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Stable isotope ratios of carbon and nitrogen in pollen grains in order to characterize plant functional groups and photosynthetic pathway types

Chantal Descolas-Gros¹ and Christian Schölzel²

¹Institut des Sciences de l'Évolution (UM2-CNRS), Université Montpellier 2, Place Eugène Bataillon, Case Courrier 061, 34095 Montpellier cedex 05, France;

²Laboratoire des Sciences du Climat et l'Environnement (LSCE/CNRS) Orme des Merisiers, Bat. 701 91191 Gif-sur-Yvette, France

Summary

Author for correspondence:

Chantal Descolas-Gros

Tel: +33 467 14 49 39

Fax: +33 467 04 20 32

Email: descolas@isem.univ-montp2.fr

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- Measurements of $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and C : N ratios on modern pollen grains from temperate plants, including whole grains as well as extracted sporopollenin, were analysed in order to characterize physiological plant types at the pollen level and to determine the variation of these parameters in modern pollen grains of the same climatic area.
- Measurements are presented for 95 batches of whole modern pollen from 58 temperate species and on the stable fraction of modern pollen grains, chemically extracted sporopollenin, for two modern species. Fourier transform infrared (FTIR) and cross-polarization and magic-angle spinning (CP/MAS) sporopollenin spectra were conducted in parallel.
- C_3 and C_4 plants can be separated by $\delta^{13}\text{C}$ measurements based on pollen. Probabilistic assignments to plant functional groups (herbaceous, deciduous woody, evergreen woody) of C_3 plants by the means of a discriminant analysis can be made for C : N ratios and for $\delta^{13}\text{C}$. The results are related to other studies on sporopollenin in order to use this method in future work on fossil samples.
- Stable isotope measurements on pollen allow improved pollen diagrams, including forms that cannot be differentiated at species level, increasing the accuracy and resolution of plant physiological type distribution in quaternary and older fossil sediments.

Key words: Fourier transform infrared (FTIR), functional groups, photosynthetic pathway, pollen, sporopollenin, stable isotopes.

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Introduction

The focus of this paper is on the question of how well stable isotope measurements on pollen grains can be related to plant functional groups and plant photosynthetic pathways. Variations in climatic parameters, such as dry-season duration, rainfall, surface temperature, and atmospheric O_2 and CO_2 concentrations, can be addressed through the geographical distribution of plant photosynthetic pathways: C_3 , C_4 ,

Crassulacean acid metabolism (CAM) (Sage *et al.*, 1999). The origin of C_4 and CAM plants, the induction of their metabolism in relation to climatic factors, and their subsequent successful expansion are important scientific challenges, which allow the improvement and validation of climate models (Ackerly & Monson, 2003; Keeley & Rundel, 2003; Monson, 2003). A simple and widely used method to scale plant physiological responses to environmental conditions (Smith *et al.*, 1997) is given by plant functional

types. They can be briefly described as groups of plant taxa with similar biological characteristics such as leaf form, habit, phenology, or bioclimatic range, as well as plant photosynthetic pathways. The exact classification, that is, the number of plant functional types, varies with the respective application. There are several applications that use the concept in order to introduce further classifications such as biomes (Prentice & Webb, 1998). Thus, the general concept of plant functional types is well developed and there are numerous applications that show their relevance in climate research, especially the forward modelling of vegetation, as well as palaeoclimate reconstructions (Prentice *et al.*, 1992, 1996; Williams *et al.*, 1998; Schölzel, 2006).

An important question is how to assign plant functional types to proxy data, for example, fossil pollen. This is well understood as far as fossil pollen spectra or macro remains are concerned. Therefore, stable isotope measurements on pollen give information that adds new insight to existing approaches. The background is that the enzyme that catalyses initial CO₂ fixation in C₃ plants produces large isotopic fractionation between inorganic and particulate carbon. The δ¹³C mean value is around -12‰ for C₄ plants and -26‰ for modern C₃ plants. C₄ plants show a limited range of variation (-10 to -14‰), while C₃ plants show a much larger range: -20 to -35‰ (Brugnoli & Farquhar, 2000). C₃ plants may then be distinguished from C₄ plants by the δ¹³C values of their organic carbon (Dawson *et al.*, 2002). Within the group of CAM plants, the C₃ and C₄ cycles are temporally separated, resulting in δ¹³C values between those for C₃ and C₄ plants, and they are therefore not considered in this work. Regarding pathways and plant functional types, C₃ plants include many plant types, whereas only herbs and a few shrubs are found in the C₄ group. Most studies on this subject use leaf characteristics (evergreen, deciduous) and associated leaf traits, wood density or seed size (Wright *et al.*, 2005; Westoby & Wright, 2006). These traits reflect different plant ecological strategies, especially soil water availability and associated abiotic factors (Ackerly & Cornwell, 2007). The δ¹³C of leaf carbon has been tested for the influence of plant types and is related to carbon and water fluxes within the corresponding ecosystem (Smedley *et al.*, 1991; Brooks *et al.*, 1997). Studies on plant types from palaeovegetation used wood characters to separate deciduous from evergreen trees and shrubs (Falcon-Lang, 2000; Poole & Van Bergen, 2006) and the mechanical architecture of growth forms (Rowe & Speck, 2005).

