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Geochimica et Cosmochimica Acta 170 (2015) 145–156

Geochimica et Cosmochimica Acta

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Leaf wax composition and carbon isotopes vary among major conifer groups

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Received 2 February 2015; accepted in revised form 25 August 2015; Available online 3 September 2015

Abstract

Leaf waxes (e.g. *n*-alkanes, *n*-alkanoic acids) and their carbon isotopes ($\delta^{13}C$) are commonly used to track past changes in the carbon cycle, water availability, and plant ecophysiology. Previous studies indicated that conifers have lower *n*-alkane concentrations than angiosperms and that ^{13}C fractionation during *n*-alkane synthesis ($\varepsilon_{n\text{-alkane}}$) is smaller than in angiosperms. These prior studies, however, sampled a limited phylogenetic and geographic subset of conifers, leaving out many important subtropical and Southern Hemisphere groups that were once widespread and common components of fossil assemblages. To expand on previous work, we collected 43 conifer species (and *Ginkgo biloba*) from the University of California Botanical Garden at Berkeley, sampling all extant conifer families and almost two-thirds of extant genera. We find that Pinaceae, including many North American species used in previous studies, have very low or no *n*-alkanes. However, other conifer groups have significant concentrations of *n*-alkanes, especially Southern Hemisphere Araucariaceae and Podocarpaceae (monkey puzzles, Norfolk Island pines, and yellowwoods), and many species of Cupressaceae (junipers and relatives). Within the Cupressaceae, we find total *n*-alkane concentrations are high in subfamilies Cupressoideae and Callitroideae, but significantly lower in the early diverging taxodioid lineages (including bald cypress and redwood). Individual *n*-alkane chain lengths have a weak phylogenetic signal, except for *n*-C_{29} alkane, but when combined using average chain length (ACL), a strong phylogenetic signal emerges. The strong phylogenetic signal in ACL, observed in the context of a common growth environment for all plants we sampled, suggests that ACL is strongly influenced by factors other than climate. An analysis of $\varepsilon_{n\text{-alkane}}$ indicates a strong phylogenetic signal in which the smallest biosynthetic fractionation occurs in Pinaceae and the largest in Taxaceae (yews and relatives). The relationship between phylogeny and $\varepsilon_{n\text{-alkane}}$ may be related to differences in carbon metabolism among conifer clades. These results have important implications for interpreting *n*-alkane $\delta^{13}C$ values in sedimentary archives, especially outside of North America.

1. INTRODUCTION

The leaf waxes of vascular plants are primarily composed of long-chain *n*-alkyl compounds including the *n*-alkanes, *n*-alkanoic acids, and *n*-alkanols (Eglinton et al., 1962; Eglinton and Hamilton, 1967; Kolattukudy et al., 1976). These leaf waxes are widely used to reconstruct environmental change because they are commonly transported and preserved in sediments from which they are easily extracted and analyzed (Cranwell, 1981; Rieley et al., 1991; Collister et al., 1994a; Freeman and Colarusso, 2001). Of the leaf wax components, *n*-alkanes have been the focus of most geologic studies, although other waxes, such as *n*-alkanoic acids and *n*-alkanols, can

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http://dx.doi.org/10.1016/j.gca.2015.08.018
0016-7037/

be informative when preserved (Feakins et al., 2007; Douglas et al., 2012; Sachse et al., 2012). Leaf waxes, especially *n*-alkanes, have been applied to answer various paleoecological questions, such as distinguishing relative changes in paleotemperature or paleohydrology (Castañeda and Schouten, 2011; Freeman and Pancost, 2014; Bush and McInerney, 2015). More recently, analyses of the carbon and hydrogen isotope composition of leaf waxes has greatly expanded their utility for paleoclimate studies, and they have been used extensively to characterize past changes in the carbon cycle, water availability, hydrology, and vegetation (e.g., Pagani et al., 2006; Schouten et al., 2007; Smith et al., 2007; Tierney et al., 2008; Castañeda et al., 2009; Tipple et al., 2011; Niedermeyer et al., 2014).

Despite the widespread use of leaf waxes, many unanswered questions about them remain. For example, the variation in wax concentration and composition among and within many major plant groups has not been quantified and is largely unknown (Diefendorf et al., 2011; Bush and McInerney, 2013). Plant traits such as leaf lifespan, cuticle thickness, and specific leaf area may influence composition of short-chain *n*-alkanoic acids (Mueller et al., 2012), but their influence on the composition of other waxes is largely unstudied. Climate may also influence leaf wax composition, as observed in studies of *n*-alkane average chain length, but relationships with climate tend to be weak and to vary by species (e.g., Dodd and Poveda, 2003; Sachse et al., 2006; Shepherd and Wynne Griffiths, 2006; Castañeda et al., 2009; Vogts et al., 2012; Hoffmann et al., 2013; Tipple and Pagani, 2013; Bush and McInerney, 2015). In addition, the timing of leaf wax synthesis may vary among species, which has important consequences for interpreting isotope signals preserved in these waxes (Sachse et al., 2006, 2009; Tipple et al., 2013). Taken together, these unanswered questions complicate interpretations of leaf waxes found in sediments, especially if plant community composition has changed in combination with climate.

In this study, we focus on understanding variation in *n*-alkane composition among conifers, a major non-angiosperm seed plant group with a long and abundant fossil record. Conifers dominate many high elevation and high latitude ecosystems in the modern world, especially in the Northern Hemisphere, but the group has a global distribution and also includes many tropical members. Prior studies have suggested that conifers differ from angiosperms in their *n*-alkane chemistry, for example, having *n*-alkane concentrations 200 times lower when grown under the same climatic conditions (Diefendorf et al., 2011; Bush and McInerney, 2013). Also, ^{13}C fractionation during *n*-alkane biosynthesis (ε_{lipid}) is ~2‰ less than in angiosperms, suggesting underlying differences in the allocation of carbon to different materials (e.g., carbohydrates, amino acids, lipids) among major plant groups (e.g. Diefendorf et al., 2011). However, Diefendorf et al. (2011) measured only a few Northern Hemisphere conifer species. Many important subtropical, tropical, and Southern Hemisphere conifers groups have been ignored, even though they were more widespread in the past and are common components of fossil assemblages.

To expand on previous studies, we collected 43 conifer species (and *Ginkgo biloba*) from the University of California Botanical Garden at Berkeley. The sample includes species from all extant conifer families and more than 60% of extant genera. By collecting all specimens at a common site we attempted to minimize the confounding effects of climate, allowing potential phylogenetic patterns in the $\delta^{13}C$ of leaf waxes to be expressed more strongly. We find that species belonging to different major conifer lineages have different *n*-alkane compositions and concentrations even when grown in the same climate. A strong phylogenetic signal is also apparent in *n*-alkane isotope fractionation values. These results have important consequences for interpreting sedimentary leaf waxes where conifers are or were part of the plant community.

