Major Evolutionary Trends in Hydrogen Isotope Fractionation of Vascular Plant Leaf Waxes

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Abstract

Hydrogen isotopic ratios of terrestrial plant leaf waxes (δD) have been widely used for paleoclimate reconstructions. However, underlying controls for the observed large variations in leaf wax δD values in different terrestrial vascular plants are still poorly understood, hampering quantitative paleoclimate interpretation. Here we report plant leaf wax and source water δD values from 102 plant species grown in a common environment (New York Botanic Garden), chosen to represent all the major lineages of terrestrial vascular plants and multiple origins of common plant growth forms. We found that leaf wax hydrogen isotope fractionation relative to plant source water is best explained by membership in particular lineages, rather than by growth forms as previously suggested. Monocots, and in particular one clade of grasses, display consistently greater hydrogen isotopic fractionation than all other vascular plants, whereas lycopods, representing the earlier-diverging vascular plant lineage, display the smallest fractionation. Data from greenhouse experiments and field samples suggest that the changing leaf wax hydrogen isotopic fractionation in different terrestrial vascular plants may be related to different strategies in allocating photosynthetic substrates for metabolic and biosynthetic functions, and potential leaf water isotopic differences.

Introduction

Hydrogen isotopic ratios (δD) of vascular plant leaf waxes are widely used for paleoclimate reconstruction from geological archives [1–9]. Leaf wax δD values preserved in lake and ocean sediments provide a record of precipitation isotopic ratios, allowing reconstruction of changes in hydrology and temperature for time scales of thousands to millions of years. However, hydrogen isotopic fractionation of terrestrial vascular leaf waxes is highly variable, differing by up to 80% across species [10–13], and the underlying controls for this variability are poorly understood, hampering quantitative paleoclimate interpretation.

Variations in vascular plant leaf wax hydrogen isotopic fractionation have been tentatively linked to growth forms [10–13]. For example, grasses typically have 30 to 50% lower δD values than trees grown in a similar environment [10–12]. Such grouping is, however, potentially problematic because growth forms are simple visual characteristics and generally do not correspond to fundamental biochemical and physiological processes, and perceived statistical differences when data are grouped by plant functional type can be misleading [14]. Published data also contain plants collected from very different geographic locations with large differences in precipitation isotopic ratios, climatic and environmental conditions [13]. Differing ambient temperature, relative humidity, soil type and other environmental factors could also potentially affect the isotopic fractionation. Most of the published fractionation values are calculated relative to modeled δD values at different localities [13,15], introducing added uncertainties. Because samples are collected based on morphological classification, there is considerable sampling bias in terms of plant phylogeny: more than 95% of the previously reported plant species are from only two major plant lineages, the eudicots and Poales [13] (Fig. S1), making general inferences across all plants difficult.

To avoid both environmental heterogeneity and poor taxonomic representation, we acquired comprehensive isotope data for plant leaf waxes, irrigation and xylem waters from 102 species from New York Botanic Garden (Table S1), and performed phylogenetically-informed analyses to critically examine the potential effects of plant types on the leaf wax hydrogen isotopic fractionation. Our objectives are thus: 1) to produce leaf wax hydrogen isotopic fractionation data with careful constraints on
plant source water and minimal variability in growth environments, and 2) to explore the potential influences of plant phylogeny and other plant characteristics (e.g., growth habits, photosynthetic pathways) on vascular plant leaf wax hydrogen isotope fractionation.

**Materials and Methods**

**Samples from the New York Botanic Garden**

All the 102 terrestrial vascular plant species were collected from the New York Botanic Garden (NYBG: Coordinates: 40.8636° N, 73.8783° W) in the USA (Table S1) following our standard lab procedures [12,16]. The majority of the samples were collected on May 28, 2009. Additional ferns were collected on July 10, 2010 (marked with * in Table S1). The lycophytes were collected on Aug 15, 2009. All the samples were collected between 12 pm and 2 pm. Fresh leaves for leaf wax analysis were stored in whirl-pak bags, while those for leaf water δD analysis were immediately sealed in glass vials. Stems (wherever available) were collected to measure the xylem water δD. The New York Botanical Garden issued the permit for sampling the 102 samples from the NYBG (Contact: Mr Jon Peter). The different sampling times were due to constraints on field trip timing, and required supplementary samples as the project progressed. Because we simultaneously determined xylem water δD values, we believe the influence of variable sampling times on our calculated isotopic fractionation values should be small and negligible.

