VARIATIONS IN LEAF WAX *n*-ALKANE CHAIN LENGTH AND CARBON ISOTOPE FRACTIONATION: APPLICATIONS FOR CHEMOTAXONOMY AND PALEOECOLOGY

A THESIS SUBMITTED TO THE FACULTY OF THE PROGRAM IN PLANT BIOLOGY AND CONSERVATION

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN PLANT BIOLOGY AND CONSERVATION

NOVEMBER 20, 2008

ABSTRACT

Because long-chain *n*-alkanes (*n*-C_{21} to *n*-C_{37}) are found in the epicuticular leaf waxes of all vascular plants and are stable, long-lived molecules, the study of their molecular and isotopic compositions stands to serve as a potentially powerful tool in the fields of modern chemotaxonomy and paleoecology. This study attempts to evaluate the usefulness of *n*-alkane distributions as identifiers of plant species and groups using data from a wide range of plant species. *n*-Alkane composition is shown here to be highly conservative within individual plants across a growing season and from different locations within a plant's canopy; composition is also largely conservative within a species but not within higher groupings. Within species, there is a correlation between the weighted mean of *n*-alkanes and the dispersion about that mean, possibly due to functional constraints on the biosynthesis of *n*-alkanes. This study also investigates the application of modern *n*-alkane data to the reconstruction of ancient plant community dynamics during the Paleocene-Eocene Thermal Maximum (PETM) and finds that the shift in paleosol *n*-alkane composition at the onset of the PETM is not related to a change in species composition of the local plant community. Finally, this study investigates the differentiation between angiosperms and gymnosperms in *n*-alkane carbon isotope composition ($\delta^{13}C$) and finds that the distinction previously reported between the two groups may be physiological rather than taxonomic. These findings suggest that the magnitude of the terrestrial PETM carbon isotope excursion may not be explained by the loss of gymnosperms species from the local plant community at the onset of the PETM.

ACKNOWLEDGEMENTS

I heartily thank all of the people who contributed to this work, without whom I could never have succeeded. I would thank firstly my advisor, Francesca Smith, for all her wise advice and infectious enthusiasm, as well as the members of my defense committee, Jim Tang and Lenny Kouwenberg, for their time and feedback throughout the process. I am also grateful to the staff of the Chicago Botanic Garden for all their assistance, and especially to Celeste VanderMey, Boyce Tankersley, and Tom Tiddens for their generous help. I thank my lab-mates Junzi Shi, Kyle Shedd , and Benjamin Farah for their tremendous assistance and ever-ready good cheer. Thanks also to Nyree Zerega for making so many things possible, and to Susan Black and Diane Huebner. I thank my fellow PBC students and my friends at Northwestern University for all their thoughtful comments as well as their support and camaraderie. And finally, I thank Mr. and Mrs. Shaw and the Plant Biology and Conservation program for invaluable financial support throughout my studies.

I dedicate my work to my grandmother, Mary T Littlejohn, whose curiosity for and enjoyment of all things intellectual have been the perennial spark of all my endeavors.

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Introduction

The phenomenon of climate change and global warming has become one of the key issues of our century. It is now well established that our species is warming the planet with the exhaust of our vehicles and our industries. Annual growing seasons are increasing in length, glaciers and polar ice are retreating, and plant and animal species of all types are rapidly responding to the myriad changes wrought by our use of fossil fuels (Walther et al. 2002, Vaughan et al. 2003, IPCC 2007). Accordingly, it is of great importance to science and to civilization to understand both the workings and consequences of global warming. It is with this end in mind that we turn to investigate climate change events of the past and the reaction of ecosystems to those events, using the past as the key to the present. One event in particular captures our attention with its resemblance our own global climate situation: the Paleocene-Eocene thermal maximum, which is thought to have been caused by a large release of carbon into the atmosphere, and it would behoove us to better understand the dynamics of the plant communities growing during that event. In order to correctly interpret the fossil remains of those ancient plant communities, we must first understand some of the molecular and ecological variety of modern plant species and communities. Here, then, the present becomes the key to the past: by examining natural variation in the composition of molecules and isotopes from a broad group of living plant species, we can build a foundation of comparison for analysis of plants long dead. Thus builds the premise for the research presented here—the examination of the diversity in molecular structure and isotopic composition of long straight-chain hydrocarbons in the leaf waxes of modern plants.

The Paleocene-Eocene Thermal Maximum

Approximately 55.8 million years ago (Ma), starting at the boundary between the Paleocene and Eocene epochs, global temperatures rose by 5 to 10°C (Zachos et al. 2003, Wing and Harrington 2001, Wing et al. 2005). This rise in temperature happened rapidly—within 10 to 20 thousand years (ka)—and it lasted ~200 ka before gradually returning to the background temperature (Zachos et al. 2003, Zachos et al. 2001, Farley and Eltgroth 2003). The evidence indicates that the global warming during this time was caused by the release into the atmosphere of an mass of carbon (~4500 Gt) roughly equivalent to today's global fossil fuel reservoir (Zachos et al. 2005). This period is known as the Paleocene-Eocene Thermal Maximum (PETM),[1] and the temperature change is associated with relatively large excursions within the benthic carbon and oxygen isotope record (Zachos et al. 2001, Zachos et al. 2003). Specifically, the event is characterized by a negative carbon isotope excursion (-2 to -7‰) of the global carbon reservoirs: marine, atmospheric, and terrestrial (Zachos et al. 2001, Bains et al. 1999, Bowen et al. 2004). The PETM was also associated with significant alteration of global carbon cycling, ocean temperatures, circulation, and chemistry, atmospheric temperatures, and both terrestrial and marine ecosystems (Zachos et al. 2001, Zachos et al. 2005, Fricke et al. 1998, Beerling 2000).

Within the terrestrial ecosystems, large migrations of both flora and fauna are observed in the fossil record, in both megafossils and palynomorphs (Fricke et al. 1998, Wing et al. 2005, Gingerich 2006, Gingerich 2003). From PETM sites in Wyoming, there is a well-documented rapid and complete turnover in floral composition: the plant

[1] It has also been called the Initial Eocene Thermal Maximum (IETM) and the Late Paleocene Thermal Maximum (LPTM).

community changes from a mix of deciduous conifers (Taxodiaceae) and angiosperms in the late Paleocene to an entirely different set of evergreen angiosperms within the PETM (dominated by Fabaceae and subtropical families and morphotypes with large leaf surfaces) and then back to an early Eocene deciduous gymnosperm/angiosperm mix resembling the original (Wing and Harrington 2001, Wing et al. 2005, S. Wing pers. comm.). Such a large body of fossil evidence for the local plant community during the PETM allows us to explore the plant community response to global warming, using preserved plant *n*-alkanes as a tool.

n-Alkanes in Epicuticular Leaf Wax

All vascular plants are known to synthesize a range of long-chain, predominantly odd-numbered unsaturated hydrocarbons (C_{21}-C_{37}), or *n*-alkanes, as a part of the wax extruded onto the exterior of the leaf cuticle (Eglinton and Hamilton 1967). This epicuticular wax protects the leaf from water loss, mechanical damage, and harmful radiation (Shepherd and Griffiths 2006, Kunst and Samuels 2003). It also allows a plant to interact with the biotic environment in remarkably complex ways, deterring herbivorous insects, for example (Müller and Riederer 2005). The structure of epicuticular wax varies between plant species, and the amount of *n*-alkanes varies as well. For example, in the gymnosperm families Pinaceae and Cupressaceae, *n*-alkanes are shown to constitute ~1% of epicuticular leaf wax (Herbin and Robins 1968b, Chikaraishi et al. 2004), compared to much larger percentages, some greater than 90%, in some angiosperm leaf waxes (Herbin and Robins 1969, Dove 1992). Although the

specific purpose of *n*-alkanes in leaf wax is unclear, the presence of *n*-alkanes as a component of plant leaf waxes appears to be universal.

Because the relative amounts of various *n*-alkanes vary so greatly between species, many authors have attempted to use *n*-alkane profiles for chemotaxonomic purposes, with varying degrees of success (Eglinton et al. 1962, Osborne et al. 1989, Osborne et al. 1993, Lockheart et al. 2000). *n*-Alkanes do not seem to be consistent within a genus or family, but they do appear to be relatively consistent within any given species. For example, the relatively well-studied *Fagus sylvatica*, across its native range, has a typical profile in which *n*-C_{27} comprises the vast majority (Lockheart et al. 1997, Collister et al. 1994; Nguyen-Tu et al. 2007; Rieley et al. 1991; Gülz et al. 1989). This high level of consistency within a species is also found in fossil leaves. For example, several *Platanus dissecta* leaves of the Miocene Clarkia Formation (17-20 Ma) have *n*-alkane profiles that are remarkably similar to one another (Lockheart et al. 2000), suggesting that this phenomenon of conservative *n*-alkane profiles within species is maintained through deep time and is potentially unaffected by diagenesis. Therefore, if alkane profiles cannot distinguish plant families from one another, they may still be useful in identifying particular species.

As noted above, *n*-alkanes are also well-known to be relatively stable, decay-resistant molecules (Eglinton and Logan 1991), and are therefore preserved in sediments and fossils from the deep past (Meyers and Ishiwatari 1993, Lockheart et al. 2000, Smith et al. 2007). This makes them potentially excellent fossil biological markers (biomarkers). *n*-Alkanes have been isolated from fossils soils and sediments as well as from fossil leaves (Smith et al. 2007, Huang et al. 1995). The carbon isotope

composition of individual alkanes can be measured, and thus *n*-alkanes can serve as a both a biomarker for vascular plants as well as evidence of the community composition and environment or, in the case of fossils, the paleoenvironment. There is evidence that the distribution of alkanes produced in a leaf is consistent across the age of a leaf (Eglinton and Hamilton 1967, Avato et al. 1984), suggesting that the alkane profile for a single plant is consistent across time (e.g. growing season) and possibly canopy location as well. This consistency is a crucial assumption in the use of alkane profiles in paleoecology. If *n*-alkane distribution varies across a growing season or is dependent on leaf position within a tree's canopy, then this would invalidate any comparisons made between modern leaves, typically sun-exposed leaves collected at the peak of the growing season, and fossil *n*-alkane profiles derived from leaves of unknown age and unknown canopy position. This assumption has until now remained largely untested, and no studies have been published comparing alkane profiles of sun and shade leaves.

The biosynthesis of *n*-alkanes begins with a process known as fatty acid elongation, where a fatty acid is enzymatically extended two carbons at a time with acetyl-CoA. This gives rise to the initial even-numbered long-chain fatty acids (C_{18}-C_{32}), which are transformed by the decarbonylation pathway first to aldehydes and then to odd-numbered alkanes through removal of a single molecule of carbon monoxide (Kunst and Samuels 2003, Post-Beittenmiller 1996). Because of this biosynthetic path, odd-numbered chains dominate heavily over even-numbered chains, though even-chained alkanes do exist in vascular plants in smaller amounts (Eglinton and Hamilton 1967). A single plant produces a range of alkanes, and this range is typically summarized as the weighted mean of the most predominant alkanes, known as average chain length (ACL),

$$\mathrm{ACL} = (25A_{25} + 27A_{27} + 29A_{29} + 31A_{31} + 33A_{33})/$$

$$(A_{25} + A_{27} + A_{29} + A_{31} + A_{33}),$$

where A is the integrated area of the chromatogram peak of each *n*-alkane (Eglinton and Hamilton 1967, Smith et al. 2007). Algae and bacteria also synthesize alkanes, but the chain lengths are significantly shorter. *n*-Alkanes with chain lengths of less than 17 carbon atoms are used as biomarkers for these organisms (Meyers and Ishiwatari 1993). Aquatic plants have also been reported to produce shorter alkanes than terrestrial plants, with average chain lengths in the mid-twenties (Chikaraishi and Naraoka 2003, Meyers and Ishiwatari 1993). The average chain lengths of vascular land plants are reported to range from 26 to 33, with the majority falling between 27 and 31 (Chikaraishi and Naraoka 2003, Bi et al. 2005, Collister et al. 1994).

Average chain length is not the only method of numerically defining *n*-alkane distribution. Various *n*-alkane ratios have been used as evidence of ecological dynamics (Zhang et al. 2006, Liu and Huang 2005). Several workers have used ratios of *n*-alkanes to identify and separate plant groups for modern agricultural as well as paleoecological purposes (Dove 1992, Brincat et al. 2000, Zhang et al. 2006). *n*-Alkanes were used to quantify known amounts of legume species in grass mixtures with a limited number of known species (Dove 1992), and the relative contribution of terrestrial plants and aquatic algae to lake sediments have been assessed using ratios of *n*-C_{29} to *n*-C_{17} (Meyers and Ishiwatari 1993, Cranwell et al. 1987).

n-Alkanes can thus provide comparisons between groups, but given that *n*-alkane distribution varies so greatly within plant families, they are most effectively used if those groups are relatively well-defined and well-separated. For example, a strong increase in

n-C_{23}/*n*-C_{31} has been shown to reflect a transition from angiosperm dicot cover to a *Sphagnum* peat bog (Nott et al. 2000, Pancost et al. 2002). In contrast, other authors have used *n*-alkane ratios to make comparisons within the vascular plants and to draw conclusions about large-scale shifts in the surrounding terrestrial ecosystems, such as an increase in *n*-C_{27}/*n*-C_{31} corresponding with a change from herbaceous vegetation to forest (Brincat et al. 2000). Others have utilized more complicated ratios (Zhang et al. 2006, Hanisch et al. 2003), but within vascular plants the comparison is typically made using *n*-C_{31} to represent grasses and *n*-C_{27} or *n*-C_{29} to represent woody plants (Zhang et al. 2006). Because these ratios involve *n*-alkanes that differ in length by only two or four carbon atoms, the comparisons drawn from them become much less distinct than comparisons based, for example, on a ratio of *n*-C_{29} and *n*-C_{17}. The leaf waxes of members of closely related vascular plant genera, or even different species within a single genus, can have very different *n*-alkane compositions, not always centered on a single *n*-alkane such as *n*-C_{27} or *n*-C_{29} (Herbin and Robins 1968a, Maffei 1994).

