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Carbon-isotopic analysis of individual pollen grains from C_3 and C_4 grasses using a spooling-wire microcombustion interface

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Abstract

Pollen grains from grasses using the C_3 and C_4 photosynthetic pathways have distinct ranges of $\delta^{13}C$ values that may be used to estimate their relative abundance in paleorecords. We evaluated a spooling-wire microcombustion device interfaced with an isotope-ratio mass spectrometer (SWiM-IRMS) for $\delta^{13}C$ analysis of individual grass-pollen grains. Pollen from four C_3 and four C_4 grass species was isolated through micromanipulation and analyzed as single grains suspended in water. A carbon yield greater than the 2σ range of the carbon content of blanks containing only water was used to distinguish samples containing pollen ("pollen present") from those not containing pollen. This criterion resulted in the exclusion of ~45% of the 946 samples applied to the wire. The average $\delta^{13}C$ values ($\pm 1\sigma$) of the remaining samples were $-26.9‰$ ($\pm 6.3‰$) and $-11.5‰$ ($\pm 9.6‰$) for C_3 grasses and C_4 grasses, respectively, after blank-correcting the $\delta^{13}C$ data. These results suggest that the SWiM-IRMS system can be used to distinguish C_3 from C_4 grass pollen. The high variability in measured $\delta^{13}C$ values is likely caused by a combination of factors. These include natural isotopic variability among individual pollen grains; the relatively poor precision that can be obtained when determining $\delta^{13}C$ values of such small samples; and the uncertainty in the magnitude, isotopic composition, and stability of the analytical blank. Nonetheless, high percentages of individual pollen grains were correctly classified as being of either C_3 or C_4 origin. On average, 90% (range = 78–100%) of pollen grains from C_3 grasses had $\delta^{13}C$ values more negative than the cutoff threshold of $-19.2‰$; while 84% (range = 77–90%) of pollen grains from C_4 grasses had $\delta^{13}C$ values more positive than $-19.2‰$. Compared with analysis using an elemental analyzer interfaced with an IRMS (EA-IRMS), the number of pollen grains required for $\delta^{13}C$-based evaluation of C_3/C_4 grass composition is many times lower with the SWiM-IRMS. Additionally, $\delta^{13}C$ data from the SWiM-IRMS does not need to be incorporated into a mixing model to derive estimates of the abundance of C_3 and C_4 grass pollen. Carbon-isotopic analysis of individual grass-pollen grains using the SWiM-IRMS system may help improve our understanding of the evolutionary and ecological significance of grass taxa in the paleorecord.

1. INTRODUCTION

Today grass-dominated communities cover about one-third of Earth's land surface, exert a large influence on global biogeochemical cycles, and provide valuable sources of food for humans and herbivores (Jacobs et al., 1999; Saugier and Roy, 2000). Within these and other communities, grasses use two major pathways of carbon fixation in plants: C_3 and C_4 photosynthesis. Based on their distinct physiologies, C_3 and C_4 grasses should respond differently to changes in important environmental variables such as atmospheric CO_2 concentrations, aridity, and temperature

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(Ehleringer, 2005). Elucidating the response of C_3 and C_4 grasses to past changes in such variables is useful for projecting how grass community composition may respond to future environmental changes. It also provides the basis for understanding the factor(s) driving the origin of the C_4 physiology in grasses, which is considered a significant evolutionary achievement (Osborne and Beerling, 2006) in large part because C_4 grasses comprise $<\sim$2% of the total number of terrestrial plant species (Sage et al., 1999) but account for ~20% of global net primary production (Lloyd and Farquhar, 1994).

Despite the importance of distinguishing the abundance of C_3 and C_4 grasses in paleorecords, such information cannot be directly derived from pollen assemblages because grass pollen cannot be morphologically resolved beyond the family level (Fægri et al., 1989). Recent studies (e.g., Clark et al., 2001; Huang et al., 2001; Smith and White, 2004; Nelson et al., 2006) have attempted to use new isotopic and morphological proxies to estimate the relative abundances of C_3 and C_4 plants in sediment records. For example, using a micromanipulator to isolate grass-pollen grains that are then analyzed using a modified elemental analyzer interfaced with a standard isotope-ratio mass spectrometer (EA-IRMS), Nelson et al. (2006) showed that differences in $\delta^{13}C$ values between C_3 and C_4 grasses could be reliably detected in grass-pollen samples containing a minimum of 600 grains. By incorporating $\delta^{13}C$ data from samples of bulk grass pollen into a simple two end-member mixing model, the relative abundance of C_3 and C_4 grasses in a sample could be estimated. This new ability to assess the relative abundance of C_3 and C_4 grasses is an important step towards improving our understanding of grass taxa in geological records.

However, there are two main drawbacks to utilizing $\delta^{13}C$ measurements made on bulk samples of grass pollen. First, because it takes ~10 h to manually isolate the 600 fossil grass-pollen grains for a single analysis, the technique greatly limits the number of samples that may be routinely analyzed. The second drawback is that the relative proportion of C_3 and C_4 grasses must be estimated from a mixing model. For example, consider the equation

$$\%\ C_4 = 100\ *(\delta_s - \delta_3)/(\delta_4 - \delta_3),$$

where δ_s is the $\delta^{13}C$ value of the 600-grain sample, and δ_3 and δ_4 are the $\delta^{13}C$ end-members for C_3 and C_4 grasses, respectively. End-member values of δ_3 and δ_4 are usually assumed to be constant ($\sim -27‰$ for C_3 and $\sim -13‰$ for C_4). However, both are known to vary greatly across environmental gradients and taxonomic groups (range = $-33‰$ to $-22‰$ for C_3 and $-15‰$ to $-10‰$ for C_4), which means that results from such mixing models have large uncertainties (Cerling, 1999). Using the extreme ranges of δ_3, a mixed pollen sample having a bulk $\delta^{13}C$ value of $-22‰$ could represent 55% abundance of C_4 grasses if δ_3 was $-33‰$ (and δ_4 was $-13‰$), or 0% C_4 abundance if δ_3 was $-22‰$. The end-member variability means that mixing models are not suitable for detecting relatively small fluctuations in C_4 abundance or the presence of C_4 grasses at abundances below ~30–40% of the total grass-pollen grains. Thus EA-IRMS analysis of bulk samples of grass pollen is not suitable for studying the origin of C_4 grasses in the geological record.