**Field samples from Blood Pond, Massachusetts and growth chamber experiments**

We also report additional data from field samples and growth chamber experiments, aimed at better understanding the hydrogen isotopic differences between grasses and dicots. We collected leaf materials for leaf wax and leaf water δD analysis for selected grasses and trees around Blood Pond (42.0814° N, 71.9618° W, 212.1 m a.s.l.), Massachusetts at different stages of the growth season (May, June, September in 2007), following the same protocol as reported in Gao et al. [12]. The sampling dates and data are given in Table S2. We followed the procedures of growth chamber experiments closely as described in Hou et al. [17]. We grew 19 trees (representing 5 species) and 19 grasses (representing 3 species) in a GC series temperature and humidity-controlled growth chamber by Environmental Growth Chamber Company (Data as shown in Table S2). The temperature in the growth chamber was kept at 20°C. RH was kept constant at 80% during the growing period (Jun 26–Jul 27, 2006). Blood Pond (Coordinates: 42.0814° N, 71.9618° W, 212.1 m a.s.l.) is private land for Jon Peter. The different sampling times were due to constraints on field trip timing, and required supplementary samples as the project progressed. Because we simultaneously determined xylem water δD values, we believe the influence of variable sampling times on our calculated isotopic fractionation values should be small and negligible.

**Analytical methods for leaf waxes**

Sample preparation, and leaf wax, leaf water and xylem water δD analysis was conducted following the same procedure as in Gao et al. [16,18]. Briefly, leaf lipids were extracted from freeze-dried leaves and quantified using a Hewlett-Packard 6890 gas chromatograph (GC) with split/splitless injector and a flame ionization detector (FID); compound identification was based on retention times of lipid standards on an Agilent 6890N GC coupled to an Agilent 5973N quadrupole mass analyzer. Data of leaf wax compositional information are presented in files Fig. S2, Table S3 and Table S4 (SI is supporting information). D/H ratio analysis was carried out on an HP 6890 GC, interfaced to a Finnigan MAT Delta+ XL isotope ratio mass spectrometer (IRMS) via a high-temperature pyrolysis reactor. The HIs factor was 2.6±0.3 during the analysis and analytical errors were <2.5 % for IRMS based on repeated analysis of our laboratory standards (mixture of n-C22, –C24, –C26, –C28, and –C30 fatty acid methyl esters and n-C22, –C24, –C26, –C28 and –C32 alkanes). The plant samples were analyzed in duplicates or triplicates to ensure repeatability (with analytical error less than 2.5 % for the majority of the samples). Leaf and xylem water was distilled using the cryogenic system at Brown University and water δD was measured on a Picarro L1102-i isotope liquid water and water vapor analyzer (Sunnyvale, CA, USA) following procedures described in Gao et al. [16]. The standard deviation for repeated standard analysis was <0.1 % for δ18O and <0.6 % for δD during our measurements, and the machine was monitored continuously with a lab standard (~38 % δD) for every 9 samples. The irrigation water used for growth experiments had a δD of ~49 %.

**Xylem water and the environment water**

We measured xylem water hydrogen isotopic ratios for the majority of plant samples except for 15 ferns and 10 lycophytes (Table S1). These fern and lycophyte samples have green, photosynthetically-active stems which contain isotopically enriched xylem water due to evapotranspiration. These plants are cultivated in moist and shaded environments with regular irrigation water at NYBG, which minimize soil water evaporation. Thus, the annual mean precipitation water δD (~57 %) was used to calculate the fractionation of these samples. This value was obtained from the Online Precipitation Isotopes Calculator (http://wateriso.utah.edu/waterisotopes/pages/data_access/oipc.html).