Changes in *n*-alkane distribution in a sediment column can be correlated with large changes in the surrounding vegetation, known from pollen records, such as angiosperm forest to peat bog or grassland or vice versa (Cranwell 1973, Cranwell et al. 1987, Brincat et al. 2000). However, before more meaning can be assigned to *n*-alkane ratios than simply corroboration for other lines of evidence, we must determine the constraints of alkane variation within plant groups and define those plant groups more clearly. This requires examining the natural variation in *n*-alkane distributions across a broad range of species from various plant groups, for example, asking whether a

dominance of *n*-C_{29} in an alkane profile is typical of all woody plants, or only specific families of angiosperm trees.

Carbon Isotope Fractionation

Carbon exists naturally as three different isotopes: ^{12}C, ^{13}C, and ^{14}C, each with one more neutron in its nucleus than the previous. ^{14}C occurs only in trace amounts and is an unstable isotope, decaying to nitrogen via beta decay with a half-life of 5730 years. By contrast, both ^{12}C and ^{13}C are stable, and most of the Earth's carbon is composed of the lighter ^{12}C, approximately 98.89%, with the remaining 1.11% being ^{13}C. The two stable isotopes are, however, distributed unevenly across different materials. This uneven distribution is the result of 'isotope effects,' which are slight differences in the chemical and physical properties due to differences in the masses of the isotopes (Dawson and Brooks 2001, Hoefs 2004). Physical, chemical, and biological reactions 'fractionate,' or change the relative proportions of the isotopes of an element between one compound and another (Hoefs 2004, Kendall and Caldwell 1998). Fractionation occurs because molecules containing heavy isotopes are more strongly bound and therefore less reactive than molecules with light isotopes. In other words, the molecule with the lighter isotope will have a lower dissociation energy and a higher zero point energy, so it will break its atomic bonds more readily and form new bonds with a higher probability than the heavier isotopes (Hoefs 2004, Kendall and Caldwell 1998). For example, $^{12}C^{16}O_2$ will react more readily than $^{13}C^{16}O_2$ at the same temperature.

Isotope effects can be classified as either thermodynamic (also called isotope exchange reactions) or kinetic, the distinction being whether the reaction is in chemical

equilibrium or non-equilibrium, respectively. Biochemical reactions tend to be kinetic, as enzymatic catalysis within a cell makes for essentially a one-way reaction and thus keeps the reactant/product balance in a constant state of disequilibrium. Because isotope effects result in fractionation of isotopes, they are known in geochemistry as fractionation factors, denoted by α which is defined as

$$\alpha = R_r/R_p,$$

where R_r is the reactant molar ratio of heavy to light isotopes of an element and R_p is that of the product. For carbon,

$$R = {}^{13}\mathrm{C}/{}^{12}\mathrm{C}.$$

The factor α can define fractionation associated with both equilibrium and kinetic exchange reactions (Kendall and Caldwell 1998). Note that this equation can be generalized to any pair of stable isotopes, e.g. D/H or $^{18}O/^{16}O$.

In plant studies, whether ecological or molecular, isotopic composition is always expressed as a δ value, which can be thought of as a comparison of ratios. Because the *absolute* isotopic composition is not easy to measure with mass spectrometry, it is instead expressed as a deviation of the sample isotope ratio from a given standard ratio

$$\delta = (R_{\text{sample}} - R_{\text{std}})/R_{\text{std}} = R_{\text{sample}}/R_{\text{std}} - 1,$$

where R_{sample} and R_{std} are the respective molar isotope ratios of the sample and standard. In typical notation, the δ value of carbon-13 would be written $\delta^{13}C$, though some slight variations can be found in older literature (Roeske and O'Leary 1984). For carbon, the international reference standard is fossil belemnite (*Belemnitella americana*) of the Cretaceous Peedee Formation in South Carolina (Park and Epstein 1961), shortened to

PDB (or the equivalent, standardized VPDB, short for the International Atomic Energy Agency, Vienna-PDB), for which $R = 0.0112372$ (Craig 1957). Being typically a very small ratio, δ is both dimensionless and conventionally expressed as 'permil' (‰). The definition of δ in permil is

$$\delta\ (‰) = (R_{sample}/R_{std}\ -1)\times 1000.$$

The factor of 1000 is superfluous to the definition per se (Farquhar et al. 1989), but it is important to remember nonetheless. The concept of permil is analogous to that of percent (%), and so it is the decimal values that are used in any calculations. For example, the average $\delta^{13}C$ of C_3 plant leaf tissue is approximately -28‰, equivalent to -0.028. Measurement precision is typically ±0.2‰, much smaller than the typically observed natural variation of $\delta^{13}C$ values (O'Leary 1988, O'Leary et al. 1992).

A negative δ value means that the $^{13}C/^{12}C$ ratio of the sample is lower than that of the standard, while a positive δ value means the opposite: that the sample ratio is higher than the standard ratio. Or to put it another way, a relatively more negative $\delta^{13}C$ means less ^{13}C relative to ^{12}C (a 'lighter' isotope ratio), and conversely a more positive $\delta^{13}C$ means more ^{13}C relative to ^{12}C (a 'heavier' isotope ratio). By definition, the δ value of the arbitrary standard is 0‰. Because all plant carbon δ values are negative relative to the PDB standard, due to the discrimination against ^{13}C during photosynthesis, it is often most expedient to compare carbon ratios in terms of more or less negative.

All carbon enters a plant as CO_2 through the stomata, and the stomata also control the flow of water in a plant. Therefore, as plants open and close their stomata in response to water stress or availability, this affects the $\delta^{13}C$ of the growing plant tissue. Knowing that $\delta^{13}C$ of leaf tissue is closely tied to water availability and usage, researchers have

discovered that plant groups with different physiologies fractionate carbon isotopes differently. For example, C_4 plants are known to have much more positive $\delta^{13}C$ values than C_3 plants (Farquhar et al. 1989, Cerling et al. 1997, Sage 2004). Angiosperms and gymnosperms are known to discriminate differently against ^{13}C, where gymnosperms often have less negative bulk $\delta^{13}C$ values than angiosperms (Leavitt and Newberry 1992, Brooks et al. 1997). Specifically, deciduous angiosperm trees tend to have an average bulk $\delta^{13}C$ ~1-3‰ lower than coniferous gymnosperm trees (Leavitt and Newberry 1992, Stuiver and Braziunas 1987).

Variation in *n*-Alkane $\delta^{13}C$

The $\delta^{13}C$ values of all components of plant tissue, including alkanes, are determined by photosynthesis, catalyzed by Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase), and subsequent biosynthesis. The maximum isotope discrimination by Rubisco was found to be approximately 29‰ (Roeske and O'Leary 1984, Park and Epstein 1960). This maximum discrimination is not usually reached within a leaf, and because the atmosphere has a $\delta^{13}C$ value of approximately -8‰ (O'Leary 1988), the leaf tissues of C_3 plants tend to have $\delta^{13}C$ values around ranging from -28‰ to -38‰ (Chikaraishi and Naraoka 2003). Carbon fractionation can also occur during synthesis of other organic molecules from PGA, the initial product of photosynthesis (Chikaraishi et al. 2004, Faure 1986), and within a C_3 plant, variation in $\delta^{13}C$ is known to exist between different classes of compounds (Bowling et al. 2008). For example, cellulose $\delta^{13}C$ values are on average approximately 2‰ more positive than total tissue values of the same tissue (Schleser 1990), while lignin appears to be more

negative (Wedin et al. 1995). The $\delta^{13}C$ values of *n*-alkanes appear to be ~2-7‰ more negative than bulk tissue values in C_3 plants, as opposed to ~6-9.5‰ in both C_4 and CAM plants (Chikaraishi and Naraoka 2003).

While much has been done to investigate the $\delta^{13}C$ values of bulk leaf tissues, there has been much less research examining compound-specific carbon isotope discrimination (Chikaraishi and Naraoka 2003, Bi et al. 2005, Collister et al. 1994). Bulk $\delta^{13}C$ values do not appear to be finely indicative of plant taxons (Kelly and Woodward 1995), but this gap can filled by chemotaxonomy of *n*-alkanes, whose distributions are known to be distinct between species and have been used to identify single species from mixed samples (Dove 1992). Compound-specific analysis of $\delta^{13}C$ can also reveal fine-scale trends often hidden in bulk tissue data (Smith et al. 2007). As shown in Figure 1, deciduous angiosperms and conifers appear to have very different $\delta^{13}C$ trends with increasing *n*-alkane chain length, beyond the overall difference in $\delta^{13}C$ values. If this divergent trend holds true for all gymnosperms and all angiosperms, then there is a great potential for its use in chemotaxonomic and paleoecological applications.

However, in defining the trend in Figure 1, little work has been done using *Ginkgo biloba* or other deciduous gymnosperms. Almost all comparisons between angiosperm and gymnosperm trees made to date, including Figure 1, involve only deciduous, broadleaf angiosperms and coniferous (i.e. evergreen needle-leaf) gymnosperms (Chikaraishi and Naraoka 2003, Leavitt and Newberry 1992, Brooks et al. 1997, Flanagan et al. 1997). In the case of Figure 1, the conifer trend is based on only four trees, while the angiosperm trend is drawn from a much larger number (Smith et al. 2007). Therefore, it remains possible that the trend assigned to systematic differences is

instead based on leaf morphology (broadleaf versus needle) or leaf habit (i.e. deciduousness.) Currently, a systematic analysis of the ^{13}C discrimination of non-coniferous gymnosperms and evergreen angiosperms has not been performed, and so the distinction between angiosperms and gymnosperms as systematic groups, in terms of both *n*-alkane and carbon isotope composition, remains relatively undefined. If the *n*-alkanes in modern plants are to be used for interpretations in paleoecology, then the range of molecular and isotopic variation in those *n*-alkanes must first be well-constrained.

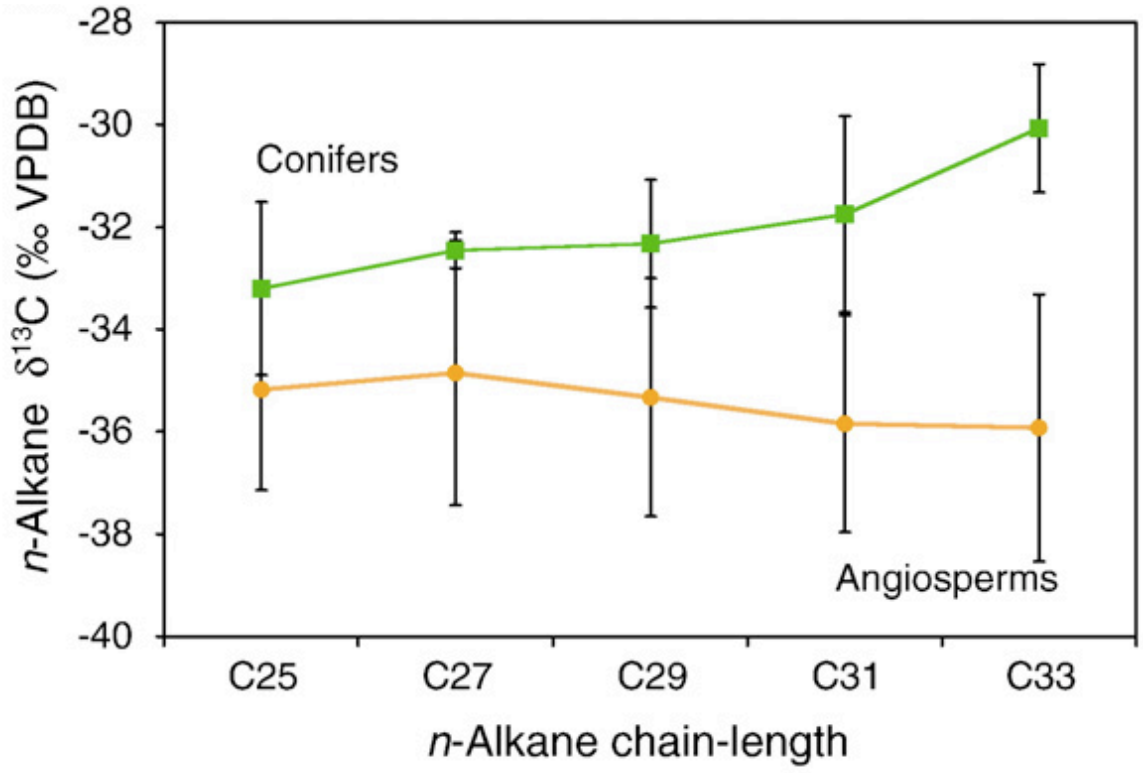


Figure 1. *n*-Alkane $\delta^{13}C$ trends of modern coniferous gymnosperms (green line) and C_3 dicot angiosperms (orange line), showing that gymnosperms have less negative values than angiosperms overall, and that where in gymnosperms, $\delta^{13}C$ increases with increasing chain length, the opposite trend is true of angiosperms (from Smith et al. 2007).

