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DOI
10.1111/pala.12523

Publication date
2021

Document Version
Final published version

Published in
Palaeontology

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Citation for published version (APA):
Jardine, P. E., Hoorn, C., Beer, M. A. M., Barbolini, N., Woutersen, A., Bogota-Angel, G., Gosling, W. D., Fraser, W. T., Lomax, B. H., Huang, H., Sciumbata, M., He, H., & Dupont-Nivet, G. (2021). Sporopollenin chemistry and its durability in the geological record: an integration of extant and fossil chemical data across the seed plants. Palaeontology, 64(2), 285-305. https://doi.org/10.1111/pala.12523
SPOROPOLLENIN CHEMISTRY AND ITS DURABILITY IN THE GEOLOGICAL RECORD: AN INTEGRATION OF EXTANT AND FOSSIL CHEMICAL DATA ACROSS THE SEED PLANTS

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Abstract: Sporopollenin is a highly resistant biopolymer that forms the outer wall of pollen and spores (sporomorphs). Recent research into sporopollenin chemistry has opened up a range of new avenues for palynological research, including chemotaxonomic classification of morphologically cryptic taxa. However, there have been limited attempts to directly integrate extant and fossil sporopollenin chemical data. Of particular importance is the impact of sample processing to isolate sporopollenin from fresh sporomorphs, and the extent of chemical changes that occur once sporomorphs enter the geological record. Here, we explore these issues using Fourier transform infrared (FTIR) microspectroscopy data from extant and fossil grass, Nitraria (a steppe plant), and conifer pollen. We show a 98% classification success rate at subfamily level with extant grass pollen, demonstrating a strong taxonomic signature in isolated sporopollenin. However, we also reveal substantial chemical differences between extant and fossil sporopollenin, which can be tied to both early diagenetic changes acting on the sporomorphs and chemical derivates of sample processing. Our results demonstrate that directly integrating extant and late Quaternary chemical data should be tractable as long as comparable sample processing routines are maintained. Consistent differences between extant and deeper time sporomorphs, however, suggests that classifying fossil specimens using extant training sets will be challenging. Further work is therefore required to understand and simulate the effects of diagenetic processes on sporopollenin chemistry.

Key words: sporopollenin, pollen, chemotaxonomy, seed plants, diagene sis, Fourier transform infrared (FTIR) microspectroscopy.

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chemical signature. This has yielded information in three key areas: (1) the chemical composition of sporopollenin, the biopolymer that makes up the outer wall (exine) of sporomorphs and gives them an exceptionally high preservation potential in the geological record (Blokker et al. 2006; de Leeuw et al. 2006; Watson et al. 2007; Fraser et al. 2012; Watson et al. 2012; Fraser et al. 2014a; Jardine et al. 2015; Li et al. 2019; Nierop et al. 2019); (2) taxonomic controls on sporomorph and sporopollenin chemistry and the possibility of using this information to classify sporomorph specimens (Pappas et al. 2003; Schulte et al. 2008; Dell’Anna et al. 2009; Steemans et al. 2010; Zimmermann 2010; Zimmermann & Kohler 2014; Julier et al. 2016; Zimmermann et al. 2016; Woutersen et al. 2018; Jardine et al. 2019); and (3) climatic controls on sporomorph chemistry, including the production of UVB absorbing compounds (UACs) which provide the basis for a palaeo-UVB proxy (Rozema et al. 1999, 2001a, b, 2002, 2009; Blokker et al. 2005, 2006; Watson et al. 2007; Lomax et al. 2008, 2012; Fraser et al. 2011, 2014b; Willis et al. 2011; Lomax & Fraser 2015; Jardine et al. 2016, 2017, 2020a; Seddon et al. 2017, 2019; Bell et al. 2018).

Despite the high potential of these methods, there are a series of unresolved issues linked to the feasibility of integrating chemical datasets derived from extant and fossil sporomorphs. (Note that here we use the term ‘fossil’ to denote sporomorphs that have been recovered from the geological record, rather than inferring any fossilization processes per se.) These issues relate mostly to how sporopollenin chemistry changes as a function of diagenesis within natural sedimentary systems, and also to the laboratory methods used to isolate sporopollenin from fresh sporomorphs. Resolving these two issues is essential if fossil material is to be classified or analysed in the context of training sets or calibrations based on extant sporomorphs.

Sporopollenin is generally thought to be robust to changes brought about by post-burial diagenetic processes. If this is correct, then sporopollenin that has been isolated from fresh material could be directly related to well-preserved fossil specimens. Fraser et al. (2012), for example, demonstrated that the chemical signature from exceptionally preserved Pennsylvanian (~310 Ma) lycopsid megaspores was highly similar to that of extant Lycopodium spores. Simulated oxidation and thermal maturation experiments have also suggested a broad taphonomic and diagenetic window for preservation of the sporopollenin chemical signature (Yule et al. 2000; Fraser et al. 2014a; Jardine et al. 2015). However, considerable changes in sporopollenin chemistry have been shown to occur with increased thermal maturation once certain temperature thresholds are reached (Watson et al. 2012; Fraser et al. 2014a; Bernard et al. 2015). It has been suggested that sporopollenin includes a hydrolysable ‘labile’ component comprising ester bound n-carboxylic acid monomers, which repolymerize in situ during diagenesis to produce a recalcitrant polyalkyl ‘geopolymer’ with a chemical signature that is distinct from the original sporopollenin biopolymer (Watson et al. 2012). In addition, the diagenetic products of plant waxes, membrane lipids or sporomorph lipids may also be incorporated during sporopollenin repolymerization (de Leeuw et al. 2006; although see also Gupta et al. 2007). While such changes to the sporopollenin chemical signature would hamper the direct integration of extant and fossil chemical datasets, relating rates and thresholds of change in experimental settings to those in natural geological systems is difficult, and so it is not known at which combination of temperature, pressure and time repolymerization occurs (Watson et al. 2012; Fraser et al. 2014a; Bernard et al. 2015).

