



Using SPIRAL (Single Pollen Isotope Ratio Analysis) to estimate C₃- and C₄-grass abundance in the paleorecord

David M. Nelson^{a,b,*}, Feng Sheng Hu^{c,d}, Daniel R. Scholes^c, Neeraj Joshi^c, Ann Pearson^b

^a Institute for Genomic Biology, University of Illinois, 1206 W. Gregory Dr., Urbana, IL 61801, United States

^b Department of Earth and Planetary Sciences, Harvard University, 20 Oxford St., Cambridge, MA 02138, United States

^c Department of Plant Biology, University of Illinois, 265 Morrill Hall, 505 S. Goodwin Ave., Urbana, IL 61801, United States

^d Department of Geology, University of Illinois, 245 Natural History Building, 1301 W. Green St., Urbana, IL 61801, United States

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ABSTRACT

C₃ and C₄ grasses differ greatly in their responses to environmental controls and influences on biogeochemical processes (e.g. water, carbon, and nutrient cycling). Difficulties in distinguishing between these two functional groups of grasses have hindered paleoecological studies of grass-dominated ecosystems. Stable carbon isotopic analysis of individual grains of grass pollen using a spooling-wire microcombustion device interfaced with an isotope-ratio mass spectrometer holds promise for improving C₃ and C₄ grass reconstructions. This technique, SPIRAL (Single Pollen Isotope Ratio Analysis), has only been evaluated using pollen of known C₃ and C₄ grasses. To test the ability of SPIRAL to reproduce the abundance of C₃ and C₄ grasses on the landscape, we measured $\delta^{13}\text{C}$ values of >1500 individual grains of grass pollen isolated from the surface sediments of ten lakes in areas that span a large gradient of C₃- and C₄-grass abundance, as determined from vegetation surveys. Results indicate a strong positive correlation between the $\delta^{13}\text{C}$ -based estimates of % C₄-grass pollen and the abundance of C₄ grasses on the landscape. The % C₄-grass pollen slightly underestimates the actual abundance of C₄ grasses at sites with high proportions of C₄ grasses, which can be corrected using regression analysis. Comparison of the % C₄-grass pollen with C/N and $\delta^{13}\text{C}$ measurements of bulk organic matter illustrates the distinct advantages of grass-pollen $\delta^{13}\text{C}$ as a proxy for distinguishing C₃ and C₄ shifts within the grass family. Thus SPIRAL promises to advance our understanding of grassland ecology and evolution.

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1. Introduction

Paleorecords are invaluable for assessing vegetational responses to varying environmental conditions, as well as the potential influence of vegetational change on important evolutionary and ecological processes, such as mammalian evolution and migration (Flessa et al., 2005). In grass-dominated ecosystems (e.g. prairie, steppe, and savanna), information about C₃ and C₄ plant variations is particularly important because these functional groups have distinct responses to controlling factors such as atmospheric CO₂ concentrations, aridity, temperature, and fire (Sage, 2005). Because these functional groups have unique $\delta^{13}\text{C}$ signatures, stable carbon isotopic analysis of numerous substrates have been commonly used to determine past shifts in the relative abundances of C₃ and C₄ plants. These substrates include bulk sediments (Meyers and Lallier-Verges, 1999), charcoal (e.g. Nelson et al., 2004), leaf waxes (e.g. Castañeda et al., 2007), paleosols (e.g. Fox and Koch, 2003), and phytoliths (e.g. Smith and White, 2004). Although the majority of C₄ plants are grasses (Lloyd and Farquhar, 1994; Sage et al., 1999), ~50% of grass species, and most

trees, shrubs, and other herbaceous taxa use C₃ photosynthesis (Sage, 2005). Thus a $\delta^{13}\text{C}$ shift in any of these substrates could involve variations between major plant life forms (e.g. C₃ trees versus C₄ grasses) and/or in functional types within a single plant form (e.g. C₃ grasses versus C₄ grasses). These variations are indistinguishable using existing $\delta^{13}\text{C}$ techniques (Ehleringer, 2005), making it difficult to tackle important questions such as when and how C₄ photosynthesis evolved (Osborne, 2008).