Research Hypotheses

ACL During the PETM

Associated with the change in plant communities in Wyoming during the PETM is a change in the ACL of *n*-alkanes in local paleosols, as seen in the graph on the right in Figure 2. ACL was observed to increase at the start of the PETM from approximately 29 to almost 31 and then decrease at the end of the PETM to its original pre-PETM values.

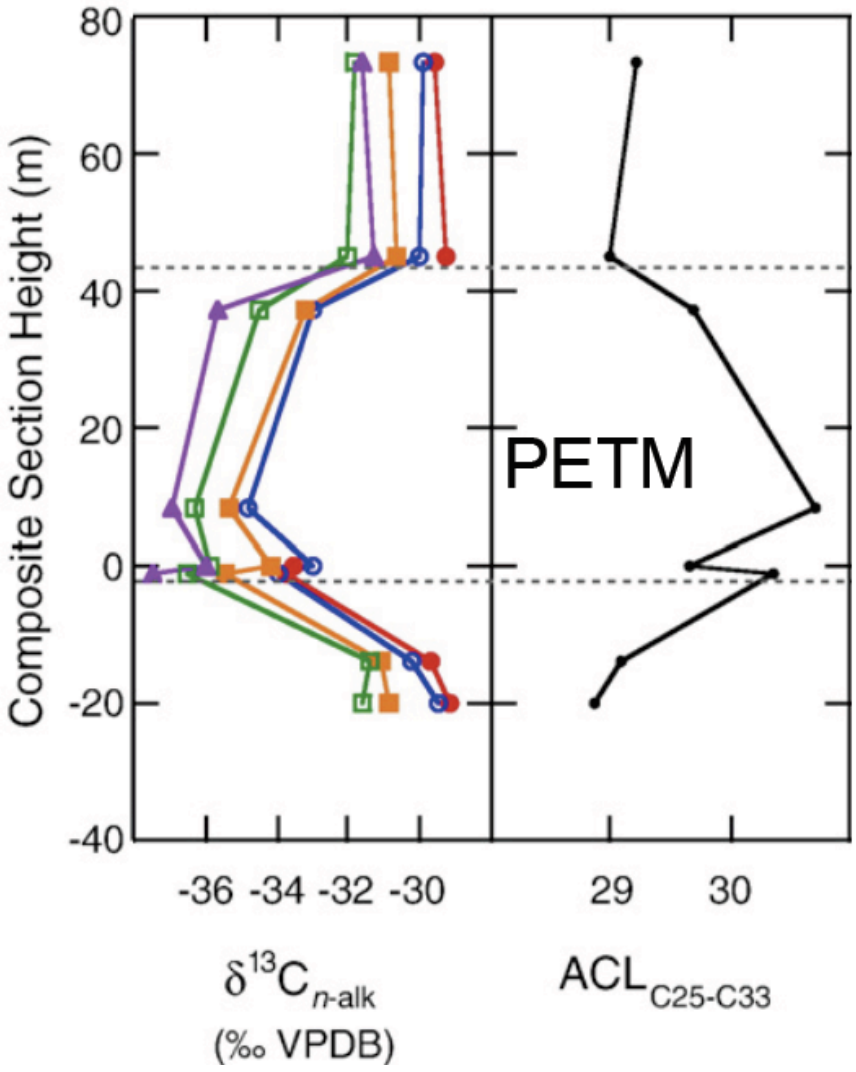


Figure 2. $\delta^{13}C$ values of *n*-alkanes (C_{25}-C_{33}) from sediments before, during, and after PETM (left). C_{25}: red solid circles, C_{27}: blue open circles, C_{29}: orange solid squares, C_{31}: green open squares, C_{33}: purple solid triangles. Average chain length (ACL) of sediments samples (right). Gray dashed lines denote beginning and end of PETM (from Smith et al. 2007).

This change in ACL can be potentially explained by one of two hypotheses: 1) the change in the plant community from species that predominantly produce shorter length *n*-alkanes to species that produce longer length alkanes and then back again, consistent with the change from mixed angiosperm/gymnosperm to entirely angiosperm and from deciduous to evergreen; 2) the change in climate to warmer conditions would increase ACL values of all species, regardless of taxonomy. If the first hypothesis were correct, modern representatives of the PETM and non-PETM communities would be expected to reflect the hypothesized differences in ACL. One would especially expect that the modern counterparts of non-PETM Bighorn Basin gymnosperms (namely, *Metasequoia glyptostroboides* and *Taxodium distichum*) would have smaller ACL values than modern angiosperms representing the PETM flora (e.g. *Gleditsia triacanthos*, *Tamarindus indica*, *Artocarpus altilis*). Therefore, this study tests the plant community change hypothesis by examining the alkane profiles of modern representatives of fossil species from the PETM and non-PETM communities.

$\delta^{13}C$ During the PETM

According to the carbon isotope record captured in oceanic sediments, the PETM is associated with a negative carbon isotope excursion (CIE), ~3.0‰ (Zachos et al. 2003). However, the terrestrial CIE is even larger, ~6-8‰ in paleosol carbonates (Koch et al. 1995, Bowen 2004, see also Figure 2). Multiple hypotheses have been proposed to explain the ~3‰ difference, the two strongest being first, that the marine sediments somehow failed to record the full magnitude of the global CIE and second, that a concurrent shift in the plant community amplified the terrestrial CIE (Smith et al.

2007). This second 'plant community change' hypothesis, based on the difference in carbon isotope values between angiosperms and gymnosperms in Figure 1, states that the marine record is the more true representation of the global CIE, and that the change in the floral community itself from an angiosperm/deciduous gymnosperm mix to 100% angiosperm amplified the terrestrial carbon isotope signature relative to the marine (Smith et al. 2007). However, if the $\delta^{13}C$ difference is related to leaf habit rather than systematic group, the plant community change hypothesis as it is now may not serve as an explanation for the terrestrial PETM carbon isotope excursion and would need to be modified to include changes in the plant community beyond taxonomic. We must be able to know with confidence that the two group trends described in Figure 1 define gymnosperms from angiosperms, and not conifers from broadleaf plants. If the plant community change hypothesis is correct, then deciduous gymnosperms representing fossil species from the late Paleocene and early Eocene should have similar $\delta^{13}C$ values as evergreen conifers. This study examines the $\delta^{13}C$ values of deciduous gymnosperms in order to evaluate the validity of the gymnosperm/angiosperm dichotomy based on *n*-alkane carbon isotope values and to test the PETM plant community change hypothesis.

Fidelity of Species n-*Alkane Profiles*

Lastly, this study tests the assumption that the *n*-alkane profile of a single tree and a single species is relatively constant, and does not depend on light exposure or leaf age. It is impossible to know where and for how long leaves contributing to sediment *n*-alkane profiles grew. For this reason, the variation of *n*-alkane distribution within a tree must be well defined in order to calibrate the modern data for proper interpretation of fossil

evidence. It is a well-established fact that the physiology of sun and shade leaves differ within a tree (Kürschner 1997), and $\delta^{13}C$ values of sun and shade leaves are also known to differ substantially (Lockheart et al. 1997, Schleser 1990, Waring and Silvester 1994). It would not be surprising to find that different sections of a canopy display differing *n*-alkane profiles, and the question of whether *n*-alkane distributions of sun and shade leaf waxes vary has not yet been definitively answered.

It is also well known that leaves collected at the height of the growing season display a different biochemistry than leaves from which the plant has withdrawn much of its biochemical structure in preparation for leaf abscission (Hopkins and Hüner 2004), and the amount of *n*-alkanes in leaf wax of some species has been shown to increase with leaf age across a growing season (Gülz and Müller 1992). If the $\delta^{13}C$ values of *n*-alkanes are also known to decrease from summer to fall (Chikaraishi et al. 2004, Lockheart et al. 1997), then *n*-alkane composition may change as well. However, this has not been explicitly tested by comparing *n*-alkane profiles from sun and fall leaves. Seasonal variation may be an important consideration in the evaluation of fossil evidence, as most leaves, especially deciduous, are shed from a tree in the autumn while most modern studies examine leaf samples collected during the summer.

Because the leaf wax forms a plant's barrier to the external environment, it can be hypothesized that the composition of that wax, and also *n*-alkane composition, would be controlled more by environmental adaptation than by phylogeny. Dodd and colleagues have studied the effect of varying environment on populations within a species (Dodd and Rafii 2000, Dodd and Poveda 2003). Because activation energies of lipid phase transitions in some industrial waxes have been shown to be related to both the weighted

chain length mean (ACL) and the dispersion of chain lengths about the mean (Reynhardt 1985), and therefore that ACL and dispersion must be related to one another, Dodd and Rafii (2000) proposed a dispersion term (d) for use in *n*-alkane distribution, defined as

$$d = \Sigma\, p_i(a_i - N)^2,$$

where p_i is the proportion of each alkane, a_i is the carbon number, and N is the weighted mean (ACL) of the alkane (Dodd and Rafii 2000, Dodd and Poveda 2003). The authors measured this on a number of species in the gymnosperm Cupressaceae (cedar family) and not only did they find that ACL for each species varied within a given range of values, they also found a strong correlation between ACL and dispersion within a species. They concluded that this correlation and the ranges of both variables were the result of local environmental adaptation by populations. A simple common garden experiment would test this conclusion, and that is provided at the Chicago Botanic Garden, where trees from different populations are planted together in the same, relatively homogeneous environment. Therefore this study also tests whether *n*-alkane profiles are consistent from sun to shade, summer to fall, and whether ACL and dispersion are correlated across multiple individuals in a single species, grown in the same environment but from various seed stock.

Materials and Methods

Study species

For this study representative trees of a number of plant families were collected from the Chicago Botanic Garden (CBG) in Glencoe, IL, with the exception of two greenhouse species (*Artocarpus altilis* and *Annona muricata*) not housed at CBG.

Artocarpus altilis and *A. muricata* were collected instead from the Lincoln Conservatory, located in Lincoln Park, Chicago. Tree species were chosen to match one or more of the following requirements: (a) that the species be a close relation to fossil species from the late Paleocene to the early Eocene, (b) that the species represent as closely as possible the morphology and postulated phylogeny of fossil species, and (c) that there be at least one species representing every possible permutation of gymnosperm/angiosperm, conifer/broadleaf, and deciduous/evergreen (with the exception of an evergreen, non-tropical angiosperm tree and needle-leaf angiosperm.) Evergreen broadleaf gymnosperms (cycads) as well as evergreen broadleaf angiosperms are both represented in this study by tropical species. Being tropical, these species necessarily grow in climate-controlled greenhouses at more temperate latitudes. The two tropical groups (gymnosperms and angiosperms) can be compared to one another, but comparisons between greenhouse and outdoor species are confounded by the strong environmental disparity between the two locations. However, the large number of plants sampled, especially deciduous conifers with their implications for the PETM plant community change hypothesis, provides a broad base for closer examination of the difference between angiosperms and gymnosperms.

The outdoor species were all located at CBG. Preference was given to native species or species adapted to the regional climate, and combined with the regular horticultural attention provided by CBG staff during the life of the plant, this should minimize stress to the plants and optimize growth. Some families may have single representative species while others have multiple species (notably, Pinaceae and Taxodiaceae.) Finally, the species were also closely matched to the corresponding fossil

taxons or morphologies and were confirmed with existing literature and an expert in PETM and Paleocene/Eocene flora (Smith et al. 2007, Wing et al. 2005, Wing and Harrington 2001, S. Wing, personal communication). The plant species chosen are listed in Table 1 (See also Appendix B).

Sample Collection

Leaves were collected from at least one individual per species, and multiple individuals for the species *Alnus glutinosa, Gleditsia triacanthos, Pinus sylvestris,* and *Taxodium distichum* (black alder, honey locust, Scotch pine, and bald cypress respectively). Five individuals were sampled in both the summer and fall for each of these four species, and all four species are abundant at CBG.

Both 'sun' and 'shade' leaves were collected for all individuals of all species where possible, in order to control for and investigate further the powerful effect of evapotranspiration on isotope discrimination. Shade leaves were not collected for all species in fall—with attenuating canopy cover, shadiness of samples could not be verified. Fall samples were selected to be as temporally close to abscission from the tree as possible, although in order to test this in some species, multiple samples were taken—either at two different dates in the fall or of two or more colors of foliage representing differing levels of leaf decay on the tree (green, yellow, or brown).

Some species do not reach full tree size (i.e. the ferns *Matteuccia pensylvanica* and *Osmunda regalis*), or are represented at CBG within a greenhouse, and for these individuals, the difference in light between different sections of the plant was not significant. Thus, the protocol was adjusted and only one leaf sample collected. The

greenhouse species also were not sampled in the fall, as there was no significant change in the artificial climate. The following species are located at the CBG tropical greenhouse: *Ceratonia siliqua, Cyathea cooperi, Cycas circinalis, Tamarindus indica,* and *Washingtonia robusta.* The greenhouses at the Chicago Botanic Garden and the Lincoln Park conservatory had comparable climates, with windows open to the outside for air circulation and daytime temperatures 80°F to 90°F in summer. All summer leaves were collected July 11-13, 2007. Fall leaves were collected from October to November 2007, with the goal of taking leaves as close to total abscission as possible while still on the tree.