Laboratory processing methods may also influence sporopollenin chemistry. Sporomorphs collected from living plants or herbarium specimens require processing to remove the external labile compounds (proteins, lipids and carbohydrates) and isolate the sporopollenin wall (Traverse 2007; Jardine et al. 2015). It is currently not well understood how processing procedures may influence sporopollenin chemistry, and therefore whether ‘pure’ sporopollenin is left over or some modified form of it (Domínguez et al. 1998; Loader & Hemming 2000; Nierop et al. 2019). Acetylation is a standard laboratory processing method used in palynological research to isolate sporopollenin. While UAC relative abundances are recoverable from acetylated sporomorphs (Jardine et al. 2016, 2017), the acetylation procedure may leave an imprint on the chemical signature of the sporomorphs (Domínguez et al. 1998; Loader & Hemming 2000; Jardine et al. 2015). This imprint would not be present in non-acetylated material, as is often the case in pre-Quaternary (i.e. Neogene and older) palynological samples where acetylation is not part of the routine processing procedure (Traverse 2007).

Most previous studies of sporomorph and sporopollenin chemistry have been carried out using a small range of samples, taxa and/or time periods. These studies have provided a wealth of information on the controls on the chemical signature of sporopollenin, but the restricted nature of their scope makes wider generalizations difficult. This is a particular problem for chemotaxonomic analysis of fossil datasets, since most research in this area to date has focused exclusively on freshly harvested or herbarium material, without additional isolation of the sporopollenin (Pappas et al. 2003; Schulte et al. 2008; Dell’Anna et al. 2009; Zimmermann 2010; Zimmermann & Kohler 2014; Julier et al. 2016; Zimmermann et al. 2016; Jardine et al. 2019; although see Woutersen et al. 2018; Nierop et al. 2019). Therefore, while freshly harvested sporomorphs are known to contain a useable
taxonomic signature in their chemical make-up, it is still not clear how much of this signal is driven by the external labile components and how much taxonomic information is recorded within the sporopollenin biopolymer. If there is only a weak taxonomic signal present in sporopollenin itself, attempts to apply chemotaxonomy to the fossil record will have limited success, even if diagenetic changes in sporopollenin are subtle enough for extant and fossil datasets to be integrated successfully.

Here, we explore these issues with a large dataset incorporating a range of taxa from different time periods, using Fourier transform infrared (FTIR) microspectroscopy to track changes in pollen chemistry. These datasets have been combined to tackle key research questions in the development of chemopalyngology. The first question focuses on testing whether chemotaxonomic information is recorded within sporopollenin. In testing this concept, we have focused on the grasses (Poaceae) as they present a classic problem in palynology: despite being highly diverse (>12,000 species) and ecologically dominant in savannah and steppe environments, grass pollen is morphologically highly similar and challenging to classify below the family level (Bush 2002; Mander et al. 2013; Julier et al. 2016; Jardine et al. 2019). Valuable palaeoecological and palaeoenvironmental information, such as relative contributions from aquatic versus terrestrial grasses, or domesticated versus wild, is therefore often lost in palynological studies (Bush 2002; Jardine et al. 2019). Our main dataset comprises extant grass pollen collected from 50 South American species in 9 subfamilies, which we use to quantify classification potential in acetylated sporopollenin. Additionally, we use this dataset to test whether the photosynthetic pathways in grasses can be determined in the broader FTIR fingerprint. Being able to reliably chemotype photosynthetic pathways of fossil pollen (C3 vs C4) would provide much needed information on the evolution of C3 and C4 grasses, and their relative dominance across different habitats (Nelson et al. 2006, 2008; Strömberg 2011).

The second question explores whether extant and fossil chemical datasets can be integrated to enable chemotaxonomic classification, by determining the impact of diagenetic processes on sporopollenin preservation within natural sedimentary settings. To answer this question, we assembled a dataset of Miocene to Pleistocene (~18 to ~0.5 Ma) grass pollen from western Amazonia (Hoorn 1994; Vink 2012; Bermúdez et al. 2017) and the Amazon Fan (Hoorn et al. 2017), to provide a direct comparison with the extant grasses examined to deliver on the first question. We also use late Pleistocene (~500 to 13 ka) grass pollen data from Lake Bosumtwi in Ghana (Miller & Gosling 2014; Jardine et al. 2016) to examine chemical changes on a 10^4 to 10^5 year timescale, providing a temporal bridge between the extant and fossil grass datasets.

Finally, we test the generality of the results obtained with grasses by using extant and Palaeogene chemical data from Nitraria (Woutersen et al. 2018) and conifer (Pinaceae and Podocarpaceae) pollen. These datasets provide additional extant–fossil comparisons across a broad phylogenetic range.

**MATERIAL AND METHOD**

**Dataset descriptions**

To deliver information on these three research topics, we assembled eight distinct yet complementary pollen datasets. These datasets, described below and organized by topic, comprise extant material harvested from field and herbaria collections, and Pleistocene to Paleocene samples retrieved from rock outcrops. An overview of the datasets is provided in Table 1.

**Chemotaxonomic signals from sporopollenin.** Dataset 1: Extant South American grasses. Pollen from 50 Poaceae species was either taken from the residue collection at the University of Amsterdam or sampled from the National Herbarium of the Netherlands at Naturalis Biodiversity Center, Leiden (Table 2; Jardine et al. 2020b, fig. S1, table S1). The pollen was extracted and the sporopollenin isolated using standard acetylation for 6 min.

**Diagenesis in non-exceptional settings.** Dataset 2: Early Miocene to Pliocene grass pollen (~18 to ~5 Ma) from western Amazonia. The data come from nine samples from six localities in Colombia (Los Chorros and Santa Sofia), Peru (Madre de Dios, Pebas and Santa Teresa) and Venezuela (the Maracaibo Basin) (Jardine et al. 2020b, fig. S1). The pollen count data from the samples are documented in Hoorn (1994), Vink (2012) and Bermúdez et al. (2017), which contain additional stratigraphic and locality information. All sample processing was carried out at the University of Amsterdam. Eight samples are clastics (very fine sands, silts and clays) and were processed using sodium pyrophosphate in a 10% solution with H2O (Na4P2O7·10(H2O)), while the one lignite sample (sample 16788 from Los Chorros) was also oxidized with Schulze reagent (2HNO3, 60% : K2C103, 7%). For all samples the organic/inorganic fraction was separated with bromoform with density 2.0 g/cm3.