We analyzed the ^{13}C composition of 946 individual grains of pollen from eight C_3 and C_4 grasses using a spooling-wire microcombustion device interfaced with an IRMS (SWiM-IRMS). Here we evaluate the potential of this technique to overcome the limitations of $\delta^{13}C$ analysis of bulk grass pollen for estimating the abundance of C_3 and C_4 grass pollen. Specifically, we expected that (1) when analyzing individual grains using the SWiM-IRMS, the total number of grains required to accurately assess C_3 and C_4 grass-pollen abundance in an unknown sample would be significantly lower than that required for analysis using the EA-IRMS, and (2) analysis of single grains would allow direct counts of the number of C_3 and C_4 grass-pollen grains in a sample (using a simple threshold to distinguish $\delta^{13}C$ values from C_3 and C_4 samples). Analysis of single pollen grains using the SWiM-IRMS would eliminate the need to estimate the abundance of C_3 and C_4 grass pollen using a mixing model.

2. MATERIALS AND METHODS

Modern pollen samples were obtained from four C_3 and four C_4 grass species (Table 1). All samples were treated following standard pollen preparation techniques modified to eliminate carbon-containing chemicals (Nelson et al., 2006). Pollen grains treated in this way are primarily composed of chemically resistant sporopollenin, the main constituent of fossil pollen grains (Loader and Hemming, 2000).

All samples were manipulated in nano-pure water stored in a pre-combusted beaker. A steel and glass syringe was used to apply discrete ~0.6-μl drops of this water to the right side of a methanol-cleaned microscope slide, as well as a larger pool (~80 μl) of water to the left side of the slide. For each species, a separate syringe was used to apply pollen samples to the large pool of water on the slide. Grass pollen was detected at 200× magnification, and individual grains were transferred from the pool of water into the ~0.6-μl drops of water using micromanipulation, as previously described (Nelson et al., 2006). A total of 423 individual pollen grains from four C_3 grass species and 523 individual pollen grains from four C_4 grass species (946 total grains) were applied to the SWiM-IRMS system in ~0.6-μl drops of water for determination of their $\delta^{13}C$ values (Table 1). The syringe (0.460 mm inner diameter) used to apply samples to the wire was rinsed between each sample. For comparison with $\delta^{13}C$ data from individual grains, $\delta^{13}C$ values of bulk aliquots of the same pollen samples from each species were analyzed using EA-IRMS, as previously described (Nelson et al., 2006). All pollen-$\delta^{13}C$ data were corrected for the post-industrial depletion of atmospheric ^{13}C, as described in Nelson et al. (2006).

The configuration and operation of a SWiM-IRMS system was previously described in detail (Brand and Dobberstein, 1996; Sessions et al., 2005; Eek et al., 2007). The system used here is a modified version built at Harvard University following the original prototype. Modifications include the use of a combustion furnace

Table 1
Summary data from individual pollen-grain samples

Name	Photosynthetic type	Samples applied to wire	Samples $>2\sigma$ of blank	$\delta^{13}C$ (1σ range)	Fraction > or <−19.2‰
Andropogon gerardii	C_4	114	70	−10.5 (9.4)	86
Sorghum halepense	C_4	143	60	−10.7 (9.3)	90
Sorghum vulgare	C_4	90	57	−13.5 (6.0)	82
Spartina pectinata	C_4	176	64	−11.1 (13.8)	77
C_4 total		523	251	−11.5 (9.6), average	84, average
Agropyron repens	C_3	101	73	−23.1 (7.7)	78
Bromus inermis	C_3	116	85	−26.8 (5.4)	95
Elymus canadensis	C_3	101	55	−30.5 (4.7)	100
Festuca elatior	C_3	105	55	−27.1 (7.3)	85
C_3 total		423	268	−26.9 (6.3), average	90, average
Unknown 1		120	67	−14.1 (8.3)	
Unknown 2		111	64	−24.7 (5.6)	

$\delta^{13}C$ data are based on samples with Vs yield $>2\sigma$ value of the "processing" blank for each species.
The fraction >−19.2‰ was used to distinguish C_4 from C_3, whereas the fraction <−19.2‰ was used to distinguish C_3 from C_4.

of smaller internal volume (0.5 mm i.d. × 14 cm length), a shorter Nafion drying tube (10 cm), and a ThermoFinnigan DeltaPlus Advantage IRMS. The wire (0.25 mm nickel wire moving at 0.8 cm/s) is passed through a cleaning oven (850 °C) purged with filtered air. A syringe is then used to transfer ~0.6 μl drops of water (containing pollen) from the microscope slide to the wire. The wire passes through a drying oven (120 °C) before entering a combustion oven (800 °C). Atmosphere is excluded from the combustion furnace by positive pressure of the helium carrier gas (6.5 psi). A portion of the combustion gases flows through a countercurrent Nafion membrane to remove H_2O, through an open split, and then to the IRMS. CO_2 reference gas is supplied via a Conflo III interface, and samples are analyzed as sets of seven injections spaced at 45-s intervals, flanked by three initial and two final pulses of reference gas. Sample yields (as CO_2 gas) are reported in units of Volts-seconds (Vs, peak area) of the mass 44 ion current. Using combustion yields of leucine injections containing 5 nmol carbon, the approximate conversion is ⩽1.4 Vs/nmol C.

To assess the contribution of background carbon we also analyzed three types of blanks: water from the beaker (called "water" blanks), water from the microscope slide (called "slide-water" blanks), and water from the microscope slide to which single grains of grass pollen were added and then subsequently removed (called "processing" blanks). "Processing" blanks were prepared for each species. These blanks were analyzed at the same time as pollen samples, which is critical in order to account for potential changes in the split ratio. All blanks were applied to the wire in ~0.6 μl aliquots. Assessing the contribution of carbon from blanks enables us to account for the possibility of pollen grains falling off the wire before reaching the combustion oven or of pollen that produces too little carbon to be distinguished from carbon-containing background (from the water or from the sample processing).