Analysis of grass-pollen $\delta^{13}\text{C}$ circumvents this problem and is emerging as a tool for distinguishing C₃ and C₄ grasses in paleorecords (e.g. Amundson et al., 1997; Jahren, 2004; Nelson et al., 2006; Descolas-Gros and Schölzel, 2007; Nelson et al., 2007). Using a spooling-wire microcombustion device interfaced with an isotope-ratio mass spectrometer (SWiM-IRMS), Nelson et al. (2007) analyzed individual pollen grains of known C₃ and C₄ grasses and showed that populations of $\delta^{13}\text{C}$ values of these two functional groups could be distinguished based on their distribution around a threshold $\delta^{13}\text{C}$ value. This new technique holds great promise for assessing questions related to the evolution of C₄ grasses and their response to environmental variations. However, the technique was based on pollen samples collected from inflorescences of herbarium specimens and modern plants in the field, and it has not been validated with geological samples.

* Corresponding author. Institute for Genomic Biology, University of Illinois, 1206 W. Gregory Dr., Urbana, IL 61801, United States. Tel.: +1 217 333 4376; fax: +1 217 333 0508.
E-mail address: dmmnelson@life.uiuc.edu (D.M. Nelson).

An important means of testing the fidelity of new indicators for paleoenvironmental studies is through surface-sediment calibration or validation across spatial environmental gradients. This approach has been commonly used to relate pollen assemblages in surface samples from lakes and peatlands to modern vegetation and climate in order to validate fossil pollen assemblages for down-core paleoenvironmental reconstructions (e.g. Davis and Webb, 1975; Davis, 2000; Whitmore et al., 2005). Similar assessments also have been performed for paleoclimate proxies such as δD of palmitic acid (Huang et al., 2002), archaeological membrane lipid composition (Schouten et al., 2002; Powers et al., 2004), and alkenone unsaturation patterns (Prah and Wakeham, 1987). Using SPIRAL (Single Pollen Isotope Ratio Analysis), we conducted $\delta^{13}C$ measurements on 1598 individual grains of grass pollen in the surface sediments of ten lakes from areas that span a large gradient of C_3 - and C_4 -grass abundance. Here we report these results and evaluate the reliability of SPIRAL for reconstructing the proportions of C_3 and C_4 grasses on the landscape.

2. Samples and methods

Nine of the lake-sediment samples used in our study came from the U.S. Great Plains and were obtained by E.C. Grimm between 1997 and 2006 from the center of each lake using a Hongve sampler. The upper ~5 cm of sediment was collected, which likely represents accumulation over several decades. The samples came from areas (Table 1) with a wide range of C_4 -grass annual net primary productivity (ANPP) (Epstein et al., 1997). Epstein et al. (1997) derived grass ANPP values at ~1700 locations throughout the Great Plains from Natural Resource Conservation Service (NRCS) range site descriptions (USDA, 1967), and used the results to map the relative productivities of upland C_3 and C_4 grasses. We visually inspected the maps of Epstein et al. (1997) to determine C_3 and C_4 grass relative productivities within a ~30 km radius of each site (e.g. Prentice et al., 1987; Sugita, 1994). The relative productivities of C_3 and C_4 grasses in the Great Plains generally correspond with their relative abundance (Grimm, 2001). To provide an additional site where C_4 grasses were abundant, we analyzed grass pollen in a pre-settlement sediment sample from Lake Annie in central Florida (Table 1), which was also provided by E.C. Grimm. The relative abundance of C_4 grasses on the modern landscape near Lake Annie was determined from Teeri and Stowe (1976).

Some errors in our estimates of C_4 -grass relative productivities are possible, because the maps of Epstein et al. (1997) are depicted in greyscale. Based on repeated inspections we estimate this error in reading the maps to be $\pm 5\%$. An additional potential source of error is that the Epstein et al. (1997) maps depict regional-scale patterns, whereas pollen assemblages typically represent plant community composition on local-to-regional scales. For example, if C_3 -grass abundance was greater locally than regionally, the estimates of C_3 -grass abundance from pollen $\delta^{13}C$ measurements may be higher than that

from Epstein et al. (1997) at a given site. We cannot quantify the error associated with this potential disparity in scale. The NRCS dataset includes non-native grasses, which is important for our validation because these species undoubtedly contribute pollen to the surface sediments of our sites. This dataset does not include aquatic (non-upland) grasses, which primarily use C_3 photosynthesis. Although aquatic grasses could bias our estimates toward C_3 , they were not observed to be abundant around any of our study sites (E.C. Grimm, personal communication).