**Calculation of hydrogen isotope fractionation between leaf lipids and water**

The raw isotopic data of the NYBG samples include δD values of leaf waxes (n-C24, C26, C28 and C30 alkanic acids and n-C27, C29, and C31 alkanes), δD of xylem water (δDxylem) and δD of environmental water (δDenvir, ~57 %; Table S1). The hydrogen isotope fractionation between leaf waxes and environmental water (δDwax-environ) was calculated by the equation

$$\Delta_{\text{wax-environ}} = \left( \frac{\delta_{\text{wax}}}{\delta_{\text{environ}} + 1} \right) - 1,$$

where δDwax is the δD value of each of the above individual lipids. Similarly, the hydrogen isotope fractionation between leaf waxes and xylem water (δDwax-xylem) was calculated by the equation

$$\Delta_{\text{wax-xylem}} = \left( \frac{\delta_{\text{wax}} + 1}{\delta_{\text{xylem}} + 1} \right) - 1.$$

**Integrating compound-specific δD values of leaf waxes**

To reveal the hydrogen isotope fractionation variations among major phylogenetic lineages, we calculated the average and standard deviation values for each lineage for individual lipids (Figs. 1A and 1C). These figures show that variations of δwax-xylem and δwax-environ values for individual lipids among different phylogenetic lineages follow similar trends. Therefore, isotopic variation in any single lipid is in general representative of the overall differences among different plant lineages. We further calculated the correlation of δwax-xylem values between different leaf lipids for all plants and found the lipid δwax-xylem values are positively correlated (Fig. S3). Such positive correlation also exists for δwax-environ values in our data (figures not shown), and has been reported previously [10–12,19].

Therefore, the best approach to compare the isotopic differences between different plant lineages is to obtain an integrated isotopic value from the measurement of seven individual
compounds (i.e., \(n\)-C_{24}, C_{26}, C_{28} and C_{30} alkanoic acids and \(n\)-C_{27}, C_{29}, and C_{31} alkanes). There are a number of advantages in obtaining an integrated isotopic value over relying on individual compounds for plant comparison: 1) integration takes advantage of multiple measurements of different compounds rather than individual compound for a given plant sample, reducing the potential influence of outliers and enhancing the likelihood of obtaining the most representative isotopic data; 2) occasionally, concentrations for certain individual compounds from a plant sample are too low or contain coeluting compounds on GC-IRMS for accurate isotopic measurements, so an integration from available measurements avoids data gaps; and 3) a greater number of isotopic observations, by considering all compounds for a single plant provides a more statistically robust representation of isotopic fractionation in certain plants.

However, we cannot simply take the mathematical means of \(\delta D\) values of different compounds in a given sample to obtain an integrated isotopic mean value, because there are some natural offsets in hydrogen isotopic values between different compound classes (e.g., alkanes, acids) and between different carbon chain length compounds of the same compound classes. For example, \(\varepsilon\) values between C_{28} \(n\)-acid and environmental water are positively correlated to those between C_{30} \(n\)-acid and environmental water (R^2 = 0.78), but the correlation is not 1:1 and the interception is not zero (Fig. S3).

To obtain integrated values for single plants, we followed the well-established statistical procedure described as follows. We first centered the \(\varepsilon_{\text{wax-xylem}}\) and \(\varepsilon_{\text{wax-environ}}\) values for each lipid by subtraction of the mean values for each lipid. We then scaled the centered values using the variable stability (VAST) scaling method [20]. This method weights each variable according to a matrix of its stability and improves the class distinction. The weights were determined by

\[
w_x = \frac{1}{n} \sum_{j=1}^{n} \frac{x_j}{\sigma_j}
\]

where \(x_j\) and \(\sigma_j\) denote the mean and standard deviation of a variable \(x\) for the \(j\)th class, respectively, and \(n\) is the total number of classes. In this way prior class information is incorporated into VAST scaling. This method is comparable to the scaling in a block fashion, utilizing the most appropriate scaling method for each variable group, particularly if different types of variables are

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**Figure 1.** Leaf wax hydrogen isotope fractionation relative to environmental water (\(\varepsilon_{\text{wax-environ}}\)) and xylem water (stem water, \(\varepsilon_{\text{wax-xylem}}\)) for all major lineages of terrestrial vascular plants. Lycop = Lycopod; Gymn = Gymnosperm; Magn = Magnoliids; Eudic = Eudicots; Monoc(-) = Monocots excluding Poales; and Poal(-) = Poales excluding BHP; BEP comprises subfamilies Bambusoideae, Ehrhartoideae and Pooidae within the graminoids. The error bars show the 1 \(\sigma\) standard deviation for all the species in individual lineages. A and C are for original data of \(\varepsilon_{\text{wax-environ}}\) and \(\varepsilon_{\text{wax-xylem}}\) values. B and D are the scaled and averaged data for the two sets of values (\(\varepsilon_{\text{wax-environ}}\) and \(\varepsilon_{\text{wax-xylem}}\)), respectively. See supplementary information on the rationale and methods for scaling the isotopic measurements.

