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CARBON ISOTOPIC FRACTIONATION BETWEEN WHOLE LEAVES AND CUTICLE

DANA L. ROYER[1] AND MICHAEL T. HREN[2]
[1]Department of Earth and Environmental Sciences, Wesleyan University, Middletown, Connecticut 06459, USA
[2]Department of Chemistry, University of Connecticut, Storrs, Connecticut 06269, USA
email:droyer@wesleyan.edu

ABSTRACT: **The $\delta^{13}C$ of fossil leaf cuticle is frequently used for paleoenvironment interpretation. A tacit assumption that is common in such studies is that the $\delta^{13}C$ of the cuticle is the same as the $\delta^{13}C$ of the original whole leaf. We tested this assumption by measuring the isotopic fractionation between cuticle and whole leaves ($\varepsilon^{13}C_{cuticle\text{-}leaf}$) in 175 phylogenetically diverse species. The average $\varepsilon^{13}C_{cuticle\text{-}leaf}$ is indistinguishable from zero (-0.04 ±1.2‰ 1σ), in keeping with the few previously published data and with studies that have tracked the evolution of leaf $\delta^{13}C$ during decomposition. Across species, $\varepsilon^{13}C_{cuticle\text{-}leaf}$ spans over 9‰: this variability does not covary with growth habit (woody vs. herbaceous) or climate, but does contain a strong phylogenetic signal. In particular, more basal groups (lycopsids and some gymnosperms, basal ferns, and basal angiosperms) tend to have negative $\varepsilon^{13}C_{cuticle\text{-}leaf}$ values. This variability should be accounted for in studies that wish to estimate whole-leaf $\delta^{13}C$ from cuticle $\delta^{13}C$.**

INTRODUCTION

The stable carbon isotopic composition ($\delta^{13}C$) of fossil plants provides considerable insight into the functioning of ancient ecosystems. Analysis of fossil plant $\delta^{13}C$ has helped to quantify the CO_2 content of the atmosphere (Schaller et al. 2011; Schubert and Jahren 2012; Franks et al. 2014) and O_2 (Beerling et al. 2002; Tappert et al. 2013), the photosynthetic pathway (C_3 vs. C_4; Fox and Koch 2003), the broad taxonomy (Fletcher et al. 2004; Boyce et al. 2007; Schouten et al. 2007; Tomescu et al. 2009), the degree of water stress on the landscape (Nguyen Tu et al. 1999), and the ocean-atmosphere-land teleconnections during severe carbon-cycle perturbations such as the end-Triassic (Bacon et al. 2011), end-Cretaceous (Jerrett et al. 2015), and Paleocene–Eocene thermal maximum (McInerney and Wing 2011).

Bulk fossil leaves are commonly used in isotopic analyses for paleoenvironment assessment (e.g., Beerling et al. 1998; Arens and Jahren 2000, 2002; Jahren et al. 2001; Bacon et al. 2011; Grein et al. 2013; Franks et al. 2014). In most cases, a required assumption is that the isotopic composition of the leaf has not changed during the fossilization process. But all fossil leaves have undergone some amount of molecular and physical degradation; frequently, just the recalcitrant cuticle is left, which is generally comprised of carbon moieties that are less readily decomposed by microorganisms (e.g., Almendros et al. 1996; Lichtfouse et al. 1997, 1998; Nierop 1998). Is the carbon isotopic composition of cuticle different than the whole leaf? If it is, fossil studies could be improved by applying a correction factor to the measured cuticle values.

There is surprisingly little information on the carbon isotopic fractionation or "discrimination" ($\varepsilon^{13}C_{cuticle\text{-}leaf}$) between cuticle and whole leaf. There is a wealth of information on the isotopic composition of individual compounds: recalcitrant components like lipids and lignin have lower $\delta^{13}C$ values relative to the bulk leaf ($\Delta^{13}C \approx$ -2 to -5‰, where $\Delta = \delta^{13}C_{substrate} - \delta^{13}C_{bulk}$ and $\Delta^{13}C_{cuticle\text{-}leaf} \approx \varepsilon^{13}C_{cuticle\text{-}leaf}$ for small values) while more labile components like sugar, starch, protein, and cellulose have higher $\delta^{13}C$ values ($\Delta^{13}C \approx +1$ to +3‰) (see Bowling et al. 2008 and references therein). Cuticle from both living and fossil plants is comprised mostly of lipids (e.g., Mösle et al. 1998; Gupta et al. 2006; Aucour et al. 2009). From this, we may expect cuticle to be isotopically depleted in ^{13}C relative to the whole leaf ($\Delta^{13}C_{cuticle\text{-}leaf} < 0$‰). However, the scant data that do exist on this topic give a mixed signal, with $\Delta^{13}C_{cuticle\text{-}leaf}$ ranging from -1.4 to +3.5‰ (Table 1); two conference abstracts by Upchurch and colleagues report a $\Delta^{13}C_{cuticle\text{-}leaf}$ of -3.5‰ from an unstated number of species (Upchurch et al. 1997; Upchurch and Marino 2007).