Pollen was extracted from ~1 ml of each sediment sample using standard techniques modified to eliminate carbon-containing chemicals (Nelson et al., 2006). All samples were manipulated in nano-pure water on a microscope slide. Grains of grass pollen were identified from a pollen-sediment slurry at 200 \times magnification and individually transferred to ~0.6- μ l drops of water using micromanipulation. Individual grains of grass pollen were then applied to the SWiM-IRMS system (Sessions et al., 2005; Eek et al., 2007) in ~0.6- μ l drops of water using a steel and glass syringe (Nelson et al., 2007).

A threshold yield of CO_2 must be established to distinguish the presence or absence of pollen, because pollen grains can be lost between the point of application to the wire and the entry of the wire into the combustion oven (a distance of ~30 cm, and an elapsed time of ~24 sec). To determine this cut-off, we analyzed drops of water to which single grains of grass pollen had been added and subsequently removed, leaving behind any residual, soluble material. These “processing” blanks were analyzed concomitantly with pollen samples from each site. As in Nelson et al. (2007), all samples with CO_2 yield (volt-seconds values, Vs) smaller than the mean $+2\sigma$ of their respective “processing” blanks were excluded from further analysis. Pollen grains from these samples likely fell off the wire before reaching the combustion oven; alternatively, these pollen grains may have been smaller than the 2σ threshold and thus indistinguishable from blanks. All final $\delta^{13}C$ data were blank-corrected by isotope mass balance, using ~25.3‰ as the $\delta^{13}C$ value of the blank (Nelson et al., 2007), and the CO_2 yield of the “processing” blanks as the mass of the blank. These “processing” blanks were determined individually for each site. The proportion of C_3 and C_4 grass pollen in each sample was determined by classifying $\delta^{13}C$ values more negative than -19.2‰ as C_3 and more positive than -19.2‰ as C_4 , following Nelson et al. (2007).

We calculated an envelope within which the true % C_4 -grass pollen would be expected to fall (hereafter in this paper, % C_4 -grass pollen refers to estimated C_4 -grass pollen abundance based on SWiM-IRMS analysis) as the sum of the error in the “true” vegetational classification ($\pm 5\%$, mentioned above) and the potential error in estimating % C_4 -grass pollen in a sample. The error in % C_4 -grass pollen classification was determined (to $\pm 2\sigma$) using a computer resampling procedure with the number of pollen grains analyzed fixed at 58, the median number of grains in our samples (see Nelson et al., 2007 for details). We used ordinary least squares (OLS) regression to determine how well the proportion of C_4 grasses on the landscape is predicted by the proportion

Table 1
Relevant information about sites and grass-pollen $\delta^{13}C$ samples from each site

Site Name (abbreviation)	Longitude °(W)	Latitude °(N)	% C_4 grasses ^a	Samples applied to wire	Samples $>2\sigma$ blank	$\delta^{13}C$ (1 σ range)
Lindsey Reservoir (LR)	-105.16	47.22	25	151	94	-22.3 (5.1)
North Lemon Lake (NL)	-102.16	46.01	30	243	103	-22.3 (8.1)
Murdo Dam (MD)	-100.72	43.92	35	118	52	-20.8 (6.9)
Little Thunder Reservoir (LT)	-105.38	43.69	40	109	57	-21.6 (7.8)
MW Lake (MW)	-104.12	43.62	40	146	45	-14.0 (10.5)
Sheridan State Fishing Lake (SS)	-100.23	39.36	60	195	94	-19.2 (7.0)
Logan City Lake (LC)	-99.58	39.63	65	149	53	-17.1 (10.3)
Lake Stockton (LS)	-99.32	39.40	65	120	51	-14.5 (10.5)
Jewel State Fishing Lake (JS)	-98.28	39.70	75	122	58	-18.6 (7.8)
Lake Annie (LA)	-81.42	27.20	80	245	142	-14.5 (10.6)
Total				1598	749	

^a Abundance of C_4 grasses estimated from vegetation surveys as described in text.

of C_4 -grass pollen in surface sediments (i.e. to calculate variance explained, R^2). Major-axis regression was used to calculate the slope, y-intercept, and the 95% confidence interval (CI) of the slope, because unlike OLS, major-axis regression accounts for error in the

independent variable (Legendre and Legendre, 1998), % C_4 -grass abundance.