**Dataset 3: Late Miocene to Pleistocene (5.85–0.54 Ma) grass pollen from the Amazon Fan.** The data come from eight samples from a hydrocarbon exploration well (Well 2) situated on the upper slope of the continental shelf beyond the Amazon River delta (latitude 3°2′59″N, longitude 47°44′46″W; Jardine et al. 2020b, fig. S1) (Hoorn et al. 2017). The samples span a depth of 1644–
3793 mbdf (metres below drill floor), which corresponds to a sediment depth of 865–3014 m once water depth (754 m) and the height of the drilling platform (25 m) are taken into account. The count data from the samples were published in Hoorn et al. (2017) and Kirschner & Hoorn (2020). The samples were processed at the University of Amsterdam with hydrochloric acid (HCl), sodium pyrophosphate, acetolysis and bromoform separation (Hoorn et al. 2017).

Dataset 4: Late Pleistocene (520 to 13 ka) grass pollen from Lake Bosumtwi in Ghana. Count data from the majority of samples were published in Miller & Gosling (2014), and FTIR-derived UAC data from the upper 130 kyr of the Bosumtwi record were published in Jardine et al. (2016). The samples were processed at the Open University using HCl, HF, KOH and acetolysis, with eight samples processed both with and without acetolysis to study the impact of this process on sporopollenin chemistry (Jardine et al. 2016).

**Data generation**

Chemical data for the South American grasses, *Nitraria* and conifer pollen (Datasets 1–3, 5–8) were generated from herbaria and collections. The sporopollenin was isolated using standard acetolysis. The FTIR data were previously published in Woutersen et al. (2018), which provides further information on the samples, as well as detailed morphological and chemical analyses of these species.

Dataset 6: Middle–late Eocene *Nitraria* pollen from the Tarim and Xining basins north of the Tibetan Plateau, which have been assigned to the form-genera *Nitrariadites* and *Nitraripollis* (Hoorn et al. 2012). The Tarim sample (sample MS-BC22) comes from the late Eocene Bashiulake Formation collected at the Mine section, and was processed at Utrecht University with HCl and HF but without heavy liquid separation or oxidation (Houben et al. 2011; Bosboom et al. 2014). The Xining sample (sample XJ88a) was taken from the middle Eocene Honggou Formation exposed at the Xiejia section, and processed at Palynological Laboratory Services Ltd, Holyhead, UK, with HCl, HF and heavy liquid separation. Further locality details are provided in Meijer et al. (2019).

Dataset 7: Extant conifer (Pinaceae and Podocarpaceae) pollen from Yunnan and Hunan provinces, China, collected in April to May 2018 directly from nine individual trees representing seven species (Jardine et al. 2020b, table S2). The sporopollenin was isolated using standard acetolysis.

Dataset 8: Paleocene fossil conifer pollen from the Nangqian Basin of east-central Tibet (NB unpub. data; see also Horton et al. 2002). Samples are from eight form-species in six genera. These samples were processed at Palynological Laboratory Services Ltd with HCl, HF and heavy liquid separation.

### TABLE 1. Overview of the sporomorph datasets used in this study.

| Dataset | Taxon | Age and type | Locality | Treatment |
|---------|-------|--------------|----------|-----------|
| 1       | Poaceae | Extant (herbarium specimens) | Amazonia/Andes | Acetolysis |
| 2       | Poaceae | Fossil (early Miocene to Pliocene) | Western Amazonia | Na₂P₂O₇ and heavy liquid separation (clastics) plus Schultze reagent (lignite) |
| 3       | Poaceae | Fossil (late Miocene to Pleistocene) | Amazon Fan | HCl, Na₂P₂O₇, acetolysis and heavy liquid separation |
| 4       | Poaceae | (Sub)fossil (late Pleistocene) | Lake Bosumtwi, Ghana | HCl, HF, KOH and acetolysis (splits of eight samples processed without acetolysis) |
| 5       | *Nitraria* | Extant (herbarium specimens) | Global | Acetolysis |
| 6       | *Nitraria* | Fossil (Eocene) | Tarim and Xining basins, China | HCl and HF, then heavy liquid separation for Xining sample only |
| 7       | Conifers | Extant (live plants) | Yunnan and Hunan provinces, China | Acetolysis |
| 8       | Conifers | Fossil (Paleocene) | Nangqian Basin, China | HCl, HF and heavy liquid separation |

### TABLE 2. Number of genera and species, and C₃/C₄ species, for each subfamily in the extant grass dataset (Dataset 1).

| Subfamily | Number of genera/species in dataset | Number of C₃/C₄ species in dataset |
|-----------|------------------------------------|----------------------------------|
| Anomochlooideae | 1/1 | 1/0 |
| Aristidoideae | 1/1 | 0/1 |
| Bambusoideae | 1/3 | 3/0 |
| Chloridoideae | 3/5 | 0/5 |
| Danthonioideae | 1/2 | 2/0 |
| Oryzoideae | 1/1 | 1/0 |
| Panicoideae | 15/24 | 6/18 |
| Pharoideae | 1/2 | 2/0 |
| Pooideae | 8/11 | 11/0 |

**Generality across taxa.** Dataset 5: Extant *Nitraria* pollen. Pollen from six different species, collected from various herbaria and collections. The sporopollenin was isolated using standard acetolysis. The FTIR data were previously published in Woutersen et al. (2018), which provides further information on the samples, as well as detailed morphological and chemical analyses of these species.
using a Thermo Scientific Nicolet iN10 MX Dual detector IR microscope at the University of Amsterdam. Atmospheric CO₂ and H₂O variations were removed by purging the system with a dry nitrogen feed. Individual pollen grains were mounted onto ZnSe windows and analysed using 128 scans and a resolution of 4; in each case the microscope aperture was matched to the size of the pollen grain being analysed. A background spectrum was taken prior to each measurement and automatically removed from the sample spectrum. While we aimed for a target of 30–40 pollen grains per species, in some cases this was not possible due to a lack of material.