The primary objective of this study is to measure $\varepsilon^{13}C_{cuticle\text{-}leaf}$ in a large, phylogenetically diverse set of species. This dataset allows us to test—much more extensively than in past studies—whether a carbon isotopic fractionation exists between cuticle and whole leaves and whether the data contain a phylogenetic signal. A strong phylogenetic signal means that more closely related species tend to have similar trait values. If a phylogenetic signal is present, then $\varepsilon^{13}C_{cuticle\text{-}leaf}$ covaries with historical factors (relatedness). Finally, given the range of environments and species sampled, we also test whether $\varepsilon^{13}C_{cuticle\text{-}leaf}$ covaries with climate (mean annual temperature and precipitation) and growth habit (woody vs. herbaceous).

MATERIALS AND METHODS

We sampled leaves from 175 species for isotopic analysis; five of these species were sampled at two sites (see online Supplemental File 1). Seventy-eight of the species come from (or near) Wesleyan University (Middletown, Connecticut), Connecticut College (New London, Connecticut), and Dinosaur State Park (Rocky Hill, Connecticut). We sampled these species in late June and early July 2015; leaf vouchers are at Wesleyan University. Most of these plants were cultivated, field-grown individuals; the main exception to this was ten tropical species from glasshouses at Connecticut College. Twenty-nine herbaceous angiosperm species come from the leaf vouchers associated with Royer et al. (2010), which are archived at Wesleyan. Most of the remaining species (n = 60) come from the vouchers associated with Peppe et al. (2011), which are archived at Wesleyan or the paleobotany collections at the Smithsonian Natural History Museum and Florida Museum of Natural History (online Supplemental File 1). For all field-grown woody plants, sun leaves were

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TABLE 1.—*Past studies that have reported the carbon isotopic difference between cuticle and whole leaves ($\Delta^{13}C_{cuticle\text{-}leaf} = \delta^{13}C_{cuticle} - \delta^{13}C_{leaf} \approx \varepsilon^{13}C_{cuticle\text{-}leaf}$).*

Study	Species	$D^{13}_{cuticle\text{-}leaf}$ (‰)	Treatment for isolating cuticle
Fletcher et al. (2004)	two mosses and six liverworts	+0.4 to +3.5	glacial acetic acid + H_2O_2 followed by chloroform + methanol
Beerling et al. (1998)	*Ginkgo biloba*	-1.2	not reported
Kowalczyk (2015)	*Ginkgo biloba*	-1.4	ethanol followed by hot H_2O_2
Kowalczyk (2015)	*Sassafras albidum*	-0.7	ethanol followed by NaOH
Milligan (2016)	*Stenochlaena palustris*	+1.4	NaOH
Willmer and Firth (1980)	*Commelina communis*	+0.5	physical separation
Nishida et al. (1981)	*Commelina communis* (abaxial)	+0.3	physical separation
Nishida et al. (1981)	*Commelina communis* (adaxial)	-1.0	physical separation
Willmer and Firth (1980)	*Tulipa gesneriana*	+1.1	physical separation
Nishida et al. (1981)	*Tulipa gesneriana*	0.0	physical separation
Willmer and Firth (1980)	*Vicia faba*	+0.2	physical separation
Willmer and Firth (1980)	*Allium vineale*	+0.2	physical separation

sampled; leaves from all field-grown herbaceous species came from the shady understory. All species were scored for growth habit (woody vs. herbaceous). For the field-grown plants, we collected climate information (mean annual temperature and precipitation) from Hijmans et al. (2005) (see also Peppe et al. 2011). Across sites, mean annual temperature (MAT) ranges from 2.5 to 26.4 °C and mean annual precipitation (MAP) from 77 to 3241 mm.