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combined [21–22]. We have further divided each data point by the average of all the values. By centering and scaling, all variables (7 lipids) can be treated equally to represent the plant (Table S5).

Using the centered and VAST scaled data, we obtained the $e^{wax-xylem}$ values of 4 individual $n$-acids and 3 individual $n$-alkanes for each plant (Table S5). We then treated $n$-acids and $n$-alkanes separately and further obtained the average values of the 4 $n$-acids ($e^{wax-xylem-n\text{-}acid}$) and the average value of the 3 $n$-alkanes ($e^{wax-xylem-n\text{-}alkane}$), respectively. Treating $n$-acids and $n$-alkanes separately is mainly based on a Principle Component Analysis of the individual $\delta D$ values of the 7 leaf lipids from each plant, suggesting $n$-acids and $n$-alkanes were separated by the 2nd major component (Fig. S4). We used the two combined values as two separate entries to represent one individual plant. We also calculated the averages and standard deviations of all entries from each lineage for both $n$-acids and $n$-alkanes (Fig. 1B). We then performed a standard analysis of variance (ANOVA) of these values among different lineages to test if the group means are equal to each other. We then performed the post hoc analysis of these values and evaluate how each two group means are different. By incorporating all available data, we take full advantage of our extensive isotopic measurements of the seven leaf wax compounds for any given plant specimen, and reduce the uncertainty associated with single compound measurements. This method allows us to focus on comparing isotopic fractionation difference between different plants (which is the main purpose of this paper), without being affected by the different isotopic fractionation of different leaf waxes in the same plants. We treated the $e^{wax-env\text{iron}}$ values in the same manner (Fig. 1D).

**Phylogenetic analysis**

The combined $n$-acid or $n$-alkane entry for each plant was used for phylogenetic analysis (Fig. 2; Fig. S5). We inferred the phylogenetic relationships of our sampled plants using the program **Phylomatic v3** ([phytodiversity.net/phylomatic](http://phytodiversity.net/phylomatic/)), and incorporated two sets of branch lengths into our analyses: branch lengths all equal to one, and branch lengths as proportional to time, adjusted with the 'bldaj' command in the program **Phylomatic** v4.2 [23]. We tested for phylogenetic signal in fractionation using Pagel’s Lambda [24], implemented in the 'phylosig' function of the R module **phytools** [25], with significance of the metrics tested using 1,000 simulations. We ran these analyses across both sets of branch lengths and with ln-transformed and untransformed datasets; in all cases we inferred significant phylogenetic signal in our hydrogen isotope fractionation data.

We also modeled the evolution of $e^{wax-xylem-n\text{-}acid}$, comparing the relative fit of different models using AIC (Aikake Information Criterion) scores. We fit our data to a pure Brownian motion model of trait evolution, as well as an Ornstein-Uhlenbeck stabilizing selection model, which allows for different regions of the tree to be evolving under different trait optima (Table 1). We tested several different O-U models after visually inspecting the patterns of $e^{wax-xylem-n\text{-}acid}$ variation across our phylogeny. First, we employed a simple O-U model that selects a single trait optimum for all taxa. Second, we modeled a two-optimum scenario, with monocots evolving under a different optimum than all other vascular plants. Finally, we designed a three-optimum scenario, where BEP grasses, all non-BEP monocots, and all non-monocot plants each were evolving under distinct optima. All phylogenetic analyses were performed in R, using the packages 'phytools' [25], ‘ape’ [26], and ‘ouch’ [27].

**Results**

**Phylogenetic patterns in hydrogen isotopic fractionation**

We first grouped hydrogen isotopic fractionation data between leaf waxes and source water (the annual precipitation ($\delta D$) at the NYBG and the xylem water of plants) according to major phylogenetic lineages, separating taxa into lycopsids, ferns, gymnospersms, magnolids, eudicots, and monocots (Fig. 1). In monocots, we have further divided the whole lineage into three sub-categories: the monocots excluding any Poales (labeled as “Monoc (–)”), the Poales excluding the BEP clade, a lineage of exclusively C3 grasses including the bamboos, rice, and the cold-adapted Pooidae (labeled as “Poal(–)”) and the BEP clade (Fig. 1; Table S1; Table S5). We also examined all the sub lineages within eudicots, but found no obvious difference in $e^D$ (thus no display in Fig 1). The fractionation computed relative to both mean annual precipitation at NYBG (used as ‘environment water’) and to xylem water shows similar trends, but the standard deviations for individual lineages are reduced by ~20% when xylem water is used for calculation. This is not surprising, as xylem water represents actual water transported by the plant vascular system for biosynthesis.