2. MATERIALS AND METHODS

2.1. Sampling location and leaf collection

Fresh leaf tissue was collected from 43 conifer species and from *G. biloba* at the University of California Botanical Garden at Berkeley (UCBGB; 37.8752°N, 122.2386°W, 210 m) in December of 2011. Evergreen leaf tissue was sampled from the 2011 leaf flush. The total rainfall for 2011 was 602 mm and the average minimum and maximum temperatures are 8.8 and 18.3 °C, respectively (PRISM, 2014). For each species, leaf tissue was collected from multiple branches on the sun-exposed side of a single mature tree per species using scissors, pruners, or pole-pruners. Sampling height ranged from 2 to 3 m except for *Sequoiadendron giganteum* and *Sequoia sempervirens*, which were sampled at ~25 m. The canopy structure at the UCBGB is very open and therefore it is unlikely that soil respiration generates a ^{13}C-depleted CO_2 layer at this sampling height. Sample collection information is reported in electronic annex Table EA-1. Samples (~10 g) were gently rinsed with deionized water, frozen (−20 °C), freeze-dried, and homogenized by powdering with a ball mill.

2.2. Lipid extraction and separation

Powdered leaves were extracted using an accelerated solvent extractor (Dionex ASE 350) with 2:1 (v/v) DCM/MeOH with three extraction cycles at 10.34 MPa (1400 psi) and 100 °C. The total lipid extract (TLE) was base saponified to cleave ester groups with 2.5 ml 0.5 N KOH in 3:1 (v/v) MeOH/water) for 2 h at 75 °C. After cooling, 2 ml of NaCl in water (5%, w/w) was added and then the solution was acidified with 6 N HCl to a pH of 1. The acidic solution was extracted with hexanes/DCM (4:1, v/v), neutralized with $NaHCO_3/H_2O$ (5%, w/w), followed by water removal over Na_2SO_4.

The saponified lipid extract was subsequently separated into four polarity fractions following a modified version of Sessions (2006). Briefly, 0.5 g of aminopropyl-bonded silica gel (Sigma Aldrich, St. Louis, USA) was loaded into 6 ml SPE glass tubes with PTFE frits on a Visiprep SPE vacuum manifold with disposable PTFE liners (Sigma Aldrich). Hydrocarbons were eluted with 4 ml of hexanes, ketones

eluted with 8 ml of hexanes/DCM (6:1, v/v), alcohols eluted with 8 ml of DCM/acetone (9:1, v/v), and acids eluted with 8 ml of DCM/formic acid (49:1, v/v).

2.3. Identification and quantification

Lipids were identified and quantified with a gas chromatograph (GC)-mass selective detector (MSD) and flame ionization detector (FID). An Agilent 7890A GC with multi mode inlet operated in pulsed splitless mode at 320 °C was interfaced to an Agilent 5975C quadrupole mass selective detector with an electron-impact ionization of 70 eV. Compounds were separated on a fused silica capillary column (Agilent J&W DB-5 ms; 30 m, 0.25 mm, 25 μm) with a 5 m guard column (Restek Rxi, 5 m, 0.32 mm) and a carrier gas (helium) flow of 1.5 ml/min. For hydrocarbons, the oven program was as follows: 60 °C for 1 min, followed by a ramp (6 °C/min) to 320 °C and held for 15 min. For all other fractions, the oven program was as follows: 60 °C for 1 min, followed by a ramp (20 °C/min) to 130 °C, then a ramp (4 °C) to 320 °C and held for 10 min. Following GC separation, column effluent was split (1:1) between the FID and MSD with a 2-way splitter with He makeup to keep pressure constant at 182 Pa. The MSD scanned a mass range of m/z 45–800 at 2 scans per second. Compounds were identified with authentic standards, library databases (NIST 2008 and Wiley 2009), published spectra, spectral interpretation, and retention times.

For quantification by FID, hydrocarbons were dissolved quantitatively in hexanes spiked with 10 μg/ml 1–1′-binaphthalene as the internal standard. For the alcohol and acid fractions, aliquots of the saponified lipid extract were dissolved in pyridine spiked with 20 μg/ml of 2-dodecanol, phthalic acid, and undecanoic acid, as the internal standards, and derivatized with *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA; Sigma Aldrich) at 70 °C for 15 min. Compound peak areas were normalized to the internal standard and converted to concentrations using external standard response curves. For hydrocarbons, the external standard was C_7–C_{40} *n*-alkanes (Sigma Aldrich) and for *n*-alkanols and *n*-alkanoic acids, the external standard was a mix of even *n*-alkanols and *n*-alkanoic acids. To determine precision and accuracy, additional analyses were made of standards treated as samples. For the *n*-alkanes, a mixture of C_{22}–C_{38} at 10.00 μg/ml was measured ($n = 5$) with a mean precision of 0.09 μg/ml (1σ) and a mean accuracy of 0.71 μg/ml. For the polar compounds, a mixture of *n*-alkanols and *n*-alkanoic acids at 25.00 μg/ml was measured ($n = 11$) with a mean precision of 3.37 μg/ml (1σ) and a mean accuracy of 2.40 μg/ml. Compound concentrations were normalized to the dry leaf mass (μg/g).

To characterize changes in long chain *n*-alkane lengths, we calculated average chain length (ACL) values:

$$\mathrm{ACL} = \frac{25[C_{25}] + 27[C_{27}] + 29[C_{29}] + 31[C_{31}] + 33[C_{33}] + 35[C_{35}] + 37[C_{37}]}{[C_{25}] + [C_{27}] + [C_{29}] + [C_{31}] + [C_{33}] + [C_{35}] + [C_{37}]} \quad (1)$$

where *n*-alkane concentrations are converted to chain length numbers. To characterize differences in odd chain length preference, the carbon preference index (CPI) was measured using the Marzi et al. (1993) equation:

$$\mathrm{CPI} = \frac{([C_{23}] + [C_{25}] + [C_{27}] + [C_{29}] + [C_{31}] + [C_{33}] + [C_{35}]) + ([C_{25}] + [C_{27}] + [C_{29}] + [C_{31}] + [C_{33}] + [C_{35}] + [C_{37}])}{2x([C_{24}] + [C_{26}] + [C_{28}] + [C_{30}] + [C_{32}] + [C_{34}] + [C_{36}])} \quad (2)$$

where larger numbers indicated a greater preference for odd chain lengths.