In order to further control transpiration and stress effects, only well-watered, healthy individual plants were chosen for sampling. To ensure equal and full light exposure, and to avoid potential complications from variations in the carbon isotope composition of atmospheric CO_2 due to canopy effects, only trees that stood free from surrounding trees—especially on the south side—were selected where possible. Sun leaves were gathered from the outer edge of the canopy, on the southern side of each plant, and typically at the maximum extent of the pole pruners (approximately 18-20 feet high). Conversely, shade leaves were taken from the bottom, northern side, and as near to the trunk as possible. Individuals were identified and marked using detailed maps and the assistance of CBG horticulturalists, as well as photographed with a digital camera, and each leaf sample was stored in a labeled paper bag. The latitude and longitude of each outdoor specimen was also recorded with GPS. All contact with plastic, wax, or paraffin was avoided, as this can contaminate leaf waxes, and bags were stapled shut immediately and allowed to dry completely before further processing.

Table 1. Plant species collected from the Chicago Botanic Garden. Summer samples were collected mid-July, and fall samples were collected October through November. Species marked in green are located in the Tropical Education Greenhouse at the CBG Regenstein Center. *Artocarpus altilis* and *Annona muricata* samples were collected from the Lincoln Conservatory greenhouses, as CBG did not house these species. Abbreviations: A: angiosperm, G: gymnosperm, F: fern; D: deciduous, E: evergreen; B: broadleaf, N: needle-leaf.

Species	Family	Period	Systematic Group	Leaf Habit	Leaf Shape
Alnus glutinosa	Betulaceae	PETM	A	D	B
Asimina triloba	Annonaceae	PETM	A	D	B
Carpinus caroliniana	Betulaceae	PETM	A	D	B
Celtis occidentalis	Ulmaceae	non-PETM	A	D	B
Ceratonia siliqua	Fabaceae	PETM	A	D	B
Cyathea cooperi	Cyatheaceae	other	F	E	B
Cycas circinalis	Cycadaceae	other	G	E	B
Fagus sylvatica	Fagaceae	PETM	A	D	B
Ginkgo biloba	Ginkgoaceae	other	G	D	B
Gleditsia triacanthos	Fabaceae	PETM	A	D	B
Koelreuteria paniculata	Sapindaceae	PETM	A	D	B
Larix decidua	Pinaceae	non-PETM	G	D	N
Lindera benzoin	Lauraceae	PETM	A	D	B
Matteuccia pensylvanica	Polypodiaceae	other	F	N/A	B
Metasequoia glyptostroboides	Taxodiaceae	non-PETM	G	D	N
Osmunda regalis	Osmundaceae	other	F	N/A	B
Picea abies	Pinaceae	non-PETM	G	E	N
Pinus sylvestris	Pinaceae	non-PETM	G	E	N
Platanus orientalis	Platanaceae	PETM	A	D	B
Populus deltoides	Salicaceae	PETM	A	D	B
Pterocarya stenoptera	Juglandaceae	non-PETM	A	D	B
Rhus typhina	Anacardiaceae	PETM	A	D	B
Salix alba	Salicaceae	PETM	A	D	B
Tamarindus indica	Fabaceae	PETM	A	E	B
Taxodium distichum	Taxodiaceae	non-PETM	G	D	N
Taxus cuspidata	Taxaceae	other	G	E	N
Thuja occidentalis	Cupressaceae	other	G	E	N
Tilia cordata	Tiliaceae	non-PETM	A	D	B
Washingtonia robusta	Arecaceae	PETM	A	E	B

Indoor and Outdoor Climate

The 2007 growing season at the Chicago Botanic Garden was 196 days long, between the last frost on 14 April and first frost on 28 October, above the 26-year average of 158 days. The total annual precipitation in 2007 was 40.99 inches, slightly above the 37-year average of 36.69 inches, with August being an exceptionally wet month (8.00 inches above average). The 2007 annual average temperature was 50.1°F, slightly above the 22-year average of 49.2°F, with the average high and low for July being 84°F and 60°F, respectively. The highest temperature recorded for the year was 96°F on 8 and 9 July. It can be safely concluded that the 2007 growing season was not extreme and thus represents a valid base of comparison to other years and other temperate zones. All outdoor weather data was provided by Celeste VanderMey, Supervisor, Plant Records, at the Chicago Botanic Garden.

Herbarium Storage

Separate sun samples were collected in the summer, one per species, for pressing and permanent storage on herbarium archival paper (Appendix A). Every attempt was made to collect specimens with either flower or fruit present, but this was not always possible. Specimens were first pressed between sheets of blotter paper, newspaper, and cardboard in a wooden plant press until completely dry. Once dried, specimens were frozen for a minimum of three days to kill any potential pests. Each dry, pressed sample was then placed on a single archival quality mounting sheet (100% cotton rag, unbuffered UC-Type) and strapped to the sheet with gummed linen tape, cut to the appropriate size. Small twigs and leaves were sewn to the sheet with linen thread. No glue was used in

mounting the specimen, so that later tissue analysis remains entirely viable and so that no glue can interfere with the molecular preservation of the tissues. Large samples were clipped and separated between two archival sheets. A gummed paper label (Figure 3) was affixed to the lower right hand corner of each sheet, and a fragment folder was also glued to each sheet.

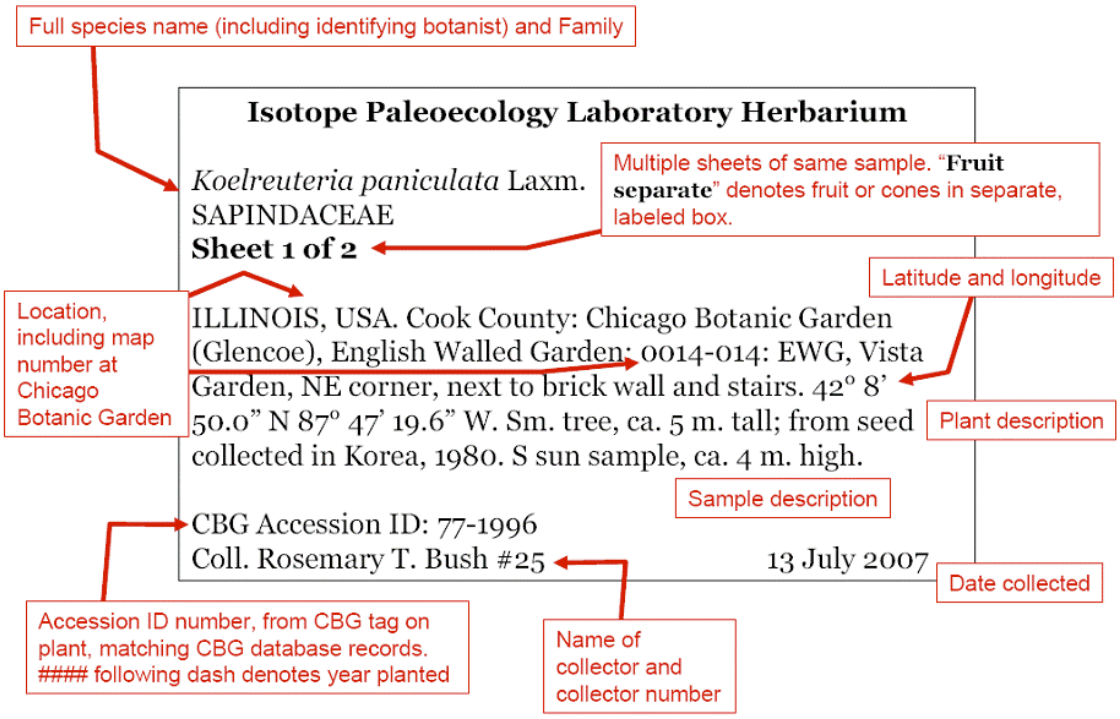


Figure 3. Sample herbarium label (in black), with descriptions of all information included in red.

Large fruits and cones were stored separately (and noted on the appropriate specimen sheet label, see Figure 3) in labeled boxes made of a single archival sheet, using an origami technique. Each pressed specimen was stored in a separate species folder, and all specimens in folders were stored in archival boxes, divided and each labeled 'Angiosperms,' 'Gymnosperms,' 'Ferns,' or 'Large Fruits and Cones.' All archival boxes have been subsequently stored in the 'Isotope Paleoecology Laboratory

Herbarium' in the Smith Lab, where they will remain for the foreseeable future. Thus, later comparisons using original plant material remain feasible.

Sample Preparation and Analysis

After being allowed to dry completely for a few weeks, leaves from coniferous species were separated and stored in clean glass jars by age cohort, e.g. first year leaves (from the 2007 growing season), second year (2006), third year (2005), etc. Only first year foliage from evergreen species will be compared with concurrent deciduous foliage so that interspecies comparisons are not confounded by age variability (Brooks et al. 1997). However, separation and storage of the different age cohorts allows for later investigation of age-dependent variation in evergreens. Dried deciduous leaves, representing only the 2007 cohort, were homogenized with grinding in a solvent-rinsed mortar and pestle in order to control for variation between single leaves (Appendix A). The number of leaves varied by species, as some species had very large leaves (e.g. *Washingtonia robusta*) and some had very small leaves, as well as by sample because shade leaves tended to fewer in number than sun leaves. A reasonable effort was made to ensure that roughly the same amount of total leaf material was used for each sample.

Lipids were extracted from all samples using a Microwave-Accelerated Solvent Extraction System 'Xpress' (MARS-X, CEM Corporation). Either 20 or 30ml of 9:1 dichloromethane (DCM):methanol was added to a portion of the dry plant material (0.2-0.4g) in clean Teflon Xpress vessels. The sample/solvent mixture was stirred with a stir bar as well as heated and pressurized using microwave radiation in clean Teflon tubes, and the lipids were extracted from the plant tissue into the solvent. The MARS-X

method is as follows: ramp to 100°C for 5 minutes, hold at 100°C for 15 minutes, and cool for 30 minutes. After being allowed to cool, the resulting total lipid extract (TLE) was then pipetted into clean glass test tubes from the MARS Xpress vessels, separating the solvent with dissolved lipids from all remaining solid plant material. The extracts were concentrated through evaporation in a TurboVap, and half of the sample archived and stored in a refrigerator. The non-polar lipids, including *n*-alkanes, were separated from the polar lipids using short column silica gel chromatography (Eglinton and Hamilton 1967), using ~1 g of activated silica gel in hexane in a Pasteur pipette. The non-polar fraction was eluted in 4 mL of hexane, followed by the polar fraction, eluted in 4 mL of 1:1 DCM:methanol.

Once isolated, the hydrocarbons were again concentrated by evaporation with nitrogen gas on an N-Evap (Organomation Associates, Inc.) and then injected into a gas chromatograph/mass spectrometer (GC/MS) (Medeiros and Simoneit 2007). Samples passed through the gas chromatograph for separation and identification (ThermoFisher Trace GC Ultra, with 15m, 0.25mm ID Thermo TR-5ms SQC column) and then to a quadrapole mass spectrometer (DSQ) and a flame ionization detector (FID) for assessment of relative abundance (ThermoFisher DSQII). The GC/MS method is as follows: initial temperature at 100°C, hold for 2 minutes, then ramp at 11°C/minute for 20 minutes to 320°C, and hold at 320°C for 5 minutes. All samples were compared to a standard containing all *n*-alkanes from C_{21} to C_{40} originally at a concentration of 40 mg/l each (1 ml, Fluka, Sigma-Aldrich). The retention time of each *n*-alkane was then known to 0.01 min from the standard and used to identify sample *n*-alkanes. *n*-Alkanes were also identified using the gas chromatograph spectra and comparison with a molecular

library. Figures 4 and 8 show resultant chromatograms. An FID integration report was created using Xcalibur software for each sample, calculating the peak area for all peaks within the retention time range for plant *n*-alkanes, as determined by the alkane standard.

Isotope results were gathered from a gas chromatograph/isotope ratio mass spectrometer (GC/IRMS, ThermoFisher Trace GC Ultra and Delta V). 1-2 μl of sample in hexane was injected into the GC (7m, 0.32mm ID Thermo RTX-5 column) via a split/splitless (SSL) injector in splitless mode with a base temperature of 220°C. The GC method is as follows: initial temperature at 80°C, hold for 1 minute, ramp at 20°C/min to 320°C, hold for 5 minutes. Results were compared with GC/MS results for *n*-alkane identification, and samples were run at varying concentrations in order to adequately evaluate alkane peaks of varying relative sizes. All statistical results were analyzed using Stata (Intercooled Stata 8.0). This includes linear regression for analysis of single plant samples and paired Student's t-test for comparison of results from two samples within a single tree.