ANOVA analysis of hydrogen isotopic fractionation demonstrates statistically significant difference among several major phylogenetic lineages (Table 2). Specifically, we find that plants representing earlier-diverging lineages (e.g., lycopsids, ferns) display smaller fractionation than later-diverging lineages (Fig. 1). A similar pattern also exists in the compiled published data [10,13] when grouped by major lineage membership (Fig. S6), but with much larger standard deviations, probably reflecting uncertainties in source water isotopic ratios.

When explicitly mapped on a phylogeny, our dataset shows strong phylogenetic patterning that more closely related species also have more similar fractionation values (Fig. 2; Fig. S5; Table 1; Table S5). The ‘Lambda metric’ analyses recovered high levels of phylogenetic signal that were statistically significant as assessed by permutation tests ($\lambda=0.83$, $p<0.001$). These results confirm the visual pattern observed on the tree. All monocots exhibit very large fractionation, and within monocots, the largest is clustered still, in the BEP clade of grasses (Fig. 1; Fig. 2; Fig. S5). These visual patterns were confirmed by modeling the evolution of hydrogen isotopic fractionation under three distinctive optima: one for BEP grasses, one for all non-BEP monocots, and one for all non-monocot vascular plants. This model was a significantly better fit than a single or two optimum model or a Browninan motion model of trait evolution [26,27] (Table 1; “Methods” Section). Overall, centering and scaling method provide better presentation of data for leaf lipid $\delta D$ analysis. It provides integrated data to represent individual plants, which can be readily incorporated to phylogenetic examination. It also presents better separation between lineages. In general, uncertainty within lineages are reduced (e.g., lycopsids, Poales, etc), which helps the detection of distinct difference between lineages.

**Leaf wax hydrogen isotope fractionation in plants with different growth forms**

Conversely, if we plot our data from NYBG in terms of plant growth forms (Fig. 3A), shrub, trees, and forbs do not show statistically different fractionation. In cases where plants exhibit different growth forms but belong to the same major phylogenetic clade, fractionation values are similar (Fig. 3B), indicating plant growth forms are not the principal determinant for the fractionation values. For example, palm trees (monocot trees) have similar
Figure 2. Phylogenetic patterns of mean leaf wax hydrogen isotope fractionation values of $n$-alkanoic acids. The tip values are the combined scaled values based on measurements of collected modern species (as in Fig. 1). The left of the two columns represent the living species values of $\delta^{18}O_{\text{wax-xylem-acid}}$ and colored branches represent inferred ancestral values. The right column represents different growth forms.
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Table 1. Evolutionary models of leaf wax hydrogen isotopic discrimination.

| Model                            | $-\ln L$ | AIC   | $q$ | $q_{\text{monocot}}$ | $q_{\text{BEP}}$ |
|----------------------------------|---------|-------|-----|----------------------|------------------|
| Brownian motion                  | $-459.78$ | $923.70$ | NA  | NA                   | NA               |
| OU: global optimum               | $-438.96$ | $884.19$ | $11.84$ | NA                   | NA               |
| OU2: monocot vs all other        | $-422.69$ | $853.82$ | $16.31$ | $-30.75$             | NA               |
| OU3: BEP vs other monocot vs all other | $-418.96$ | $848.60$ | $16.31$ | $-27.82$             | $-56.44$         |

$q = $ modeled ‘optimum’ trait value under a stabilizing selection evolutionary model. AIC = Akaike Information Criterion scores accounting for sample size. L = Likelihood scores. OU = the Ornstein-Uhlenbeck stabilizing selection model.
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Table 2. ANOVA and post hoc analysis of leaf wax hydrogen isotope fractionation relative to xylem water (centered and VAST-scaled for both $n$-acids and $n$-alkanes).

