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### Morphometrics of modern and fossil Poaceae pollen from South America

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**Publication date**  
2023

[Link to publication](#)

#### **Citation for published version (APA):**

Wei, C. (2023). *Morphometrics of modern and fossil Poaceae pollen from South America*. [Thesis, fully internal, Universiteit van Amsterdam].

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Grasses pollen. Caixia Wei.

## Chapter 2

### **Is Poaceae pollen size a useful proxy in palaeoecological studies?**

– New insights from a Poaceae pollen morphological study in the Amazon

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*Review of Palaeobotany and Palynology*, 308, 104790, with open access

Doi: 10.1016/j.revpalbo.2022.104790

**Abstract**

Poaceae (the grass family) is highly diverse, geographically widespread, and an important component of many terrestrial ecosystems. Poaceae pollen size has previously been suggested as a proxy to reconstruct the past vegetation and climates in the Amazon area, but it is still unclear if this variable can be used at broader spatial and deep-time scales. Here we set out to perform a comprehensive assessment and to test the robustness of this proxy. We generated pollen size data, as well as associated measurements of the pollen wall and pore, using 127 plant specimens from 58 species across the Poaceae phylogeny from the Amazon drainage basin (ADB). We tested the relationship between pollen size and a range of abiotic and biotic variables (vegetation type, soil composition, climate conditions, photosynthetic pathway and genome size), and fitted models using phylogenetic generalized least squares (P-GLS) and linear mixed models (LMM). Our measurement data shows that Poaceae pollen size presents a very wide range (18.77–71.62  $\mu\text{m}$ ), and varies not only among genera and species but also within species. There is no obvious relationship between pollen size and the explanatory variables considered here. We suggest that pollen size cannot be used as generally applicable proxy to reconstruct past vegetation and climate.

**Keywords**

Grasses; Palynology; Pollen morphology; Past vegetation; Climate reconstruction

## 1 Introduction

Poaceae (the grass family) originated in the Cretaceous (c. 100 million years ago; Ma) and diversified in the early Miocene (c. 23–16 Ma) (Linder, 1987; White et al., 2000; Prasad et al., 2005, 2011; Strömberg, 2011; Samant and Mohabey, 2014; Wu et al., 2018). The Poaceae family currently has approximately 11,783 species in over 700 genera (Dahlgren et al., 1984; Soreng et al., 2022), with grassland ecosystems extending across latitudes and in different climates and soil types (Jacobs et al., 1999), ranging from mountain to lowlands, aquatic habitats to deserts, cold to temperate grasslands, and from floodplains to coastlines (Gibson, 2009; Linder et al., 2018). Grasslands cover around 40% of the Earth's surface (Blair et al., 2014) and can thus be regarded as the most common vegetation type with Poaceae the most widespread angiosperm family around the globe (Linder et al., 2018). Nevertheless, substantial knowledge gaps remain about the timing and geographic expansion of grass-dominated habitats.

The Poaceae evolutionary and ecological history can be reconstructed from micro- and macrobotanical fossil material (e.g. Edwards et al., 2010; Prasad et al., 2011; Kirschner and Hoorn, 2020). Poaceae are wind pollinators and thus great pollen producers, which should make them ideal candidates when studying the microbotanical fossil record (Linder, 1987). In general, pollen morphology provides important information on the taxonomic boundaries and affinities between different taxa. However, Poaceae pollen present a challenge as their pollen have a relatively stenopalynous morphology both in modern and fossil species (Page, 1978; Salgado-Labouriau and Rinaldi, 1990; Beug, 2004; Halbritter et al., 2018). This problem is common to almost all Poaceae members, which makes it difficult to use them as palaeoclimate or palaeoecological indicators.

Mature pollen grains of Poaceae range in size from ~20 to >100  $\mu\text{m}$  (Ferguson et al., 1977; Beug, 2004), and there has been considerable interest in using this pollen size variation as a direct indicator of past ecologies and environments. For example,

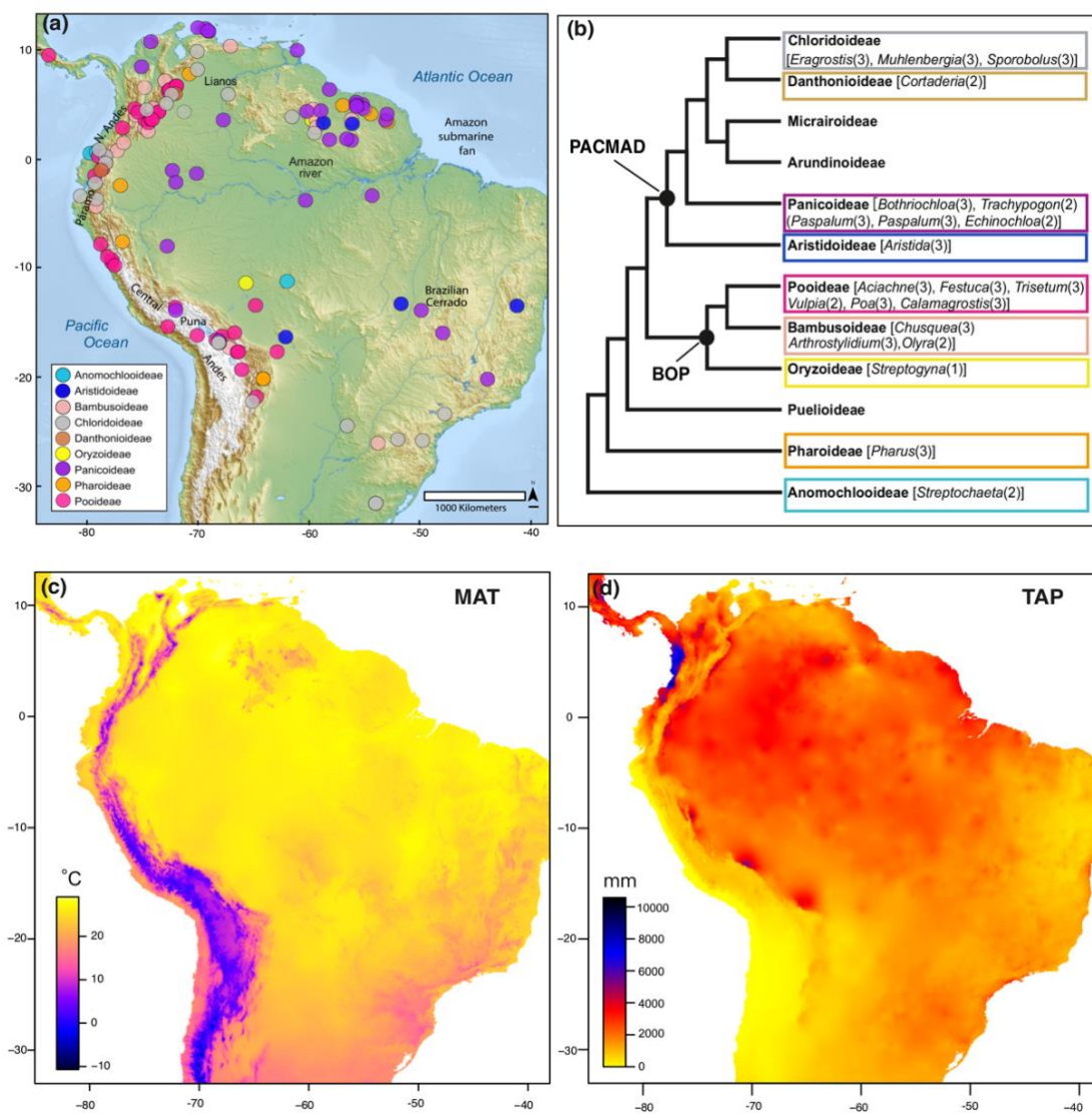
pollen size has long been used to differentiate between domesticated and wild grasses in Holocene pollen records (Salgado-Labouriau and Rinaldi, 1990; Beug, 2004; Holst et al., 2007). Size has also been suggested as a biological parameter to estimate ploidy (Katsiotis et al., 1995; Jan et al., 2015), to determine the mode of reproduction (Kelly et al., 2002), and quantify the level of diplogamete formation in plants (de Storme and Geelen, 2013). Furthermore, Schüler and Behling (2011) proposed that grass pollen size could be used as a tool to distinguish past grasslands in South America. Subsequently, Jan et al. (2015) concluded that pollen grain size is larger in grasses with a C4 photosynthetic pathway when compared to C3 species, and Radaeski et al. (2016, 2020) suggested that arboreal forest species and grassland and herbaceous forest species from southern Brazil could be differentiated based on pollen morphology. In spite of this, pollen size has been deemed controversial as a proxy to reconstruct past vegetation and climates. For instance, Griener and Warny (2015) suggested that *Nothofagus* pollen grain size could be used to reconstruct moisture availability in the fossil record. However, Jardine and Lomax (2017) re-evaluated their analysis and suggested that it is premature to use pollen size as a moisture indicator in the fossil record, especially because the impacts of genome size variations on pollen size are not well understood (Den Nijs et al., 1980; Knight et al., 2010).