2.4. Compound-specific carbon isotope analyses

Compound-specific carbon isotope analyses of the *n*-alkanes were determined by GC-combustion (C)-IRMS. Prior to analysis, saturated hydrocarbons were isolated from the hydrocarbon fraction on 0.5 g of 5% Ag-impregnated silica gel (w/w) and eluted with 4 ml of hexanes. GC-C-IRMS was performed with a Thermo Trace GC Ultra coupled via an Isolink combustion furnace and Conflo IV open split interfaced to a Thermo Electron Delta V Advantage IRMS. GC conditions were similar to the above with the exception of a faster ramp rate (8 °C/min). Isotopic abundances were determined relative to a reference gas calibrated with Mix A (Arndt Schimmelmann, Indiana University) and sample size dependency (linearity) monitored with Mix B. Carbon isotope values of samples are reported in delta notation relative to the standard Vienna Pee Dee Belemnite (VPDB) as $\delta^{13}C = [(^{13}R_{sample}/^{13}R_{VPDB}) - 1]$ where $^{13}R = {}^{13}C/{}^{12}C$. Carbon isotope accuracy was monitored with co-injected *n*-C_{41} alkane and the precision and accuracy were 0.03‰ (1σ; $n = 144$) and −0.17‰, respectively, over the course of analysis.

Isotope fractionation that occurs during leaf wax synthesis was estimated using ε_{lipid} notation (Chikaraishi et al., 2004) with the following equation:

$$\varepsilon_{n\text{-alkane}} = \left(\frac{\delta^{13}C_{lipid} + 1000}{\delta^{13}C_{leaf} + 1000} - 1\right) \quad (3)$$

with the assumption that $\delta^{13}C_{leaf}$ approximates the reactant (pyruvate) utilized to synthesize *n*-alkanes. $\varepsilon_{n\text{-alkane}}$ values were calculated for each individual *n*-alkane chain length,

concentration permitting, between n-C_{23} and n-C_{37}. In addition, a weighted $\varepsilon_{n\text{-alkane}}$ value was calculated for each species ($\varepsilon_{n\text{-alkane weighted}}$) that takes into account the concentration of each odd n-alkane between n-C_{25} and n-C_{37} and the $\delta^{13}C$ value of the respective chain length.

2.5. Bulk carbon isotope analyses

$\delta^{13}C$ of bulk organic carbon and weight percent total organic carbon (wt.% TOC) were determined via continuous flow (He; 120 ml/min) on a Costech combustion elemental analyzer (EA) interfaced with a Thermo Electron Delta V Advantage isotope ratio mass spectrometer (IRMS) with a Conflo IV. $\delta^{13}C$ values were corrected for sample size dependency and normalized to the VPDB scale using a two-point calibration with IAEA calibrated (NBS-19, L-SVEC) in-house standards (from −38.26‰ to −11.35‰) following Coplen et al. (2006). Error was determined by analyzing additional independent standards with a precision of 0.05‰ ($n = 18$; 1σ) and accuracy of 0.04‰ ($n = 14$).

2.6. Phylogenetic data and statistical analyses

In order to assess the effect of evolutionary relationships on n-alkane patterns, we first grouped conifer species according to major clades as recognized by previous phylogenetic studies (Rai et al., 2008; Leslie et al., 2012). These generally correspond to traditionally recognized conifer families, and include the Araucariaceae, Cupressaceae, Pinaceae, Podocarpaceae, Sciadopityaceae, and Taxaceae. We also analyzed three additional subclades of Cupressaceae: the Cupressoideae (Northern Hemisphere cypresses and junipers), the Callitroideae (Southern Hemisphere cedars), and the taxodioid Cupressaceae, an informal group of early-diverging lineages that includes species such as redwoods and bald cypresses.

In the context of this initial phylogenetic grouping, we used a principal components analysis (PCA) of n-alkane concentration, ACL, and CPI for all species to identify basic patterns in the data. We also performed a phylogenetically corrected PCA, a statistical correction which accounts for the phylogenetic non-independence of the data (Revell, 2009). This method uses a phylogenetic tree and a Brownian motion (BM) model of trait evolution to calculate expected trait covariance simply due to relatedness; that is, closely related species should have similar trait values while more distantly related taxa should have more divergent values. This phylogenetic covariance matrix is then used in the PCA analysis of the correlation matrix of trait values. In essence, the analysis uses the non-phylogenetic residual variation among the taxa to compute eigenvalues and eigenvectors, so that taxa are more appropriately ordinated on principal component axes (Revell, 2009). Studies suggest this method can reduce error in statistical results, even if the data is subsequently used in further phylogenetic analyses (see Revell, 2009).

We next directly tested for phylogenetic signal in these variables (n-alkane concentration, ACL, and CPI), as well as for Principal Components Axis 1 (PC 1) scores (in both traditional PCA and phylogenetically corrected PCA), using the K-statistic of Blomberg et al. (2003). Blomberg's K is a measure of how well the distribution of observed trait values follows expectations from a BM process given the phylogenetic relationships among the species. A value of 1.0, for example, indicates that a trait exhibits the distribution expected to occur under a BM process given the phylogenetic tree, while a value much less than 1 indicates that it is distributed without respect to observed phylogenetic relationships. We also used the K-statistic to test for phylogenetic signal in $\varepsilon_{n\text{-alkane}}$ values for n-alkanes n-C_{29}, n-C_{31}, n-C_{33} (the most common n-alkanes measured in sedimentary archives), and for the weighted $\varepsilon_{n\text{-alkane}}$ average for each species.

All phylogenetic analyses in this study used a time-calibrated molecular conifer phylogeny from Leslie et al. (2012). This tree was subsampled to include only the 43 conifer species used in this analysis. *Ginkgo* was not included in the (Leslie et al., 2012) study and was therefore excluded from the phylogenetic analyses. The phylogenetically-adjusted PCA and phylogenetic signal tests were performed using the package phytools (Revell, 2012) in the open source statistical software R version 3.1.2 (R core team, 2014). Other statistical analyses were preformed using R and JMP Pro 11.0 (SAS, Cary, USA).

3. RESULTS AND DISCUSSION

3.1. *n*-Alkanoic acid and *n*-alkanol concentrations among conifers

The n-alkanoic acids (even chains from C_{24} to C_{30}) are the least abundant of the leaf wax n-alkyl lipids, having a mean concentration of 50 ± 62 μg/g (1σ, $n = 43$) among all species, although they are not necessarily the least abundant n-alkyl lipids in each subfamily (Fig. 1). The n-alkanols (even chains from C_{24} to C_{34}) have roughly twice the concentration of the n-alkanoic acids when compared among all species ($\bar{X} = 99 \pm 252$ μg/g, 1σ, $n = 43$), but concentrations are much more variable among subfamilies. *Taxodium distichum* is unique among all species in having exceptionally high n-alkanol concentrations (1590 μg/g). There is no *a priori* reason to suspect such high concentrations in this species given that the two closely related species, *Glyptostrobus pensilis* and *Cryptomeria japonica*, as well as the rest of the Cupressaceae, have low concentrations. Overall, the n-alkanoic acid and n-alkanol concentrations measured here are similar to previous studies of conifer leaf waxes (Diefendorf et al., 2011).