The resolution and accuracy of isotopic data may be improved if measurements are made on specific plant biomarkers that are stable through geological times (De Leeuw *et al.*, 1991), such as wood cellulose (Schleser *et al.*, 1999), phytoliths (Alexandre *et al.*, 1997; Scott, 2002), cuticles (Beuning & Scott, 2002; Wooler, 2002), long-chain leaf waxes (Huang *et al.*, 1999) and pollen (Amundson *et al.*, 1997; Beerling & Jolley, 1998; Loader & Hemming, 2000, 2001, 2004; Descolas-Gros *et al.*, 2001; Jahren, 2004). The latter is especially

interesting because it contains a particularly resistant biopolymer (sporopollenin), the exine part of pollen grain, which is insoluble in organic solvents and well preserved in sediments (Van Bergen *et al.*, 1995). Stable isotope ratio measurements on sporopollenin of pollen grains therefore appear to be a powerful and accurate tool for comparing the impact of climatic factors on modern and fossil distribution of physiological plant types. It is an independent proxy with a time constant shorter than vegetation biomass modifications (Loader & Hemming, 2004). However, no precise and reliable calibration yet exists that could be useful for both modern and fossil material.

In this study, stable carbon (δ¹³C) measurements on modern pollen grains from temperate plants (whole grains and extracted sporopollenin) were combined with stable nitrogen (δ¹⁵N) measurements and C : N ratios, with the following specific objectives: to validate the characterization of physiological plant types at the pollen level; and to determine the variation of these parameters in modern pollen grains of the same climatic area (the temperate zone) and on functional types within the C₃ plants. Since this is the first study in this direction, only the major groups, herbaceous plants, deciduous trees and evergreen trees, are considered.

Materials and Methods

Pollen

Pollen was collected from plants in the temperate zone. Most of them come from an area of 60 km² around Montpellier (south of France). Not all of them are native plants of this area and some of them are cultivated plants. The batches used are monospecific and individually checked, and only those containing > 99% of pollen grains of the species named were kept. For eight of these species, a number of different sampling batches were measured to study intraspecific variability. Each batch was made up by sampling pollen grains from one to 10 individual plants. For two species, several batches were sampled over a week in the same year on different individual plants in a small area around Montpellier in order to study regional-scale variations. A total of 95 batches are studied, corresponding to 58 different species (Supplementary material, Table S1). For the analysis of results, species are grouped into four categories: C₄ plants (all herbaceous), and three groups of C₃ plants, herbs (including Poaceae), deciduous woody plants, and evergreen woody plants.

Sporopollenin extraction

The future application of this work is in the analysis of fossil pollen where intine and cytoplasm of pollen grains are not preserved during fossilization and only the exine, composed of sporopollenin, remains. To isolate sporopollenin, two treatments were carried out: (i) acetolysis (Erdtman, 1960),

followed by repeated rinsing with distilled water and dehydration in an alcohol gradient as described by Descolas-Gros *et al.* (2001); and (ii) H_2SO_4 treatment (8 h), following the protocol described by Loader & Hemming (2000), followed by rinsing with distilled water and drying for 24 h. The C : N, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ parameters were measured on the resulting sporopollenin. Two pollen batches were used, one from a C_3 plant (*Platanus* sp.), and one from a C_4 plant (*Sorghum* sp.).

Stable isotope measurements $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and C : N values were calculated by simultaneous measurements on a Finnigan Delta spectrometer coupled to an elemental analyser. The values were obtained by using the following equation:

$$\delta^{13}\text{C} (\text{‰}) = ((R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}) \times 10^3$$

(R , $^{13}\text{CO}_2/^{12}\text{CO}_2$ value).

Spectroscopic analysis

Sporopollenin structures, after the different chemical treatments of the pollen grains, are compared by two solid spectroscopic methods: Fourier transform infrared (FTIR) spectroscopy and ^{13}C cross-polarization and magic-angle spinning nuclear magnetic resonance (^{13}C CP/MAS NMR) spectroscopy. FTIR results are obtained, using a Perkin-Elmer spectrometer. Samples were prepared by the KBr method; spectra have been extracted in the wave region of $370\text{--}4000\text{ cm}^{-1}$ and are expressed as transmittance (%). ^{13}C CP/MAS NMR spectroscopy was done with a Bruker spectrometer. Chemical shifts are expressed in parts per million (ppm).

Data analysis

In order to study the dependence of the C : N ratio, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ on plant functional group, three categories of C_3 plants were selected: (i) herbaceous including Poaceae; (ii) deciduous trees; and (iii) evergreen trees (Table S1). A finer subdivision, especially with respect to the known definition of plant functional types (PFTs) as given in, for example, Barboni *et al.* (2004) or Prentice *et al.*, 1992), would be interesting but is not possible because of the comparably small number of measurements and the limited geographic area. The differences among the groups were tested by a one-way analysis of variance (ANOVA). To assign measurements of the parameters (C : N ratio, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) to one of the plant functional groups, that is, to make forward predictions, a quadratic discriminant analysis (QDA) has been applied. The result is a set of posterior probabilities for each group given a certain state of the parameters. The quality of these QDA predictions is tested by the probability of detection (POD) of the correct group, which is defined as the number of classes correctly predicted divided by the total number of observations. To obtain independent estimates of the POD, a leave-one-out cross-validation (CV) is used.

Results

Observed variation of measured C : N, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of plant functional groups

For the estimation of the methodological variations, measurements were made in triplicate on three subsamples within the same pollen batch for two different species. Standard deviation for measurements and subsampling within the same species batch are not more than 1 for C : N ratios, 0.5‰ for $\delta^{13}\text{C}$ and 1.5‰ for $\delta^{15}\text{N}$. The results were analysed from two different points of view. First, the C : N, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are discussed on the basis of a single species. Second, the same parameters are analysed for three functional groups (herbs, deciduous trees and evergreen trees) by ANOVA and QDA.