For each broad-leaf species, we sampled the central portion of a single leaf with a hole-punch (typically 4–6 leaf disks). For needle-leaf species, we sampled multiple, adjacent whole leaves. For species with minor or non-photosynthetic leaves (Lycopodiales, Psilotales, and Equisetales), we sampled the photosynthetic stems. For each species, we isolated the cuticle with bleach (1:1 household bleach to DI water; e.g., Kerp and Krings 1999). This treatment took between hours to days, depending on reactivity. All processed cuticle was uniform in its clear color and little-to-no residual mesophyll. Although different chemical treatments can be better at clearing cuticle, depending on the species (e.g., Table 1), we consider it more important for data fidelity to apply a uniform methodology. We note that we sampled the same *Ginkgo* tree as Kowalczyk et al. (2015) (Table 1), and although different methods were used to isolate the cuticle, the isotopic fractionations are very similar (-1.2 vs. -1.4‰).

All whole leaf and cuticle samples were weighed (0.5–4 mg) into tin capsules prior to stable isotopic analysis. The stable carbon isotopic composition of leaf and cuticle samples were measured via high temperature combustion in a Costech Elemental Analyzer attached to a Thermo MAT 253 IRMS using He as a carrier gas. The isotopic compositions of unknown leaf samples were corrected for size and scale compression effects using a suite of international reference materials that span the range of carbon isotopic variability and sample intensities. Reproducibility of standards over a range of sample sizes was < 0.2‰ 1σ. Replicate cuticle samples differed by up to 0.7‰ (mean = 0.4‰).

We calculated carbon isotope discrimination between leaf and cuticle ($\varepsilon^{13}C_{cuticle\text{-}leaf}$) following Coplen (2011) with the multiplier of one thousand [$\varepsilon = (\alpha - 1) \times 1000$], where $\alpha = \delta^{13}C_{cuticle} + 1000 / \delta^{13}C_{leaf} + 1000$. In per mil notation, this is comparable to the formulation for discrimination that describes the fractionation between substrate (atmospheric CO_2) and product (leaf biomass) (e.g., O'Leary et al. 1981) defined as $\Delta^{13}C_{cuticle\text{-}leaf} = (\delta^{13}C_{cuticle} - \delta^{13}C_{leaf}) / (1 + \delta^{13}C_{leaf} / 1000)$.

We constructed an order-level phylogeny following Zanne et al. (2014) (online Supplemental File 2). We then used three tools to test for phylogenetic signal in the isotopic data. The first two, Blomberg et al.'s (2003) *K* and Pagel's (1999) λ, test how closely trait distributions follow the Brownian motion model of evolution. Under the Brownian model, trait values for phylogenetically close species will be more similar to one another than distantly related species. For both *K* and λ, a value of zero is conformable with no phylogenetic signal (the null hypothesis), while a value of one is consistent with the Brownian model. We computed λ and *K* using the phytools R package (Revell 2012).

K and λ are each a single statistic that describe trait patterning across an entire tree. To explore finer-level patterns, we computed phylogenetically independent contrasts (PICs) at each node in the phylogeny using the APE package in R (Paradis et al. 2004). A PIC is the expected difference in trait values between the two distal branches of a node after taking into account relatedness by assuming a Brownian model (Felsenstein 1985). PIC values are influenced by phylogenetic patterning and sample size.

RESULTS

The mean $\varepsilon^{13}C_{cuticle\text{-}leaf}$ in 180 species-site pairs is -0.04‰ (±1.2‰ 1σ). The data appear to be normally distributed (Fig. 1; one-sample Kolmogorov-Smirnov normality test: $D = 0.04$; $P = 0.79$), and the mean