Approximately 1 ml of sediment that was not treated for pollen extraction was used for analysis of total carbon and nitrogen elemental

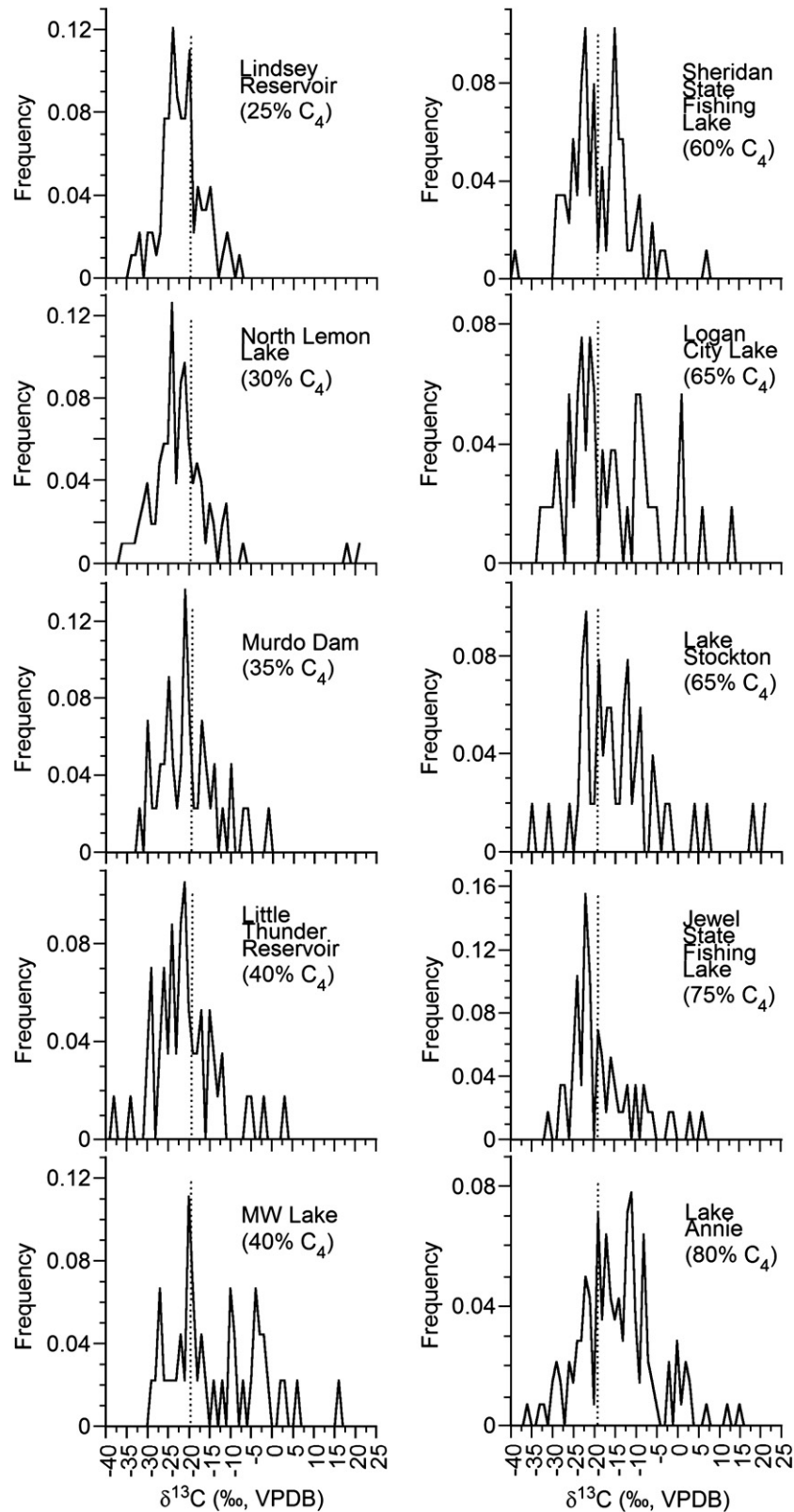


Fig. 1. Histograms of grass-pollen $\delta^{13}C$ data from the surface sediments of each site used in this study. The dotted lines at -19.2‰ signify the threshold used to distinguish C_3 from C_4 pollen. The numbers on parentheses indicate the abundance of C_4 grasses at each site as determined from vegetation surveys.