Results and Discussion

n-*Alkane Variation in Modern Plants and Species*

The results of the GC/MS analyses are displayed in Table 2. The *Ceratonia siliqua* (carob) sample was excluded due to its high impurity and the very low confidence of *n*-alkane identification and quantification. Three samples from the *Taxodium distichum* (bald cypress) replicates also had to be excluded due to their extremely low concentrations of *n*-alkanes, namely the shaded summer samples from trees #2,3 and 5. Cypresses #1 and 4 had sufficient amounts of *n*-alkanes in their shaded leaves to be

quantified. Also, ACL could not be calculated for the ferns *Matteuccia pensylvanica* and *Cyathea cooperi*, as the *n*-C_{31} peak was obscured by a much larger peak of an unknown compound. Interestingly, this same compound was observed in all samples of *M. pensylvanica* and *C. cooperi*, but not in the more distantly related *Osmunda regalis* (see Appendix B). Finally, some sample chromatograms displayed spectra which are possibly the result of residues of unconsolidated hydrocarbons on the leaves deposited from fossil fuel fumes, ubiquitous in anthropogenic settings (Wakeham 1976, Meyers and Ishiwatari 1993).

One difference between angiosperms and gymnosperms was the amounts of *n*-alkanes each group produces in its leaf waxes. Although this was not quantified in absolute terms, all gymnosperm leaves contained much smaller quantities of *n*-alkanes than did most angiosperm leaves, consistent with previous findings (Herbin and Robins 1968, Herbin and Robins 1969). Average chain length (ACL) was calculated in two ways (Table 2). For the first method, ACL was calculated following Smith et al. (2007), as the weighted mean of C_{25} to C_{33} odd chain lengths. This method was found in some cases to significantly skew interpretation of the data, especially for species with dominant *n*-alkanes outside that range (e.g. *Thuja occidentalis*). Thus, a second ACL was calculated, using data of all *n*-alkane peaks detected, C_{21} to C_{37}. In many cases, this extension of the weighted mean does not affect the result, e.g. where the dominant *n*-alkane constituted a very large majority of the distribution (*Alnus glutinosa*, *Gleditsia triacanthos*), but this wider ACL better represents the distribution of *n*-alkanes and is the measurement used in all subsequent figures and discussion. This highlights the caveat that ACL is only as good as the data it from which it is calculated.

Table 2. *n*-Alkane distribution, average chain length (ACL), dominant alkane, and peak dominance of all samples collected. Those samples with data marked 'n/a' had *n*-alkane compositions that were either so contaminated with other non-polar molecules or so dilute that they could not be confidently interpreted. ACL (25-33) denotes ACL calculated using *n*-C_{25} to *n*-C_{33}, and ACL (21-37) denotes ACL calculated using all measured *n*-alkanes (C_{21} to C_{37}). Not all alkanes were detected in all samples. Botanical names were confirmed with The International Plant Names Index (www.ipni.org) and the USDA Plants Database (plants.usda.gov). For each outdoor plant, there are a minimum of three samples (except in the case of Honey Locust 2, for which no fall sample could be collected)—two samples in the summer and one in the fall. For several species, multiple samples were collected in the fall, e.g. confidently known shaded samples or foliage in various stages of decay on the tree (ranging from green to brown foliage).

Species Common Name	Species Botanical Name	Sample	ACL (25-33)	ACL (21-37)	Dominant Alkane	Peak Dominance: C_n/(Sum of Odd Alkanes)					
						C_{25}	C_{27}	C_{29}	C_{31}	C_{33}	C_{35}
OUTDOOR ANGIOSPERMS:											
Black Alder 1	*Alnus glutinosa* (L.) Gaertn.	Summer, Sun	28.25	28.25	29		0.373	0.627			
		Summer, Shaded	28.13	28.12	29	0.016	0.416	0.552	0.013		
		Fall, Sun	28.32	28.32	29	0.013	0.342	0.616	0.028		
Black Alder 2	*Alnus glutinosa* (L.) Gaertn.	Summer, Sun	28.30	28.26	29	0.019	0.320	0.644	0.009		
		Summer, Shaded	28.45	28.30	29	0.026	0.246	0.666	0.033		
		Fall, Sun	28.36	28.25	29	0.024	0.278	0.668	0.011		
Black Alder 3	*Alnus glutinosa* (L.) Gaertn.	Summer, Sun	27.37	27.12	27	0.160	0.456	0.335			
		Summer, Shaded	27.76	27.41	27	0.011	0.556	0.365			
		Fall, Sun	27.73	27.23	29	0.086	0.408	0.420			
Black Alder 4	*Alnus glutinosa* (L.) Gaertn.	Summer, Sun	28.05	28.03	29	0.010	0.456	0.526	0.004		
		Summer, Shaded	28.14	28.10	29	0.020	0.411	0.532	0.028		
		Fall, Sun	27.89	27.81	27	0.020	0.511	0.458			
Black Alder 5	*Alnus glutinosa* (L.) Gaertn.	Summer, Sun	27.77	27.77	27	0.040	0.536	0.424			
		Summer, Shaded	27.95	27.92	27	0.023	0.493	0.464	0.015		
		Fall, Sun	28.07	28.07	29	0.018	0.436	0.540	0.006		
Pawpaw	*Asimina triloba* Dunal	Summer, Sun	28.94	28.94	29	0.028	0.192	0.560	0.220		
		Summer, Shaded	29.31	29.31	29	0.016	0.134	0.537	0.303	0.009	
		Fall, Sun	29.09	29.09	29	0.026	0.168	0.540	0.265		
		Fall, Shaded	29.31	29.31	29	0.016	0.136	0.536	0.305	0.007	
American Hornbeam	*Carpinus caroliniana* Walter	Summer, Sun	28.55	28.50	29	0.045	0.255	0.572	0.118	0.002	
		Summer, Shaded	28.32	28.28	29	0.051	0.293	0.592	0.056		
		Fall, Sun	28.50	28.50	29	0.045	0.230	0.652	0.073		

Species Common Name	Species Botanical Name	Sample	ACL (25-33)	ACL (21-37)	Dominant Alkane	Peak Dominance: C_n/(Sum of Odd Alkanes)					
						C_{25}	C_{27}	C_{29}	C_{31}	C_{33}	C_{35}
Hackberry	*Celtis occidentalis* L., 'Chicagoland'	Summer, Sun	29.18	29.18	29	0.023	0.083	0.676	0.219		
		Summer, Shaded	28.96	28.88	29	0.029	0.109	0.700	0.148		
		Fall, Sun	29.30	29.30	29	0.012	0.089	0.646	0.240	0.012	
European Beech	*Fagus sylvatica* L., 'Pendula'	Summer, Sun	27.08	27.08	27	0.019	0.922	0.059			
		Summer, Shaded	27.04	27.03	27	0.031	0.916	0.048	0.002		
		Fall, Sun	27.04	27.04	27	0.029	0.922	0.049			
		Fall, Shaded	27.07	27.04	27	0.021	0.918	0.055			
Honey Locust 1	*Gleditsia triacanthos* L., f. *inermis*	Summer, Sun	29.06	29.06	29	0.002	0.013	0.936	0.049		
		Summer, Shaded	29.07	29.07	29		0.019	0.926	0.055		
		Fall, Sun	29.15	29.15	29		0.012	0.901	0.087		
Honey Locust 2	*Gleditsia triacanthos* L., f. *inermis*	Summer, Sun	29.09	29.09	29		0.013	0.929	0.059		
		Summer, Shaded	29.05	29.05	29		0.015	0.945	0.040		
Honey Locust 3	*Gleditsia triacanthos* L., f. *inermis*	Summer, Sun	29.06	29.06	29		0.015	0.939	0.046		
		Summer, Shaded	29.11	29.11	29		0.012	0.918	0.069		
		Fall, Sun	29.20	29.20	29		0.014	0.871	0.115		
Honey Locust 4	*Gleditsia triacanthos* L., f. *inermis*	Summer, Sun	29.05	29.05	29		0.018	0.937	0.045		
		Summer, Shaded	29.10	29.10	29		0.013	0.922	0.064		
		Fall, Sun	29.11	29.11	29		0.021	0.902	0.077		
Honey Locust 5	*Gleditsia triacanthos* L., f. *inermis*	Summer, Sun	29.04	29.04	29	0.002	0.016	0.942	0.040		
		Summer, Shaded	29.13	29.13	29		0.013	0.910	0.077		
		Fall, Sun	29.07	29.07	29		0.018	0.931	0.051		
Golden Rain Tree	*Koelreuteria paniculata* Laxm.	Summer, Sun	30.92	30.91	31	0.010	0.028	0.155	0.601	0.202	0.002
		Summer, Shaded	30.87	30.87	31	0.009	0.046	0.175	0.543	0.227	
		Fall, Sun	31.04	31.02	31	0.009	0.031	0.142	0.561	0.250	0.003
Spicebush	*Lindera benzoin* (L.) Blume	Summer, Sun	28.17	28.17	29	0.055	0.386	0.477	0.081		
		Summer, Shaded	28.99	28.96	29	0.003	0.233	0.523	0.236		
		Fall, Sun	28.14	28.14	29	0.058	0.398	0.459	0.084		
Plane Tree	*Platanus orientalis* L.	Summer, Sun	29.10	29.16	31	0.184	0.245	0.095	0.255	0.204	0.014
		Summer, Shaded	29.28	29.31	33	0.199	0.217	0.060	0.226	0.262	0.021
		Fall, Sun	28.94	28.65	31	0.199	0.228	0.085	0.256	0.170	0.009

Species Common Name	Species Botanical Name	Sample	ACL (25-33)	ACL (21-37)	Dominant Alkane	Peak Dominance: C_n/(Sum of Odd Alkanes)					
						C_{25}	C_{27}	C_{29}	C_{31}	C_{33}	C_{35}
Cottonwood	*Populus deltoides* Bartram ex Marshall	Summer, Sun	28.75	28.71	29	0.016	0.134	0.806	0.039		
		Summer, Shaded	28.86	28.86	29	0.008	0.088	0.870	0.034		
		Fall, Sun	28.84	28.84	29	0.007	0.114	0.829	0.050		
Chinese Wingnut	*Pterocarya stenoptera* C. DC.	Summer, Sun	29.55	29.52	29	0.011	0.056	0.612	0.286	0.032	
		Summer, Shaded	29.39	29.39	29	0.005	0.065	0.687	0.217	0.026	
		Fall, Sun	29.54	29.46	29	0.019	0.056	0.589	0.288	0.035	
		Fall, Shaded	29.56	29.47	29		0.039	0.662	0.254	0.031	
Staghorn Sumac	*Rhus typhina* L.	Summer, Sun	26.76	26.55	25	0.403	0.356	0.097	0.062	0.024	
		Summer, Shaded	27.20	27.07	27	0.296	0.407	0.165	0.080	0.022	
		Fall, Sun	26.98	26.78	25	0.362	0.377	0.106	0.076	0.031	
White Willow	*Salix alba* L.	Summer, Sun	27.54	27.47	27	0.098	0.530	0.350	0.006		
		Summer, Shaded	27.71	27.59	27	0.056	0.536	0.369	0.016		
		Fall, Sun	27.59	27.47	27	0.110	0.488	0.367	0.016		
Littleleaf Linden	*Tilia cordata Mill., 'Greenspire'*	Summer, Sun	28.25	28.11	29	0.142	0.213	0.485	0.134		
		Summer, Shaded	29.07	29.01	29	0.028	0.080	0.709	0.173		
		Fall, Sun	28.41	28.28	29	0.121	0.207	0.486	0.161		
OUTDOOR GYMNOSPERMS:											
Ginkgo	*Ginkgo biloba* L.	Summer, Sun	26.93	26.72	27	0.180	0.635	0.118	0.014		
		Summer, Shaded	26.98	26.75	27	0.149	0.666	0.115	0.012		
		Fall, Sun	26.69	26.49	27	0.234	0.626	0.087			
European Larch	*Larix decidua* Mill.	Summer, Sun	27.76	27.32	27	0.205	0.266	0.331	0.110		
		Summer, Shaded	28.09	27.41	29	0.147	0.237	0.357	0.133		
		Fall, Sun	28.50	28.50	29	0.093	0.255	0.457	0.194		
Dawn Redwood	*Metasequoia glyptostroboides* Hu & Cheng	Summer, Sun	28.03	27.42	29	0.181	0.253	0.300	0.124	0.030	
		Summer, Shaded	27.93	27.14	29	0.163	0.247	0.328	0.117		
		Fall, Sun	27.48	27.38	29	0.157	0.436	0.393			
		Fall, Shaded	28.01	27.75	29	0.133	0.323	0.372	0.120		
Norway Spruce	*Picea abies* (L.) Karst.	Summer, Sun	27.12	25.97	25	0.283	0.202	0.197	0.046	0.013	
		Summer, Shaded	27.58	26.66	29	0.221	0.226	0.276	0.091		
		Fall, Sun	28.18	26.89	29	0.068	0.267	0.366	0.081		