Adding to the above, palynological processing methods and storage time are also key factors that can impact pollen size, and add another level of uncertainty when using pollen size as a palaeoecological tool. Treatment with KOH, acetolysis and hydrofluoric acid (HF) (Christensen, 1946; Reitsma, 1969; Dickson, 1988; Faegri et al., 1989; Moore et al., 1991) are thought to cause size changes in pollen, as is storage and mounting in glycerin jelly, which can lead to a general increase in pollen size (Christensen, 1946; Andersen, 1960; Cushing, 1961; Reitsma, 1969; Faegri et al., 1989; Moore et al., 1991; Sluyter, 1997).

## Chapter 2

In this study, we test whether Poaceae pollen size is a robust proxy to be applied over broad spatial and long temporal (i.e. deep-time) scales. To achieve this, we measured 2540 Poaceae pollen grains that were collected from the Amazon drainage basin (ADB) from 127 plant specimens belonging to 58 species from nine subfamilies across the Poaceae phylogeny (Fig. 1a, b). The ADB comprises a wide variety of climates and environments, and a diverse phylogenetic range of grasses, in addition to being a key focus area of (palaeo) biogeographic and macroevolutionary research (Hoorn et al., 2010; Cheng et al., 2013; Kirschner and Hoorn, 2020) and is thus an ideal setting to test the broader utility of Poaceae pollen size as palaeoenvironmental proxy. We test if Poaceae pollen size differs among taxa, and in relation to six abiotic and biotic variables, specifically vegetation type, soil composition, climate (temperature and precipitation), photosynthetic pathway, and genome size. We also assess when and how pollen size changes when stored in glycerin jelly. Our study provides a comprehensive assessment of grass pollen size, integrating multiple abiotic and biotic variables, and suggests that there is no obvious relationship between Poaceae pollen size and any of these variables.

## Chapter 2



**Fig. 1.** Study area and the sample sites. (a) collection localities for the herbarium specimens sampled for this study. Symbol colours denote the Poaceae subfamily, and the base map was download from <https://mapswire.com/>; (b) Poaceae subfamily cladogram, redrawn from Soreng et al. (2015). The genus names and numbers in square brackets are those included in this study, with the numbers denoting the number of species sampled for each genus. (c and d) Current climate data estimates from WorldClim 2 (Fick and Hijmans, 2017); (c) mean annual temperature (°C) and (d) total annual precipitation (mm).

## **2 Materials and methods**

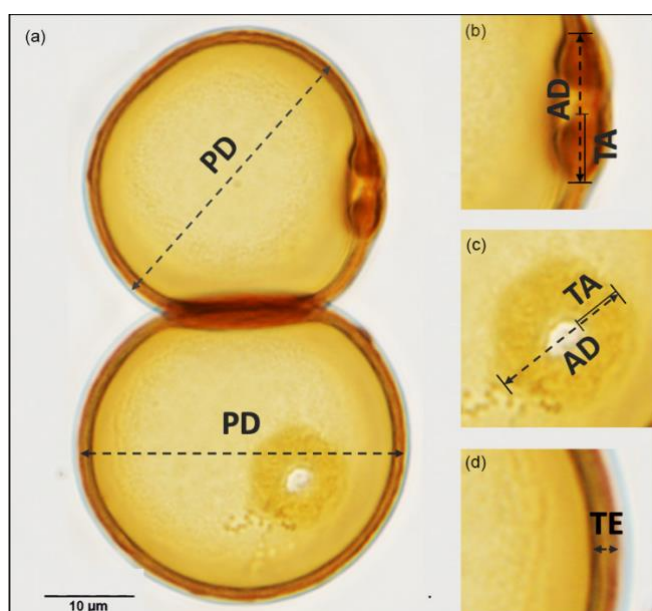
### **2.1 Sample collection and processing**

We sampled pollen grains from 58 species belonging to 9 different Poaceae subfamilies. For each species, a target of three plant specimens was chosen to incorporate the morphological variation within species, however considering the restrictions on the amount of material available, only one or two specimens were available for some species. Anthers were harvested from 127 plant specimens obtained from the National Herbarium of the Netherlands (Naturalis) (L), that were all collected between 1808 and 2012 in South America (see Fig. 1a, b). Some of these samples were previously collected and processed for the University of Amsterdam modern pollen reference collection; further sampling was carried out for this study to provide a broad phylogenetic, geographic and climatic range within the dataset. For each specimen, we provide the country of origin, the year of collection, collector information, location (latitude and longitude), elevation, photosynthetic pathway, climate data, vegetation type, soil type, genome size, and the storage time in glycerin jelly prior to measurement (see Appendix S1 in Supporting Information).