3. RESULTS AND INTERPRETATIONS

3.1. Precision versus sample size

To assess the shot-noise limited precision of average-sized grains of grass pollen we analyzed the $\delta^{13}C$ composition of two dissolved organic standards, leucine and dextrose, which have $\delta^{13}C$ values similar to those of C_3 and C_4 plants, respectively. Results show that the analytical precision of $\delta^{13}C$ values from leucine and dextrose is no worse than ±5‰ at sample sizes similar to those of pollen grains (Fig. 1). Thus, the observed scatter in the pollen $\delta^{13}C$ data (Section 3.4, $\delta^{13}C$ composition of blank-corrected samples) cannot be caused solely by analytical imprecision or sample handling blanks.

3.2. Peak areas of samples

The peak areas, corresponding to combustion yield of CO_2 gas, do not differ between "water" and "slide-water" blanks (Fig. 2). However, the peak areas of "processing" blanks are significantly greater than "water" blanks (Fig. 2), suggesting the addition of water-soluble carbon to the sample during chemical treatment. This excess carbon amounts to ~0.1 Vs (⩽0.07 nmol carbon) per grain (Fig. 2). Because no carbon-containing chemicals were used during treatment, the origin of the excess carbon is not immediately clear. There are three possible sources. First, the soluble component derives from traces of carbon removed from the inner part of the grains (intine) during treatment (Nelson et al., 2006) that remained with the sample through the treatment process. Second, airborne particles in the laboratory contribute the excess carbon. Third, soluble material from the surfaces of the plastic tubes in which the samples were treated was released during treatment and remained with the sample through the treatment process. The two latter possibilities imply that the $\delta^{13}C$ value of "processing" blanks would be uniform between

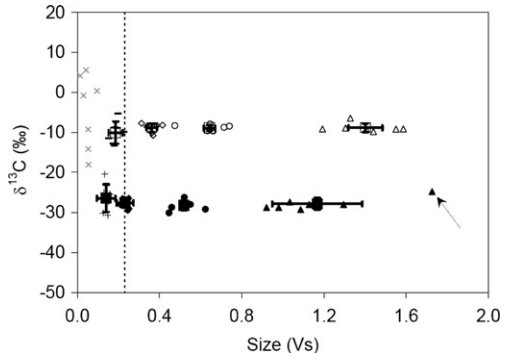

Fig. 1. Isotopic data for organic standards. Hollow symbols, dextrose ($\delta^{13}C_{true} = -9.8‰$); solid symbols, leucine ($\delta^{13}C_{true} = -29.8‰$). Data are shown for samples of nominal mass 1.25 nmol C (triangles), 0.625 nmol C (circles), 0.312 nmol C (diamonds), and 0.156 nmol C (+ and − symbols); $n = 7$ replicates for each standard. CO_2 area data are in Volt-second units, Vs. The means and sample standard deviations are shown for each set. One of the 1.25-nmol C leucine samples appears to be an outlier (arrow). The dashed line at ~0.2 Vs indicates the approximate 2σ threshold used to distinguish the presence or absence of pollen in samples. Isotopic values for pure water blanks (gray '×' symbols) also are shown.

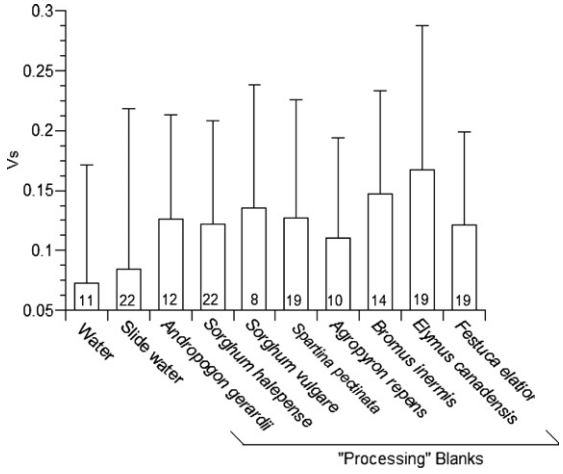

Fig. 2. CO_2 area data of blanks. Means and 2σ ranges are shown. The numbers inside the bars indicate the number of samples analyzed. "Water" indicates water applied directly to the spooling-wire. "Slide water" indicates water applied to a microscope slide and then to the spooling-wire. For the blanks of each species ("processing" blanks) a pollen grain was dispensed and then removed from water on a microscope slide and 0.6 μl of the water was subsequently applied to the spooling-wire. The "water" and "slide water" blanks are not statistically different ($p = 0.30$). The blanks of each species are statistically different from the "water" ($p < 0.05$, with the exception of *Agropyron repens*, for which $p = 0.09$).

C_3 and C_4 samples, whereas the first possibility implies that the blanks would differ between C_3 and C_4 samples. The isotope data presented below (Section 3.3) suggest that the latter possibilities are the most likely explanations.

Regardless of the precise origin of the carbon in the "processing" blanks, their peak areas were used to determine the presence or absence of a pollen grain in a sample. Of the 946 individual pollen-grain samples applied to the moving wire, 303 (32%) and 427 (45%) have total peak areas within $\pm1\sigma$ and $\pm2\sigma$, respectively, of the peak areas of "processing" blanks. For each species we defined a sample as "pollen present" if its Vs yield was greater than the 2σ value of the "processing" blank and "pollen absent" if its Vs yield was less than or equal to the 2σ value of this blank. The more conservative $\pm2\sigma$ criterion results in better separation of $\delta^{13}C$ values from C_3 and C_4 grains than the $\pm1\sigma$ range. The improvement in separation of $\delta^{13}C$ values from C_3 and C_4 grains when using an even more conservative $\pm3\sigma$ criterion is minimal (improvement of ±2%), but the percentage of eliminated samples (an additional 12%) is much greater at a $\pm3\sigma$ cutoff. In addition, the significant positive relationship between mean grain diameter and peak areas ($n = 8$, $r = 0.73$, $p = 0.038$) means that setting the "pollen present" criterion too high (e.g., $\pm3\sigma$ or higher) would weight paleo-reconstructions toward species that produce larger grains. Thus the $\pm2\sigma$ criterion appears optimal. Finally, the sizes of the "processing" blanks for each species are not significantly different from the average size of the blank for all species combined (average p-value = 0.36, all p-values >0.02). Thus the difference is minimal between using the individual $\pm2\sigma$ ranges of the "processing" blanks for each species and using the average $\pm2\sigma$ range of all of the species as the threshold for "pollen present."