Species Common Name	Species Botanical Name	Sample	ACL (25-33)	ACL (21-37)	Dominant Alkane	Peak Dominance: C_n/(Sum of Odd Alkanes)					
						C_{25}	C_{27}	C_{29}	C_{31}	C_{33}	C_{35}
Scotch Pine 1	*Pinus sylvestris* L.	Summer, Sun	28.06	27.95	29		0.463	0.521			
		Summer, Shaded	27.81	27.81	29	0.103	0.390	0.508			
		Fall, Sun	27.99	27.77	29		0.489	0.480			
Scotch Pine 2	*Pinus sylvestris* L.	Summer, Sun	27.84	27.12	29	0.171	0.233	0.386	0.076		
		Summer, Shaded	28.21	26.96	29	0.109	0.165	0.436	0.074		
		Fall, Sun	28.29	27.94	29	0.131	0.253	0.407	0.123	0.028	
Scotch Pine 3	*Pinus sylvestris* L.	Summer, Sun	27.88	27.79	29	0.127	0.296	0.563			
		Summer, Shaded	28.54	28.04	29	0.063	0.181	0.581	0.094		
		Fall, Sun	27.94	27.73	29	0.155	0.279	0.444	0.080		
Scotch Pine 4	*Pinus sylvestris* L.	Summer, Sun	28.25	28.05	29		0.367	0.606			
		Summer, Shaded	28.21	27.47	29	0.064	0.277	0.489	0.054		
		Fall, Sun	28.11	27.86	29		0.431	0.534			
Scotch Pine 5	*Pinus sylvestris* L.	Summer, Sun	27.82	27.30	29	0.114	0.300	0.484			
		Summer, Shaded	27.89	27.58	29	0.102	0.327	0.527			
		Fall, Sun	27.91	27.77	29	0.148	0.326	0.418	0.088		
Bald Cypress 1	*Taxodium distichum* (L.) Rich., 'Shawnee Brave'	Summer, Sun	31.23	31.51	33	0.052	0.116	0.072	0.122	0.564	0.075
		Summer, Shaded	29.31	27.66	33	0.106	0.174	0.179	0.109	0.199	
		Fall, Sun	30.06	30.38	33	0.071	0.230	0.142	0.115	0.377	0.065
		Fall, Shaded	29.92	29.20	33	0.053	0.199	0.186	0.164	0.272	0.022
Bald Cypress 2	*Taxodium distichum* (L.) Rich.	Summer, Sun	29.02	28.57	33		0.447	0.268		0.229	
		Summer, Shaded	n/a*	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
		Fall, Sun	29.87	29.45	33	0.074	0.210	0.173	0.156	0.299	0.020
Bald Cypress 3	*Taxodium distichum* (L.) Rich.	Summer, Sun	31.15	31.10	33	0.057	0.103	0.085	0.095	0.531	0.085
		Summer, Shaded	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
		Fall, Sun (green)	29.89	29.05	33	0.062	0.215	0.152	0.092	0.309	0.037
		Fall, Sun (brown)	30.07	29.86	29	0.025	0.086	0.407	0.181	0.226	0.029
Bald Cypress 4	*Taxodium distichum* (L.) Rich.	Summer, Sun	31.51	31.53	33	0.053	0.084	0.049	0.095	0.600	0.086
		Summer, Shaded	29.13	28.26	27	0.103	0.231	0.219	0.137	0.179	
		Fall, Sun-exposed	29.38	29.03	27	0.068	0.336	0.171	0.108	0.274	
Bald Cypress 5	*Taxodium distichum* (L.) Rich.	Summer, Sun	30.37	30.55	33	0.080	0.204	0.107	0.117	0.452	0.039
		Summer, Shaded	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
		Fall, Sun	29.71	29.27	33	0.072	0.243	0.163	0.156	0.277	0.017

Species Common Name	Species Botanical Name	Sample	ACL (25-33)	ACL (21-37)	Dominant Alkane	Peak Dominance: C_n/(Sum of Odd Alkanes)					
						C_{25}	C_{27}	C_{29}	C_{31}	C_{33}	C_{35}
Japanese Yew	*Taxus cuspidata* Siebold & Zucc., 'Cuspidata'	Summer, Sun	27.86	27.69	27	0.095	0.489	0.295	0.048	0.040	
		Summer, Shaded	28.89	28.81	27	0.057	0.332	0.292	0.184	0.103	0.009
		Fall, Sun	28.40	28.33	27	0.075	0.420	0.285	0.102	0.087	0.009
American Arborvitae	*Thuja occidentalis* L. 'Klehm's Strain'	Summer, Sun	31.88	34.44	35		0.005	0.033	0.024	0.127	0.771
		Summer, Shaded	31.52	34.37	35	0.006	0.008	0.029	0.019	0.108	0.787
		Fall, Sun (green)	31.79	34.53	35	0.005	0.005	0.024	0.020	0.117	0.790
		Fall, Sun (yellow)	31.84	34.54	35		0.007	0.024	0.017	0.100	0.803
		Fall, Sun (brown)	31.79	34.42	35		0.007	0.027	0.018	0.102	0.798
		Fall, Shaded (green)	31.95	34.52	35		0.005	0.024	0.018	0.108	0.802
		Fall, Shaded (brown)	31.50	34.45	35		0.011	0.032	0.018	0.091	0.802
OUTDOOR FERNS:											
Ostrich Fern	*Matteuccia pensylvanica* (Willd.) Raymond	Summer	n/a	n/a	25	0.247	0.373	0.234	0.127	n/a	
		Fall	n/a	n/a	27	0.168	0.222	0.214	0.175	n/a	
Royal Fern	*Osmunda regalis* L.	Summer	28.25	28.04	29	0.065	0.283	0.565	0.052		
		Fall	28.26	28.00	29	0.060	0.275	0.584	0.039		
GREENHOUSE PLANTS:											
Angiosperms:											
Soursop	*Annona muricata* L.		28.17	27.65	29	0.030	0.317	0.564			
Breadfruit	*Artocarpus altilis* (Parkinson) Fosberg		28.60	28.60	29		0.239	0.725	0.033	0.003	
Carob	*Ceratonia siliqua* L.		n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Tamarind	*Tamarindus indica* L.		28.43	28.33	29	0.078	0.296	0.458	0.129	0.021	
Mexican Fan Palm	*Washingtonia robusta* H. Wendl.	Tip of Leaf	28.53	27.85	29	0.069	0.237	0.435	0.115	0.026	
		Base of Leaf	28.26	27.31	29	0.087	0.236	0.410	0.100		
Gymnosperms:											
Queen Sago	*Cycas circinalis* L.	Tip of Leaf	27.60	27.13	27	0.128	0.461	0.246	0.046	0.020	
		Tips of Leaflets at Leaf Base	27.88	27.56	27	0.084	0.410	0.280	0.051	0.025	0.024
		Bases of Leaflets at Leaf Base	27.80	27.54	27	0.081	0.459	0.266	0.047	0.024	0.021
Ferns:											
Australian Tree Fern	*Cyathea cooperi* (F. Muell.) Domin	Tip of Leaf	n/a	n/a	29	0.089	0.212	0.368	0.274	n/a	
		Base of Leaf	n/a	n/a	29	0.084	0.158	0.331	0.334	n/a	

* 'n/a' denotes that the data could not be confidently assessed for the sample. In the case of the *Taxodium distichum* shaded samples, this is due to the extremely low concentrations of *n*-alkanes in the samples which made identification and qualification in the samples chromatograms difficult to impossible. In the case of *Ceratonia siliqua* and the two fern species *Matteuccia pensylvanica* and *Cyathea cooperi*, this was due to the presence of other unidentified organic compounds which inhibited proper identification of *n*-alkane peaks.

ACL values and dominant alkanes presented here also compare well with values reported in the literature for *Fagus sylvatica* (Lockheart et al. 1997, Collister et al. 1994; Nguyen-Tu et al. 2007; Rieley et al. 1991; Gülz et al. 1989), *Alnus glutinosa* and *Salix alba* (Rieley et al. 1991), and *Ginkgo biloba* (Nguyen Tu et al. 2003). The alkane distribution of *Cycas circinalis* here resembles alkane distributions from a large number of other cycad genera (Osborne et al. 1989, Osborne et al. 1993). However, ACL does not fully encapsulate the distribution of *n*-alkanes across species and samples, nor the variation therein. For example, *Platanus orientalis* and *G. triacanthos* have similar ACL values, close to 29, but this hides very different *n*-alkane distributions, shown in Figure 4 (see also Table 2).

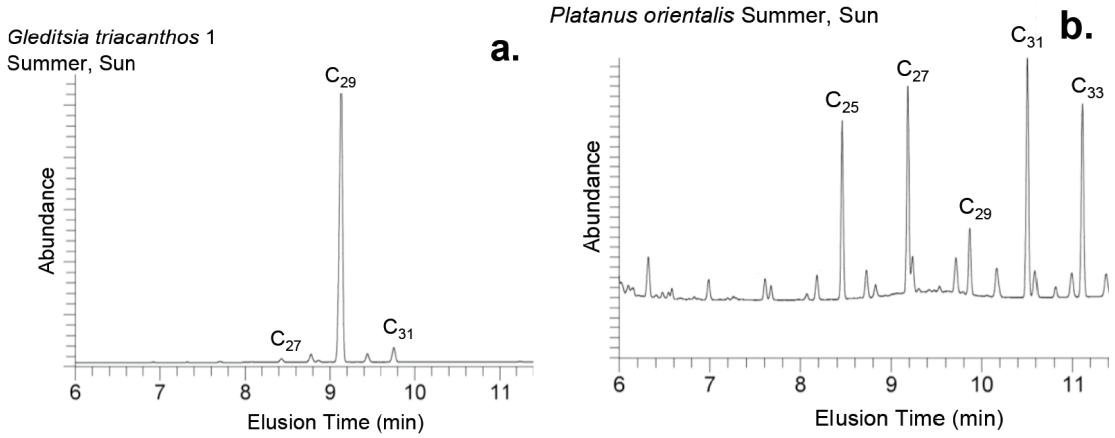


Figure 4. Chromatograms of summer sun samples from *Gleditsia triacanthos* 1 (a) and *Platanus orientalis* (b), showing odd-chain *n*-alkane peaks, each labeled at the top. Abundance is relative, with comparisons within a chromatogram only.

Gleditsia triacanthos displays an extremely dominant C_{29} peak, with very small amounts of other *n*-alkanes, thus averaging very close to 29, while *P.orientalis* leaves appear to have relatively little *n*-C_{29} but equal amounts of other *n*-alkanes so that the average of all *n*-alkanes roughly equals 29. This example highlights a second caveat in the use of ACL in that it does not distinguish between some patterns of variation in plant leaves. Most species produce a series of *n*-alkanes with a single apparent dominant chain length or two co-

dominant peaks, but not all species, and this variation, as exemplified by Figure 4, is overlooked in presenting just an average value.

Only two species in this study had ACL values outside of the previously reported range of 26 to 33 (Chikaraishi and Naraoka 2003, Bi et al. 2005, and Collister et al. 1994), namely *Picea abies*, and *T. occidentalis*. Only one sample of *P. abies* leaves (summer, sun) fell below 26 (at 25.97), but the ACL values of *T. occidentalis* leaves in all seven samples collected fell consistently near 34.4-34.5, with the C_{35} alkane being strongly dominant, well above any previously reported values. Diverse species such as *Rhus typhina, Ginkgo biloba,* and *P. abies* with low ACL values (<27) demonstrate a lack of clear distinction between the *n*-alkane distributions of terrestrial plants and aquatic plants, which appear to have a range of ACL of 23 to 26.5 (Chikaraishi and Naraoka 2003). There was no significant difference between the average chain lengths and *n*-alkane compositions of any plant groups, including angiosperms and gymnosperms (paired Student's t-test, $p = 0.8308$) as well as evergreen and deciduous species (paired Student's t-test, $p = 0.9354$) or broadleaf plants and conifers (paired Student's t-test, $p = 0.7360$). Indeed, the variation seen in angiosperm ACL (26.55-31.02) and *n*-alkane distribution appears to fall within that for gymnosperms (ACL range 25.97-34.54). No plant groups were distinguished by a signature *n*-alkane distribution or ACL, with the possible exception of *T. occidentalis* representing gymnosperms with scale leaves. Although every species displayed a consistent unique pattern of *n*-alkane molecular composition, ACL alone cannot distinguish either groups or species. This is consistent with previous findings (Maffei 1994).

Despite a lack of consistency across large plant groups, the composition of *n*-alkanes within individual plants was highly consistent. Within all species and plant groups, there was

a very strong correlation and no significant difference observed between sun and shaded samples within a season (linear regression $R^2 = 0.754$, $p < 0.0005$; Figure 5) or between seasons for a single canopy location (linear regression $R^2 = 0.865$, $p < 0.0005$; Figure 6). The correlation between sun and shaded samples becomes even stronger when all *Taxodium* samples are excluded (linear regression $R^2 = 0.963$, $p = <0.0005$). *Taxodium distichum* is also the only species which shows a consistent decrease in ACL from sun to shaded samples (paired Student's t-test, $p = 0.038$) (Figure 5). As part of a closer look at molecular transformation during leaf color change in the fall, multiple samples of different leaf colors (green, yellow, and brown) from three species were sampled: *Asimina triloba*, *Taxodium distichum*, and *Thuja occidentalis*. In support of the lack of difference between summer and fall leaves, as demonstrated in Figure 6, there appears to no trend in ACL from summer green leaves through color change to entirely dead, brown leaves (Figure 7), with the exception of *T. distichum*, which has varying ACL values but also demonstrates no obvious correlation with leaf color. The lack of difference between opposite locations on the canopy and between seasons provides evidence for the validity of comparisons made between modern leaves taken from trees during the summer with fossil *n*-alkanes from leaves grown at an unknown position in a plant canopy and deposited on the ground, to be incorporated into the sediment, at an unknown time of year.