The pollen grains were collected from anthers of herbarium specimens. These subsamples were processed to clean the exine and remove the cytoplasmic content by using standard acetolysis (9 parts acetic anhydride to 1 part concentrated sulfuric acid) with samples heated to 100 °C for 5 min (Erdtman, 1986; Faegri et al., 1989). Residues were then transferred to glycerin jelly and placed in an oven until the remaining water had evaporated. Acetolyzed pollen material was mounted in glycerin jelly on slides and the coverslip sealed with paraffin. This process was performed at the Institute for Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, Netherlands.

## 2.2 Palynological analysis and microphotography

The pollen grains were observed using a Leica microscope and a 5.1 megapixel USB camera (AmScope) under 400 × magnification (× 40 objective, and × 10 eyepieces), the position of the grass pollen grains was recorded with an England Finder (EF). For each sample, we measured pollen diameter (PD), thickness of the exine (TE), annulus diameter (AD), and thickness of annulus (TA) in 20 randomly selected pollen grains using the software ImageJ (National Institute of Health, USA) (Fig. 2), resulting in a total of 2540 pollen grains measured.



**Fig. 2.** Four measured parameters of Poaceae pollen grain were considered in the study. (a) pollen diameter (PD) of two pollen grains with different position of annulus; (b) thickness of exine (TE) and (c) annulus diameter (AD); and (d) thickness of the annulus (TA).

Microphotography of Poaceae was carried out using Nomarski Differential Interference Contrast (DIC) following Bercovici et al. (2009). While making the photos, the varying z-axis was recorded then images were aggregated through manual z-stacking in software Helicon Focus 6.0 (Helicon Soft Ltd., Kharkiv, Ukraine) and Adobe Photoshop 2021 (Adobe Systems Inc., San Jose, CA, USA). This stacking technique aggregates different layers to provide more detailed images. The plates of

grass pollen grains were made with CorelDRAW 2020 (Corel Corporation, Ottawa, Canada).

## **2.3 Selected variables and data collection**

### **2.3.1 Vegetation type**

Poaceae occur in a wide range of ecosystems in South America, ranging from mountain to lowlands, cold to temperate grasslands, floodplains and coastline (Gibson, 2009; Linder et al., 2018). We assigned each sampling locality to one of the following five vegetation types, following Burkart (1975) and Kirschner and Hoorn (2020): desert, montane grassland shrubland, savanna, tropical dry forest, and tropical moist forest. The vegetation type corresponding to each sampled plant specimen is listed in Appendix S1.

### **2.3.2 Soil type**

The geographic distributions of soils encompass a considerable diversity across the ADB, and they are usually associated with large-scale geomorphologic features (Hoorn et al., 2010; Quesada et al., 2011). We used the Soil and Terrain Database (SOTER) for Latin America and the Caribbean (SOTERLAC), version 2.0, at a scale of 1:5000000 (Dijkshoorn et al., 2005) to define soil categories for each site, using the WRB (World Reference Base) Soil Groups to assign each sampling locality to one of 19 soil types. The soil type corresponding to each sampled plant specimen is provided in Appendix S1.

### **2.3.3 Modern climate data**

A climatic gradient spans the ADB ranging from the continuously rainy northwest to the wet/dry climate and long dry season of the southern and eastern regions, including the Cerrado (woodland/savannah) in the southeast (Davidson et al., 2012). We used the WorldClim 2.1 database (Fick and Hijmans, 2017) and Climate Research Unit (CRU) TS4.04 database (Harris et al., 2020) to extract the mean annual

temperature (MAT) and total annual precipitation (TAP) from the latitude and longitude of each sample site. The WorldClim database gives monthly climate values as averages for the years 1970 to 2000; the information was extracted using the 2.5-min dataset. The CRU data gives monthly values for each year from 1901 to 2019 at 0.5 degrees spatial resolution; for each sample the information was extracted for the specific year of collection. No data were therefore available for unknown sampling years or sampling years before 1901. The climate estimates from both datasets were strongly positively correlated, and we therefore used the CRU data in further analyses since these are year specific estimates for the sampling localities. All data are listed in Appendix S1 of the Supporting Information.

### **2.3.4 C3/C4 photosynthetic pathway**

Today's C4 grasses are mostly confined to low latitudes and elevation, whereas C3 species dominate at high latitudes and elevation (Boom et al., 2002; Edwards et al., 2010). Photosynthetic pathways assigned to each species came from Klink and Joly (1989), Giraldo- Canas (2010), Bremond et al. (2012) and references therein. Of the 58 species in our dataset, 23 are C4 and 35 are C3 (see summary in Appendix S1).

### **2.3.5 Genome size**

Genome sizes are highly variable across the grasses (Feuillet and Keller, 2002). In our study, we took genome size information from Kew Garden's C-values Database (Leitch et al., 2019). However, only six of our taxa were present in the C-values Database, which limits the scope of analyses with this variable. The genome size data for these six taxa are listed in Appendix S1.

## **2.4 Data analysis**

### **2.4.1 Preliminary data exploration**

We used pairs plots for basic data visualization and exploration, which allows variable distributions, and their pairwise bivariate relationships, to be visually and quantitatively (via the Pearson correlation coefficient) displayed on the same plot. The pairs plots were used for determining the strength of the relationship among the four pollen grain parameters (pollen diameter, the thickness of exine, annulus diameter, and the thickness of the annulus), and for preliminary exploration of pollen size and the continuous explanatory variables (latitude, longitude, elevation, MAT, TAP, genome size, and sample storage time in glycerin jelly).