A total of 268 C_3 and 251 C_4 samples (63 and 48% of the total number of C_3 and C_4 grains applied, respectively) have peak areas exceeding their respective $\pm2\sigma$ thresholds (Fig. 2). It is not surprising that nearly half (45%) of the individual analyses have to be excluded. The most likely explanation is that pollen grains fall off the moving wire in transit before reaching the combustion oven. Natural variability in carbon content of individual grains may also cause the peak areas of some combusted grains to be genuinely smaller than the $\pm2\sigma$ threshold and thus indistinguishable from blanks. We also saw no evidence for grains being unintentionally carried-over in the syringe (resulting in no grains in some samples and more than one grain in others). However, because it is impossible to discriminate between these options, all Vs data smaller than the $\pm2\sigma$ cutoffs were excluded.

3.3. Blank corrections

The $\delta^{13}C$ value of the blank could not be measured directly because its low carbon content led to poor precision (Fig. 1), and the cause of the poor precision (e.g., true variability versus instrumental variability) could not be assessed. However, we were able to determine the average isotopic composition of the "processing" blanks by comparing the $\delta^{13}C$ values of bulk pollen aliquots from EA-IRMS with the average values of individual pollen grains obtained from SWiM-IRMS. All of the C_3 species fall near a 1:1 line, whereas $\delta^{13}C$ values for three out of four C_4 species fall below this line (Fig. 3, hollow symbols). The one C_4

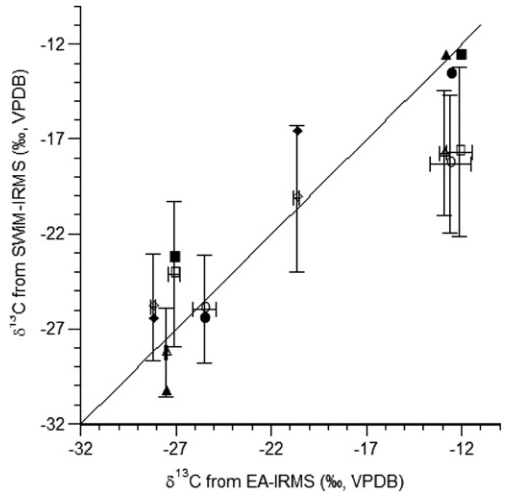

Fig. 3. Comparison of mean $\delta^{13}C$ data from C_3 and C_4 grass pollen obtained using EA-IRMS and SWiM-IRMS. Hollow symbols represent raw data; closed symbols represent blank-corrected data as described in Section 3.3. Error bars ($\pm 1\sigma$) are displayed only on hollow symbols. C_3 species (on left): square, *Agropyron repens*; circle, *Bromus inermis*; triangle, *Elymus canadensis*; diamond, *Festuca elatior*. C_4 species (on right): square, *Andropogon gerardii*; circle, *Sorghum halepense*; triangle, *Sorghum vulgare*; diamond, *Spartina pectinata*. The solid line represents a 1:1 relationship between $\delta^{13}C$ EA-IRMS and SWiM-IRMS.

species falling on the 1:1 line (*Spartina pectinata*) has a much more negative $\delta^{13}C$ value (−20.7‰, based on EA-IRMS) than is typical of C_4 species. These results suggest that if the size of the "processing" blank is similar among all samples (which it appears to be, based on Fig. 2, and as stated above in Section 3.2), the greater negative bias on the C_4 data means that the $\delta^{13}C$ value of the blank is likely close to the typical isotopic composition of C_3 species. Thus we suggest that the greater peak areas of "processed" blanks than of "water" blanks results from traces of water-soluble carbon from the plastic tubes in which the samples were treated or traces of carbon from airborne particles.

To estimate the mass and ^{13}C composition of the blank we used the equation,

$$\delta_{SWiM} = \delta_{ea} + (M_b/M_{SWiM})(\delta_b - \delta_{ea}),$$

where δ_{SWiM} and M_{SWiM} denote the average $\delta^{13}C$ and Vs values, respectively, for a species obtained using SWiM-IRMS, δ_{ea} equals the average $\delta^{13}C$ value obtained using EA-IRMS, and M_b and δ_b signify the average Vs and $\delta^{13}C$ value of the blank. We solved for M_b and δ_b iteratively by minimizing the sum of the absolute difference between δ_{SWiM} predicted using the above equation and the average $\delta^{13}C$ value measured for each species using the SWiM-IRMS. This process (excluding the anomalous *S. pectinata* data) yields values of 0.16 Vs and −25.3‰ for M_b and δ_b, respectively. The value of M_b calculated this way (0.16 Vs) is very similar to the average mass of all of the "processing" blanks (0.14 Vs, Fig. 2) as determined by direct measurements. When the SWiM-IRMS data are corrected using these calculated values of M_b and δ_b, they showed a much closer fit (slope of regression improves from 0.57 to 0.95) with the EA-IRMS data (Fig. 3, solid symbols). This result suggests that the interpretation that M_b and δ_b represent a generally uniform addition of exogenous carbon is reasonable; however, we acknowledge that this average $\delta^{13}C$ value of the "processing" blank could reflect a wide range of true variability. The precise source of the excess carbon is uncertain, although it appears more likely to be from plasticware than airborne particles, because if airborne particles were the sole component of the blank our "slide-water" and "processing" blanks should have peak areas of similar size, in contrast to our results (Fig. 2). If the *S. pectinata* data are included in the above calculations, M_b and δ_b are 0.16 Vs and −24.9‰, respectively. Using −24.9‰ rather than −25.3‰ for δ_b results in a difference of only 0.16‰ in δ_{SWiM}; thus the effect of excluding *S. pectinata* is minimal. We blank-corrected all of the SWiM-IRMS data using values of 0.16 Vs and −25.3‰ for M_b and δ_b, respectively.