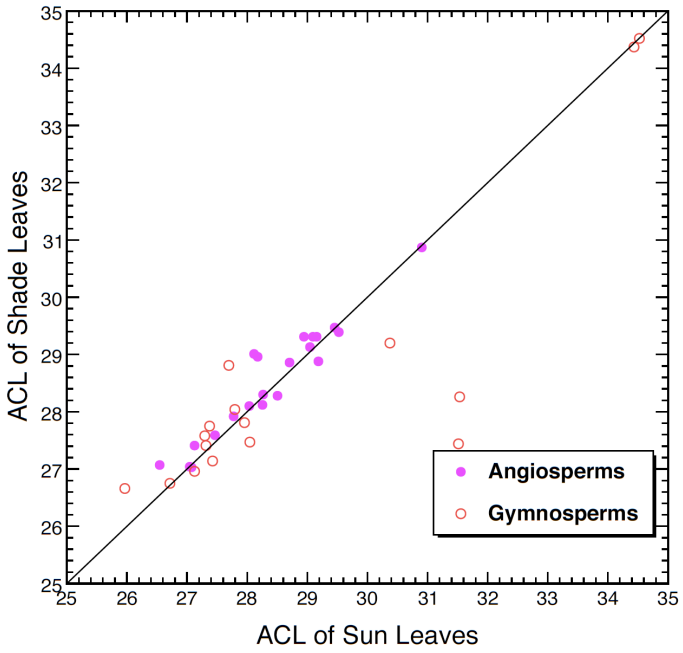


Figure 5. Average chain lengths (C_{21}-C_{37}) of sun-exposed leaves compared to shaded leaves of all outdoor species sampled, controlling for season (R^2 = 0.754, p = <0.0005). The line shown is a one-to-one line. The two points well above the line both represent *Taxodium distichum*, which is the only species sampled to display a consistent decrease in ACL from sun to shade leaves (paired t, p = 0.038).

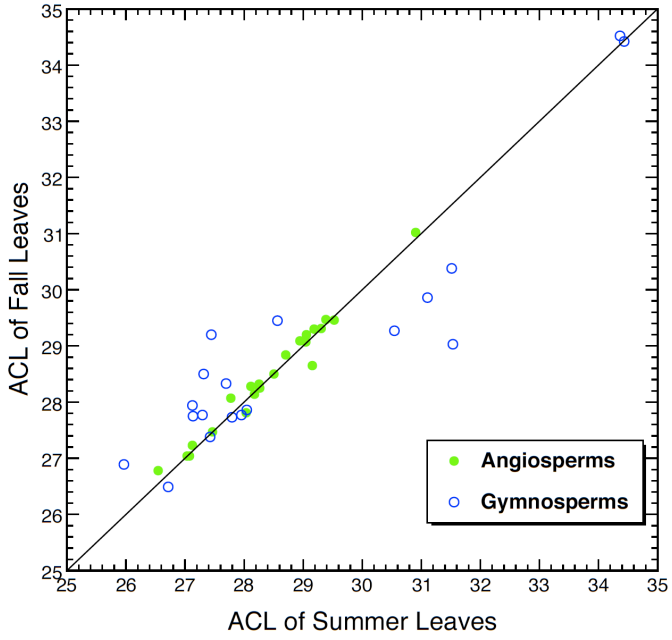


Figure 6. Average chain lengths (C_{21}-C_{37}) of summer leaves compared to fall leaves of all outdoor species sampled, controlling for light exposure (R^2 = 0.865, p = <0.0005). The line shown is a one-to-one line.

Within a single species, the consistency of *n*-alkane distribution appears to be somewhat more mixed. For both the angiosperms species for which replicate individuals were sampled (*A. glutinosa* and *G. triacanthos*), there is an overall similarity in the ACL values of individuals. The range of *A. glutinosa* ACL values was 27.13-28.32, reflecting variation in the co-dominance of *n*-C_{27} and *n*-C_{29} (Figure 8). *Gleditsia triacanthos* samples have an even smaller range: 29.04-29.20, reflecting the extreme dominance of *n*-C_{29} (Table 2, Figure 4a). In the gymnosperms, the ranges of ACL values within species were much larger. The ACL range of *Pinus sylvestris* trees was 26.96-28.04. For *Taxodium distichum* trees it was even larger: 27.44-31.53, although the dominant alkane was typically *n*-C_{33}. However, the data for *T. distichum* is drawn from the smallest sample set of all replicate tree species because of the paucity of *n*-alkanes in the shaded samples. It may therefore be that the ACL range for *T. distichum* is even larger than what is shown here. Overall, the results presented here appear consistent with previously reported *n*-alkane distributions for the same species, suggesting that despite some variation possibly due to environmental pressures or genetic polymorphism, *n*-alkane production within a species can be relatively well constrained by genetic predisposition.

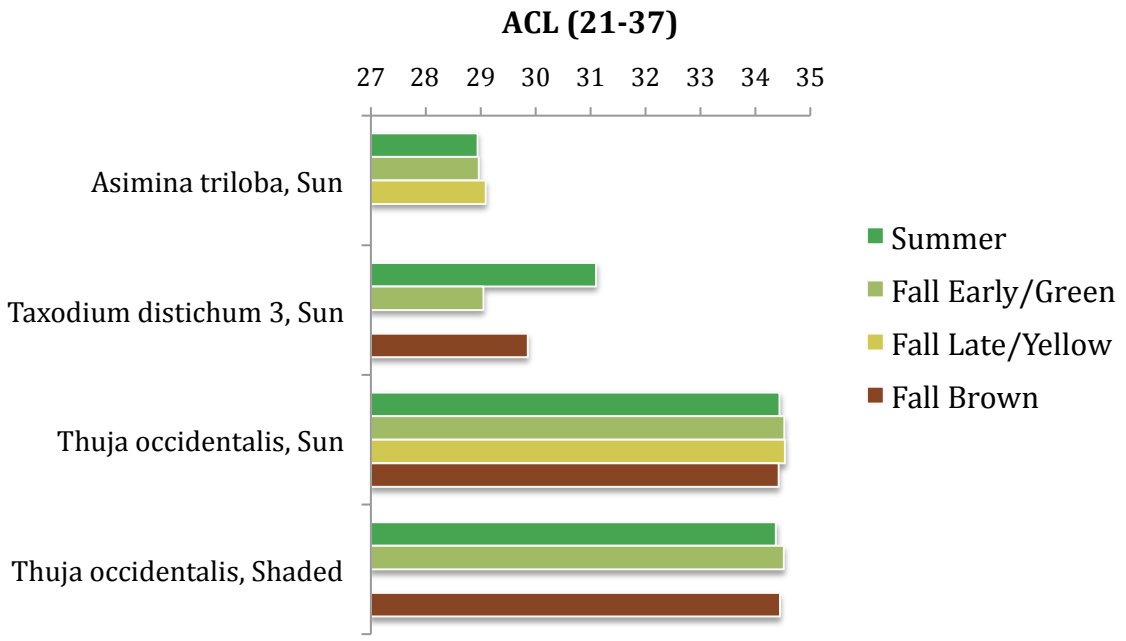


Figure 7. Average chain lengths of variously colored summer and fall leaf samples of three species, showing no trend in changing ACL through the stages of leaf maturation and decay.

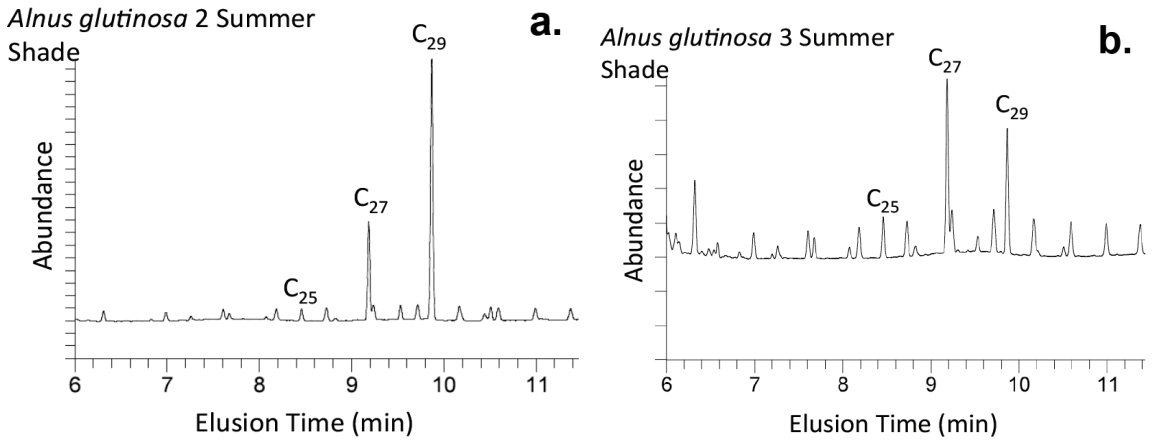


Figure 8. Chromatograms of summer shaded samples of *Alnus glutinosa* #2 (a) and #3 (b), showing odd-chain *n*-alkane peaks, each labeled at the top. Abundance is relative, with comparisons within a chromatogram only.

n-*Alkanes Through Deep Time: Chemotaxonomic Implications*

Remarkably, the pattern of *n*-alkane molecular composition appears to be conserved across time: the unusual *n*-alkane distribution pattern observed in *P. orientalis* (Figure 4) strongly resembles the pattern observed in *Platanus* fossil leaves (*P. dissecta*) from the Miocene Clarkia Formation (17-20 Ma) (Huang et al. 1995, Lockheart et al. 2000). This does not appear to hold for all species, however. Despite great consistency among modern *Fagus sylvatica* samples (this study, Lockheart et al. 1997, Collister et al. 1994; Nguyen-Tu et al. 2007; Rieley et al. 1991; Gülz et al. 1989), *n*-alkane distributions of two fossil *Fagus* leaves from the Miocene Clarkia did not resemble modern *F. sylvatica n*-alkane distributions but appeared more similar to other Pliocene *Fagus* fossils (Lockheart et al. 2000, Zanetti et al. 2007). The alkane distribution of Miocene Clarkia *Salix* fossils also differed from modern *Salix alba* values (Table 2, Yang and Huang 2003). This lack of temporal consistency in the *Fagus* and *Salix* genera may be because the fossil specimens are more distantly related to their modern counterparts than the modern and fossil *Platanus* specimens are to one another. The Fagaceae and Salicaceae families are much larger and more diverse than the Platanaceae (which only contains the genus *Platanus*), and the distribution of their *n*-alkane compositions appears to be more varied (Lockheart et al. 2000, Lockheart et al. 1997). Overall this, then, suggests that in angiosperms the pattern of *n*-alkane distribution can be very highly conserved in some genera, possibly with closely related species. *n*-Alkane profiles may therefore serve as a reliable chemotaxonomic indicator on the genus level as well as species if properly constrained. In large angiosperm families, the amount of variation in *n*-alkane distribution among species may be too great to reliably constrain for use in fossil chemotaxonomy.

The results of comparisons between modern and fossil gymnosperms is somewhat more mixed. *n*-Alkane distributions from Miocene Clarkia *Taxodium* fossils do not mirror their modern counterparts analyzed here (Lockheart et al. 2000), and *Taxodium* fossil alkane distributions from ~12.8 Ma and 17-20 Ma also bore little resemblance to one another, although the range of alkanes is similar (Lockheart et al. 2000). Pliocene *Ginkgo* samples from France have a different dominant alkane (*n*-C_{29}) than modern *Ginkgo biloba* samples (Nguyen Tu et al. 2003). Conversely, *Metasequoia* examples from both the Miocene Clarkia Formation and the Paleocene and Eocene in the Canadian Arctic compare closely with modern *Metasequoia* samples (Yang 2005, Lockheart et al. 2000). Thus the evidence suggests that some species in both the angiosperms and gymnosperms may be more conservative over long periods of time in their *n*-alkane distributions than others.

Group Trends: n-*Alkanes as Ecological Indicators*

Turning to examine group trends in alkane profiles, Figure 9 demonstrates the dominant alkane for each individual in this study, as taken from the summer sun samples, and it emphasizes again the point that the range of variation for angiosperms falls within the range of variation for gymnosperms. Figure 10 shows the dominant alkane for grass samples collected in 1996-1997 across the Great Plains area (F. Smith, unpublished data). As seen in Figure 10, there is some evidence that *n*-C_{31} dominates in C_3 grasses and therefore would likely be a major component of alkanes preserved in sediments in temperate grassland areas, while *n*-C_{27} or *n*-C_{29} dominate in most deciduous angiosperm trees which tend to be—but are not necessarily always—the dominant life form of modern temperate forests (Meyers and Ishiwatari 1993). This suggests that there may be a basis for the use of ratios comparing

amounts of *n*-C_{31} in sediments to *n*-C_{29} for use as evidence of ecosystem shifts from grassland to wooded (Zhang et al. 2006). As shown by Figure 9, however, not all woody plants produce *n*-C_{27} or *n*-C_{29} as the most dominant in their leaf waxes. Notably some gymnosperms have dominant alkane peaks greater than *n*-C_{31}. Other studies suggest that a large number of herbaceous angiosperms produce predominantly *n*-C_{31} (Maffei 1994, Nott et al. 2000, Mimura et al. 1998; Eglinton et al. 1962). Furthermore, not all grasses produce *n*-C_{31} in greater amounts than other *n*-alkanes (Figure 10). C_3 grasses appear to produce predominantly *n*-C_{31}, but C_4 grasses do not. Thus, the use of an *n*-C_{29}/*n*-C_{31} or similar ratio may only apply to a much narrower suite of life forms than previously suggested. For example, it may apply specifically to a change from C_3 grassland to deciduous angiosperm forest dominated by known species with known alkane profiles.