### **2.3.4 Piecewise regression analysis in pollen size and sample storage time**

Storing samples in glycerin jelly is known to cause swelling in pollen grains (Christensen, 1946; Andersen, 1960; Cushing, 1961; Reitsma, 1969; Faegri et al., 1989; Moore et al., 1991; Sluyter, 1997). We therefore compared the measured pollen size with the sample storage time in years between sample preparation and pollen size measurement, including 0, 1, 3, 6, and 7 years. This analysis revealed a nonlinear increase in pollen size with sample storage time, with a faster rate of increase initially and then a lack of change in size after 3 years (see Section 3.2 for more information). We therefore used piecewise regression to account for the nonlinear relationship between the response and explanatory variables, using one break-point to divide the relationship into two segments, each with its own slope (McZgee and Carleton, 1970). We carried out the piecewise regression using the segmented function in the segmented package (Muggeo, 2008) for R, which finds the position of the break-point by minimizing the negative log-likelihood function. The residuals from the fitted piecewise regression were then used as the response variable during model fitting and selection (see below), which is referred to as

'corrected pollen size' with the influence of sample storage time removed (McZgee and Carleton, 1970).

### **2.3.5 Trait evolution on phylogenies**

From a total of 58 taxa in our dataset, 32 are present in the most recent grass molecular phylogeny (Spriggs et al., 2014). These 32 taxa are representative of most of the variation in pollen size, climate, photosynthetic pathway, latitude, longitude, and elevation contained in our full dataset (see Fig. S3.1. in Appendix S3), hence for phylogenetically informed analyses we limited our dataset to these 32 taxa. Spriggs et al. (2014) considered two hypotheses for dating the origin of Poaceae, one with a Paleocene origin of grasses based on macrofossil evidence, and one dated with a Cretaceous origin based on phytolith evidence. Since a Cretaceous origin is consistent with the fossil record as it is currently understood (Edwards et al., 2010; Prasad et al., 2011), we used this phylogeny for our analyses, although preliminary analysis suggested that using the alternative phylogeny did not alter our results.

Mapping sample traits directly onto the molecular phylogeny using ancestral character estimation (Revell, 2013) can be used to show trait evolution along the phylogeny. We carried this out for pollen diameter, photosynthetic pathway, MAT and TAP, because these were the variables selected for model fitting (see below).

For the continuous characters (pollen diameter, MAT and TAP), ancestral character estimation was carried out via maximum likelihood estimation assuming a Brownian motion model of trait evolution, using the species mean values in each case. For photosynthetic pathway, which is a binary discrete character (C3 versus C4), we used stochastic character mapping (Huelsenbeck et al., 2003), assuming an equal rates model of trait evolution, and estimating the posterior probability of the character state across the tree being C4 from a sample of 100 stochastic maps (Revell, 2013). We also measured Blomberg's K in order to estimate a phylogenetic signal for the three continuous characters. A value of Blomberg's K close to zero indicates

phylogenetic independence, whereas values close to one indicate that the observed variation in the trait data is predicted by the structure of the phylogenetic tree (Blomberg et al., 2003).

Both vegetation type and soil type are unbalanced in terms of the distribution of samples across the factor levels; vegetation type also correlates with climate (see Fig. S3.2. in Appendix S3). We therefore focused on photosynthetic pathway, MAT and TAP as explanatory variables for model fitting, using a multiple regression framework. We constructed a candidate set of models comprising all combinations of the three explanatory variables with additive effects only, resulting in seven main models. We also included a null model in the candidate set which contains only an intercept, so that the main models being tested could be directly compared to a model without any explanatory variables.

### **2.3.6 Model construction and validation**

Ordinary least squares (OLS) regression assumes that the residuals are independent and identically distributed (Zuur et al., 2009). Our dataset violates the independence assumption in two keyways. First, the sampling scheme is nested, with pollen grain measurements nested within plants, and plants nested within species. Second, the species are not independent because they are descended from a common ancestor, and a level of phylogenetic autocorrelation in the residuals is therefore expected. We therefore fitted models in two different ways to account for these structures in the data. To account for nestedness we used linear mixed models (LMM) with plant specimens nested within species as a two-level random effect (Laird and Ware, 1982; Zuur et al., 2009). Since graphical model validation (see below) of the analysis of corrected pollen size revealed some skewness in the residuals, we also performed the LMM model fitting on the corrected log pollen size (calculated by taking the natural logarithm of pollen size and taking the residuals of a piecewise regression of pollen size on sample storage duration, as described above). To account for phylogenetic non-independence, we fitted the models in a phylogenetic generalized

least squares (P-GLS) framework (Grafen, 1989), using the mean corrected pollen size for the 32 species present in the molecular phylogeny as the response variable. For each round of model fitting, we also explored variance structures to account for heterogeneity in residual variance (i.e. increases or decreases in variance along a continuous explanatory variable, or among factor levels of a categorical variable) (Zuur et al., 2009).

Models were ranked using the Akaike information criterion, corrected for small sample sizes (AICc) (Anderson, 2008), where the lowest AICc value indicates the greatest support for a model, relative to other models in the same candidate set. The  $R^2$  value (the coefficient of determination, defined as the proportion of variance in the response variable explained by the explanatory variables) was used to assess the explanatory power of the models. While calculating  $R^2$  values has previously been challenging outside of an OLS setting, recently  $R^2$  metrics have been developed for other classes of models as well. For the LMM models, we used the  $R^2$  formulation of Nakagawa et al. (2017), which provides an  $R^2$  for the fixed effects (the marginal  $R^2$ , or  $R^2_m$ ), and an  $R^2$  for the entire model, including both fixed and random effects (the conditional  $R^2$ , or  $R^2_c$ ). For the P-GLS models, we used the  $R^2_{pred}$  measure of Ives (2019). We checked that the linear model assumptions were not violated by studying the model residuals (Zuur et al., 2009). Specifically, we checked the homogeneity of the variance by plotting fitted values against residual values, the normality of the model residuals by plotting a histogram of the residuals, as well as the independence of the model residuals by plotting residuals vs each explanatory variable.