|                  | Lycop | Fern | Gymn | Magn | Eudic | Monoc(-) | Poal(-) |
|------------------|-------|------|------|------|-------|----------|---------|
| e$^\theta$ wax-xylem-acid |       |      |      |      |       |          |         |
| Fern             |       |      |      |      |       |          |         |
| Gymn             | 0.01  | 0.04 |      |      |       |          |         |
| Magn             | 0.00  | 0.00 | 0.50 |      |       |          |         |
| Eudic            | 0.00  | 0.00 | 1.00 | 0.13 |       |          |         |
| Monoc(-)         | 0.00  | 0.00 | 0.00 | 0.33 | 0.00  |          |         |
| Poal(-)          | 0.00  | 0.00 | 0.00 | 0.04 | 0.00  | 0.75     |         |
| BEP              | 0.00  | 0.00 | 0.00 | 0.00 | 0.00  | 0.00     | 0.00    |
|                  |       |      |      |      |       |          |         |
| e$^\theta$ wax-xylem-alkane |       |      |      |      |       |          |         |
| Fern             |       |      |      |      |       |          |         |
| Gymn             | 0.01  | 0.47 |      |      |       |          |         |
| Magn             | 0.00  | 0.03 | 0.77 |      |       |          |         |
| Eudic            | 0.00  | 0.20 | 1.00 | 0.46 |       |          |         |
| Monoc(-)         | 0.00  | 0.00 | 0.03 | 1.00 | 0.00  |          |         |
| Poal(-)          | 0.00  | 0.00 | 0.00 | 0.64 | 0.00  | 0.58     |         |
| BEP              | 0.00  | 0.00 | 0.00 | 0.00 | 0.00  | 0.00     | 0.01    |

Lycop = Lycopod; Gymn = Gymnosperm; Magn = Magnoliids; Eudic = Eudicots; Monoc(-) = Monocots excluding Poales; and Poal(-) = Poales excluding BHP. The values in bold show the corresponding two group means are not significantly different at 95% confidence level. The ANOVA test suggests that Magnoliids are not significantly different from eudicots, gymnosperms, monocots (-) and Poales(-) (in the case of $n$-alkanes), although this could be a result of small sample numbers (3) for this plant group.

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fractionation values to those of forb/herbs in Liliaceae (monocot herbs). Tree ferns have significantly smaller fractionation than palm trees, but have similar values to those of other ferns (Fig. 2; Fig. S5) [10]. The highest fractionation values within the ‘graminoids’ are found in bamboos, which might be better described as having a shrub-like habit, with multiple, tall growing shoots.

When including graminoids as a plant growth form, a standard ANOVA analysis supports an effect of growth form on the absolute $\varepsilon_{wax-water}$ values ($F = 6.91$, $p < 0.01$). However, ‘graminoids’ is a standard growth form category used by ecologists that actually refers to a plant lineage, in this case grasses and sedges. Thus, the perceived hydrogen isotopic difference between different plant growth forms is primarily due to the coincidence of graminoids as a growth form that occurs solely in the monocots, which is a phylogenetic clade with distinct fractionation.

**Influence of photosynthetic pathways**

In contrast to the strong phylogenetic patterns of leaf wax hydrogen isotopic fractionation, photosynthetic pathways exert a minor impact, as found in many previous studies [28–30]. Leaf wax hydrogen isotopic fractionation relative to xylem water (absolute values) in C₄ grasses is $\sim 20\%$ ($17\%$ in the composite dataset from [13]) smaller than that in C₃ grasses (Fig. 4). Such difference is thought to originate from different venation patterns and physiological characteristics between C₃ and C₄ grasses [20]. Similarly, species exhibiting Crassulacean Acid Metabolism (CAM) show smaller absolute leaf wax hydrogen isotopic fractionation values than C₃ species within individual major lineages (Fig. 4), though our sample number of CAM species is admittedly small (a total of 5 species). Despite the hydrogen isotopic fractionation difference between C₃ and C₄ plants, C₄ grasses still have $\sim 30\%$ larger fractionation than C₃ eudicots. Therefore, isotope effects derived from photosynthetic methods are subordinate to the very large difference between monocots and other plants. Fractionation in C₃ grasses ($n = 3$) was $\sim 30\%$ smaller than that in C₄ grasses ($n = 11$) (Fig. 4), although this could also be largely a clade effect, as 10 of our 11 C₃ grasses were sampled from the BEP lineage (Fig. 1; Fig. 2; Fig. S5).