The C : N weight ratios vary from 5 (*Sambucus nigra*) to 87 (*Cupressus arizonica*), while the C : N values for different batches of a given C_3 species appear to be very similar (Fig. 1). *Cupressus sempervirens* is an exception, as the range is between

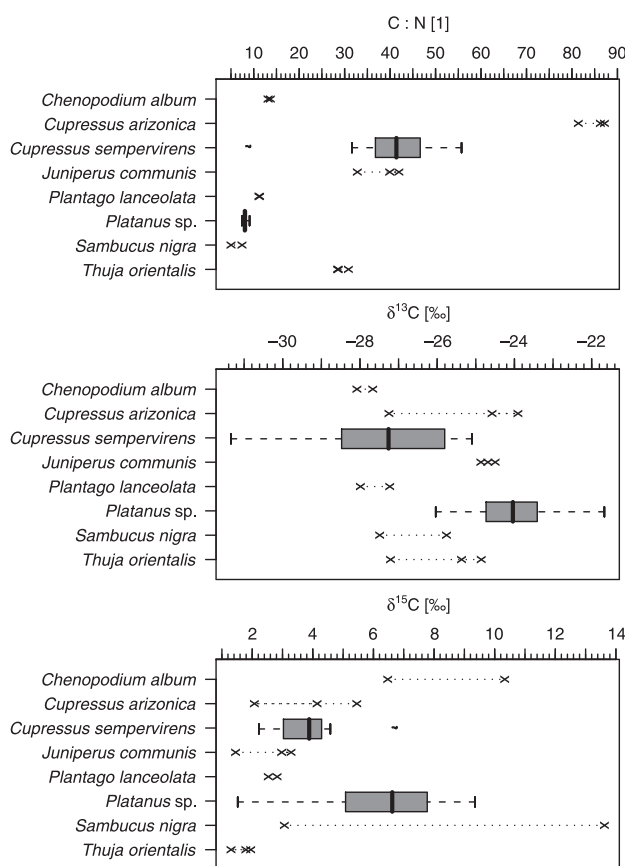


Fig. 1 Measurements of the C : N ratio, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ for modern whole pollen grains in different batches of C_3 species. Standard box-and-whisker plots are given for *Cupressus sempervirens* (11 measurements) and *Platanus* sp. (19 measurements), describing median, upper and lower quartiles, and smallest and largest observations or $1.5 \times \text{IQR}$ (interquartile range) and outliers, respectively.

Table 1 Results of the one-way ANOVA for C : N ratio, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, carbon content and nitrogen content for three physiological groups of C_3 species: herbaceous including Poaceae (A); deciduous trees (B); and evergreen trees (C) (also see Table S1)

Parameter	$F_{2,89}$	P -value	μ_A	μ_B	μ_C
C : N [1]	47.34	9.89×10^{-15}	12.11	8.68	36.55
$\delta^{13}\text{C}$ [‰]	18.85	1.49×10^{-07}	-26.74	-24.55	-26.06
$\delta^{15}\text{N}$ [‰]	3.18	0.046	3.83	4.52	2.71
C [1]	4.39	0.015	45.74	46.99	44.46
N [1]	99.06	2.20×10^{-16}	3.87	5.66	1.86

nine and 56. For this species, nitrogen weight content variability is greater than for other species in this study (Table S1). Nevertheless, the relative stability of values between the different batches for a species confirms the conservative behaviour of this parameter. The $\delta^{13}\text{C}$ values for modern pollen organic carbon range from -31.3‰ (*C. sempervirens*) to -10‰ (*Sorghum vulgare* and *Zea mays*). C_3 plant pollen values range between -31.3 and -21.6‰ , while C_4 plant pollen range between -15.9‰ and -10‰ (Table S1). The difference between the highest and the lowest $\delta^{13}\text{C}$ values is up to 6‰ for *C. sempervirens*, 4‰ for *Platanus* sp., and 3‰ for *C. arizonica* and *Thuja orientalis* (Fig. 1). Values of $\delta^{15}\text{N}$ are between -3.6‰ for *Fagus sylvatica* and 13.6‰ for *Sambucus nigra*. Large differences between batches of the same species were observed for this parameter, with ranges of up to 10‰ for *S. nigra* and 8‰ for *Platanus* sp. (Fig. 1). Within the C_4 group, the $\delta^{15}\text{N}$ mean value (6.9‰) is higher than for C_3 plants (2.9‰). This result needs to be considered with caution, as very few C_4 species were sampled in this study. Owing to the small number of measurements, C_4 plants are not included in the following statistical analyses. Nevertheless, it should be pointed out that the $\delta^{13}\text{C}$ values for C_4 plants are clearly outside of the ranges of the other three functional groups (Fig. 2).

The ANOVA results for the three functional groups are given in Table 1 and the corresponding boxplots in Fig. 2. Mean C : N values are significantly different among the three functional groups ($F_{2,89} = 47.34$, $P < 0.001$). The mean value for evergreen trees (36.55) is greater than for deciduous trees (8.68) and for herbaceous plants and Poaceae (12.11). For example, C : N values > 20 are found only in the evergreen trees group. These differences are linked to variations in nitrogen rather than in carbon (Table 1, Fig. 2). The variations in the $\delta^{13}\text{C}$ values are less extreme than for the C : N ratio, but still significant at the 0.001 level. The group mean value is highest for deciduous trees (-24.55‰) and more similar for herbs (-26.74‰) and evergreen trees (-26.06‰). The group means of the $\delta^{15}\text{N}$ values vary between 2.71 and 4.52‰ and are significant at the 0.05 (5%) level. The widest variation, including negative values for pollen, is observed for trees, especially the deciduous group. The values for herbaceous plants and Poaceae are all positive (3.83‰) and the range of values is smaller than for tree species.