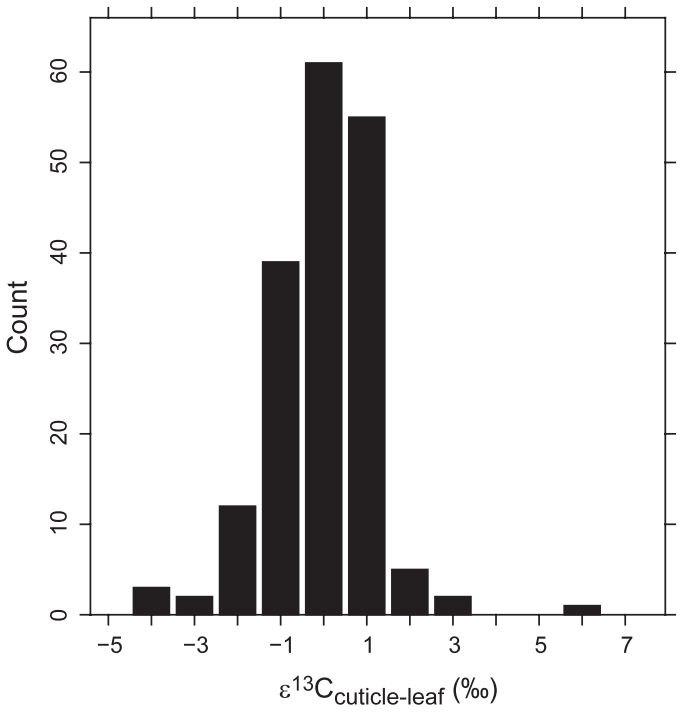

FIG. 1.—Species-level frequency of the carbon isotopic offset between cuticle and bulk leaves ($\varepsilon^{13}C_{cuticle\text{-}leaf}$).

value of -0.04‰ is not significantly different than zero (one-sample t-test: $t_{179} = -0.43$; $P = 0.66$).

Although the mean tendency of $\varepsilon^{13}C_{cuticle\text{-}leaf}$ is approximately zero, what can account for the 9.9‰ range in individual values (Figure 1)? Growth habit and climate do not appear important: the null hypothesis that $\varepsilon^{13}C_{cuticle\text{-}leaf}$ is the same between woody and herbaceous species cannot be rejected with 95% confidence, although it comes close (means = -0.13‰ and +0.22‰, respectively; two-sample t-test: $t_{81} = 1.69$; $P = 0.09$), and correlations in $\varepsilon^{13}C_{cuticle\text{-}leaf}$ with MAT and MAP are both very weak ($r^2 < 0.08$).

Can relatedness partly explain the distribution in $\varepsilon^{13}C_{cuticle\text{-}leaf}$? The phylogenetic patterning of the order-level means is shown in Figure 2. Both the K and λ statistics strongly support the presence of phylogenetic signal ($K = 0.76$, $P = 0.006$; $\lambda = 1.22$, $P < 0.001$). More basal groups—especially the lycopsids (Lycopodiales), the basal fern order Psilotales, and some gymnosperms (Cycadales, Ginkgoales) and basal dicot angiosperms (Chloranthales, Canellales)—have a tendency for negative $\varepsilon^{13}C_{cuticle\text{-}leaf}$ values. The PICs in part support this notion: the node that separates Psilotales from all other more derived ferns has the largest contrast (-0.19‰; n = 16 species in clade; see highlighted value in Fig. 3). This means that—after taking relatedness into account—the expected difference in $\varepsilon^{13}C_{cuticle\text{-}leaf}$ between Psilotales and other ferns is -0.19‰. Similarly, the contrast between Chloranthales (a basal dicot) and all remaining more derived groups is -0.13‰ (n = 111 species; see highlighted value in Fig. 3). The only PICs with larger absolute values are associated with sparsely sampled nodes (< 10 species), and so are less representative of broader patterns (Santalales vs. Caryophyllales, n = 3 species, PIC = -0.17; Magnoliales+Laurales vs. Canellales, n = 8 species, PIC = +0.15). Across the entire tree, the average absolute difference in measured $\varepsilon^{13}C_{cuticle\text{-}leaf}$ for each contrast in Figure 3 is 0.84‰; after taking relatedness into account with the PICs, this difference shrinks to 0.06‰.