**Discussions**

Possible factors that contribute to the phylogenetic patterns in plant leaf wax hydrogen isotopic fractionation

What are the mechanisms underlying these strong phylogenetic patterns? From xylem water to leaf waxes, there are numerous biosynthetic steps that may impart different hydrogen isotopic fractionation [13,31], and unraveling the exact mechanism will require comprehensive examination of each point where reactants are not 100% transformed into products. One explanation would be that plants have different evapotranspiration rates, as has been suggested previously [10,13,32], and recently further demonstrated by analyzing plant leaf water from across a large relative humidity gradient [33–34]. To test if leaf water $\delta D$ difference can fully explain the contrasting leaf wax $\delta D$ difference between eudicots and grasses we analyzed a total of 110 eudicot and grass samples from growth chamber or field (Table S2; Fig. 5; Fig. S7). We find in all cases, grasses have $\sim 10$ to $15\%$ higher leaf water $\delta D$ values than eudicots, but $\sim 30$ to $50\%$ lower leaf wax $\delta D$ values than eudicots (Fig. 5). Additionally, the leaf water $\delta D$ values in our plant samples from NYBG vary only $\sim 16\%$ and show little
correlation with leaf wax δD values (Table S1). For example, four fern species have slightly higher leaf water δD values than Poales, but have much heavier leaf wax δD values than Poales (Fig. 5). We do acknowledge, however, that sampling leaf water may carry inherent uncertainties because of the variable nature of leaf isotopic composition during the diurnal cycle [35], though we did sample our leaves at nearly the same time of day (12pm–2pm). While leaf water δD values must affect the leaf wax isotopic values [16,18], our results suggest other factors are likely also important in determining the different hydrogen isotopic fractionation between eudicot trees and grasses (Fig. 5, Fig. S7).

Another explanation for the isotopic difference among different lineages is that plants have evolved divergent strategies of allocating photosynthetic products among metabolic processes. Our recent studies of leaf wax regeneration rates from a monocot (the grass *Phleum pratense*) and a eudicot (the tree *Fraxinus americana*) in greenhouse experiments show that leaf wax production appears to begin significantly earlier in the grass during a diurnal cycle [16,18]. If eudicots in general tend to show lower rates of wax regeneration, their isotopic signal may be controlled primarily by the rapid leaf expansion phase in the early growth season [16,18,32,36–37]. Such scenario is consistent with our data of naturally occurring plants collected around Blood Pond in May, July and September, respectively (Fig. S7). Leaf waxes from eudicot trees remained relatively constant over the growth season, probably mostly inheriting an early season isotopic signal [18], and grasses maintain lower δD values than trees throughout the season (Table S2, Fig. S7). These observations suggest that synthesis of leaf waxes in eudicots likely use isotopically enriched sugars that are already partially used for other metabolic and biosynthetic functions, or stored in roots and trunks over the winter season, contributing to the relatively high leaf wax δD values [38]. We also concur with the proposal of Kahmen et al. [33] that differential degree of hydrogen atoms derived from water versus from NADPH may also be important in regulating the hydrogen isotopic fractionation of plant leaf waxes.

Evolution and leaf wax hydrogen isotope fractionation

If our experiments with grasses and trees can be extrapolated more broadly, it is possible that the observed phylogenetic trend is related to a change in the rate and timing of leaf wax synthesis during seasonal and/or diurnal cycles. Such changes in biosynthetic strategy may have resulted in differences in prioritizing leaf wax synthesis and utilization of fresh/residual sugars. Additionally, leaf wax synthesis could have been significantly altered by the evolution of the monocot leaf, which is developmentally very different than leaves in other plant groups, with the leaf lamina possibly being derived from the leaf base [39] and also having an extended period of growth and development via an intercalary meristem. Our observations of exceedingly high isotopic fractionation in the ‘BEP’ grass lineage have no reasonable explanation at this time, and warrant more research.

In summary, our study shows clearly that growth form or plant ‘functional type’ bears little on values of hydrogen fractionation in leaf waxes. Variation in hydrogen isotopic fractionation among vascular plants is best explained by plant lineage differences, and may reflect, at least in part, evolutionarily driven changes in biosynthetic priorities and leaf developmental programs. Palco-
mate reconstruction utilizing leaf wax δD values must take into consideration these significant isotopic differences between plant lineages.

Supporting Information

Figure S1 Sampling species numbers in each major phylogenetic lineage (data from Sachse et al. (2012) for the upper panel and this study for the lower panel). The published sampling pool, reviewed by Sachse et al. (2012), is heavily biased toward Poales and Eudicots.