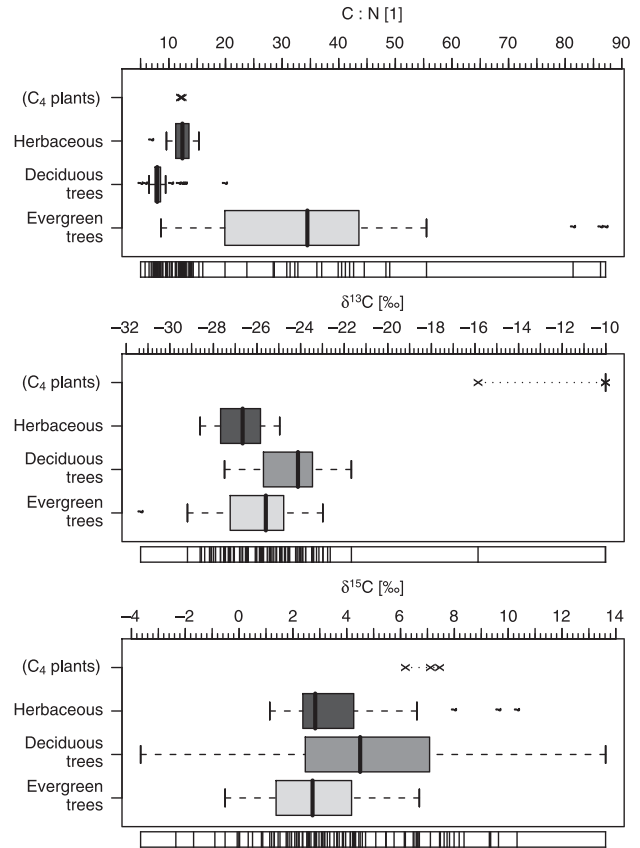


Fig. 2 Variation ranges for modern whole pollen grains of three groups of C_3 species: herbaceous including Poaceae, deciduous trees, and evergreen trees (also see Table S1). Additionally, direct measurements for the three C_4 plants are given. See Fig. 1 for an explanation of the boxplots. The bars on the right-hand side of the plot show the distribution of the direct measurements.

For the QDA, results for each of the three groups are given in terms of the posterior probability (Fig. 3). For the C : N ratio, the QDA the cross-validation estimate of the POD (see earlier) is 0.82, which allows relatively good classifications for the three functional groups. For evergreen trees, the posterior probability converges to one for C : N going to -8 , which is an artificial effect and quite typical for quadratic discriminant functions. For the naturally positive range of C : N ratios, this does not affect the QDA predictions. The classification of each group by $\delta^{13}\text{C}$ is less precise than for C : N, but is still possible with a CV-estimated POD of 0.55 for the correct class. With a POD of 0.43, the $\delta^{15}\text{N}$ has a low predictability for the correct group and is therefore not shown.

In addition to the univariate QDA, a bivariate QDA has been applied to the C : N ratio and the $\delta^{13}\text{C}$. The bivariate posterior probability for each of the three groups is given in Fig. S1. It is included for the sake of completeness, but we do not want to overemphasize the results. The sample size is comparably small and the POD only increased to 0.85, compared with 0.82 for the C : N ratio in the univariate case.

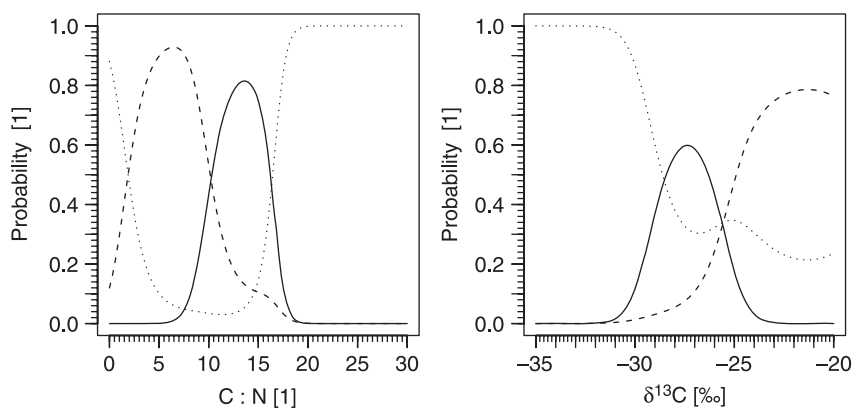


Fig. 3 The results of the quadratic discriminant analysis (QDA) for C : N ratio and $\delta^{13}\text{C}$ applied to the three functional groups: herbaceous including Poaceae (solid line), deciduous trees (dashed line) and evergreen trees (dotted line). The plots show the posterior probability for each group.

Sporopollenin extracted from whole modern pollen grains

The use of $\delta^{13}\text{C}$ organic carbon variability in studying the processes responsible for CO_2 fixation by plants and physiological type characterization, not only for the modern period, but also over geological time, requires measurements only on the stable part of pollen grains preserved within sediments (sporopollenin). To extract sporopollenin, acetolysis and H_2SO_4 treatments are used on pollen grains of a C_3 species (*Platanus* sp.) and a C_4 species (*Sorghum* sp.). Acetolysis produces an important lowering of the $\delta^{13}\text{C}$ value (-5‰ for *Sorghum* sp. and -2‰ for *Platanus*). This decrease is smaller with the H_2SO_4 treatment (only -1‰) (Table 2).

For the two species studied, spectra obtained by two spectroscopic methods, FTIR and CP/MAS, were compared to examine the molecular components of pollen grains and sporopollenin (Fig. S2). Changes in the FTIR spectra between untreated and treated samples are observed between 1100 and 1200 cm^{-1} . This may reflect the removal of polysaccharides for the treated samples (Fig. S2). The ^{13}C CP/MAS spectra for the two treatments show differences between the two species; the spectrum obtained with H_2SO_4 on *Sorghum* sp. does not show the presence of cellulose ($\Delta 55\text{--}110\text{ ppm}$) (Fig. S2). All results show aliphatic and aromatic structures in differing amounts. When the nitrogen content of sporopollenin is considered, some nitrogen is detected (Table S1). The occurrence of nitrogen in isolated sporopollenin is a subject of controversy (discussed in the following section)

Discussion

Whole pollen grains (untreated)