### **2.4 Software and data availability**

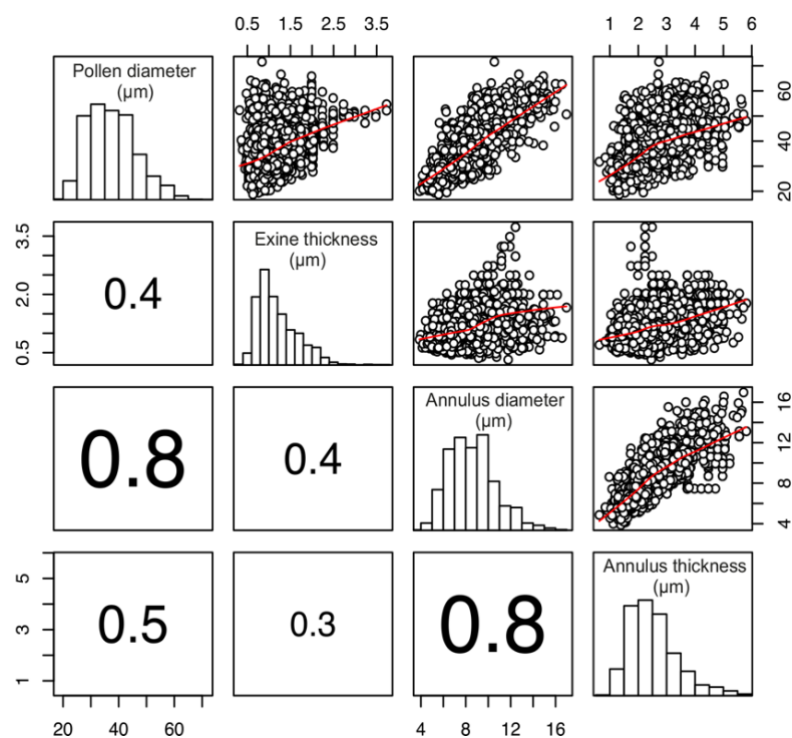
All analyses were carried out in R v.4.1.1 (R Core Team, 2021), using the package “segmented” v. 1.3.4 (Muggeo, 2008) for piecewise regression; package “ape” v. 5.5 (Paradis and Schliep, 2019) and “phytools” v. 0.7-90 (Revell, 2012) for phylogenetic analyses; package “nlme” v. 3.1.153 (Pinheiro et al., 2007) for mixed effects models and generalised least squares; package “MuMIn” v. 1.43.17 (Bartoń, 2020) for AICc

calculation and  $R^2$  for LMM models; package “rr2” (Ives, 2019) for  $R^2$  for the P-GLS models. All data files and code used in this analysis are available in the supplementary information and can be download for peer review in Dryad at: <https://datadryad.org/stash/share/SbJT0yK0zeL0dWxOoHmyywctRtJvCFqkBQHHRZg02F4>

### 3 Results

#### 3.1 Pollen measurement data

The pollen grains exhibited a wide range of pollen diameters: 18.77  $\mu\text{m}$  for *Eragrostis maypurensis* (subfamily Chloridoideae) to 71.62  $\mu\text{m}$  for *Echinochloa polystachya* (subfamily Panicoideae) (see Table S2.1. in Appendix S2). The pairs plot shows a broadly positive relationship among the four pollen grain parameters (Fig. 3). Since all variables are positively correlated, and pollen diameter is the most straightforward of the four to measure consistently in fossil samples, we focus on pollen diameter for subsequent analyses.

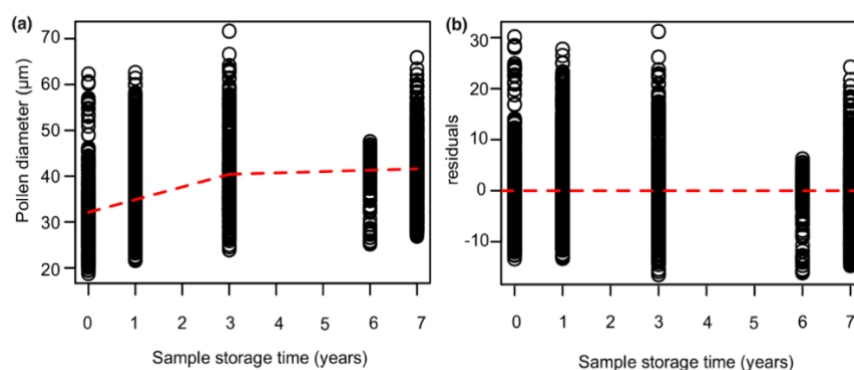


**Fig. 3.** Pairs plot of the four parameters measured from Poaceae pollen grains in this study. Histograms on the diagonal show the distribution of each parameter, and the lower half of the plot shows correlation coefficients (Pearson's  $r$ ) for each pair of parameters.

Preliminary exploration of pollen size and the continuous explanatory variables shows that pollen size is only weakly correlated with the spatial and climatic variables (See Fig. S3.3. in Appendix S3). Pollen size shows a slightly stronger positive correlation with both genome size and storage time in glycerin jelly.

### 3.2 Poaceae pollen size in relation to the sample storage time

Our piecewise regression of pollen size on sample storage time shows that there is a breakpoint at 3 years, which separates the regression line into two different slopes. Pollen grain size distinctly increases in the first 3 years of storage in glycerin jelly, and changes more gradually after this (Fig. 4a). The residuals from the fitted piecewise regression were taken and used as corrected pollen size, without the influence of sample storage time (Fig. 4b). The logarithm of pollen size in relation to sample storage time was also corrected in this way (See Fig. S3.4. in Appendix S3).



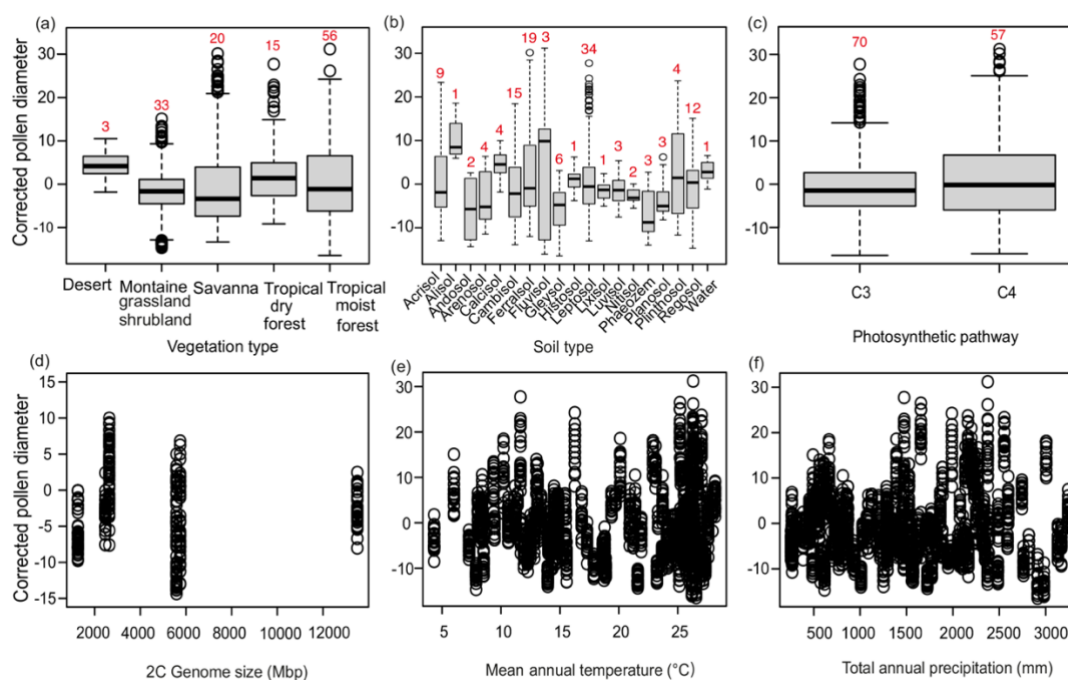
**Fig. 4.** Poaceae pollen size in relation to the sample storage time (the gap between sample preparation and pollen size measurement). (a) Pollen size plotted against sample storage time, the red dashed line is the fitted model from a piecewise regression with the breakpoint at 3 years. (b) Residuals from the fitted piecewise

regression model, which are used as corrected pollen size in the subsequent analyses.