Chemical data for the Lake Bosumtwi grass pollen (Dataset 4) were generated at the Open University, UK, using a Thermo Nicolet FTIR bench unit interfaced with a Continuum IR-enabled microscope, which was fitted with a 15 × reflachromat objective lens and a liquid nitrogen-cooled MCT-A detector. A purge unit was installed during data generation. Therefore, the uppermost 69 samples from the Bosumtwi sedimentary sequence were analysed with an unpurged system, while for the lower 62 samples the entire system was purged using a Peak Scientific ML85 purge unit. The lower 12 unpurged samples were also reanalysed with the purged system, to check the impact of the purging status on the results. To generate the data individual pollen grains were picked from the sediment samples and mounted on ZnSe windows in groups of 8–10 grains with 3 replicate groups per sample. FTIR spectra were generated using a microscope aperture of 100 × 100 μm, 256 scans per sample and a resolution of 4. For further details see Jardine et al. (2016).

Data analysis

FTIR spectra from individual pollen grains are prone to high levels of Mie-type scattering and other distorting effects (Zimmermann et al. 2015a, 2016; Zimmermann 2018). This was the case with the individual grain spectra generated in this study, although high quality spectral data were obtained in the majority of cases for the 1800 to 800 cm⁻¹ region (Jardine et al. 2020b, fig. S2). We therefore followed previous studies (Zimmermann & Kohler 2014; Bagcioglu et al. 2015, 2017; Zimmermann et al. 2015b, 2016) in focusing on the lower end of the spectra, here limiting the spectral range to the 1800 to 800 cm⁻¹ region, and discarded any spectra that contained significant noise or distortions in this interval. IR absorbance band assignment was carried out with reference to the published literature (Dominguez et al. 1998; Coates 2000; Mayo et al. 2003; Fraser et al. 2012; Bagcioglu et al. 2015; Jardine et al. 2015, 2017; Julier et al. 2016; Woutersen et al. 2018). Data analysis was carried out on non-differentiated spectra as well as first and second-derivative spectra. Since taking derivatives of spectra not only enhances smaller spectral features but also adds noise (Varmuza & Filzmoser 2009), the spectra were differentiated using the Savitzky–Golay (SG) smoothing algorithm, using a second degree polynomial and a window size (w, with a larger value leading to more smoothing) determined by leave one out cross validation coupled with k-nn (k-nearest neighbour) classification (described below; see also Julier et al. 2016; Jardine et al. 2019). All spectra (differentiated and non-differentiated) were processed using extended multiplicative signal correction (EMSC), a model based spectral processing technique for baseline correction, noise removal and normalization (Kohler et al. 2009; Zimmermann et al. 2015a, 2016; Liland et al. 2016). Non-differentiated spectra were preprocessed with an EMSC model incorporating third order polynomial effects, whereas differentiated spectra were preprocessed with an EMSC model incorporating first order polynomial effects. EMSC allows for a reference spectrum to be incorporated to guide the baseline correction (Zimmermann et al. 2015a; Liland et al. 2016). Here we followed standard practice and used the mean spectrum for each dataset as the EMSC reference spectrum, except for the Nitraria and Lake Bosumtwi spectra (Datasets 4–6) which all showed a pronounced baseline shift. Therefore, the baseline corrected mean spectrum for each of the three datasets was used as the EMSC reference spectra. For the Nitraria datasets we used a second-order polynomial baseline, and for the Bosumtwi data a first-order polynomial baseline (i.e. a straight line).

We tested classification potential using k-nn classification on the extant grass spectra (Dataset 1). Unknown objects (here IR spectra) are classified based on the identities of the most similar objects in the training set, with similarity in this case being the lowest Euclidean distance. The parameter k is the number of most similar objects (nearest neighbours) used for classification, and based on past research (Jardine et al. 2019) this was kept to k = 1 because increasing k beyond this does not improve classification success. To provide a thorough test of classification success we divided the dataset into a training set (2/3) and a validation set (1/3) by holding back every third sample for validation. We then used the training set to select the best combination of processing parameters (i.e. those that gave the highest classification success rate), comparing EMSC corrected non-differentiated, first-derivative and second-derivative spectra, varying the SG smoothing window (w) between 5 and 43. Classification success for the training set was tested using leave one out cross validation, where each spectrum is treated as an unknown and classified in turn, based on the identities of the rest of the spectra in the training set. The combination of processing parameters that produced the highest classification success rate was then used to process both the training set and the validation set;
the spectra in the validation set were classified using the identities of the most similar spectra in the training set. Since most genera are represented by just one species and one individual, we focus primarily on subfamily level classifications, although genus level classification success rate is also given for comparison.

We ordinated the FTIR spectra using principal components analysis (PCA), which enables complex multivariate data to be viewed on a small number of axes (Varmuza & Filzmoser 2009). The PCA plots presented in the main paper are based on non-differentiated spectra to facilitate interpretation of loadings plots; for comparison PCA plots based on second derivative spectra are provided in Jardine et al. (2020b, figs S3–S8).

All data analysis was carried out using R version 3.5.2 (R Core Team 2018) with the packages ‘baseline’ version 1.2-1 (Liland & Mevik 2015), ‘caret’ version 6.0-85 (Kuhn 2020), ‘class’ version 7.3-15 (Venables & Ripley 2002), ‘corrplot’ version 0.84 (Wei & Simko 2017), ‘EMSC’ version 0.9.0 (Liland 2017), ‘maps’ version 3.3.0 (Becker et al. 2018), and ‘rworldmap’ version 1.3-6 (South 2011). The raw data, R code and supplementary information are available for download from figshare (Jardine et al. 2020b, tables S1, S2 and figs S1–S9).