Pollen synthesis is linked to gametogenesis and secondary metabolism (Wiermann *et al.*, 2001). Secondary isotopic fractionation linked to metabolic processes should be integrated, resulting in masking of the differences between C_3 and C_4 plants (Brugnoli & Farquhar, 2000; Hobbie & Werner,

2004). Our results clearly show that this is not the case and that it is possible to differentiate the various photosynthetic pathways in modern plants, at the pollen level, using $\delta^{13}\text{C}$ data (Table S1). The values obtained are within the respective ranges generally observed for C_3 and C_4 plants. They confirm the results obtained by Amundson *et al.* (1997), as well as those of Loader & Hemming (2000) on Poaceae pollen. Most of the isotopic data obtained on modern plants have been measured in leaves, where major isotopic fractionation takes place because of the different CO_2 fixation processes. The $\delta^{13}\text{C}$ variability observed in this study is in the same range as those for leaves (for an individual plant) in relation to the leaf position, their physiological state, incident light (Berry *et al.*, 1997; Guehl *et al.*, 1998) and re-assimilation of respired CO_2 (Schleser & Jayasekera, 1985). Jahren (2004) found that the carbon isotope difference between pollen and leaf tissue values and between pollen and stem values within the same species is $\pm 3\text{‰}$.

In this study, the C : N data range for pollen is smaller and narrower (Fig. 1) than most data obtained on other plant parts. The largest variations appear to be between the different parts of the plants (Meyers & Ishiwatari, 1995; Melillo, 1996). The nature of the plant constituent from which the C : N-values are obtained appears to have an important influence: C : N ratios for lignin and cellulose are higher than our data on pollen, which is principally composed of sporopollenin (Brugnoli & Farquhar, 2000). Measurements of pollen grain C : N ratios add to the knowledge of organic sources found in lake sediments, in discriminating aquatic from land sources or anthropogenic effects.

The observed $\delta^{15}\text{N}$ pollen values are in the same range as those quoted in the literature for plants from various ecosystems (Figs 1, 2). The lowest values are generally interpreted as characteristic of plants growing on soils depleted in ^{15}N (Shearer & Kohl, 1989; Handley *et al.*, 1999). The measured $\delta^{15}\text{N}$ value for plants represents an integration of the various sources of plant nitrogen, as well as the different mechanisms of N uptake and metabolism. It is especially valuable in characterizing root uptake of inorganic nitrogen (NH_4^+ , NO_3^-),

Table 2 Literature data on $\delta^{13}\text{C}$ values for whole pollen grains, sporopollenin and calculated Δ obtained after different chemical treatments on various pollen species

Species	Whole pollen grains	Sporopollenin	Δ	Treatment	References
<i>Zea mays</i> C ₄	-10.45	-18.44	-7.99	Erdtman	Loader & Hemming (2000)
<i>Populus trichocarpa</i> C ₃	-23.97	-33.27	-9.3	Erdtman	Loader & Hemming (2000)
<i>Sorghum vulgare</i> C ₄	-10.02	-14.6	-4.58	Erdtman modif	This work
<i>Platanus</i> sp. C ₃	-23.16	-25.04	-1.88	Erdtman modif	This work
<i>Zea mays</i> C ₄	-12.1	-14.4	-2.3	KOH + Erdtman	Amundson <i>et al.</i> (1997)
<i>Cynodon dactylon</i> C ₄	-13.7	-17.6	-3.9	KOH + Erdtman	Amundson <i>et al.</i> (1997)
<i>Hilaria cenchroides</i> C ₄	-16.5	-18.9	-2.4	KOH + Erdtman	Amundson <i>et al.</i> (1997)
<i>Orcuttia californica</i> C ₄	-15.3	-23	-7.7	KOH + Erdtman	Amundson <i>et al.</i> (1997)
<i>Pennisetum clandestinum</i> C ₄	-18.2	-28.8	-10.6	KOH + Erdtman	Amundson <i>et al.</i> (1997)
<i>Opizia stolonifera</i> C ₄	-18.8	-25.5	-6.7	KOH + Erdtman	Amundson <i>et al.</i> (1997)
<i>Lolium multiflorum</i> C ₃	-25.1	-27.5	-2.4	KOH + Erdtman	Amundson <i>et al.</i> (1997)
<i>Lolium multiflorum</i> C ₃	-27.5	-30.1	-2.6	KOH + Erdtman	Amundson <i>et al.</i> (1997)
<i>Zea mays</i> C ₄	-10.45	-12.26	-1.81	H ₂ SO ₄	Loader & Hemming (2000)
<i>Populus trichocarpa</i> C ₃	-23.97	-27.52	-3.55	H ₂ SO ₄	Loader & Hemming (2000)
<i>Pinus sylvestris</i> C ₃	-27.39	-29.57	-2.18	H ₂ SO ₄	Loader & Hemming (2000)
<i>Sorghum vulgare</i> C ₄	-10.02	-11.2	-1.18	H ₂ SO ₄	This work
<i>Platanus</i> sp. C ₃	-23.16	-24.07	-0.91	H ₂ SO ₄	This work
<i>Cedrus deodara</i> C ₃	-27	-26.21	0.79	NaOH	Jahren (2004)
<i>Pinus coulteri</i> C ₃	-29.4	-28.18	1.22	NaOH	Jahren (2004)
<i>Pinus echinata</i> C ₃	-30.2	-28.65	1.55	NaOH	Jahren (2004)
<i>Pinus rigida</i> C ₃	-30.5	-29.54	0.96	NaOH	Jahren (2004)
<i>Cedrus deodara</i> C ₃	-27	-27.15	-0.15	H ₂ O ₂	Jahren (2004)
<i>Pinus coulteri</i> C ₃	-29.4	-29.57	-0.17	H ₂ O ₂	Jahren (2004)
<i>Pinus echinata</i> C ₃	-30.2	-30.62	-0.42	H ₂ O ₂	Jahren (2004)
<i>Pinus rigida</i> C ₃	-30.5	-31.18	-0.68	H ₂ O ₂	Jahren (2004)
<i>Cedrus deodara</i> C ₃	-27	-27.11	-0.11	Schulze modif	Jahren (2004)
<i>Pinus coulteri</i> C ₃	-29.4	-29.44	-0.04	Schulze modif	Jahren (2004)
<i>Pinus echinata</i> C ₃	-30.2	-30.46	-0.26	Schulze modif	Jahren (2004)
<i>Pinus rigida</i> C ₃	-30.5	-31.02	-0.52	Schulze modif	Jahren (2004)
<i>Agropyron repens</i> C ₃	-24.1	-27.1	-3.0	HCl + KOH + HF + H ₂ SO ₄	Nelson <i>et al.</i> (2006)
<i>Bromus inermis</i> C ₃	-24.5	-25.5	-1.4	HCl + KOH + HF + H ₂ SO ₄	Nelson <i>et al.</i> (2006)
<i>Sorghum halapense</i> C ₄	-11.4	-12.6	-1.2	HCl + KOH + HF + H ₂ SO ₄	Nelson <i>et al.</i> (2006)
<i>Sorghum vulgare</i> C ₄	-9.2	-12.9	-3.7	HCl + KOH + HF + H ₂ SO ₄	Nelson <i>et al.</i> (2006)
Pollen concentrate		-30.39 to -29.49		(Unknown)	Eriksson <i>et al.</i> (1996)
Pollen concentrate		-28 to -23		(Probably Schulze)	Beerling & Jolley (1998)