DISCUSSION

Across the full data set, we cannot reject the null hypothesis that $\varepsilon^{13}C_{cuticle\text{-}leaf} = 0$‰. We also find no significant covariation with growth habit (woody vs. herbaceous) and climate (mean annual temperature and precipitation). Because all of the field-grown herbs were in the understory but leaves from the field-grown trees and shrubs were exposed to full sun, we also infer little covariation with irradiance, which at most is the statistically non-significant mean difference between herbaceous and woody species (+0.35‰). These findings are in line with the limited published data on $\varepsilon^{13}C_{cuticle\text{-}leaf}$ (Table 1), but at odds with previously published data (e.g., Bowling et al. 2008; Mendez-Millan et al. 2011) that suggest lipid-rich cuticle should be isotopically depleted in ^{13}C ($\varepsilon^{13}C_{cuticle\text{-}leaf} < 0$‰; see also Introduction). We do recognize, however, that a growing body of literature demonstrates that not all lipid components are ^{13}C-depleted relative to whole leaves (e.g., Diefendorf et al. 2011, 2012, 2015).

The variance in $\varepsilon^{13}C_{cuticle\text{-}leaf}$ across species is large, with a 1σ of 1.2‰ and a total range of 9.9‰. This distribution across plant orders has a strong phylogenetic signal (Figs. 2, 3). Most notably, some basal groups such as the lycopsids (Lycopodiales), the basal fern Psilotales, the gymnosperms Cycadales and Ginkgoales, and the basal dicots Chloranthales and Canellales have distinctively low $\varepsilon^{13}C_{cuticle\text{-}leaf}$ values. These low values contrast with those of their sister groups, a pattern that is broadly corroborated by the PICs (Fig. 3). It is important to note that not all basal groups have low values, including most ferns and the heavily sampled Pinales (Fig. 2).

Recognition of phylogenetic signal in $\varepsilon^{13}C_{cuticle\text{-}leaf}$ is broadly consistent with the findings of Diefendorf et al. (2015). These authors studied 43 species within Pinales and found phylogenetic signal in the carbon isotopic fractionation of *n*-alkanes, a common component of cuticle. Together with the findings reported here, this raises the possibility that historical factors may often be important for controlling—across species—carbon isotopic partitioning within leaves.

The low $\varepsilon^{13}C_{cuticle\text{-}leaf}$ values for the Lycopodiales and Psilotales may be due to sampling stems, not leaves, but we also sampled stems for Equisetales and did not find low values. Although we did not detect a significant correlation between $\varepsilon^{13}C_{cuticle\text{-}leaf}$ and growth habit (herbaceous vs. woody), we do note that the two most basal orders with low $\varepsilon^{13}C_{cuticle\text{-}leaf}$ values—Lycopodiales and Psilotales—are herbaceous. But herbaceous taxa follow the same general pattern as the full data set, with most of the derived herbaceous groups having $\varepsilon^{13}C_{cuticle\text{-}leaf}$ values near zero (e.g., Asterales, Apiales). This reinforces the interpretation that in this data set growth habit does not influence $\varepsilon^{13}C_{cuticle\text{-}leaf}$.

If these data are representative of plants in general, they support the notion that not all lipids—especially those in the most recalcitrant fraction of the cuticle—have low $\delta^{13}C$ values (e.g., Diefendorf et al. 2011, 2012, 2015). In most plants, the bulk of the compounds that comprise the cuticular wax originate from long-carbon chain fatty acids and include alkanes, aldehydes, alcohols, ketones and esters (e.g., Yeats and Rose 2013). Other secondary metabolites such as triterpenoids, flavonoids, and tocopherols may also be significant components for some plant species (Jetter et al. 2006; Diefendorf et al. 2012). The isotopic composition of these compounds can vary dramatically within a single plant (e.g., Bowling et al. 2008; Dungait et al. 2008, 2010; Diefendorf et al. 2012). Ultimately, lipid composition can vary dramatically between plant species, ontogeny, and growth conditions (e.g., Jenks and Ashworth 2010; Diefendorf et al. 2015). A variety of external factors such as pathogens, irradiance, temperature, and water availability interact with the regulatory genes to influence cutin or wax biosynthesis (Yeats and Rose 2013). These environmental factors, and differences in physical biochemical growth strategies, drive variations in the makeup of cuticle and resultant $\varepsilon^{13}C_{cuticle\text{-}leaf}$. Indeed, the isotopic fractionation between leaves and normal alkanes can vary up to 10‰ both across species and across different alkane chain lengths (Diefendorf et al. 2011; Eley 2016). We speculate that the observed differences in $\varepsilon^{13}C_{cuticle\text{-}leaf}$ reflect both variations in the distribution of compounds produced in the cuticle by different plants, as well as differences in the magnitude of carbon isotope discrimination between plants for an individual compound class (fatty acids, normal alkanes, wax esters, etc.).