RESULTS

Chemotaxonomic signals from sporopollenin

The mean FTIR spectrum for each grass subfamily (Dataset 1) shows absorbance bands associated with carboxyl groups at 1710 cm$^{-1}$ (C=O stretching), and aromatic compounds at 1590 cm$^{-1}$ (C=C stretching), 1430 cm$^{-1}$ and 1370 cm$^{-1}$ (C–H bending) (Fig. 1). There are prominent peaks at 1170 and 1030 cm$^{-1}$, with the 1170 cm$^{-1}$ peak having a shoulder at 1230 cm$^{-1}$. While peaks at or close to these wavenumbers are present in the phenolic compounds para-coumaric and ferulic acid that form part of the sporopollenin biopolymer (Baçıoğlu et al. 2015), similar peaks have also been interpreted as new C–O bonds forming during acetylation (Dominguez et al. 1998). The small peak at 1650 cm$^{-1}$ is the amide I peak (C=O stretching), and relates to proteins external to the pollen wall that were not fully removed during acetylation (Jardine et al. 2015, 2017). While the spectra of the different grass subfamilies are consistent in the peaks present, there are subtle variations in both peak height and position.

The maximum subfamily classification success rate with the training dataset was 97%, with SG smoothed second derivative spectra and $w = 25$ (Table 3). The next best combination of parameters was with SG smoothed first derivative spectra (96%), and then unprocessed spectra (90%). The genus level classification success rate was 5–10% below the subfamily rate (Table 3). Processing the validation dataset with second derivatives and classifying the spectra based on the identities of the training dataset gave a classification success rate of 98% at the subfamily level, and 93% at the genus level. Confusion matrices for the training and validation datasets both show a similar pattern of misclassifications at the subfamily level, but without any obvious phylogenetic signal (i.e. specimens are not preferentially misclassified into more closely related subfamilies) (Jardine et al. 2020b, fig. S9).

The PCA shows clustering at subfamily level, despite large amounts of overlap among the different taxa (Fig. 2A). C$_3$ and C$_4$ grasses do not group consistently separately from each other when all taxa are considered (Fig. 2B), although it is challenging to distinguish between photosynthetic pathway and taxonomic patterns with this broad scale analysis, since most subfamilies are represented by either all C$_3$ or all C$_4$ species (Table 2). Both Panicum and Paspalum contain a mix of C$_3$ and C$_4$ species, and running a PCA on just these genera reveals both within-genus and within-species clustering (Fig. 3A), as well as a clearer C$_3$/C$_4$ separation, with the C$_3$ species having higher scores on PC 2 (Fig. 3B). While most species in the dataset are represented by only one individual plant, both Panicum laxum and Panicum pilosum are represented by two individuals. In both cases the individuals within each species cluster together in the ordination space, demonstrating a broadly consistent chemical signature among individuals of the same species (Fig. 3A).

Diagenesis in non-exceptional settings

There are substantial chemical differences between the extant (Dataset 1) and fossil (Datasets 2 and 3) South American grass pollen grains (Fig. 4). A PCA of these data divides the extant and fossil data on PC 1, with some additional separation between the Amazon Fan and western Amazonia spectra (Fig. 4A). Both the mean spectrum from each dataset (Fig. 4B) and the PC 1 loadings (Fig. 4C) show that the fossil spectra have relatively higher 1600 and 1370 cm$^{-1}$ aromatic peaks, and a much reduced 1710 cm$^{-1}$ carboxyl peak. Additional differences occur in the lower wavenumbers and probably relate at least in part to laboratory processing procedures: while all spectra have a peak at $\sim$1030 cm$^{-1}$, this is more pronounced in the extant grains and also to some extent in the Amazon Fan spectra, all of which were acetyolysed. The peak at 1170 cm$^{-1}$ with a shoulder at 1230 cm$^{-1}$ is strongly expressed in the extant spectra but very weakly in the fossil ones; conversely a peak at 1115 cm$^{-1}$ is present in the fossil spectra but not those from the extant grasses. As with the peaks at $\sim$1370, 1230, 1170 and 1030 cm$^{-1}$, a peak at $\sim$1115 cm$^{-1}$ is present in both
para-coumaric acid and ferulic acid (Bağcioğlu et al. 2015).

Thermal maturation of palynomorphs causes them to darken in colour, which forms the basis of thermal maturation indices (Traverse 2007; Fraser et al. 2014a). Comparison of light microscope (LM) and scanning electron microscope (SEM) images of extant and early Miocene grass pollen, however, demonstrates that the early Miocene pollen is well-preserved and not visibly darkened (Fig. 5). (Since the processing protocol used for the western Amazonian samples (see Material and Method, above) will not have caused bleaching of the material this is taken to be the primary sporomorph colour.) This suggests that thermal maturation of the host rock is minimal, and the chemical differences between the extant and fossil pollen (Fig. 4) are not related to particularly poor preservation conditions.

There are some differences detectable among localities in the western Amazonia fossil spectra (Dataset 2), with samples from Santa Sofia having a more pronounced 1370 cm\(^{-1}\) peak and samples from Los Chorros having higher peaks at ~1040 and 1115 cm\(^{-1}\) (Fig. 6A). Multiple samples from the same locality have highly similar spectra, which is also the case for Los Chorros despite these samples representing different lithologies and sample processing methods (sample 16835 is clastic that was processed with sodium pyrophosphate, while sample 16788 is a lignite that was processed with both sodium pyrophosphate and Schultze reagent; see Material and Method, above, for further details). A PCA shows grouping of spectra within localities, but no consistent changes with time (Fig. 6B). Similarly, there are differences among samples in the Amazon Fan fossil spectra (Dataset 3) that leads to within-sample grouping in a PCA, but no obvious changes through time (Fig. 7).