nitrogen fixation in symbiotic systems (mycorrhiza), use of organic nitrogen sources (urea, amino acids) and use of atmospheric N₂ (Virginia *et al.*, 1989; Yoneyama *et al.*, 1998). Handley *et al.* (1999) show that foliar $\delta^{15}\text{N}$ also appears to be related to rainfall.

The parameters ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, C : N) measured on whole pollen grains appear to be sufficiently reproducible in the same climatic and geographical area within the same year (Table 2) to use them as proxies for climatic reconstruction purposes, corroborating the findings of Loader & Hemming (2004). On a latitudinal gradient, they show the relationship between temperatures 4 and 6 wk immediately before pollen release and $\delta^{13}\text{C}$ values for *Pinus* sp. pollen grains. The separation and analysis of individual genera from modern pollen assemblages will be a necessary first step if robust environmental correlations are to be quantified with precision.

Finally, for the pollen traits used in this study, herbaceous plants appear to be more similar to evergreen trees than to deciduous trees (Fig. 2). Ehleringer & Cooper (1988) found a negative correlation between plant longevity and discrimination of leaf carbon isotope in desert plants. Long-lived species have a greater probability of experiencing severe drought and have to employ more conservative water-use techniques to survive. The observed differences can be explained by foliage longevity, especially for evergreen trees. The timescales of the environmental parameters, which are integrated in these two plant parts (leaves and pollen), are different. Pollen of evergreen and deciduous trees integrate short timescales, relative to the duration of pollen synthesis and development. As a consequence, different root systems and local soil water availability also contribute to the observed differences (Schenk & Jackson, 2002). The pollen traits considered in this study

therefore appear to be well adapted for the study of trait shifts along gradients of environmental factors and for the study of variation among co-occurring species (Ackerly & Cornwell, 2007).

Sporopollenin extracted from whole modern pollen grains

In the literature, few studies compare results from the same samples on whole pollen grains and sporopollenin after chemical treatment. In this case, the largest $\delta^{13}\text{C}$ shifts are observed with acetolysis (up to 10‰). The observed shifts are smaller than those generally mentioned using this method (Table 3). Alcohol dehydration treatment, used after acetolysis, appeared to reduce the shift in values, corroborating previous observations made by Dominguez *et al.* (1998) and Ahlers *et al.* (2000). In all studies, H_2SO_4 treatment produces a smaller decrease in $\delta^{13}\text{C}$ values than acetolysis. The amplitude of the shift is not the same for different species and varies with treatments. Nevertheless, values obtained on pollen exine remain in the range for C_3 and C_4 plants (Table 3). To interpret these variations between results, it is necessary to know if stable isotope measurements have been performed on the same structural molecule (after the different chemical treatments of the pollen grains) and if these treatments induce chemical alterations of this polymer or not. The quality and reproducibility of stable isotope data depend on the answers obtained.

What is the sporopollenin structure? Unfortunately, data on sporopollenin structure have been obtained using different chemical treatments and protocols (Rorrer *et al.*, 1990; Tarlyn *et al.*, 1993; Shaw & Apperley, 1996; Dominguez *et al.*, 1998; Wiermann *et al.*, 2001), making comparisons between results difficult. The exact molecular structure of sporopollenin remains unclear and is complicated by the fact that solubilization of this polymer is problematic. Only solid spectroscopic methods may be used routinely to obtain data on the molecular structure (Table 3).

Until recently, it has been generally believed that the structural units of this complex polymer are carotenoid or carotenoid esters. Later investigators have indicated that carotenoids do not contribute to sporopollenin. ^{13}C -NMR solid-state studies show that the main building blocks are oxygenated aromatics and aliphatics originating from the phenylpropanoid pathway (Meuter-Gerhards *et al.*, 1995; Guilford *et al.*, 1988; Wehling *et al.*, 1989; Willmesmeier *et al.*, 1993). These findings are corroborated by tracer biosynthesis (Gubatz & Wiermann, 1992, 1993; Gubatz *et al.*, 1993; Meuter-Gerhards *et al.*, 1999), and the occurrence of aromatic groups was confirmed by pyrolysis GC techniques (Van Bergen *et al.*, 1993). *p*-Coumaric acid appears to be one of the constituents of sporopollenin (Wehling *et al.*, 1989). Degradation experiments and immunocytochemical studies show that phenols are also

integral compounds (Schulze Osthoff & Wiermann, 1987; Niester-Nyveld *et al.*, 1997). All the different components appear to have a tendency toward self-assembly (Collinson *et al.*, 1993; Hemsley *et al.*, 1996, 1998, 2004; Thom *et al.*, 1998; Gabarayeva & Hemsley, 2006). For instance, there is no clear molecular sporopollenin reference pattern that may be characterized by a single technique and it is not known which part of this molecule is similar among species. To our knowledge, no study has attempted to link structural modifications with the $\delta^{13}\text{C}$ values obtained.

