxonomical and environmental signal.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.revpalbo.2019.104114.

Acknowledgements

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