The Lake Bosumtwi grasses (Dataset 4) show limited evidence for consistent chemical changes over the last 500 kyr. The first two axes of a PCA, which together account for 56% of the variance in the dataset, show no obvious temporal trends, apart from a possible slight increase in PC 2 scores at ~350 ka BP (Fig. 8A). Higher principal components also show no clear change through time (Jardine et al. 2020b, fig. S8). The main chemical shift occurs ~50 to 90 ka BP with a transient increase in PC 1 scores, driven by increases and decreases in the relative heights of peaks and troughs across the spectra (Fig. 8B). There are no clear differences associated with whether the FTIR system was purged or unpurged.
FIG. 2. Principal components analysis (PCA) of extant South American grass pollen FTIR spectra. A, symbols coloured by subfamily. B, symbols coloured by photosynthetic pathway. Values in parentheses provide the percentage of variance explained by each principal component. Subfamily colours in A are the same as those in Figure 1.
FIG. 3. PCA of Panicum and Paspalum FTIR spectra. A, symbols coloured by species; B symbols coloured by photosynthetic pathway. Circles = Panicum; triangles = Paspalum. Values in parentheses provide the percentage of variance explained by each principal component.
**FIG. 4.** Comparison of extant and fossil South American grass pollen. A, PCA of extant and fossil South American grasses, with extant grass pollen grains shown as orange circles, Amazon Fan fossil grains shown as light blue triangles, and western Amazonia fossil grains shown as dark blue triangles; values in parentheses provide the percentage of variance explained by each principal component. B, stacked mean spectra for the three datasets, with colours as in A and shaded regions showing ±1 standard deviation about the mean. C, loadings plot for PC 1.
(Jardine et al. 2020b, fig. S8). Comparing acetylated and non-acetylated spectra reveals the presence of peaks at 1170 and 1030 cm\(^{-1}\) in the acetylated spectra, and a more pronounced 1710 cm\(^{-1}\) peak in the non-acetylated spectra, as well as a range of smaller peaks in the 1250 to 1450 cm\(^{-1}\) range (Fig. 8C). The acetylated Bosumtwi spectra are similar to those of the extant South American grasses (Dataset 1; Figs 1, 4B), with the major differences being the lack of protein-related peaks in the Bosumtwi spectra, which suggests the full removal of these during burial and/or processing. The non-acetylated Bosumtwi spectra are distinct from the (also non-acetylated) western Amazonia fossil spectra (Dataset 2; Fig. 4B) in having a much more pronounced 1710 cm\(^{-1}\) peak and a greater number of clearly identifiable peaks in the < 1450 cm\(^{-1}\) region, although both contain 1600 and 1370 cm\(^{-1}\) aromatic peaks.

**Generality across taxa**

Both the *Nitraria* (Datasets 5 and 6) and conifer (Datasets 7 and 8) pollen show large differences between the extant and fossil spectra (Fig. 9), with similar changes to those in the South American grasses (Datasets 1–3; Fig. 4B). While the spectra for the extant taxa are broadly similar, the Palaeogene *Nitraria* and conifer pollen spectra are simpler than the Miocene to Pleistocene South American grass spectra, with less clearly defined peaks. The fossil *Nitraria* spectra are broadly similar across the two samples and basins (Tarim vs Xining), but show some differences in the 1000 to 1100 cm\(^{-1}\) range (Fig. 9A).

**DISCUSSION**

Our results demonstrate a mix of promising aspects and challenges for sporopollenin chemistry-based palynology. The high classification success rate demonstrated by the extant grass taxa (Dataset 1, 98% at subfamily level for the validation dataset) indicates that sporomorph chemotaxonomy can be applied effectively with isolated sporopollenin, including with acetylated grains and larger numbers of taxa. Even reliably classifying grass pollen grains to subfamily level represents a major advance from what is possible with standard light microscopy, and our genus level results suggest that classifying to finer taxonomic levels may also be achievable (Julier et al. 2016). Our results also suggest that the photosynthetic pathways of plants may be identifiable via FTIR analysis of sporomorphs (Fig. 3B), and this would provide a new and efficient means of quantifying the relative abundance of C\(_3\) versus C\(_4\) grasses through time (Amundson et al. 1997; Loader & Hemming 2000; Nelson et al. 2006, 2008).

Nevertheless, we stress that further replication is needed across taxonomic scales, including multiple individuals within species, to gain a fuller understanding of likely classification success at genus and species level, and better assess the potential for distinguishing among C\(_3\) and C\(_4\) grasses.

The consistency of the signal through time in the Lake Bosumtwi grass pollen spectra (Dataset 4, Fig. 8), and the broad chemical similarity with the extant South American grasses (Fig. 4C), suggests that chemotaxonomy using extant calibration datasets has potential in late Quaternary settings. The late Quaternary timeframe encompasses questions regarding vegetation changes across glacial-interglacial cycles (Torres et al. 2005, 2013; Melles et al. 2012; Miller & Gosling 2014; Miller et al. 2016), human impacts on natural systems (Bush 2002; Bush et al. 2015; Loughlin et al. 2018), and the spread of agriculture (Piperno et al. 2004; Weiss et al. 2004; Fuller 2007; Crowther et al. 2016; Jardine et al. 2019), which could be addressed using a chemotaxonomic approach. In addition to increasing the taxonomic resolution of morphologically cryptic taxa (such as grasses), chemotaxonomy may allow for automated pollen counting, which is a long-standing goal in palynology (Holt et al. 2011; Holt & Bennett 2014).

In spite of the promising results demonstrated by the extant and late Quaternary datasets, considerable differences occur between the spectra of extant and fossil pollen. These differences are caused partly by the laboratory processing methods used, and partly by post-burial processes that result in the transformation of biological material into geological/palaeobotanical samples. Comparison of the acetylated and non-acetylated grass pollen from Lake Bosumtwi (Dataset 4), in which sample age and processing are otherwise identical, reveals a number of differences across the spectra (Fig. 8C). Encouragingly, the non-acetylated spectra are highly similar to published FTIR spectra of sporopollenin that has been chemically isolated for microencapsulation, for example the ‘high temperature switched treatment’ of Gonzalez-Cruz et al. (2018; see their fig. 7). This method employs treatments with acetone (overnight), orthophosphoric acid (7 days) and KOH (12 h), with interim washes with water, acetone, HCl, NaOH and ethanol, to isolate sporopollenin. This similarity suggests that the non-acetylated Bosumtwi spectra are a reasonable measurement of ‘naturally’ isolated sporopollenin before diagenetic processes have caused further changes, which provides a benchmark to compare the extant and fossil FTIR spectra against.