Total n-alkanol concentrations among extant conifer species do not show significant phylogenetic signal, although the distribution of total n-alkanoic acids do show some phylogenetic structure ($K = 0.62$, $p = 0.01$). This appears to be due to the consistently higher total n-alkanoic acid concentrations in the Pinaceae clade, which imparts a weak phylogenetic structure to the data. It is also important to note here that n-alkanoic acids are indeed present, especially in Pinaceae (105 ± 89 μg/g, 1σ, $n = 10$), but are generally lower in concentration than in previously reported angiosperms (335 ± 294 μg/g, 1σ, $n = 29$,

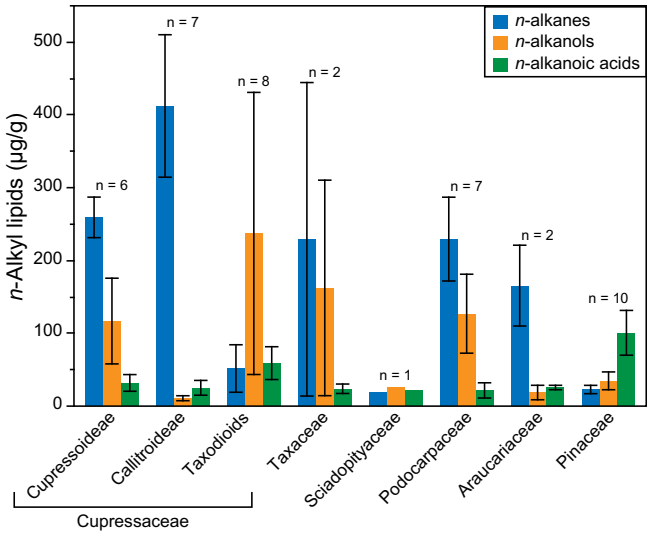

Fig. 1. The average *n*-alkane (C_{25} to C_{38}), *n*-alkanol (even chains from C_{24} to C_{34}), and *n*-alkanoic acid (even chains from C_{24} to C_{30}) concentrations for each of the major conifer groups. Concentrations are reported in µg/g dry leaf material with one standard error indicated.

Diefendorf et al., 2011). The *n*-alkanols have much lower concentrations in conifers studied here (99 ± 252 µg/g, 1σ, $n = 43$) than in angiosperms (1167 ± 1190 µg/g, 1σ, $n = 29$, Diefendorf et al., 2011).

The similar concentration of *n*-alkanoic acids in Pinaceae and angiosperms suggests that sediments will contain leaf waxes from each group in rough proportion to their abundances. Since conifers and angiosperms fractionate carbon differently during photosynthesis and biosynthesis, and have different responses to water availability, this mixing will complicate interpretation of the isotopic composition of *n*-alkanoic acids from sediments (Collister et al., 1994b; Chikaraishi and Naraoka, 2003; Bi et al., 2005; Vogts et al., 2009; Diefendorf et al., 2010, 2011; Polissar and Freeman, 2010; Sachse et al., 2012; see Section 3.3 for further discussion).

3.2. *n*-Alkane characteristics and phylogenetic relationships

The *n*-alkanes are on average higher in concentration (C_{25}–C_{38}; $\bar{X} = 173 \pm 193$ µg/g, 1σ, $n = 43$) than the *n*-alkanols and the *n*-alkanoic acids (Wilcoxon rank-sum test, $p < 0.005$). This pattern extends to most conifer families except for Pinaceae, which tends to have more *n*-alkanoic acids (Fig. 1). This pattern was also observed in a previous study (Diefendorf et al., 2011). Interestingly, there is no correlation between *n*-alkane concentrations and *n*-alkanols or *n*-alkanoic acids, e.g., high concentrations of *n*-alkanes do not necessarily indicate high concentrations of *n*-alkanols or *n*-alkanoic acids. This may be attributed to the different functions of *n*-alkyl lipids and their relatives in controlling the physiochemical properties of leaf waxes, especially with respect to crystal structure, morphology, chemical behavior, plasticity, and permeability (Riederer and Schneider, 1990; Müller and Riederer, 2005; Jetter et al., 2006).

n-Alkane concentrations vary among the major conifer lineages (Figs. 1 and 3). For example, *n*-alkane concentrations are high in the Araucariaceae, Podocarpaceae, and Taxaceae, as well as the Callitroideae and Cupressoideae clades within Cupressaceae. In contrast, Pinaceae, Sciadopityaceae, and taxodioid conifers (early diverging lineages of Cupressaceae) all have low concentrations. ACL and CPI also vary among conifer clades, with the highest values in the Callitroideae and Cupressoideae (Fig. 3). Notably, these two groups have much higher *n*-alkane concentrations, ACL values, and CPI values than do representatives of early-diverging lineages within the Cupressaceae. Total *n*-alkane concentrations in Podocarpaceae are similar to Callitroideae and Cupressoideae, but their composition is different; species in Podocarpaceae tend to have much lower ACL values.

A PCA of total *n*-alkanes (C_{25} to C_{38}), ACL, and CPI further indicates these variables are phylogenetically structured (Fig. 4a). Cupressoideae and Callitroideae species plot negatively on Principal Component Axis 1 (PC 1), which summarizes 66% of the variation in the data and is negatively correlated with all three variables. Araucariaceae and Podocarpaceae show intermediate values on PC I, while Pinaceae and many taxodioid Cupressaceae (although not all) plot more positively. Principal Component Axis 2, which explains 16% of the variation in the data and is positively correlated with CPI and negatively correlated with ACL, shows less obvious phylogenetic structure. Correcting for the phylogenetic non-independence of the data when calculating principal components scores does not significantly change these results, although in this case species show more variation on PC 2, with Cupressoideae and Callitroideae plotting somewhat negatively compared to other conifers (Fig. 4b).

Direct tests of phylogenetic signal in these variables (*n*-alkane concentration, ACL, CPI, and PC 1 scores) further supports the idea that evolutionary history influences their values. ACL, CPI, and PC 1 scores show significant phylogenetic signal, where the distribution of their values across