(DOC)

Figure S2 Comparison of chain-length distributions of n-alkanes between all trees of any phylogenetic lineages and all Poaceae and between C4 Poaceae and C3 Poaceae from NYBG. n-Alkanes of majority of trees and grasses are dominated by C29 n-alkanes, whereas 4 out of 9 C3 grasses and 2 out of 3 C4 grasses have C31 n-alkane as the most abundant n-alkane lipid. The error bars show the 1σ standard deviation for each leaf lipid.

(DOC)

Figure S3 Correlation of hydrogen isotope fractionation values between individual leaf lipids and xylem water.

(DOC)

Figure S4 The Principle Component Analysis (using MatLab) of the δD data of 4 individual n-acids and 3 individual n-alkanes.

(DOC)

Figure S5 Leaf wax hydrogen isotope fractionation values for all plant species from NYBG on a phylogenetic tree. The tip values are the combined scaled values of n-alkanes *wax-xylem-alkane*, based on measurements of collected modern species (as in Fig. 1), and branches are colored according to the inferred ancestral values of *wax-xylem-alkane* across the phylogenetic tree.

(DOC)

Figure S6 Replotting three datasets by phylogeny (This study; Sachse et al. (2012); and Hou et al. (2007), respectively). Only hydrogen isotope fractionations of C29 n-alkane and C29 n-acid relative to mean annual precipitation (MAP) are shown for comparison, while other leaf wax compounds show similar patterns. δD values of MAP were calculated from the Online Precipitation Isotopes Calculator. The error bars show the 1σ standard deviation for all the available species in individual lineages. Numbers show the species numbers for individual lineages. The gray boxes represent the box-whisker plots, whereas the magenta lines represent category mean values with 1σ standard deviation.

(DOC)

Figure S7 Seasonal variations in δD values of leaf water, xylem water and leaf waxes for plant samples collected from Blood Pond, Massachusetts (USA). Only C29 n-acid is shown for comparison, while other n-acids show similar patterns.

Note in all cases, grasses have higher leaf water δD values, but lower leaf wax δD values than trees (Table S2). The error bars show the 1σ standard deviation for all the species in each season.

(DOC)

Table S1 δD values of leaf waxes, leaf water and xylem water for all the plant samples from the NYBG. C21, C26, C28 and C30 are n-alkanoic acids and C27, C29 and C30 are n-alkanes. Leaf δD and xylem δD values are for water distilled from leaf and stem sampled during 12pm-2pm of the sampling day, respectively. Lycopsids (lycop), Gymnosperms (gymn), Magnoliids (magn), Eudicots (eudic), monocots (monoc), Poales (Poal); Forb/herb (F/H), climbing vine (CV) and Graminoids (Gram).

(DOC)

Table S2 The δD values of leaf lipid C29 n-acid, leaf water and xylem water for the field (Blood Pond) and growth chamber plant samples. The left side are for tree species and the right are for grasses. The irrigation water used for growth chamber experiments has a δD value of −49‰.

(DOC)

Table S3 The leaf lipid abundances for each n-alkane lipid for plant samples collected from the New York Botanic Garden. The unit for single lipids and sums is μg/g d.w. leaf. ACL is the average chain length (ACL = ∑Cn/sum, where n is carbon number 21, 23, 25, 27, 29, 31 and 33; Cn is lipid with n carbon number and sum is total mass of C21–C33 n-alkanes). R is the ratio of total n-acids (C20–C32) to total n-alkanes (C21–C33).

(DOC)

Table S4 The leaf lipid abundances for each n-alkanoic acid lipid for plant samples collected from the New York Botanic Garden. The unit for single lipids and sums is μg/g d.w. leaf. ACL is the average chain length (ACL = ∑Cn/sum, where n is carbon number 24, 26, 28, 30, and 32; Cn is lipid with n carbon number and sum is total mass of C24–C32 n-acids).

(DOC)

Table S5 The centered and VAST-scaled *wax-xylem* values for the 7 leaf lipids for each plant species. The detailed description for the scaling and combination is described in “Methods”.

(DOC)

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Author Contributions

Conceived and designed the experiments: YH. Performed the experiments: LG YH EE YZ. Analyzed the data: LG YH EE YZ. Contributed reagents/materials/analysis tools: LG YH EE YZ. Wrote the paper: LG YH EE.

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