The spectra of acetylated extant and late Quaternary pollen (Figs 1, 4B, 8C, 9) contain prominent peaks at 1170 and 1030 cm\(^{-1}\) that are only weakly expressed in the non-acetylated material. A similar effect was demonstrated by Dominguez et al. (1998), and the presence of additional carbon compounds following acetylation was
FIG. 5. Extant and fossil grass pollen under LM and SEM. A–C, extant grains from *Paspalum fasciculatum* (subfamily Panicoideae). D–F, fossil grains from the early Miocene Santa Teresa locality in Peru. Scale bars represent: 10 μm (A, D); 5 μm (B, E); 1 μm (C, F).

FIG. 6. Western Amazonia fossil grasses. A, mean FTIR spectrum for each sample, ordered by time interval and with shaded regions showing ±1 standard deviation about the mean. B, PCA of individual spectra, with symbol style representing time period, and colours representing locality; values in parentheses provide the percentage of variance explained by each principal component.
highlighted by Amundson et al. (1997) and Loader & Hemming (2000). Amundson et al. (1997) put their presence down to the incomplete removal of glacial acetic acid or cellulose acetate following acetolysis. Domínguez et al. (1998) found that refluxing the acetolysed material in methanol removed the additional peaks, so this should be explored as an extra step in palynological processing, although it is not clear if this would remove all differences between acetolysed and non-acetolysed material (Fig. 8C). Otherwise, if the samples to be compared are all extant or late Quaternary, it will be necessary to ensure that all have undergone the same chemical treatments to make them fully comparable.

Interestingly, the effect of acetolysis was less pronounced in the fossil material. Comparing the Miocene to Pliocene dataset from western Amazonia (Dataset 2, not acetolysed) with the Miocene to Pleistocene Amazon Fan dataset (Dataset 3, acetolysed) reveals broadly similar spectra (Fig. 4C). The additional peaks that are present in the extant and late Quaternary acetolysed material are only subtly expressed in the Amazon Fan spectra. As yet, it is not clear whether this is down to different base materials (i.e. extant sporopollenin vs sporopollenin that has undergone a degree of burial across longer geological timescales) or to additional steps in the processing of the Amazon Fan samples that could have removed the impact of acetolysis. While the extant pollen grains were only acetolysed, and the Lake Bosumtwi samples were acetolysed as the last step in their processing, the Amazon Fan samples underwent heavy liquid separation after acetolysis. It is not clear how this may have impacted upon the sporopollenin chemistry, but even further additional washing steps may have removed the chemical imprint of the acetolysis.

FIG. 7. Amazon Fan fossil grasses. A, mean FTIR spectrum for each sample, ordered by core depth and with shaded regions showing ±1 standard deviation about the mean. B, PCA of individual spectra, with symbol style representing time interval, and colours representing sample; values in parentheses provide the percentage of variance explained by each principal component. Sample numbers are metres below drill floor (mbdf) sample depth.
FIG. 8. FTIR data from Lake Bosumtwi, Ghana. A, PCA sample scores from PC 1 and PC 2 plotted against time; values in parentheses provide the percentage of variance explained by each principal component. B, loadings from PC 1 and PC 2. C, mean spectra for subset of eight samples, both acetolysed and non-acetolysed; shaded regions show ±1 standard deviation about the mean.
procedure. Clearly, more work needs to be done in this area.

Beyond acetolysis, the other processing reagents used in this study may have led to inter-dataset differences. While the extant datasets were simply acetolysed, the fossil datasets were variously treated with KOH, HF, HCl, sodium pyrophosphate, Schulze reagent and heavy liquid separation, as well as acetolysis in some cases (Table 1). KOH has been shown to have a minimal impact on sporopollenin chemistry (Lutzke et al. 2020), and the same is thought to be true for HF (Domínguez et al. 1998) but the effect of the other reagents has not been explicitly tested. The Bosumtwi samples (Dataset 4) were treated with HF, HCl and KOH, and the overall similarity of the extant (Figs 1, 4) and acetolysed Bosumtwi (Fig. 8C) spectra suggests that the impact of these reagents on sporopollenin chemistry is likely to be limited. This is supported by the similarity between the non-acetolysed Bosumtwi spectra (Fig. 8C) and published FTIR spectra from sporopollenin isolated for pharmaceutical microencapsulation applications (see previous discussion and Gonzalez-Cruz et al. 2018). If correct, this suggests that the HF and HCl used in processing the fossil Nitraria (Dataset 6) and conifer (Dataset 8) pollen should also have caused minimal changes to the sporopollenin chemistry, although we cannot rule out subtle responses to these reagents.

The two samples from the Miocene Los Chorros locality in western Amazonia (Dataset 2) represent two different lithologies (clastic and lignite) and processing approaches: while both samples were processed with sodium pyrophosphate and bromoform heavy liquid separation, the lignite sample (sample 16788) had an additional processing step with the use of Schulze reagent. The pollen from both samples are chemically highly similar and plot together in ordination space (Fig. 6), which suggests that (fossil) sporopollenin chemistry is robust to Schulze reagent, although the impact of length of treatment with Schulze reagent requires further study. The effect of heavy liquid separation is harder to evaluate, but some of the subtle differences between the fossil Nitraria samples (Fig. 9A) may be the result of this procedure being used in processing the Xining sample but not the Tarim sample. However, given the short exposure time to the reagent, we view this scenario as being plausible but unlikely. The two fossil Nitraria samples are also much more similar to each other than they are to the fossil conifer sample (Fig. 9B), despite the use of heavy liquid separation with this sample as well. In summary, while more research needs to be carried out in this area, our results indicate that acetolysis leaves a relatively stronger imprint on sporopollenin chemistry, while other reagents appear to alter the sporopollenin chemical signature much less.