The FTIR spectra obtained in this study (Fig. S2) have similar trends as those previously obtained (Wiermann *et al.*, 2001; Willmesmeier *et al.*, 1993; Meuter-Gerhards *et al.*, 1995). They confirm the importance of complete cellulose extraction, as previously demonstrated with calcofluor fluorescence (Dominguez *et al.*, 1998; Loader & Hemming, 2000). To visualize the effects of chemical artefacts induced by acetolysis treatment and control the complete extraction of sporopollenin, spectra should be obtained after different treatment times in order to follow the evolution of the various peaks. Cellulose residue is probably not the only contaminant, as complex mixtures of waxes, including hydrocarbonate, accumulate on and in the exine (Gubatz *et al.*, 1998). Another problem linked to sporopollenin structure is the occurrence of nitrogen as an elemental constituent of this macromolecule. Ahlers *et al.* (2000) detected some nitrogen by elemental analysis and XPS measurements, but considered this result an artefact, corroborating the previous results of Jungerferman *et al.* (1997). Kawase & Takahashi (1995) found similar results. Nitrogen may be the result of the presence of residual amino acids, as protein is associated with exine (Chay *et al.*, 1992), and/or contamination by solvents. If the fact that the sporopollenin molecule has no nitrogen can be clearly confirmed, following C : N ratios of samples may be an additional means to control the extraction procedure for this molecule.

In all cases, this does not prevent examination of the structural characteristics of the molecule in order to control the chemical interactions with the solvents. Our data show that it is necessary to associate stable isotope measurements with control of the polymer structure. Further work is needed on the effects of the different chemical treatments on the structure of this polymer, so as to ensure that the differences between $\delta^{13}\text{C}$ values do not reflect different extraction amounts.

Further suggestions for the use of fossil pollen To adequately characterize physiological types of terrestrial plants preserved in sediments as palaeorecords, it is important to control the effect of chemical treatments in extracting sporopollenin from the sediment. The same problems were encountered in previous comparisons between whole pollen grains and sporopollenin, and they are complicated by the fact that the solvents used to extract pollen from various sediment types are different (HCl for calcareous, HF for siliceous and HNO_3 for lignites). On modern pollen, Jahren (2004) used three chemical treatments,

Table 3 Bibliographic data on sporopollenin structure obtained by solid spectrometric methods, isolation and purification procedure

Isolation and purification procedures	Structure control	Species	Comparisons with:	References
Enzymatic hydrolysis Phosphoric acid	FTIR ¹³ C NMR	<i>Pinus mugo</i> <i>Triticum aestivum</i> , <i>Pinus thumberghiana</i> , <i>Zea mays</i> , <i>Ambrosia trifida</i>	<i>Chlorella</i> , <i>Lycopodium</i>	Schulze Osthoff <i>et al.</i> (1987) Guilford <i>et al.</i> (1988)
Acetolysis/acetone	¹³ C NMR	<i>Pinus sylvestris</i> <i>Dioon edule</i> , <i>Typha angustifolia</i> , <i>Betula pubescens</i> , etc.	Fossils	Hemsley <i>et al.</i> (1992, 1993, 1995, 1996)
Phosphoric acid (50°C)	FTIR, ¹³ C NMR	<i>Cycas circinalis</i> , <i>Typha angustifolia</i> , <i>Tulipa</i>	Spores (Bryophyta, Pteridophyta)	Willmesmeier <i>et al.</i> (1993)
Acetolysis Solubilization (2-aminoethanol)	XPS, FTIR, UV-VIS	<i>Magnolia grandifolia</i> , <i>Hibiscus syriacus</i>		Kawase & Takahashi (1995)
Methanol, chloroform, acetone, ethylene glycol, phosphoric acid	FTIR, ¹³ C NMR	<i>Cucurbita Tulipa</i>	Comparison between development stages	Meuter-Gerhards <i>et al.</i> (1995)
Hot methanol, chloroform/hot KOH and H ₂ SO ₄	C-NEXAFS	<i>Lycopodium clavatum</i>	Fossil material (coal)	Cody <i>et al.</i> (1996)
Hot phosphoric acid, hot H ₂ SO ₄	¹³ C NMR	<i>Lycopodium clavatum</i>	Comparison between treatments	Shaw and Apperley (1996)
Enzymatic hydrolysis	FTIR, ¹³ C NMR	<i>Typha angustifolia</i>	Reaggregated sporopollenin	Jungfermann <i>et al.</i> (1997)
Anhydrous hydrogen fluoride in pyridine, acetolysis and acetolysis/methanol	FTIR	<i>Betula alba</i> , <i>Ambrosia eliator</i> , <i>Zea mays</i> , <i>Pinus pinaster</i>	Untreated pollen grains	Dominguez <i>et al.</i> (1998)
Enzymatic hydrolysis, extraction using solvent of increasing polarity, solubilization (2-aminoethanol)	¹ H NMR	<i>Typha angustifolia</i>		Ahlers <i>et al.</i> (1999a)
Enzymatic hydrolysis, extraction using solvent of increasing polarity, solubilization (2-aminoethanol)	¹ H NMR	<i>Torreya californica</i>	<i>Typha angustifolia</i>	Ahlers <i>et al.</i> (1999b)
Enzymatic hydrolysis, extraction using solvent of increasing polarity without acetone use	FTIR, ¹³ C NMR, XPS	<i>Typha angustifolia</i>	Peracetylated sporopollenin	Ahlers <i>et al.</i> (2000)
Hot treatment	FTIR	<i>Lycopodium clavatum</i>	Fossils	Yule <i>et al.</i> (2000)
Enzymatic hydrolysis, extraction using solvent of increasing polarity without acetone use	FTIR	<i>Pinus mugo</i> , <i>Typha angustifolia</i> , <i>Torreya californica</i> , <i>Taxus baccata</i> , <i>Cedrus atlantica</i>		Wiermann <i>et al.</i> , 2001)
Methanol/HCl 0.1 N (70°C), BaCO ₃ different times	FTIR, ¹³ C NMR, XPS	<i>Typha angustifolia</i>	Untreated pollen grains between treatments	Bubert <i>et al.</i> (2002)
Acetone, 5 M NaOH, 3 M HCl 2-aminoethanol (80°C)	MALDI-TOF, MS, ¹ H NMR	<i>Pinus sylvestris</i> , <i>Selaginella</i> <i>pallescens</i> , <i>Lycopodium clavatum</i>	GC-MS	Moore <i>et al.</i> (2006)