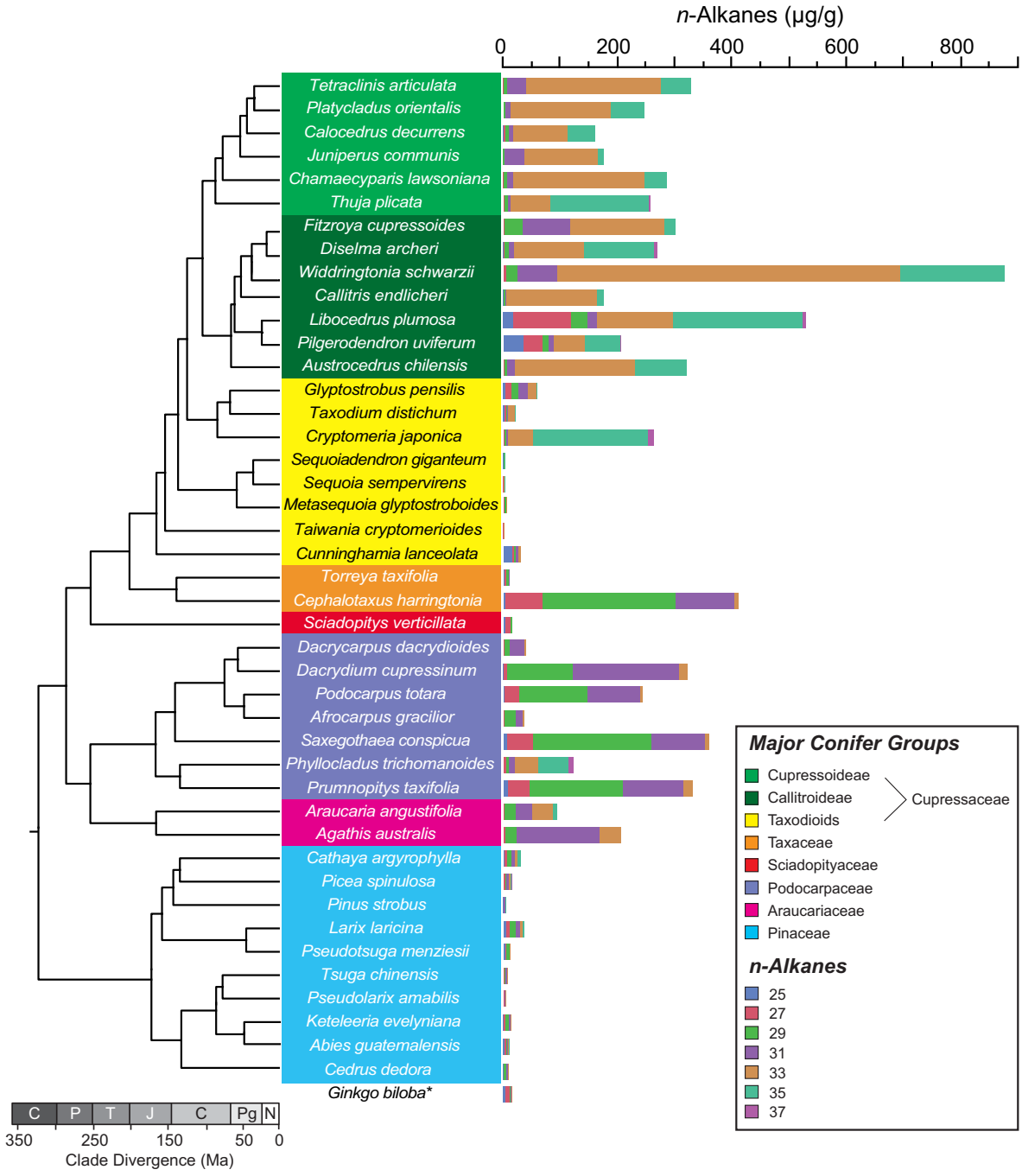

Fig. 2. *n*-Alkane concentrations for the 43 conifer species and *Ginkgo biloba*. Conifer species are grouped into major clades with a DNA sequenced-based phylogeny that is age-calibrated using the fossil record Rai et al. (2008), Leslie et al. (2012). The *n*-alkane concentrations (µg/g dry leaf material) are reported for odd *n*-alkanes from C_{25} to C_{37}.

conifers is consistent with expectations from a Brownian motion evolutionary process operating across the observed phylogenetic relationships among the species (Table 1). Phylogenetic signal is particularly strong in ACL and PC 1 (both in regular and phylogeny-adjusted values). In contrast, total *n*-alkane concentration does not show a statistically significant phylogenetic signal. Concentrations of individual *n*-alkanes likewise do not generally show evidence of phylogenetic signal, except for C_{28} and C_{29} (Table 1).

Overall, the concentrations of *n*-alkanes in these conifers are significantly different from previous studies that indicated that *n*-alkanes are 200 times higher in angiosperms than conifers (Diefendorf et al., 2011). This prior study focused on North American conifer species only and did not include any representatives from major lineages that

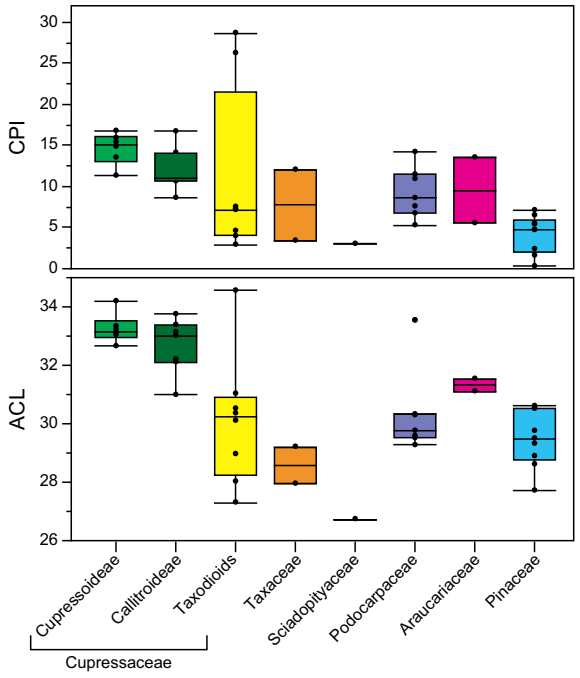

Fig. 3. Carbon preference index (CPI) and average chain length (ACL) for major conifer groups. Box and whisker plots show the median, upper and lower quartiles, and maximum and minimum values, with outliers shown. Each black dot represents one species.

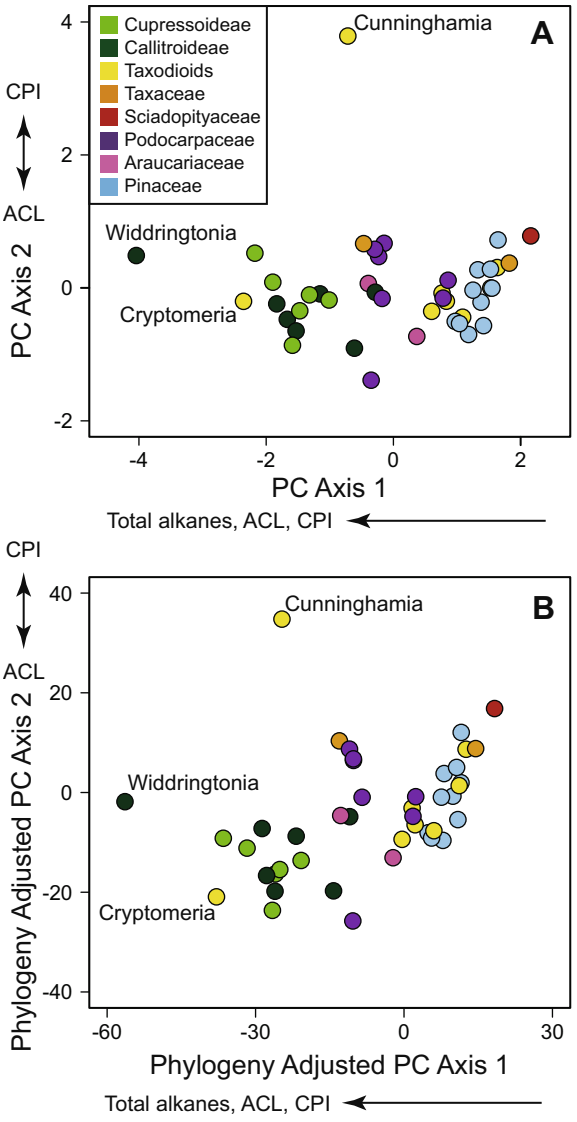

Fig. 4. (A) Principle components analysis (PCA) of *n*-alkanes concentrations, ACL, and CPI for all species. (B) Phylogenetically corrected PCA that accounts for the phylogenetic non-independence of the data.