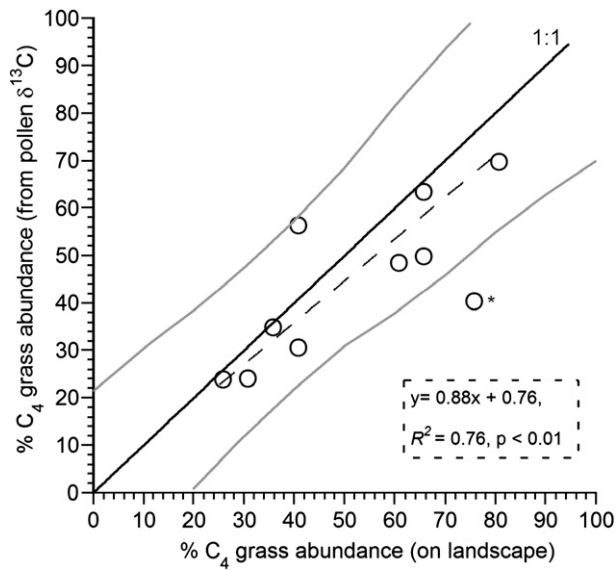


Fig. 2. Relationship between % C₄ grasses estimated from $\delta^{13}\text{C}$ data of grass pollen in lake sediments and % C₄ grasses in the vegetation around the lakes. The solid line represents a 1:1 relationship, and the grey lines define the envelope of errors related to $\delta^{13}\text{C}$ analysis and vegetation classification. The dashed line represents a linear regression through the data (excluding the outlier, Jewel State Fishing Lake, which is labeled with an asterisk). The major-axis slope is 0.88 (95% CI=0.51–1.47). If the outlier is included, the dashed line remains significant ($R^2=0.57$, $p=0.01$, major axis slope=0.76, 95% CI of slope=0.31–1.56).

ratios and for bulk $\delta^{13}\text{C}$ analysis. These subsamples were treated with 10% HCl to remove carbonates prior to analysis. Total carbon and nitrogen content was determined using a Costech 4010 CHN analyzer, and $\delta^{13}\text{C}$ values were determined using a Europa Hydra 20/20 IRMS.

3. Results and discussion

The number of individual grains of grass pollen applied to the wire for each of our ten sites ranged from 109 to 245. The resulting number of these samples with peak areas greater than the 2σ range of “processing” blanks (those deemed to contain pollen) ranged from 45 to 142. Of the 1598 grass pollen grains applied to the moving wire, 749 (47%) gave large enough CO_2 yields that they were deemed to contain pollen (Table 1). This percentage is similar to that obtained previously from analysis of eight different grass species (55%, Nelson et al., 2007). The mean $\delta^{13}\text{C}$ value from each of our ten surface-sediment sites falls within or between the ranges expected for $\delta^{13}\text{C}$ values of C₃ (~–33 to –22‰) and C₄ (~–15 to –10‰) plants (Table 1; Cerling, 1999). However, 16% of all of the individually-measured $\delta^{13}\text{C}$ values are <–33‰ or >–10‰ (Fig. 1). For example, although the mean $\delta^{13}\text{C}$ value of individual grass-pollen grains from Logan City Lake (–17.1‰, Table 1) is between the typical range of $\delta^{13}\text{C}$ values expected for C₃ and C₄ plants (Cerling, 1999), the absolute range of the $\delta^{13}\text{C}$ values (–34 to 13‰) exceeds these natural ranges (Fig. 1). A similarly large range of variation occurred in the measured $\delta^{13}\text{C}$ values of individual grass-pollen grains from known species. The scatter was attributed primarily to poor measurement precision and uncertainty in the magnitude, composition, and variability of the analytical blank, although intracellular variation in carbon allocation may also exist (Nelson et al., 2007). Despite this previously observed variation, Nelson et al. (2007) found a bimodal distribution of $\delta^{13}\text{C}$ values: 90% of the values from C₃ grasses were more negative than –19.2‰ whereas 84% of the values from C₄ grasses were more positive than –19.2‰. Thus for the lake samples analyzed here, we classified $\delta^{13}\text{C}$ values falling below –19.2‰ as indicating pollen of C₃ origin, and values above as being of C₄ origin.