FTIR, Fourier transform infrared; NMR, nuclear magnetic resonance; XPS, X-ray photoelectron spectrometry; UV-VIS, UV-visible spectroscopy (UV, ultra violet); C-NEXAFS, carbon-near edge X-ray absorption fine structure; MALDI-TOF, matrix-assisted laser desorption/ionization – time of flight mass spectrometry; GC-MS, gas chromatography-mass spectrometer.

which have frequently been employed to extract pollen from rocks and measure $\delta^{13}\text{C}$. The most common treatment (HF + HCL) does not affect $\delta^{13}\text{C}$ values, but no studies on the resulting sporopollenin structure are shown. The occurrence of a diagenetic effect on sporopollenin during fossilization and its effect on $\delta^{13}\text{C}$ values is a further problem. Diagenetic processes can lead to carbon loss via various chemical transformations of the biomolecule, such as the removal of functional groups or depolymerization, or selective preservation of some resistant constituents of organic matter relative to more degradable compounds (Hedges & Prahl, 1993; Hemsley *et al.*, 1993; Fogel & Tuross, 1999; Schleser *et al.*, 1999).

Structural investigations on fossil sporopollenins were principally performed with ^{13}C solid-state NMR (Hemsley *et al.*, 1992, 1995, 1996). The results show that, even if the structures are modified with the same diagenetic history, differences between species may be shown. In diagenesis, heating appeared to be the main process altering sporopollenin composition (Yule *et al.*, 2000). The impact of these diagenetic processes on sporopollenin structure and associated $\delta^{13}\text{C}$ values needs to be studied.

Conclusion

Pollen provides an integrated view of plant metabolism and allows the study of plant biomes using small-volume samples. Our results show that plant physiological types can be well characterized, both on whole pollen grains and on extracted sporopollenin. This is an interesting tool for comparing herbaceous and tree formations, especially in transition zones. Furthermore, $\delta^{13}\text{C}$ measurements allow for separation of C_3 and C_4 plants based on pollen. Probabilistic assignments to functional groups of C_3 plants are given for C : N ratios and with less precision for $\delta^{13}\text{C}$. A clear relationship for $\delta^{15}\text{N}$ cannot be confirmed. The direct characterization of physiological types based on $\delta^{13}\text{C}$ measurements of fossil sporopollenin may be particularly well suited for older geological periods when the species were not the same as at present (Vad Odgaard, 1999; Kellogg, 2001). This is also useful in improving the results of pollen diagrams, including forms that cannot be differentiated at species level, and where it is important to determine the relative contributions of C_3 and C_4 plants (distribution limits) and to improve PFT determination. It appears that the pollen traits used in this study provide information on the relationship between environment and plant types on timescales different from those obtained with leaves (Reich *et al.*, 1999). Inter-calibration between pollen and leaf traits on altitudinal or latitudinal gradients should be of interest for climate and vegetation modelling.

More work is needed to improve our understanding of $\delta^{15}\text{N}$ variations in modern plants and in the associated pollen, especially to clearly identify the links with variations in environmental factors (Körner, 1999; Adams & Grierson, 2001). A

study of the impact of climatic factors in C_3 plants should also be considered, as the variability of the measured parameters is sufficiently reproducible in a small geographic area. A wider geographic area would allow for an assignment to classical PFTs (Prentice *et al.*, 1992).

Finally, stable isotope measurements on pollen appear to be a powerful tool in improving the accuracy and resolution of plant physiological type distribution in quaternary and older fossil sediments, leading to an independent palaeobiodiversity index. Comparisons at this level are of interest because they simplify the calibration of palaeo-proxies on recent data based on the same material.

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Supplementary material

The following supplementary material is available for this article online:

Table S1 Analysis and physiological characteristics (pathway types) of modern pollen species: carbon and nitrogen weight content, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and C : N ratio

Fig. S1 The results of the bivariate quadratic discriminant analysis (QDA) for C : N ratio and $\delta^{13}\text{C}$ applied to the three

functional groups: herbaceous including Poaceae, deciduous trees, and evergreen trees. The plots show the bivariate posterior probability for each group.

Fig. S2 Fourier transform infrared (FTIR) for *Sorghum* (a) and *Platanus* (b), and ^{13}C -CP/MAS NMR (cross-polarization and magic-angle spinning nuclear magnetic resonance) data for *Sorghum* (c) and *Platanus* (d), for untreated pollen (lower curves), acetolysis (middle curves), and H_2SO_4 (upper curves) treatments.

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