### 3.3 Pollen size variation in relation to explanatory variables

Boxplots and scatterplots were used to explore the relationship between corrected pollen size and the six explanatory variables. Median pollen size is similar across the different vegetation types, with substantial overlap among the size distributions (Fig. 4a). While the pollen from the desert samples is slightly larger than the other vegetation types, this environment is only represented by three samples, which is not enough to support a firm conclusion. The pollen size of the same species from the same vegetation type can also vary quite substantially, for example *Trachypogon spicatus* plants from Suriname and British Guiana, both belonging to tropical moist forests, differ in their average pollen size by more than 10  $\mu\text{m}$  (Fig.5a; Appendix S1).

The soil type is quite similar to the vegetation type in that there is substantial overlap among the pollen size distributions and a broad similarity of the group medians (Fig. 5b). The soil types where the median pollen size does appear to be larger, such as the Alisols and Fluvisols, are only represented by a limited number of samples (one and three samples respectively) (Fig.5b). The mean pollen size of C4 species is larger than the C3 species but just by 1.94  $\mu\text{m}$ , and also their pollen size range is slightly wider, but there is considerable overlap in the distributions of the two groups (Fig.5c). Pollen size increases in variance along the MAT gradient, and its variance is bigger towards the middle of the TAP gradient, although there are no clear positive or negative correlations between pollen size and climate (Fig.5d-e). Although the values of genome size are reported in just six taxa, the results suggest that the corrected pollen size does not show a clear relationship with genome size (Fig.5f).

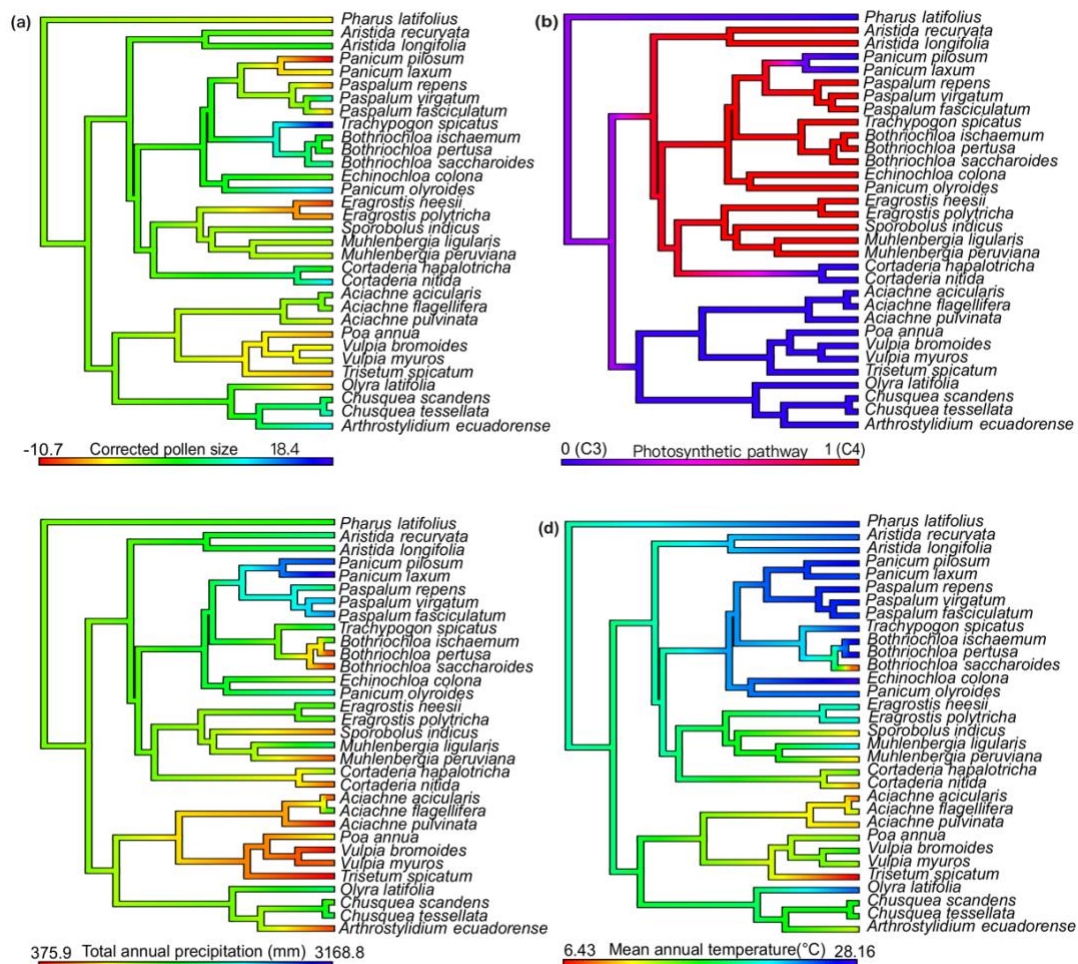


**Fig. 5.** Corrected Poaceae pollen grain size plotted against the six explanatory variables used in this study. (a – c) Boxplots of pollen size against (a) vegetation type, (b) soil type, and (c) photosynthetic pathway. (d – f) Scatterplots of pollen size against (d) genome size, (e) mean annual temperature, and (f) total annual precipitation. The red number above the boxes represent the number of samples.

### 3.4 Trait evolution on phylogenies

The phylogenetic signal in corrected pollen size is relatively low, and mapping this trait onto the molecular phylogeny shows limited evidence for larger, clade-wide tendencies towards larger or smaller pollen (Fig. 6a, Table 1). For the photosynthetic pathway, we simulated the posterior probability of the lineage being C4 species at any specific point, with 0 representing C3 species, which shows that C3 and C4 grasses are concentrated in the PACMAD and the BOP clades, respectively (Fig. 6b). Similar to corrected pollen size, the phylogenetic signal is low for TAP (Fig. 6c, Table 1). There appears to be some grouping for MAT, specifically in the PACMAD clade and especially for Panicoideae, which is mostly distributed in high-temperature, low elevation areas, and the BOP clade which mostly occurs in low temperature, high

elevation areas (Fig. 6d). The Blomberg's  $K$  value for MAT is accordingly slightly higher than for corrected pollen size and TAP, although it is still moderate (Table 1).