3.4. $\delta^{13}C$ composition of blank-corrected samples

The mean $\delta^{13}C$ values of the blank-corrected individual pollen grains obtained using the SWiM-IRMS system fall within the expected ranges for C_3 and C_4 plants for each species (Fig. 4). Thus the SWiM-IRMS may be used to distinguish populations of individual C_3 and C_4 pollen grains. However, there is large variation around these means, and many individual data points exceed the ranges expected for $\delta^{13}C$ values of C_3 and C_4 plants, in both positive and negative directions. For example, although the mean $\delta^{13}C$ value for *Sorghum halepense* (−10.7‰) is within the typical range of $\delta^{13}C$ values expected for C_4 plants, both the standard deviation (±9.3‰) and the absolute range of values measured (−34.8‰ to 19.7‰; Fig. 4) exceed the −10 to −15‰ natural range. The breadth of the scatter in $\delta^{13}C$ values at a given peak size also varies among species (Fig. 4), showing that some species have more natural or analytical variability than others.

Sporopollenin-rich organic matter could be expected to deviate from the bulk plant isotopic composition by 5–7‰ in the negative direction, because of the predominance of lipidic carbon in the biosynthesis of sporopollenin (Hayes, 2001; Shaw and Yeadon, 1964). However, this difference is not a large enough effect to explain the occasional observations of C_4 samples with $\delta^{13}C$ values <−22‰. Similarly, there is no (known) concomitant evidence for significant isotopic enrichment that could result in $\delta^{13}C$ values more positive than ∼−8‰. A possible explanation for either of these sets of outliers would require intracellular partitioning of carbon to be governed by branched pathways, allowing some pools of biosynthetic intermediates to become isotopically very heavy, or very light (Hayes, 2001). Although such variation in intracellular allocation of carbon may explain some of the excess isotopic variability observed here, it is unlikely to account fully for the wide range of values observed in the data.

The wide range of scatter in the data must have a significant analytical component. Individual grains of grass pollen contain very small amounts of carbon, and thus some of

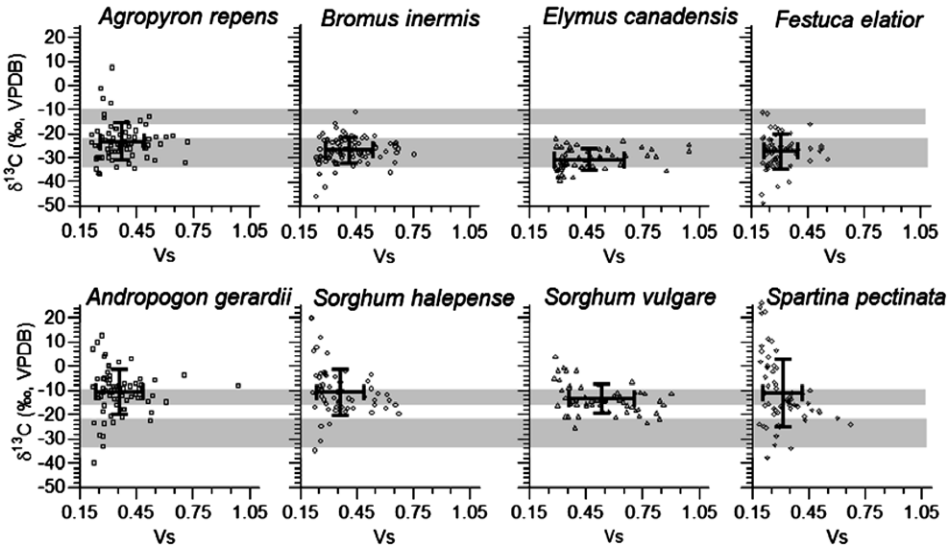

Fig. 4. $\delta^{13}C$ and Vs data from individual grains of pollen from C_3 (top row) and C_4 (bottom row) grasses. Only samples exceeding the 2σ range of the mean Vs value of blanks are shown. The mean value for each species is shown with 1σ error bars. The shaded boxes encompass the typical ranges (Bender, 1971; Cerling, 1999) of $\delta^{13}C$ values for C_3 and C_4 plants.

the variation in $\delta^{13}C$ values may be caused by relatively poor precision and accuracy of the measurements made with the SWiM-IRMS. However, the average grain of treated grass pollen is ~0.38 Vs, and the isotopic precision that can be obtained for replicate injections of leucine and dextrose in this size range is no worse than ±5‰ (Fig. 1), suggesting that poor precision is not the only cause of variation in $\delta^{13}C$ values. The variation in final reported values also incorporates a large amount of uncertainty in both the isotopic composition of—and more importantly, the isotopic variability of—the analytical "processing" blank. Calculating $\delta^{13}C_b$ by the methods above yields only the average value but not the absolute range of $\delta^{13}C_b$. Together, these factors would be expected to most greatly affect the $\delta^{13}C$ values of the samples with the smallest reported peak areas. The observed range of variation in $\delta^{13}C$ values (Fig. 4) is indeed greatest for samples with Vs values below ~0.3, and the uncertainty in the blank correction has the largest impact on these samples. The fact that none of the species trend toward an average δ_{blank} value (or a species-specific δ_{blank} value) at small sample sizes suggests that $\delta^{13}C_b$ may have greater variability than the natural $\delta^{13}C$ variability of the pollen grains. However, we cannot currently test this hypothesis because along with variability in the "processing blank," analytical imprecision and true natural variation may also influence the wide range of scatter in the data.