If the abundance of C₃ and C₄ grass pollen in sediments determined with SWiM-IRMS indicates their proportions on the landscape as

estimated from vegetational surveys (Teeri and Stowe, 1976; Epstein et al., 1997), then there should be a positive relationship between our measurements and the survey data. Consistent with this expectation, the % C₄-grass pollen in the lake samples is significantly correlated with the proportion of C₄ grasses in the surrounding vegetation (Fig. 2). Furthermore, all but one of the data points fall within the defined error envelope. The outlying site is Jewel State Fishing Lake where the % C₄-grass pollen is 35% lower than the abundance of C₄ grasses present on the surrounding landscape. The most likely explanation for this discrepancy is that the Epstein et al. (1997) maps of regional C₃ and C₄ grass productivity do not capture the local abundances of C₃ and C₄ grasses around this site.

Although the 95% CI of the major-axis slope includes the 1:1 line, all but one of the data points falls below the 1:1 line, indicating that the % C₄-grass pollen tends to underestimate the proportion of C₄ grasses on the landscape. The under-estimation occurs to a greater extent at sites with high proportions of C₄ grasses (Fig. 2). This pattern cannot be accounted for by differential transport or preservation of C₃- and C₄-grass pollen grains because they are morphologically indistinct. Other possible explanations are preferential production of C₃- and C₄-grass pollen and/or disparity between the regional scale of the Epstein et al. (1997) dataset and local-to-regional scale of pollen assemblages, which are difficult to

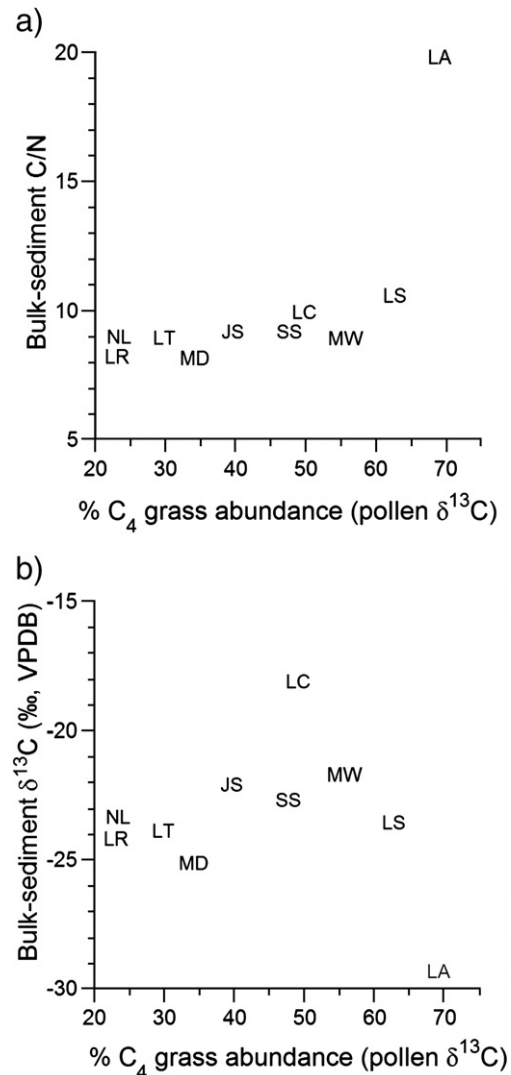


Fig. 3. Comparison of the abundance of C₄ grasses at each site derived from grass-pollen $\delta^{13}\text{C}$ data with a) bulk-sediment C/N, and b) bulk-sediment $\delta^{13}\text{C}$. Site abbreviations are as in Table 1.

evaluate. Nelson et al. (2007) also found that C₃-grass pollen tend to be more accurately classified than C₄-grass pollen using SPIRAL at the cutoff value of −19.2‰. Regardless of the potential causes, under-estimation of the C₄-grass proportion can be corrected simply by using the equation of the major-axis regression defined by the data in Fig. 2. Overall these results indicate that SPIRAL classifies the sedimentary abundance of C₃ and C₄ grass pollen to within the window of expected uncertainty (Fig. 2) and is useful for reconstructing the proportions of C₃ and C₄ grasses on the landscape.