A chemical treatment (bleach) to isolate cuticle may be a poor surrogate—in terms of $\delta^{13}C$—for the taphonomic process of producing fossil cuticle. Carbon is often resorbed in aging leaves (Vergutz et al. 2012); these leaves eventually drop and then undergo some amount of decay in the soil (e.g., Nguyen Tu et al. 2003). Subsequent burial greatly curtails decomposition, but biomolecules can continue to polymerize (e.g., Gupta et al. 2006, 2007). In addition, once buried in soil, cuticle from different plant species may degrade at different rates and the distribution of compounds within these components may change through time (Macnamara et al. 1983). There is a rich literature on the evolution of $\delta^{13}C$ in soil organic matter. In soils, organic matter at depth is often more enriched in ^{13}C than younger organic matter towards the soil surface; this pattern is thought to arise not from an isotopic fractionation in the plant matter itself but from the addition of bacterial and fungal biomass, which tend to have higher $\delta^{13}C$ values (Balesdent et al. 1993; Ehleringer et al. 2000). However, any isotopic patterns in disseminated organic matter from aged soils do not have direct relevance for leaf fossils because they are different materials and because leaves typically fossilize in settings where there is rapid burial (i.e., not in stable soils). Along these lines, Arens and Jahren (2000) found little difference in $\delta^{13}C$ between leaf cuticle and co-existing disseminated organic matter (OM) in a rapidly deposited sedimentary succession: using the regression between the two measured variables, $\Delta^{13}C_{cuticle\text{-}OM}$ ranged only from -0.3 to +0.1‰. Separate from disseminated organic matter, what about the decomposition of the leaves