The $\delta^{13}C$ values of individual pollen grains from C_3 and C_4 grasses obtained using the SWiM-IRMS system exhibit large variations and overlap with one another. Therefore, it is not feasible to distinguish individual grains of C_3 and C_4 grass pollen by simply classifying the data into the typical ranges of C_3 and C_4 plants (Fig. 4, −33 to −22‰ and −15 to −10‰, respectively). The SWiM-IRMS method cannot at present be used to count single occurrences of "a C_3 grain" or "a C_4 grain," because even the largest grains (e.g., those with a CO_2 yield >0.75 Vs) occasionally produced $\delta^{13}C$ values outside of their typical ranges and could be misclassified (Fig. 4). However, the vast majority of $\delta^{13}C$ values from C_3 grasses is more negative than the typical range for C_4 grasses; and conversely, most $\delta^{13}C$ values from C_4 grasses are more positive than a typical C_3 range. In an approximately equal distribution of data points (268 C_3 and 251 C_4), the data are clearly bimodal (Fig. 5). Because the data are normally distributed we attempted to use a Gaussian mixture model (http://cobweb.ecn.purdue.edu/~bouman/software/cluster/) to fit distributions to the C_3 and C_4 populations, deconvolute the populations, and directly determine the proportion of the data in each distribution. When C_4 abundance was <50% the mixture model classified the proportion of C_4 grains well. However, the mixture model did a poor job of classifying the proportion of C_4 grains when their abundance was >50%, likely because the C_4 data are noisier than the C_3 data. Thus we decided to distinguish populations of C_3 and C_4 grass pollen based on their distribution around a $\delta^{13}C$ threshold value.

We explored three ways for establishing this threshold value. We first used the mid-point between the average $\delta^{13}C$ values of the EA-IRMS pollen data (excluding *S. pectinata* data), which yields a threshold value of −19.7‰ (Fig. 3). However, the greater variation associated with the SWiM-IRMS data suggests that EA-IRMS data from bulk samples may not be appropriate for establishing the threshold. This is especially true if the SWiM-IRMS imparts a systematic offset to the data (accuracy issue) for which we currently have no means of assessing or correcting. We next binned the SWiM-IRMS data by increments of 1.0‰ and chose a threshold that minimized the sum of C_3 bins with $\delta^{13}C$ values more positive than the threshold and C_4 bins with $\delta^{13}C$ values more negative than the threshold. This approach produces a threshold value of −20.0‰. However, this value may be biased by individual species

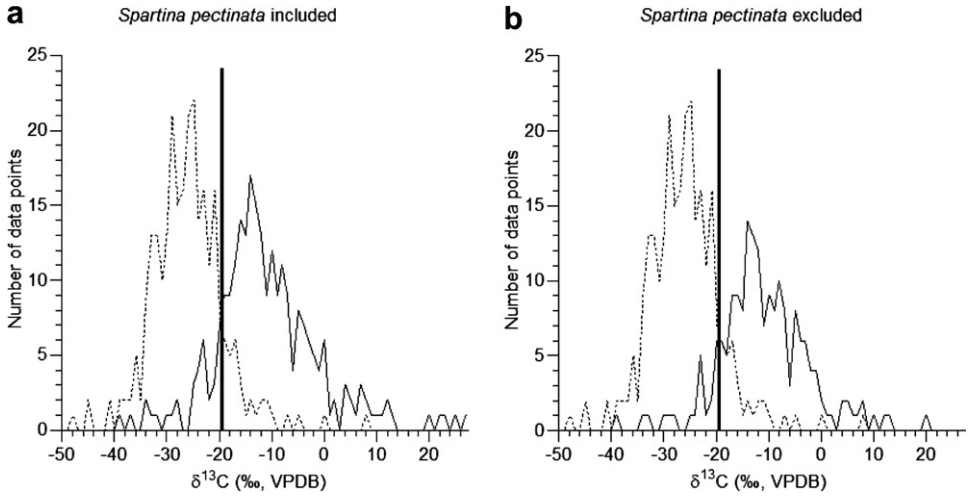

Fig. 5. Histograms of data presented in Fig. 4. The dashed lines represent data from C_3 grasses and the solid lines data from C_4 grasses. The thick lines at −19.2‰ signify the threshold distinguishing C_3 from C_4 grass pollen, as discussed in the text.

that are represented more frequently in the data set. Therefore, we also calculated the mid-point (−19.2‰) between the average $\delta^{13}C$ values for the C_3 (−26.9‰) and C_4 (−11.5‰) grass-pollen samples from the SWiM-IRMS (Table 1). This approach is more comparable to the approach using the EA-IRMS data, and unlike the binning method, the result of −19.2‰ partially removes the effect of unequal numbers of grains for all species. Regardless, the three approaches yielded similar threshold values (−19.7‰, −20.0‰, and −19.2‰); thus they would not lead to significantly different estimates of the abundance of C_3 and C_4 grass pollen. We chose to retain −19.2‰ as the final threshold value because it results in a slightly more conservative estimate of the proportion of C_4 grass pollen, which is critical when C_4 grass pollen is at low abundance (<∼30%). The threshold value of −19.2‰ remains the same whether the mean value of *S. pectinata* (−11.1‰, Table 1) is included (Fig. 5a) or excluded (Fig. 5b) from the average $\delta^{13}C$ value of individual grains of C_4 grass pollen.

3.5. Samples with unknown C_3 and C_4 proportions

To estimate the number of individual pollen-grain measurements that would be necessary to accurately estimate the abundance of C_3 and C_4 grass pollen in an unknown sample we developed subroutines in Matlab (The MathWorks, Inc.) to resample and count data points. The master dataset used for the calculations included the 268 C_3 and 251 C_4 samples classified as "pollen present." From the master dataset a series of new datasets ($dataset_{new}$) were created. Each $dataset_{new}$ contained specified percentages of C_3 and C_4 pollen grains (e.g., 0%, 10%,…,100%). The maximum possible number of grains was included in each $dataset_{new}$. For example, the $dataset_{new}$ with <48.4% (=251/[251 + 268]) C_4 grains contained all 268 of the C_3 data points; the $dataset_{new}$ with >48.4% C_4 contained all 251 of the C_4 data points. From each $dataset_{new}$ 50, 100, or 150 grains were randomly selected. The abundances of C_3 and C_4 grains in each $dataset_{new}$ were then predicted using the threshold value of −19.2‰. The accuracy of predictions (e.g., ±10% means that the predicted distribution reproduced the known distribution to within ±10%) was then evaluated by comparison with the true proportions of C_3 and C_4 grass pollen in each $dataset_{new}$. A subroutine was created to repeat the evaluation process 250 times for each of the 30 $datasets_{new}$ that were randomly created for each proportion of C_3 and C_4 grass pollen from the master dataset.