grow today in the Southern Hemisphere (Araucariaceae, Callitroids, Podocarpaceae) or from a number of Northern Hemisphere genera now restricted to Asia but formerly more widespread (e.g., *Cunninghamia*, *Keteleeria*, *Pseudolarix*). Representatives from these genera and families were components of North American floras at various times in the past; for example, Araucariaceae is found in North America and Europe until the end-Cretaceous (Kunzmann, 2007; Stults et al., 2012) and *Cunninghamia* was present until at least the Middle Miocene (Otto et al., 2003). This study confirms that Pinaceae and taxodioid Cupressaceae have significantly lower concentrations of *n*-alkanes compared to angiosperms, as was concluded by Diefendorf et al. (2011). However, many conifer taxa do indeed produce significant concentrations of *n*-alkanes. This has important implications for interpreting sedimentary leaf waxes and their carbon and hydrogen isotopes in locations where Cupressoideae, Callitroideae, Araucariaceae and Podocarpaceae are common today or were common in the past.

Previous leaf wax studies have indicated that leaf lifespan appears to be an important control on leaf waxes and that longer-lived leaves tend to have greater concentrations (Diefendorf et al., 2011). In this study, seasonally deciduous species tend to plot higher on PC Axis 1 (Fig. 5a), which is associated with fewer total alkanes, lower ACL, and lower CPI. The small number of deciduous conifers makes the significance of these patterns difficult to interpret, however; evergreen species show a greater average concentration of *n*-alkanes than deciduous species (Fig. 5b), but this difference is not statistically significant ($p = 0.10$ with Mann–Whitney *U*-test). When examined within a proper phylogenetic context, these differences become even less pronounced. Deciduous conifers are found only in the Pinaceae clade (*Larix*, *Pseudolarix*) and among the early-diverging taxodioid Cupressaceae lineages (*Glyptostrobus*, *Metasequoia*, *Taxodium*). When these deciduous species are compared to related evergreen taxa from their respective groups, they do not have lower *n*-alkane concentrations (Fig. 5b). In fact, deciduous taxodioid species have higher average *n*-alkane concentrations than evergreen taxodioid species, although this difference is not significant ($p = 0.55$ with Mann–Whitney *U*-test). The higher average *n*-alkane concentrations of evergreen conifers as a whole is essentially an artifact of sampling; it is due to the abundance of Cupressoideae, Callitroideae, and Podocarpaceae species that are uniformly evergreen

Table 1
Phylogenetic signal in conifer alkane data.

Category	Trait	Blomberg K[a]
Individual *n*-alkanes	C_{22}	0.22
	C_{23}	0.16
	C_{24}	0.24
	C_{25}	0.32
	C_{26}	0.49
	C_{27}	0.29
	C_{28}	**0.64***
	C_{29}	**0.66***
	C_{30}	0.52
	C_{31}	0.41
	C_{32}	0.40
	C_{33}	0.43
	C_{34}	0.45
	C_{35}	0.34
	C_{36}	0.29
	C_{37}	0.29
	C_{38}	0.19
n-Alkane statistics	Total alkanes	0.41
	ACL	**1.03****
	CPI	**0.67****
	PC1[b]	**0.84****
	$PC1_{phy}$[c]	**0.85****
n-Alkane fractionation	$\varepsilon_{n\text{-alkane}}$ C_{29}	**0.94****
	$\varepsilon_{n\text{-alkane}}$ C_{31}	**0.81****
	$\varepsilon_{n\text{-alkane}}$ C_{33}	**0.77****
	$\varepsilon_{n\text{-alkane weighted}}$	**0.91****

[a] A K value of 0.0 indicates that a trait shows no phylogenetic signal while a value of 1.0 indicates that a trait shows the expected distribution given the phylogeny and a Brownian motion model of trait evolution; values greater than 1.0 are also possible. Statistical significance is assessed by a randomization test and significant values are indicated in bold.
[b] PC1 is the individual species scores on the first Principal Component Axis.
[c] $PC1_{PHY}$ is the individual species scores after accounting for phylogenetic non-independence in the data.
* Indicates significance at $p < 0.05$,
** Indicates $p < 0.01$.

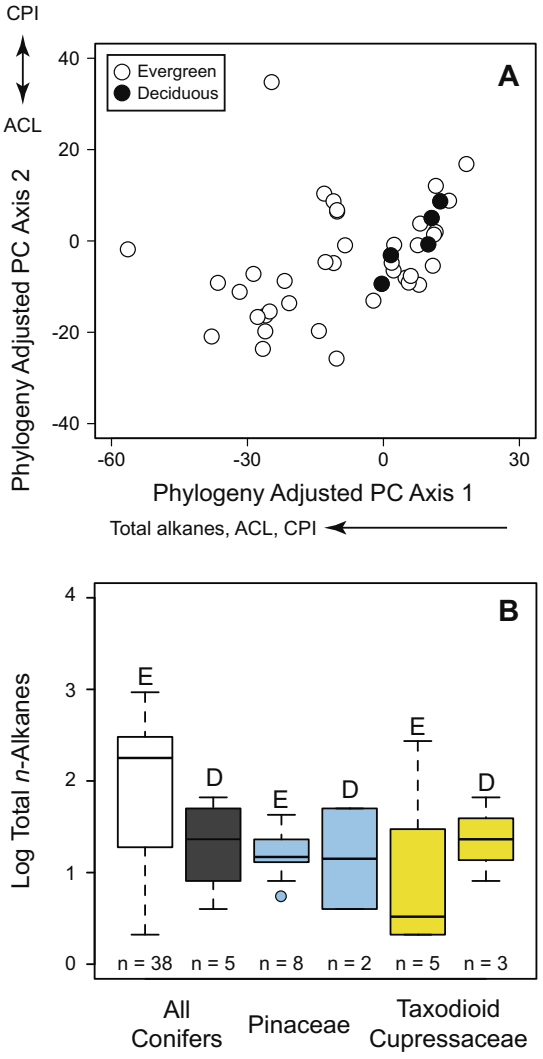

Fig. 5. (A) The phylogenetically corrected PCA from Fig. 4 with leaf lifespan indicated. (B) Total *n*-alkane concentrations (μg/g dry leaf, log scale) for all conifers, Pinaceae and taxodioid Cupressaceae with leaf lifespan indicated (E, evergreen; D, deciduous). See Fig. 3 caption for description of box and whiskers plots.

and have clade-specific high *n*-alkane concentrations. Leaf life span in conifers ranges from <1 year to many decades (e.g., Niinemets and Lukjanova, 2003), and *n*-alkane concentrations have so far only been contrasted between deciduous and "evergreen" species, leaving more refined analyses for the future.