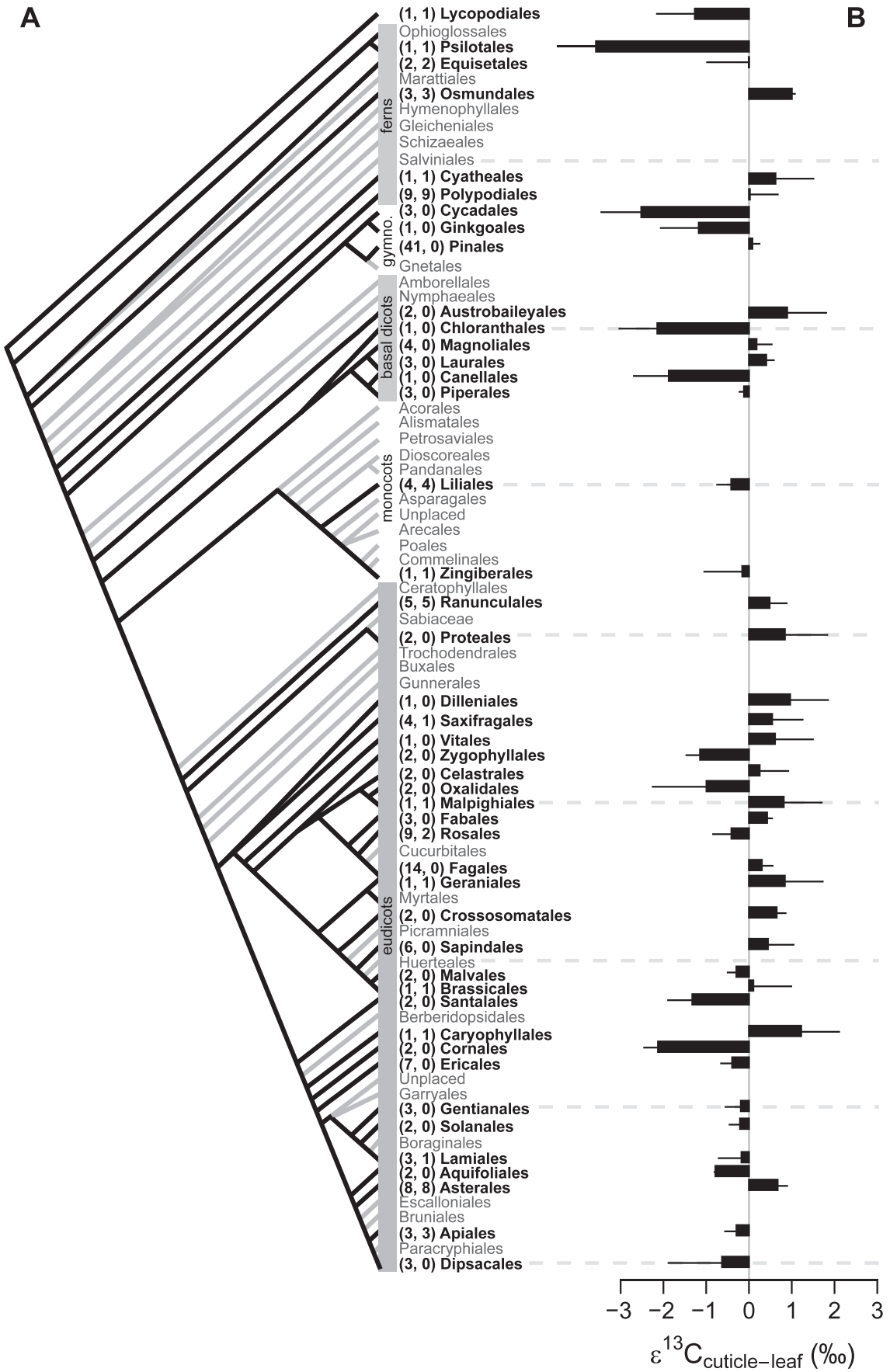

FIG. 2.—Order-level patterns in the carbon isotopic offset between cuticle and bulk leaves ($\varepsilon^{13}C_{cuticle\text{-}leaf}$). **A**) Order-level phylogeny for vascular plants (APW v.13: Stevens 2001). Names in bold are represented in the isotopic data set used here, with the total number of species and number of herbaceous species in parenthesis. Abbreviation: gymno. = gymnosperms. **B**) Order-level means of $\varepsilon^{13}C_{cuticle\text{-}leaf}$. Errors are the standard errors of the mean; errors for orders with only one sampled species are the mean standard deviation of all other sampled orders (0.91‰).