Carbon isotopic analysis of individual grains of grass pollen provides distinct advantages over $\delta^{13}\text{C}$ analysis of other substrates. To illustrate that information on C₃ and C₄ grass variations may be masked by proxies such as bulk-sediment $\delta^{13}\text{C}$, we compared % C₄-grass pollen with C/N and $\delta^{13}\text{C}$ values of total organic matter measured on the same sediment samples. Bulk-sediment C/N values ranged from 8.1 to 10.6 at nine of the ten sites (Fig. 3a), indicating that at these sites bulk organic matter is primarily of aquatic origin (Meyers and Lallier-Verges, 1999) and thus does not reflect the proportions of C₃ and C₄ plant biomass on the terrestrial landscape. Lake Annie was an exception, with a C/N ratio of 19.8 (Fig. 3a). This high ratio suggests that Lake Annie contains organic matter produced primarily by land plants; thus the $\delta^{13}\text{C}$ value of the total sediment sample should indicate the relative proportions of C₃ and C₄ plant biomass (but not necessarily grasses) on the surrounding landscape. The bulk-sediment $\delta^{13}\text{C}$ value (−29.3‰) at Lake Annie (Fig. 3b) suggests that organic matter derived exclusively from C₃ plants, even though ~80% of the grasses on the landscape at Lake Annie are C₄ grasses. C₃ tree taxa such as *Pinus* and *Quercus*, which dominate pollen assemblages at Lake Annie, are likely the primary contributors of the total sedimentary organic matter. This interpretation is supported by the fact that grass pollen comprises only ~5% of the pollen spectrum (Watts, 1975).

Although not unexpected, the example from Lake Annie illustrates the inability of bulk-sediment $\delta^{13}\text{C}$ measurements to distinguish C₃ and C₄ shifts within the grass family, or alternatively, to detect the presence of C₄ grasses when grasses are a fractionally small proportion of the overall vegetation. Such complications also affect inferences of C₃ and C₄ abundances from $\delta^{13}\text{C}$ measurements of other bulk-phase materials, such as charcoal or leaf waxes. These limitations can be overcome using $\delta^{13}\text{C}$ analysis of individual grass-pollen grains, which provides the most straightforward approach for distinguishing C₃ from C₄ grasses in the paleorecord. Nevertheless, bulk-phase $\delta^{13}\text{C}$ measurements may contain information about the overall proportions of C₃ and C₄ biomass on the landscape, providing complementary information to that available using SPIRAL.

One potential complication of SPIRAL is the inability to distinguish pollen from aquatic versus terrestrial grasses in down-core samples. However, this complication can largely be avoided by selecting sites where aquatic grasses are unlikely to be abundant. For example, lakes with steep sides tend to have small littoral zones and limited habitats for aquatic grasses, regardless of the specific lake water-level. Another potential challenge is to account for variation in the cutoff value (−19.2‰) arising from changes in the ^{13}C content of atmospheric CO₂. However, the cutoff value may be shifted for geological samples based on estimates of the past ^{13}C composition of atmospheric CO₂ (Zachos et al., 2001). Additionally, it is necessary to measure $\delta^{13}\text{C}$ values of grass pollen from field collections and surface samples on other continents in order to extend the geographic applicability of SPIRAL.

Our results indicate that SPIRAL can help address questions about grassland evolution and ecology that previously have been difficult to answer. For example, when and why did the C₄ physiology evolve, and what is the relative importance of atmospheric CO₂ concentrations, climate, and fire on C₃ and C₄ grass variations? Molecular-clock evidence suggests that C₄ grasses originated during the late Oligocene, coincident with a precipitous drop in atmospheric CO₂ concentrations. However, there is currently no geological evidence for C₄ plants prior to the middle Miocene (Tippie and Pagani, 2007). SPIRAL may help

address this discrepancy because it focuses on a single plant life form and can detect the presence of C₄ grasses at lower abundance than bulk-phase $\delta^{13}\text{C}$ -based approaches. SPIRAL also offers a new tool to assess the influence of global versus regional drivers of C₄-grass fluctuations after the emergence of C₄ photosynthesis. Such information may be important for constraining dynamic vegetational models, guiding management and conservation strategies in grass-dominated ecosystems, and testing hypotheses regarding environmental controls (e.g., atmospheric CO₂ concentrations, fire) on C₃ and C₄ grasses in the geological record (e.g. Keeley and Rundel, 2005).

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