The results from this resampling exercise show that the proportion of correctly classified trials increased as a function of the number of pollen grains included in a sample (Fig. 6). For example, in a sample of 50 individual grains in which the true abundance of C_4 grass pollen is 50%, the counted abundance of C_4 grass pollen would be expected to fall within ±10% of the true abundance with only 1σ (68%) confidence. But if the number of grains in the sample is increased to 100, the counted abundance would be expected to fall within ±10% of the true abundance with 2σ confidence (or 95% of the time). This shows the power of analyzing greater numbers of pollen grains within a given sample. Conversely, the proportion (e.g., 68% or 95%) of correctly classified trials is larger if the acceptable range of error (e.g., ±10%) is larger (Fig. 6). In the example above, the counted abundance of C_4 grass pollen would be within ±10% of the true abundance with 1σ (68%) confidence; but that same 50-grain sample would be classified to within ±15% of the true abundance with 2σ (95%) confidence.

The most stringent classification is achieved by measuring a 150-grain sample, which under certain conditions can be classified to within ±10% of its "true" C_3/C_4 distribution if the sample falls in the range of ∼30–60% total C_4 abundance (Fig. 6b). The accuracy of classification is also better at low, rather than high, relative abundance of C_4 grass pollen, regardless of the number of grains analyzed (Fig. 6). For example, in a sample of 50 individual grains the counted abundance of C_4 grass pollen would be within ±20% of the true abundance at the 95% (2σ) confidence

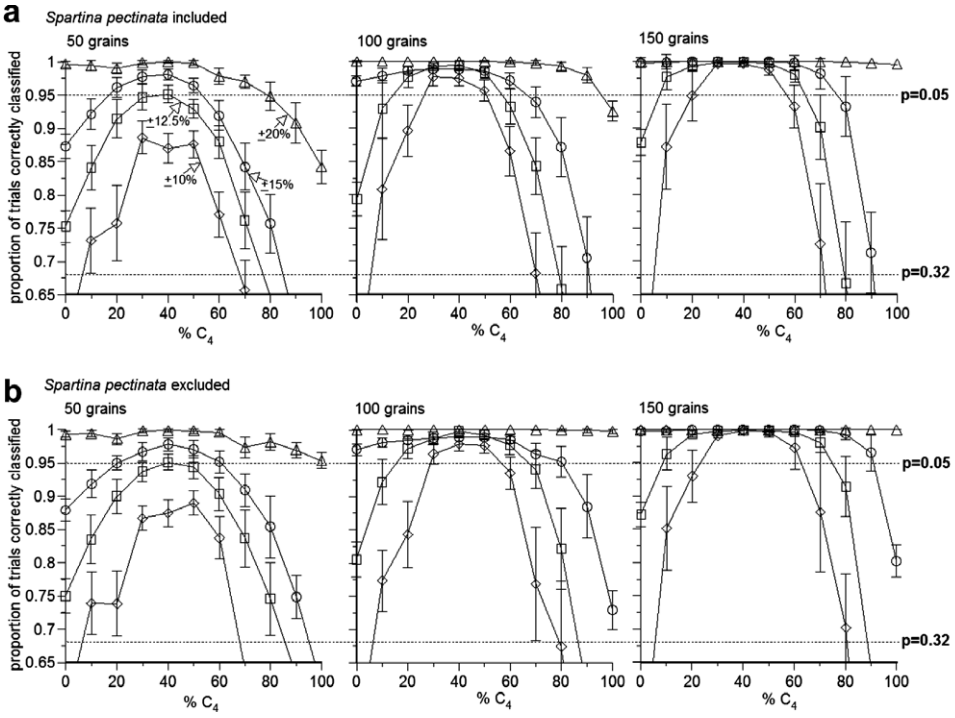

Fig. 6. Proportion of randomized trials in which C_4 pollen abundance of a sample dataset was correctly identified. Each dataset contained 50, 100, or 150 grains randomly selected from the master dataset, as described in Section 3.5. The accuracy of estimated C_4 abundance in relation to the true C_4 proportion of each dataset is shown with different symbols: diamonds, squares, circles, and triangles represent accuracy of ±10%, ±12.5%, ±15%, and ±20%, respectively. Lines for 1σ and 2σ confidence limits of the classifications are shown.

level if the true abundance of C_4 grass pollen is 0%, but correspondingly only at the 68% (1σ) confidence level if the true abundance is 100%. This effect is not as large if the data from *S. pectinata* are excluded (Fig. 6b).

To further evaluate our ability to count the number of C_3 and C_4 grass-pollen grains in a sample with an unknown C_3 and C_4 grass proportion, we created and then analyzed two "unknown" samples. The first unknown sample contained 20% pollen from *F. elatior* (C_3) and 80% pollen from *S. halepense* (C_4) and the second contained 80% pollen from *F. elatior* and 20% pollen from *S. halepense*, but these proportions were unknown to the analyst (a "blind" trial). Of the 67 data points with Vs values greater than the 2σ range of blanks from the first unknown (Table 1), 70% are classified as from a C_4 grass, and of the 64 data points from the second unknown, 13% are classified as from a C_4 grass. These estimated C_4 proportions are within the ±1σ ranges of the true abundance of C_4 grass pollen in both unknowns, consistent with what would be predicted by our resampling method. The accuracy of the counted abundance of C_4 pollen in the unknowns decreases by 1% if a 3σ criterion is used instead of the 2σ criterion.

4. DISCUSSION

Our results demonstrate the potential for SWiM-IRMS analysis to help distinguish C_3 from C_4 grass pollen in paleorecords. The number of grains required for $\delta^{13}C$-based evaluation of the composition of C_3 and C_4 grass-pollen grains is much lower with SWiM-IRMS than with EA-IRMS. A single $\delta^{13}C$ analysis of grass pollen using EA-IRMS requires ~600 grains, whereas estimates of C_3 and C_4 grass-pollen abundance can be obtained using SWiM-IRMS with as few as 50 samples, each representing a single pollen grain. The one drawback to using the SWiM-IRMS is that, on average, ~45% of samples applied to the spooling-wire did not yield adequate carbon above the ±2σ threshold of "processing" blanks (see Section 3.2). Thus if 50 data points are required to address a specific research question, ~91 grains must be applied to the wire. Nonetheless, the time required to obtain reliable $\delta^{13}C$ data from pollen grains is still substantially lower with SWiM-IRMS than with EA-IRMS analysis. If ~60 grains can be isolated from a sediment sample per hour (Nelson et al., 2006), using the SWiM-IRMS results in a reduction of ~8 h of isolation time. Thus the entire process of sample preparation and analysis remains substantially faster with SWiM-IRMS than with EA-IRMS.