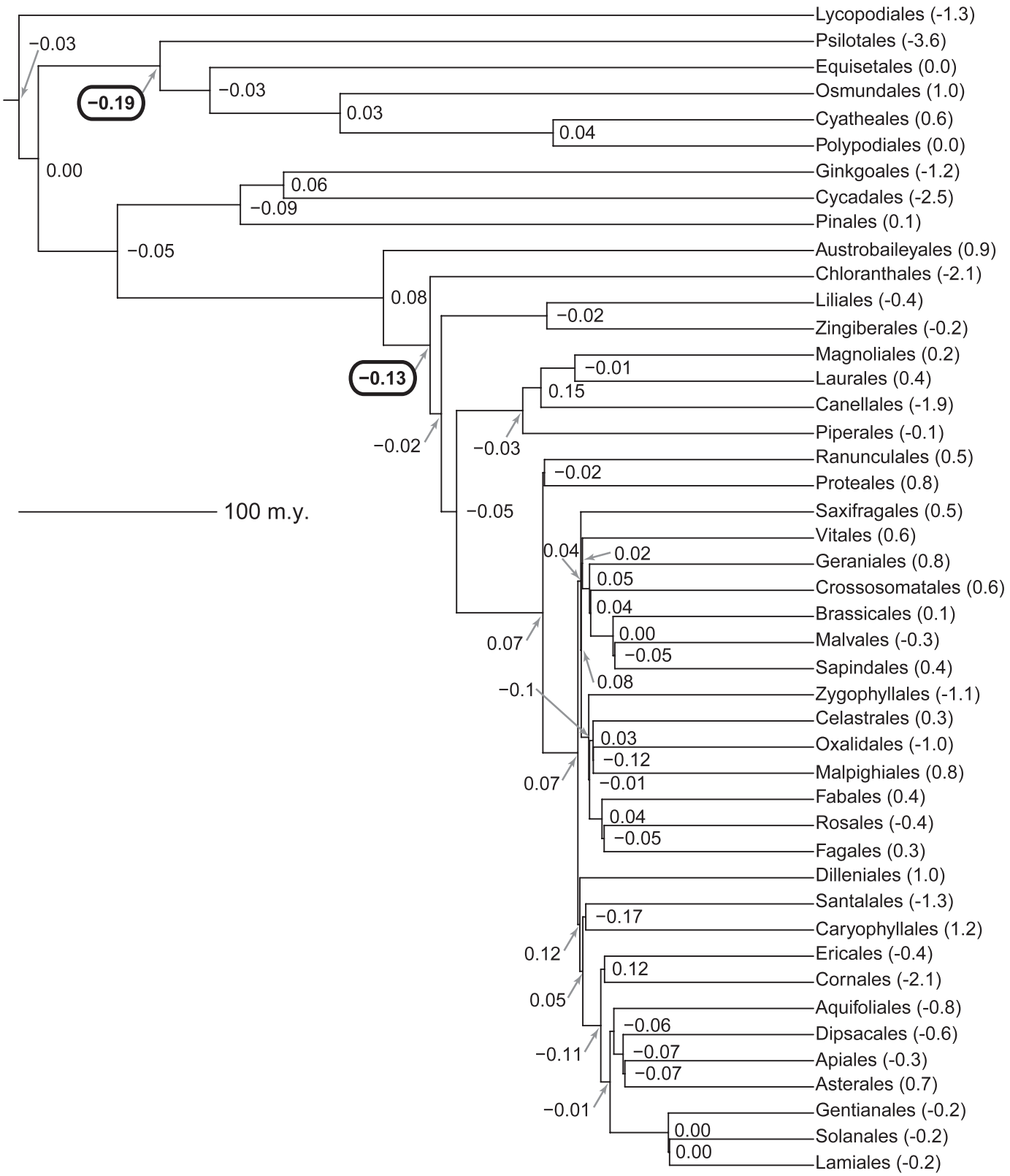

FIG. 3.—Phylogeny of orders present in data set (after Zanne et al. 2014); this phylogeny has a similar structure to the undated APW phylogeny (compare with Fig. 2A). The numbers in parentheses that follow the order names are the $\varepsilon^{13}C_{cuticle\text{-}leaf}$ means (identical to Fig. 2B). The numbers at the nodes are the phylogenetically independent contrasts (PIC); the two circled values in bold are large contrasts that are discussed in the text.

themselves? Experiments and field observations of litter decay consistently find little-to-no temporal patterns in leaf $\delta^{13}C$ (Balesdent et al. 1993; Ehleringer et al. 2000; Nguyen Tu et al. 2004; but see Fernandez et al. 2003). In sum, results from most taphonomic studies are consistent with the expectation for very little carbon isotopic difference between fresh leaves and cuticle.

Although the mean $\varepsilon^{13}C_{cuticle\text{-}leaf}$ is near-zero in the data set, the range is almost 10‰. For fossil studies where whole-leaf $\delta^{13}C$ is inferred from cuticle $\delta^{13}C$, uncertainty in $\varepsilon^{13}C_{cuticle\text{-}leaf}$ poses a challenge. One approach for recourse is to use Figure 2 and online Supplemental File 1 to apply an appropriate correction to measured values of cuticle $\delta^{13}C$. Another option is to make new $\varepsilon^{13}C_{cuticle\text{-}leaf}$ measurements on a nearest living relative (Kowalczyk et al. 2015; Milligan et al. 2016). A third option is to include an error term that reflects the uncertainty in assuming a 1:1 relationship between whole-leaf and cuticle $\delta^{13}C$. In this data set, 68% of the $\varepsilon^{13}C_{cuticle\text{-}leaf}$ values fall between -1.1 and +1.0‰ (similar to ±1 standard deviation) and 95% of the values fall between -2.7 and +1.8‰ (similar to ±2 standard deviations).

CONCLUSION

We find no support for the hypothesis that the central tendency of $\varepsilon^{13}C_{cuticle\text{-}leaf}$ is different than zero. This interpretation is in keeping with the scant literature on $\varepsilon^{13}C_{cuticle\text{-}leaf}$ and with the more established literature on plant taphonomy. Despite the mean tendency, there is considerable scatter in $\varepsilon^{13}C_{cuticle\text{-}leaf}$ across species, and this scatter has phylogenetic signal. As a result, care should be taken when reconstructing whole-leaf $\delta^{13}C$ from cuticle $\delta^{13}C$.

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SUPPLEMENTAL MATERIAL

Data are available from the PALAIOS Data Archive: http://www.sepm.org/pages.aspx?pageid=332.

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