**Fig. 6.** Poaceae phylogeny of Spriggs et al. (2014) pruned to the 32 taxa present in both the phylogeny and our dataset, with four traits mapped on to the phylogeny: (a) corrected pollen size; (b) photosynthetic pathway; (c) total annual precipitation; (d) mean annual temperature. Note that in (b), the colors represent the posterior probability of the character state being C4.

**Table 1.** Phylogenetic signal values (Blomberg's  $K$  and  $p$ -value) of the bioclimatic traits measured in Poaceae in South American.

Trait	Blomberg's $K$	$p$ -value
Corrected pollen size	0.395	0.026
Total annual precipitation	0.352	0.045
Mean annual temperature	0.425	0.017

### 3.5 Model fitting and selection

From the candidate set of models (seven different combinations of explanatory variables and one null model with just an intercept included), fit using either LMM based on the corrected pollen size or corrected log pollen size, or a P-GLS model based on the corrected species mean pollen size, the null model has the lowest AICc value, and therefore represents the best trade off of model complexity and fit (Table 2). This means that a strong relationship between pollen size and the selected explanatory variables - mean annual temperature (MAT), total annual precipitation (TAP) and photosynthetic pathway (PP) – is not supported. Model validation plots are provided in Appendix 3 (Fig. S3.5-3.7). These show that the null model provides an adequate fit for the data, with no obvious patterns in the residuals, aside from a skewness in the basic LMM model that is removed when corrected log pollen size is used as the response variable.

For the LMM models, the  $R^2_m$  value, which gives the proportion of variance explained by the fixed effects (MAT, TAP, and PP), is  $\leq 0.01$  for models M1 to M7, which shows that virtually none of the variance was explained by these variables ( $R^2_m$  for the null model (M8) is 0 because there are no fixed effects in this model) (Table 2). The  $R^2_c$  value, which gives the proportion of variance explained by the entire model, is about 0.75 for models M1 to M8, which means that the nested structure represented by the random effects explains a substantial proportion of the pollen size variation. The results are quite similar in the LMM.log model, except that here the  $R^2_c$  values are  $\sim 0.9$ , indicating a higher proportion of variance accounted for

by the random effects. For the P-GLS models, the  $R^2$  values are much lower (Table 2). This shows the importance of random effect structure in the LMM models, which is not fully compensated for in the phylogenetic structure included in the P-GLS models to account for non-independence of the residuals.

**Table 2.** AICc and  $R^2$  values for the fitted models. AICc = corrected Akaike information criterion;  $R^2$  = proportion of variance in the response variable explained by the model;  $R^2_m$  = marginal variance explained by the fixed effects;  $R^2_c$  = conditional variance explained by both fixed and random effects. MAT = mean annual temperature; MAP = total annual precipitation; PP = photosynthetic pathway. The lowest AICc values for each set of models are shown in bold.

Model number	Variables included	LMM			LMM.log			P-GLS	
		AICc	$R^2_m$	$R^2_c$	AICc	$R^2_m$	$R^2_c$	AICc	$R^2$
M1	TAP + MAT + PP	11584.16	0.0106	0.7478	-6049.35	0.0134	0.9039	219.38	0.2511
M2	TAP + MAT	11583.08	0.0008	0.7483	-6050.50	0.0029	0.9041	216.55	0.2281
M3	TAP + PP	11582.53	0.0082	0.7488	-6050.88	0.0091	0.9041	217.42	0.2103
M4	MAT + PP	11582.24	0.0102	0.7480	-6051.37	0.0134	0.9039	216.37	0.2571
M5	TAP	11581.22	0	0.7488	-6052.28	0.0008	0.9041	214.70	0.1973
M6	MAT	11581.11	0.0007	0.7485	-6052.51	0.0029	0.9040	213.82	0.2336
M7	PP	11580.52	0.0082	0.7488	-6052.70	0.0081	0.9042	215.13	0.2030
M8	-	<b>11579.21</b>	0	0.7488	<b>-6054.14</b>	0	0.9041	<b>212.65</b>	0.1858

#### 4 Discussion

The main focus of our work is to test the expectation raised in earlier studies (Schüler and Behling, 2011; Griener and Warny, 2015; Jan et al., 2015; Radaeski et al., 2016, 2020) that pollen size could be used as a proxy to reconstruct key abiotic or biotic variables in the fossil record. Although there have been a number of attempts to extract information from grass pollen size beyond the traditional wild *versus* domesticated distinction (e.g. Schüler and Behling, 2011; Jan et al., 2015; Radaeski et al., 2016, 2020), the datasets used in these studies lacked an explicit spatial or

phylogenetic context, and each focused on a small number of (i.e. one or two) explanatory variables. In our study we aimed to address these problems through extensive sampling across the ADB, including a wide climatic variation and diverse phylogenetic range of grasses, while evaluating the relevance of several key explanatory variables via model selection. We found that the use of grass pollen size as a proxy to retrieve information about past vegetation history and climate change is not supported—pollen size does not vary with the explanatory variables tested here, specifically temperature, precipitation, photosynthetic pathway, vegetation type, soil type, and genome size. Therefore, despite the abundance of grass pollen in the fossil record, and the fact that pollen size can be readily measured in both fossil and extant material, it does not qualify for a widely applicable palynological proxy.

We found that pollen has a wide size range both at genus and species level, which is consistent with other studies (Rohde, 1959; Salgado-Labouriau and Rinaldi, 1990; Beug, 2004). We note that size variation also occurs among plant specimens within species, even if they are from the same vegetation type. We also observe that pollen size ranges overlap between vegetation and soil types, with their average size being quite similar when tested at a broader scale; yet the phylogenetic signal on pollen size is quite weak so that it is hard to classify grass even at subfamily level. From this we conclude that Poaceae pollen cannot be separated into distinct taxonomic categories based on size measurement results alone.