All of the samples collected in this study were grown at the same site and thus the same climate, although variations in water availability across the site cannot be ruled out (UCBGB occasionally waters plants, *pers. comm.*). Nonetheless, the strong phylogenetic clustering in the *n*-alkanes suggests that climate alone may not be the primary factor influencing *n*-alkane biochemistry at the time of synthesis, at least at the broadest scale. For example, Cupressaceae species differ dramatically in their physiology and the types of environments they inhabit, from drought adapted species living in arid regions to drought intolerant species living in wet habitats (Pittermann et al., 2012; Brodribb et al., 2014), but our data suggests all species have relatively high ACL values that they appear to have inherited from a common ancestor (Figs. 2 and 3). A possible implication of our data, then, is that *n*-alkane chemistry and ACL values do not have any strong functional or adaptive significance, and major differences among clades simply reflect drift over hundreds of millions of years of independent evolution. On the other hand, some studies suggest that alkane chemistry does play a functional role; for example, longer chain waxes may act as protection against water loss within Cupressaceae species (Dodd et al., 1998; Dodd and Poveda, 2003). One way to reconcile these results could be that phylogenetic relationships broadly structure patterns of *n*-alkane chemistry regardless of climate, but climate or other environmental effects still plays a role in determining specific ACL values within or among species. Our results do strongly suggest, however, that searching for such influences has to be done in a phylogenetic context.

Regardless of the exact relationship between climate and ACL, which has been investigated in a number of studies (Dodd et al., 1998; Hoffmann et al., 2013; Tipple and Pagani, 2013; Bush and McInerney, 2015), one implication of our results is that interpreting alkane data, such as ACL values, from the fossil record may be complicated by changes in species composition. For example, the Eocene Thunder Mountain Flora (Erwin and Schorn, 2005) and the Oligocene Creede Flora (Axelrod, 1987; Wolfe and Schorn, 1990) both record upland environments with abundant conifers, but the latter is dominated by a single species of *Juniperus* while the former is predominantly Pinaceae. Regardless of whatever actual climatic differences may have existed between these floras, their ACL and CPI values are likely to be affected by differences in species composition, in this case the high ACL and CPI values of Cupressaceae relative to Pinaceae. Of course, differences in species composition are also likely to be associated with differences in moisture or temperature regimes, but our results suggest that the phylogenetic relationships are important to consider when interpreting *n*-alkane chain lengths.

3.3. Carbon isotope fractionation during *n*-alkane biosynthesis

Fractionation during decarboxylation of pyruvate discriminates against ^{13}C and results in acetyl-CoA being 4.5‰ lower on average than bulk tissue (Park and Epstein, 1961; Monson and Hayes, 1982; Melzer and Schmidt, 1987). Previous studies focusing on *n*-alkanes have measured *n*-C_{29} ε_{alkane} values of -4.4 ± 2.3‰ (1σ, $n = 3$) for conifers and angiosperms grown at a single location in Pennsylvania (Diefendorf et al., 2011) and other studies have found similar results for different combinations of species (Collister et al., 1994b; Chikaraishi and Naraoka, 2003; Chikaraishi et al., 2004; Bi et al., 2005). In this study, we find conifers have *n*-C_{29} ε_{alkane} values of -4.1 ± 3.1 (1σ, $n = 24$) and $\varepsilon_{n\text{-alkane weighted}}$ values of -3.9 ± 2.5 (1σ, $n = 41$). These results are similar to prior studies and also suggests that fractionation occurs primarily during decarboxylation of pyruvate.

Interestingly, $\varepsilon_{n\text{-alkanes-weighted}}$ values are distinct among major conifer groups, with the highest values in Pinaceae and the lowest values in Taxaceae (Fig. 6; ANOVA, $R^2 = 0.65$, $p < 0.001$). We also observed a significant phylogenetic signal in $\varepsilon_{n\text{-alkanes}}$ values for the *n*-C_{29}, *n*-C_{31}, and *n*-C_{33} alkanes, as well as for $\varepsilon_{n\text{-alkanes-weighted}}$ (Table 1). This suggests that the evolutionary history of these conifers shapes biosynthetic fractionation for *n*-alkanes.

Positive linear correlations have been observed between total amount of leaf lipids and enrichment factors (Park and Epstein, 1961; Hobbie and Werner, 2004). However, we do not observe this relationship between $\varepsilon_{n\text{-alkane}}$ and total *n*-alkanes or total *n*-alkyl lipids in this study. Also, individual *n*-alkane chain length concentrations and $\varepsilon_{n\text{-alkane}}$ values are not correlated, even within an individual family. For example, the subfamilies within Cupressaceae have similar $\varepsilon_{n\text{-alkanes}}$ values, but a large range in *n*-alkane concentrations. We speculate that a correlation is not observed because *n*-alkanes represent a small portion of the total leaf lipid pool and are not continuously produced (e.g., Tipple et al., 2013). Thus, enzymatic isotope effects between reactant and product are not clearly related to lipid production flux as is observed in the total lipid pool (Park and Epstein, 1961; Hayes, 2001).

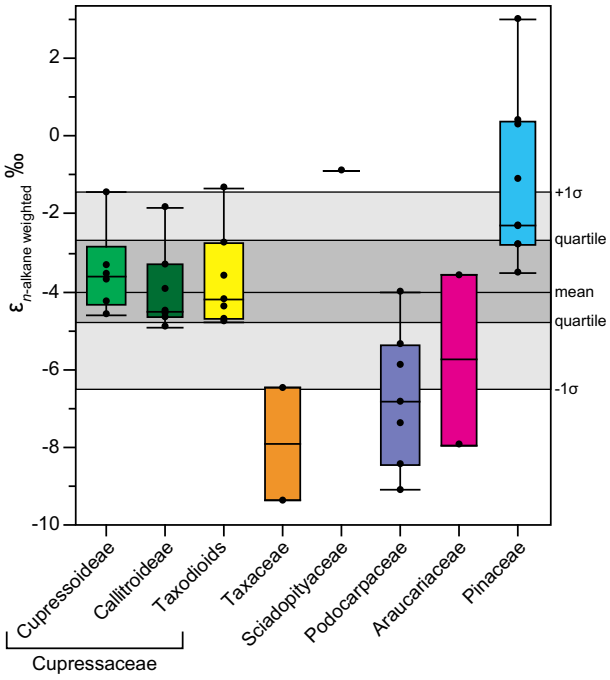

Fig. 6. Fractionation during *n*-alkane biosynthesis weighted by *n*-alkane concentration ($\varepsilon_{n\text{-alkane weighted}}$). Black dots represent one species and box and whisker plots represent the median, upper and lower quartiles, and maximum and minimum values for each major conifer group. For reference, the mean, upper and lower quartiles, and upper and lower 1 standard deviation about the mean are shown for all conifers.