There are also changes in pollen chemistry that are not attributable to the processing methodology. In particular, the loss of the 1710 cm\(^{-1}\) carboxyl peak, the relative dominance of the 1600 cm\(^{-1}\) aromatic peak, and the general simplification of the spectra demonstrated in the fossil samples is more likely to be down to diagenetic processes acting on the sporopollenin chemistry. This is consistent with the finding that sporopollenin comprises a more labile component comprising carboxylic acid monomers (Watson et al. 2012), with the major differences between the extant/late Quaternary and fossil material being due to the loss or repolymerization of this labile fraction during early diagenesis. This loss is not recreated with acetolysis or burial over short-timescales (i.e. 10\(^5\) years) and/or at shallow depths (150 m for the
oldest Lake Bosumtwi samples, vs 865 m for the sample of a similar age from the Amazon Fan). Our results suggest that this repolymerization can happen relatively early once sporomorphs enter the geological record, but do not rule out the possibility that the resultant geopolymer can be altered by further diagenetic processes. Such an effect may account for the continued simplification of the spectra shown by the Palaeogene *Nitraria* and conifer pollen (Datasets 6 and 8; Fig. 9) compared to the Neogene grasses (Datasets 2 and 3; Fig. 4). These results also suggest that exceptional preservation of the kind reported by Fraser et al. (2012) is likely to be rare in the fossil record, with some level of chemical change to be anticipated even over short geological timescales.

Similar changes (i.e. the loss carboxylic acids and a concomitant reduction in the 1710 cm\(^{-1}\) C=O peak) have been recreated in thermal maturation experiments (Yule et al. 2000; Watson et al. 2012; Bernard et al. 2015), suggesting that such experiments are successfully simulating early diagenetic processes and the repolymerization of the labile sporopollenin fraction. It is not clear, however, how well they can recreate longer term changes following the formation of the sporopollenin geopolymer. Nevertheless, a similar approach with extant material is likely to provide a better match for deep time fossil specimens than is possible with acetylization (or other laboratory processing) alone, which may enable the development of calibration datasets from extant samples that can be used with fossil material. Using the chemical signature of fossil specimens with known affinities may also be viable as a means of classifying problematic fossil material. Such as approach has been used with Silurian cryptospores to demonstrate their relationship with land plants rather than marine palynomorph groups (Steemans et al. 2010), and with fossil cuticle fragments with ambiguous phylogenetic relationships (Vajda et al. 2017). Therefore, as long as a taxonomic signature is still present in deep time sporopollenin (or cuticular material), there is potential to use it for classification purposes even if it is modified from its original state.

We have taken a broad-scale exploratory approach with this study, analysing different datasets that incorporate a range of taxa, time periods and laboratory processing procedures. This has enabled us to consider a number of variables that may impact upon sporopollenin chemistry, using samples that are representative of the majority of routine palynological investigations and existing sample sets. However, it also means that separating out the impact of these different variables, and understanding how they act individually and in combination with each other, is more challenging than in a manipulative experimental setting. Further work is therefore needed to test and validate the results presented in this paper. This research should be targeted towards dedicated experimental approaches where processing procedures can be harmonized across extant and fossil datasets, and the potential controlling factors can be manipulated and tested independently of each other. This will be particularly useful for disentangling the impact(s) of different processing reagents, how they may interact in processing protocols, and whether and how they act differently on sporopollenin from extant sporomorphs vs fossil sporopollenin of different diagenetic grades. A further area of uncertainty in our analysis is to what extent ambient growing conditions, aside from variations in UVB (Lomax et al. 2008), exert a control on sporopollenin chemistry, and whether that could lead to differences among datasets. A better understanding of other potential environmental controls on sporopollenin chemistry is therefore needed, ideally both from controlled growth experiments as well as collecting from natural environmental gradients.

**CONCLUSION**

We have shown that that extant sporomorphs can be successfully classified using the chemical signature of sporopollenin. Diagenetic repolymerization of sporopollenin happens relatively soon after burial, however, meaning that chemical datasets from extant and fossil sporomorphs are challenging to integrate directly. Further differences between extant and fossil material can occur via the processing methods used to isolate sporopollenin from fresh sporomorphs. Taken together, these results show that linking extant and fossil sporopollenin chemistry datasets is not a trivial task. In late Quaternary settings sporopollenin appears chemically highly similar to extant material, suggesting great potential to integrate across datasets if the laboratory processing methods are similar. This supports the idea of using FTIR-based chemotaxonomy to increase the taxonomic resolution in morphologically cryptic groups (Julier et al. 2016; Jardine et al. 2019), and to develop automated classification systems for generating pollen counts (Zimmermann et al. 2016). For deeper time material, however, changes in sporopollenin chemistry are likely to have occurred leading to systematic differences between the extant and fossil chemical signature. Further experiments in laboratory processing and thermal maturation (Watson et al. 2012; Fraser et al. 2014a; Bernard et al. 2015; Jardine et al. 2015), applied to a range of taxa, will therefore be necessary to study the impact of geological processes on classification ability and the phylogenetic and environmental signals in sporopollenin. It remains to be seen whether spectra close to those from pre-Quaternary specimens can be recreated from extant sporomorphs, and therefore whether classification from extant training sets is feasible for deep time fossil
material. Where this is not possible, generating training sets based on fossil morphotaxa will still be of great value for assigning specimens of unknown affinity to plant lineages, which in itself may help unravel some of the key developments in land plant evolution and diversification.

Acknowledgements. We are deeply indebted to Antoine Cleef for collecting the grass sample material from Naturalis Biodiversity Center in Leiden. We also thank Annemarie Philip and Malcolm Jones for sample processing, and Caixia Wei for pollen photography. Sally Thomas and four anonymous reviewers are thanked for detailed reviews that greatly increased the quality of this paper. This research was funded by ERC grant MAGIC 649081 (GDN), and NERC grant NE/K005294/1 (WDG, WFT, BHL). BHL and WFT’s ongoing work on sporopollenin chemistry is currently supported by NERC grants NE/P013724/1 and NE/R001324/1. PEJ is currently funded by DFG grant 443701866.

DATA ARCHIVING STATEMENT

Research Data and R code associated with this article, as well as an appendix that contains the supplementary figures and tables, can be accessed at: https://doi.org/10.6084/m9.figshare.11382102

Editor. Evelyn Kustatscher

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