Prior to our study, and before coming to the above conclusions, we also tested the effect of glycerin jelly on pollen size in order to eliminate this as a confounding factor. Our results show that residence time in glycerin jelly indeed causes swelling of pollen grains and clearly influences pollen grain size, which is consistent with the existing literature (Christensen, 1946; Cushing, 1961; Moore et al., 1991; Jan et al., 2015). Hamilton (1972) reported that grass pollen size noticeably increased when storing for 6 to 7 years in glycerin jelly. However, we found that swelling occurs much earlier, and that pollen size clearly increased during storage in glycerin jelly in

the first 3 years. In this timespan, swelling caused pollen to increase by around 7  $\mu\text{m}$ , after that, the pollen size no longer swells significantly. In our research, we corrected grass pollen size affected by storage in glycerin jelly by using a piecewise regression model. For future studies, we recommend standardizing the sample preparation to avoid grain size changes from different chemical treatments, and measuring pollen grains shortly after sample preparation in glycerin jelly, which is in agreement with Jan et al. (2015). An alternative is to use silicon oil as a mounting medium, which avoids the pollen swelling issue (Andersen, 1960; Sluyter, 1997).

Our analysis differs from previous pollen size studies because these have typically focused on testing one explanatory variable at a time, often using simple null hypothesis testing approaches (Griener and Warny, 2015; Jan et al., 2015; Radaeski et al., 2020). Here, we have used a model fitting and selection approach, and we argue that this is a more fruitful avenue for understanding the drivers of morphological trait variation, and the potential of this variation to form the basis of proxies to use in palaeo-settings. For example, using multiple regression coupled with information theoretic criteria such as AICc allows a range of non-nested models with different combinations of explanatory variables, each representing a specific biological hypothesis, to be directly compared, ranked and their relative levels of support assessed (Anderson, 2008; Zuur et al., 2009). Extensions of ordinary least squares regression, such as the LMM and P-GLS frameworks used here, allow the structures in the data that violate assumptions of independence to be directly incorporated into the model fitting process, which is not possible with standard statistical tests. The use of summary statistics such as  $R^2$  also allows the explanatory power of the model to be directly assessed, and the partitioning of this value into marginal and conditional components in the LMM  $R^2$  (Nakagawa et al., 2017) provides further insights: the fixed effects (i.e. the explanatory variables) explain a very limited proportion of variance in pollen size, while the random effects, which account for the nested structure of pollen grains within plants with species, are sufficient to explain the majority of the variance across the dataset.

Since for each of our three candidate sets of models the null model was ranked the highest, we did not investigate effect sizes of the explanatory variables (i.e. the slope of the fitted regression lines for continuous variables, and differences between means for categorical variables). The importance of focusing on effect sizes rather than  $p$  values to evaluate the biological significance of relationships has been well-documented in the literature, not least because  $p$  values are in part driven by sample size (see Holland, 2019 for a thorough discussion of this point). In the context of our pollen size data, we note that the large sample size, with 2540 grains measured, means that simple statistical tests are likely to indicate statistically significant differences even if the effect size is negligible. For example, as noted above the difference in means between C3 and C4 corrected pollen size is just 1.94  $\mu\text{m}$ , yet a  $t$ -test suggests a statistically significant difference with a very low  $p$  value ( $t = -6.07$ ,  $d.f. = 2011.8$ ,  $p = 1.509 \times 10^{-9}$ ). This may explain why some previous studies have reported substantial relationships between pollen size and explanatory variables such as photosynthetic pathway and vegetation type (Jan et al., 2015; Radaeski et al., 2020), in contrast to the results reported here. We therefore encourage palynologists and palaeoecologists to carefully evaluate the strength of relationships and the predictive power of measured variables, especially where the aim is to develop proxies to reconstruct environmental or biotic change in the fossil record.

The use of corrected pollen size in the analyses may have reduced the strength of the relationships with the explanatory variables, if these variables correlated with sample storage time and useful information was removed during the piecewise regression (Fig. 4). However, we note that the correlations between uncorrected pollen size and both MAT and TAP are very weak (Fig. S3.3), and the difference in means between C3 and C4 pollen size is just 1.05  $\mu\text{m}$  (although a  $t$ -test still indicates a statistically significant difference:  $t = -2.98$ ,  $d.f. = 2246.5$ ,  $p = 0.003$ ). The only variable that shows a stronger correlation with both pollen size and sample storage time is genome size, and this is possibly worth further investigation with a larger dataset.

Taken together, pollen size does not seem a generally applicable proxy for reconstructing past vegetation and climates. Julier et al. (2016) proposed that extant Poaceae pollen can be identified at the subfamily level by using FTIR spectroscopy; Jardine et al. (2021) integrated extant and fossil sporopollenin chemical data, validating Julier's conclusion for modern materials but demonstrating consistent differences between extant and fossil sporomorphs. They therefore suggested that classifying pre-Quaternary fossil specimens using extant training sets will be challenging. Other options, such as analysis of the ultrastructure of Poaceae pollen grains, possibly in combination with deep learning, show promise for extracting further information from pollen samples. Mander et al. (2013), for instance, established quantitative morphometric methods to characterize surface ornamentation and classify Poaceae pollen grains using scanning electron microscope (SEM) images. More recently, Romero et al. (2020) developed three convolutional neural network (CNN) classification models to explore biological affinity by using Airyscan confocal superresolution microscopy. For future analyses, we suggest scientists consider variation at different taxonomic levels and combine optical superresolution imaging with deep-learning classification methods, which may provide new and more promising avenues for Poaceae pollen classification, and subsequently new information with which to explore past vegetation and climate change.

## 5 Conclusion

We have carried out a broad-scale, biogeographic analysis to test the proposition that grass pollen size could be a useful proxy to reconstruct key biotic and abiotic variables. Our results suggest that Poaceae pollen size does not respond to explanatory variables such as vegetation type, soil composition, climatic conditions, photosynthetic pathway, and genome size. We therefore propose that pollen size is not a robust proxy as previously suggested, and it cannot be used to reconstruct past vegetation or climate parameters. The weak phylogenetic signal in pollen size also

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suggests that linking grass pollen grains to specific clades (e.g. subfamilies) will also be challenging. For future analyses of pollen size, we recommend standardizing sample preparation approaches, and measuring pollen grains shortly after sample preparation. Careful consideration of variation at different levels of the sampling hierarchy – within individuals, within species, and among species – will also be important. We also recommend exploring the surface ornamentation of Poaceae pollen grains (using, for example SEM or Airyscan confocal superresolution microscopy) as a basis for developing new tools to reconstruct past plant history and climate.

### **6 Support Information**

The data that support the findings of this study are currently openly available for peer review in Dryad at: <https://doi.org/10.5061/dryad.0rxwdb3k>.

Supplementary data to this article can be found online at:

<https://doi.org/10.1016/j.revpalbo.2022.104790>