Although, on average, the $\varepsilon_{n\text{-alkanes weighted}}$ values are close to −4.5‰ (Park and Epstein, 1961; Monson and Hayes, 1982; Melzer and Schmidt, 1987), several species in Pinaceae are more than 1σ above the mean, and Taxaceae, Podocarpaceae, and one species in Araucariaceae are more than 1σ below (Fig. 6). Some of this variability in $\varepsilon_{n\text{-alkanes weighted}}$ values could be caused by phenological differences among species. If leaf waxes and bulk leaf tissue are not synthesized at the same time, species that produce new growth at different times of year could have different $\varepsilon_{n\text{-alkane}}$ values. For example, leaf wax synthesis could coincide with periods of either high or low photosynthetic demand or water availability. This directly translates into a control on pyruvate $\delta^{13}C$ values with high values during periods of greatest photosynthetic demand and/or decreased water availability (Farquhar et al., 1989; Diefendorf et al., 2010). Alternatively, carbon metabolism could be different among species such that the source of pyruvate utilized in the acetogenic pathway varies (Zhou et al., 2010) or the timing of synthesis varies. For example, if a large percentage of leaf waxes are synthesized during bud break and leaf flush, as suggested by Tipple et al. (2013), then leaf wax $\delta^{13}C$ may ultimately be controlled

by the proportion of stored sugars utilized to produce pyruvate and thus cause ^{13}C enrichment (Terwilliger and Huang, 1996; Jäggi et al., 2002), as has been suggested for non-photosynthetic tissues (Cernusak et al., 2009). On the other hand, if leaf wax synthesis occurs throughout the growing season (Sachse et al., 2006, 2009), then stored sugars could be less important as source of pyruvate. Differences in carbon metabolism therefore could be an important factor controlling variations in $\varepsilon_{n\text{-alkanes}}$.

Different species of plants produce different amounts of various *n*-alkane chain lengths, and that the isotopic composition of these chain lengths is affected both by taxon-specific biosynthetic fractionation factors and common environmental effects that influence photosynthetic fractionation. In order to extract an environmental signal from sedimentary leaf waxes, it then becomes necessary to know the proportion of the wax in the sample that comes from the different taxa. For example, if the conifers in a mixed conifer/angiosperm forest are *Juniperus* or *Thuja*, then interpreting the $\delta^{13}C$ of *n*-C_{29} alkane as an angiosperm biomarker would be justified; *Juniperus* and *Thuja* produce almost entirely *n*-alkanes longer than C_{31}. Interpreting sedimentary *n*-alkanes in mixed forests of angiosperms and Podocarpaceae will be challenging given that both produce abundant *n*-C_{29} alkanes. Plant communities with grasses and Callitroideae and/or Cupressoideae will be more problematic given the predominance of grasses with chain lengths longer than *n*-C_{31} and thus overlapping in chain length with these conifer subfamilies. Choosing the correct *n*-alkane chain length for interpreting isotope signals will be critical and must be constrained with independent information about vegetation.

4. CONCLUSIONS

We provide new measurements of leaf wax composition and $\varepsilon_{n\text{-alkanes}}$ values for 43 conifer species (and *G. biloba*) from the University of California Botanical Garden at Berkeley. This sampling provides information at the same site, thereby minimizing effects of climate, for all extant conifer families and most extant genera. We find that *n*-alkanes are indeed common in many conifer families, especially families that are common today in the Southern Hemisphere and Asia, including Araucariaceae, Podocarpaceae, and many species of Cupressaceae, especially in the subfamilies Cupressoideae and Callitroideae. Pinaceae, Sciadopityaceae, and the early diverging taxodioid lineage within Cupressaceae, however, are characterized by low concentrations of *n*-alkanes.

The concentration and chain length of *n*-alkanes both are strongly influenced by evolutionary history; a strong phylogenetic signal is present in the *n*-C_{29} alkane concentrations, average chain length, and carbon preference index. The role of the environment in shaping *n*-alkane composition is, however, unclear. Average chain length significantly varies among conifer families and strongly indicates that species composition will have a critical control on average chain length in sedimentary archives, thus interpretations of average chain length as a proxy for climate must be done cautiously and with careful regard for phylogenetic context.

We found that the values of *n*-C_{29} ε_{alkane} and $\varepsilon_{n\text{-alkane weighted}}$ in conifers to be broadly consistent with previous studies. $\varepsilon_{n\text{-alkanes-weighted}}$ values, along with C_{29}, C_{31} and C_{33} ε_{alkane}, however, are highly correlated with phylogenetic relationships among conifers. This suggests that biosynthetic fractionation may be related to differences in carbon allocation or metabolism among conifer groups, although the exact mechanism is unknown and will require further research.

Overall, our findings show that some conifers have the potential to make major contributions to *n*-alkanes in sediments. The possibility that such conifers have contributed to the *n*-alkanes in any given sediment sample will have to be determined from other sources of information, such as pollen and/or macrofossils. If *n*-alkane producing conifers are present, it may still be possible to separate angiosperm from conifer biomarkers by selecting a particular *n*-alkane chain length that is produced mostly by either angiosperms or conifers. This is particularly important in the context of interpreting the $\delta^{13}C$ of *n*-alkanes because conifers and angiosperms fractionate differently during photosynthesis (e.g. Diefendorf et al., 2010). Mixing of conifer and angiosperm derived *n*-alkanes could cause variation in sedimentary $\delta^{13}C_{alkane}$ values that could be misinterpreted as change in the $\delta^{13}C$ of the carbon cycle, changes in vegetation, or changes in water use efficiency.

ACKNOWLEDGEMENTS

We thank Holly Forbes and the staff at the University of California Botanical Garden at Berkeley for access to specimens and assistance with sample collection. We thank Cindy Looy for assistance with sample collection for earlier preliminary sampling not used in this study. We also thank Justin Dodd and Kevin Mueller for assistance with sampling. Sharmila Giri, Douglas Sberna, Keegan McClannahan, Brian Simpkins, and Katherine Finan are thanked for assistance with sample preparation. We also thank Jeremy Beaulieu and Elisabeth Forrestel for their support with data analysis methods. This research was supported by the National Science Foundation (EAR-1229114 to A.F.D.) and the American Chemical Society Petroleum Research Fund (PRF #51787-DNI2 to A.F.D.).

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gca.2015.08.018.

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Associate editor: Alex L. Sessions