In addition to reducing the sample size requirements, the SWiM-IRMS technique also avoids the assumption of end-member constancy that is required when using a two end-member mixing model to decouple the fractions of C_3 and C_4 pollen contained in a set of 600-grain samples (e.g., Nelson et al., 2006). Because of potential variation in end-member $\delta^{13}C$ values, changes in the abundance of C_4 grass pollen derived from a mixing model may reflect changes in the abundance of C_4 grass pollen or simply changes in the $\delta^{13}C$ value of the C_3 and/or C_4 end-member. Thus detecting small changes in the abundance of C_4 grass pollen is difficult based on bulk samples of grass pollen,

particularly when the overall fraction of C_4 grains is low. In contrast, fluctuations in the fraction of C_4 grass pollen can be detected with higher confidence by counting the number of individual grains using the SWiM-IRMS technique. For example, in a sample of 100 individual grains in which the true abundance of C_4 grass pollen is 20%, the counted abundance of C_4 grass pollen is within 10% of the true abundance at the 68% (1σ) confidence level, or within 12.5% of the true abundance with 95% (2σ) confidence (Fig. 6). Thus there is 95% (2σ) certainty that such a sample contains at least 8% C_4 grass pollen. This example also illustrates another advantage of our new technique over calculating the abundance of C_4 grass pollen using a mixing model: the ability to quantify the uncertainty in the estimated abundance of C_4 grass pollen.

There are numerous potential applications for $\delta^{13}C$ analysis of grass pollen using SWiM-IRMS. Recent $\delta^{13}C$ evidence from carbonate and organic matter in paleosols suggests that C_4 plants were present in low abundance ($<\sim$30%) in North America by 21 Ma (Fox and Koch, 2003), which is broadly consistent with molecular clock evidence for the appearance of C_4 grasses between 25 and 32 Ma (Gaut and Doebley, 1997). However, interpretation of $\delta^{13}C$ data from such materials is often difficult because of the potential for a varying C_3 end-member and the possibility that the ^{13}C content of the carbonates and/or bulk organic matter has been altered after deposition. In addition, previous studies may be confounded by contributions to the substrate being analyzed (e.g., carbonates and organic matter) from non-grass taxa, which predominantly use C_3 photosynthesis. Our method illustrates the potential for using $\delta^{13}C$ analysis of individual grains of grass pollen to determine the timing of C_4-grass evolution with greater confidence than was previously available. Analysis of $\delta^{13}C$ values of individual grains of grass pollen could also be used to study C_3 and C_4 grass dynamics after the well-characterized rise to dominance of C_4 plants during the late-Miocene (Cerling et al., 1993). For example, uncertainties in the distribution of tall-, mid- and, short-grass prairie communities in the North American Great Plains during the late-Quaternary could be resolved through knowledge of the abundance of C_3 and C_4 grasses. Finally, $\delta^{13}C$ analysis of individual grains of pollen could be used to estimate variability in pollen $\delta^{13}C$ and provide information about the biosynthesis of pollen in different species.

One challenge to determining the numbers of C_3 and C_4 grass-pollen grains in an unknown sample is how to account for variation in the threshold value (−19.2‰) used to distinguish C_3 from C_4 data. Such variation may result from changes in the ^{13}C content and/or partial pressure of atmospheric carbon dioxide. However, our method of distinguishing C_3 and C_4 grass pollen using the SWiM-IRMS should be more robust than the EA-IRMS method (Nelson et al., 2006) against a shift in the threshold value. The bimodal distribution of data points (Fig. 5) means that such a shift should be detected, assuming that at least one representative sample in any given period of the geological record contains an approximately even abundance of C_3 and C_4 grass pollen. If the distribution of individual SWiM-IRMS data points were at all bimodal, the data could be used to revise the threshold value to one more appropriate to the geologic interval of interest. For time periods when an approximately bimodal distribution is unavailable, the threshold value may be adjusted based on other independent proxy data, such as estimates of the concentration (Pagani et al., 2005) and ^{13}C composition (Zachos et al., 2001) of atmospheric carbon dioxide.

Improved isotopic estimates of C_4-grass abundance in the geological record can help elucidate potential global (e.g., atmospheric CO_2 concentrations) or local (e.g., aridity, fire) factors favoring the emergence, expansion, and fluctuations of C_4 grasses (e.g., Cerling et al., 1994; Cerling et al., 1997; Pagani et al., 1999; Pagani et al., 2005; Osborne and Beerling, 2006). For example, the origin of C_4 plants has been hypothesized to have occurred in response to declining atmospheric CO_2 concentrations during the Oligocene (Pagani et al., 2005). Yet, the timing of the origin of C_4 plants is equivocal (Osborne and Beerling, 2006). An evaluation of factors controlling the origin of C_4 plants is best done by considering only one plant life-form (e.g., grasses) (Ehleringer, 2005), and our new method is promising for that purpose. Previous studies have also linked the well-documented expansion of C_4 dominated communities during the late-Miocene to declining atmospheric CO_2 concentrations (e.g., Cerling et al., 1997). The lack of evidence to support this linkage (Pagani et al., 1999) has led others to suggest an important role of fire in the late-Miocene expansion of C_4 dominated ecosystems (Keeley and Rundel, 2005). The fire hypothesis involves the replacement of C_3 woodlands by C_4 grasslands as the result of dry fire-seasons. However, it remains unclear whether fire specifically favored C_4 grasslands *per se* rather than just grasslands, or whether C_4 grasslands replaced C_3 grasslands instead of C_3 woodlands. Analysis of $\delta^{13}C$ values of individual grains of grass pollen would help resolve these and other uncertainties about the response of C_3 and C_4 grasses to environmental changes in the geologic record.

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