Combined with pollen records, the comparison between *n*-C_{29} and *n*-C_{31} may still be a useful tool in paleoecological reconstruction. Carbon isotope ratios preserved in sediments and animal tissue can be used to distinguish C_3 and C_4 ecosystems from one another. Isotope values alone cannot distinguish between C_3 grasses and C_3 dicots, however, and so comparison of alkane distributions may assist here in teasing apart the components of a C_3 ecosystem. Again, the comparison can only be made within the framework of other evidence of species presence, e.g. pollen records, and the alkane ratios of the plant groups to be investigated must be known with a relatively high degree of confidence in order that the extrapolation to preserved alkane distributions be valid. A large body of work exists describing *n*-alkane variation across a large range of plant species and habits (Maffei 1994, Mimura et al. 1998), and this can be used to build the necessary foundation of modern support for confident interpretation of data from sediments.

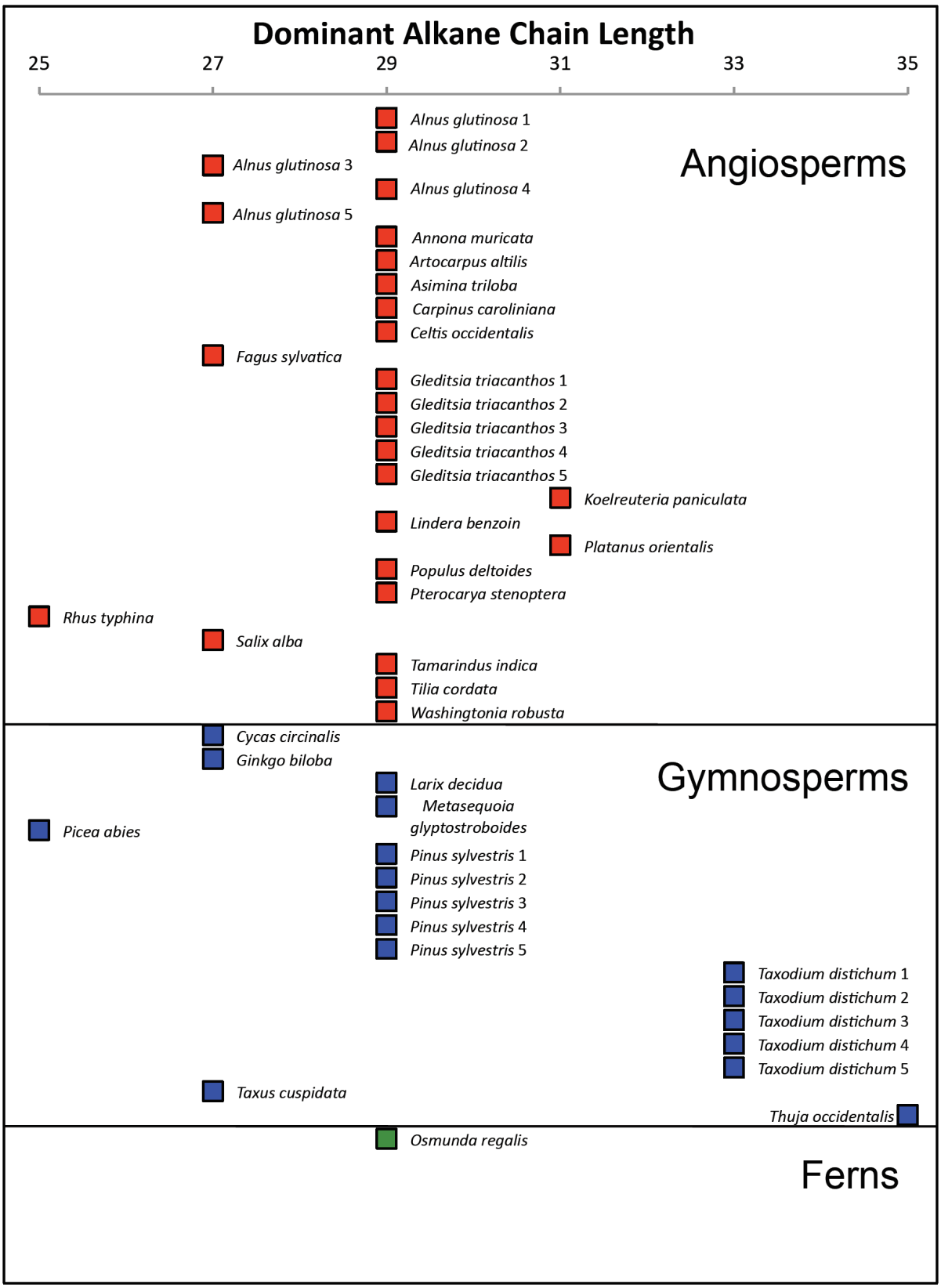


Figure 9. Dominant alkane of all summer (sun) samples of all species. Red denotes angiosperm species, blue gymnosperms, and green ferns.

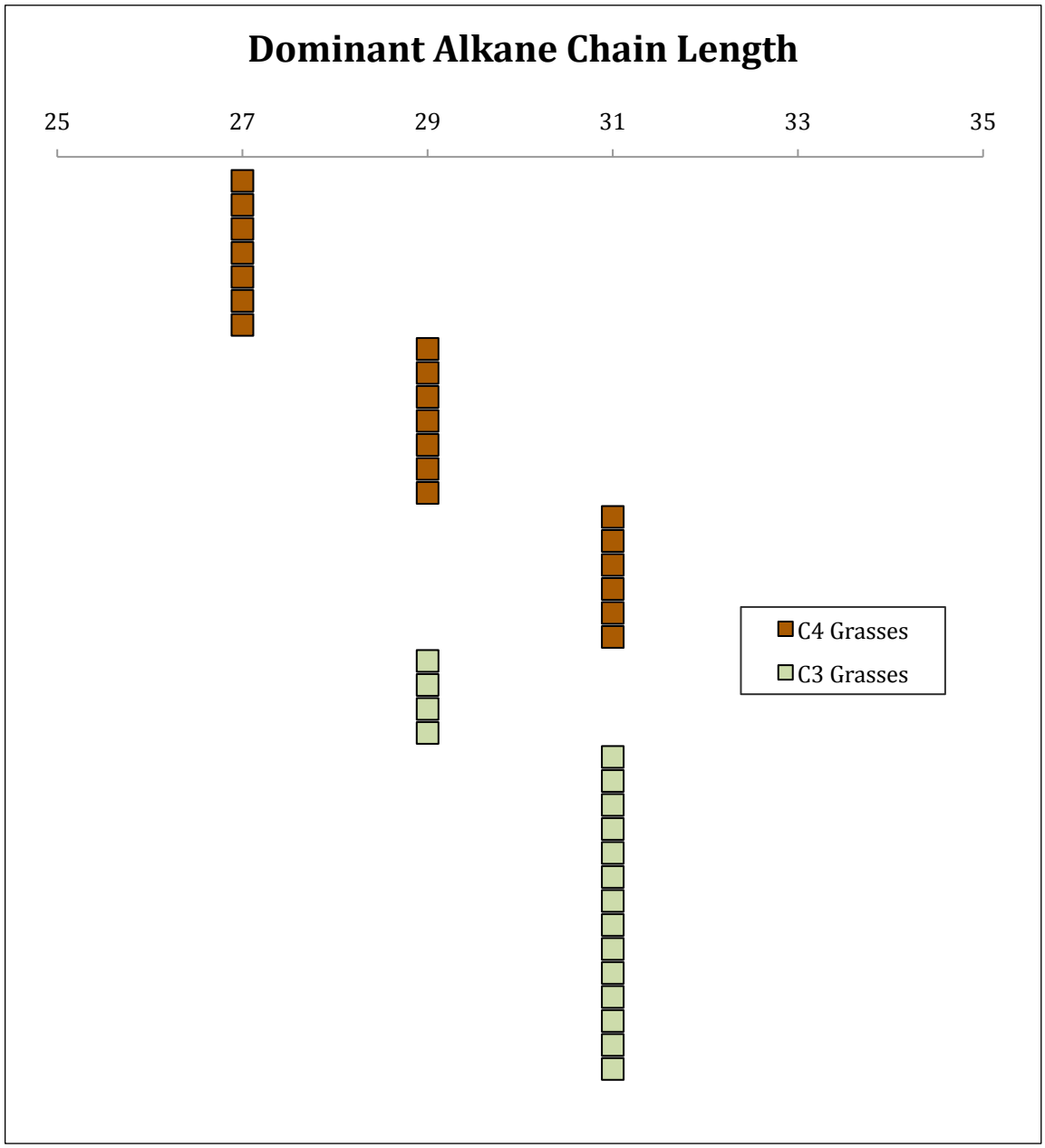


Figure 10. Dominant alkane chain lengths from samples of grass species collected across the Great Plains area during 1996-1997 (F. Smith, unpublished data, Smith and Freeman 2006). Dark orange denotes C_4 grasses, light green C_3 grasses.

PETM ACL and Plant Community Change

The similarity in ACL between angiosperms and gymnosperms can itself be applied to data from the PETM Bighorn Basin sediments, which shows an increase in ACL concurrent with the decrease in $\delta^{13}C$ values at the beginning of the PETM and then a decrease in sediment ACL towards the end of the PETM (Smith et al. 2007, Figure 2). Figure 11 demonstrates what the change in PETM ACL would look like if it was composed of the ACL values of the modern representatives of the plant community.

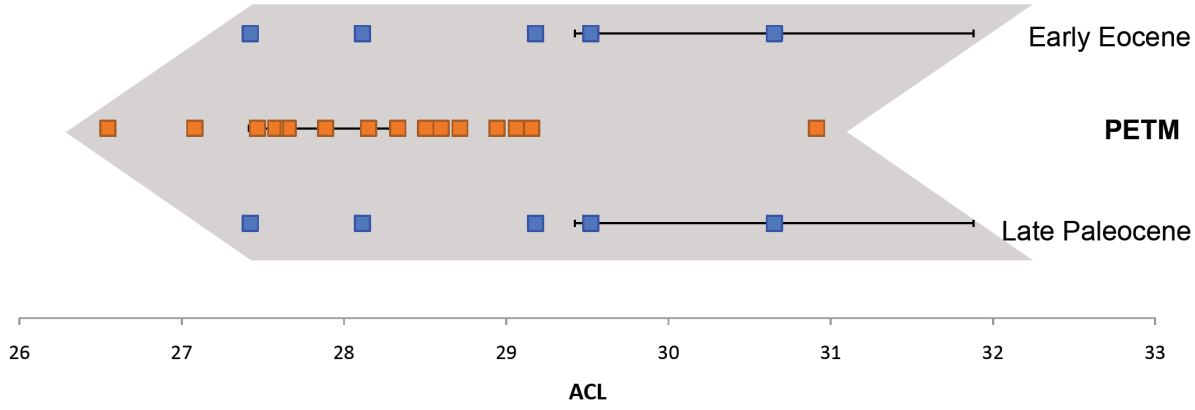


Figure 11. Average chain length (ACL) of modern representatives of the non-PETM communities of the late Paleocene and early Eocene and of the PETM community, showing no overall increase from non-PETM to PETM ACL values. Error bars represent standard deviation for species with multiple individuals, i.e. *Taxodium distichum* in the "non-PETM" communities and *Gleditsia triacanthos* (not visible due to small size) and *Alnus glutinosa* in the "PETM" community.

The data, especially from *T. distichum*, would suggest that if the plant community change hypothesis were true, then the PETM trend in ACL should have decreased (Table 2, Figures 11 and 9) rather than increased as observed in the Bighorn Basin (Figure 2). The alkane distribution of *M. glyptostroboides* is not significantly different from the distributions of PETM angiosperm representatives. Therefore, the data here does not support this explanation of the increase in ACL during the PETM. Other studies of modern plants do

suggest that the climate change-related hypothesis may be correct, however. Sachse et al. (2006) showed an increase in ACL across species with both increasing mean annual temperature (MAT) and precipitation (MAP). While not conclusive, these data suggest that if ACL and MAT are positively correlated, then the change in ACL across the PETM was likely driven by climate rather than plant community change.

ACL and Dispersion

One of the shortcomings of using only ACL and/or simple alkane ratios to describe trends in *n*-alkane distributions among plants is that they fail to encapsulate the variation in the range and the proportions of all alkanes (Figure 4). Therefore, the dispersion term (d) is introduced. Dodd and coworkers demonstrated a strong correlation between ACL and d in species from the Cupressaceae (Dodd and Poveda 2003, Dodd and Rafii 2000), and Figure 12 shows the same strong correlation in the species in this study with multiple sampled individuals. Table 3 describes the equations of the linear regressions, as well as their R^2 and p values. The variation within *Gleditsia triacanthos* is exceptionally small, but the same lack of consistency in regression slope across species is seen in the three species sampled by Dodd and Rafii (2000), suggesting that while there appears to be a relationship between ACL and dispersion, its character cannot be generalized for all species. The same correlation also exists for grasses (Poaceae) collected from the Great Plains area during a separate study (Figure 13, Smith and Freeman 2006). Within the grasses, the correlation holds across species.

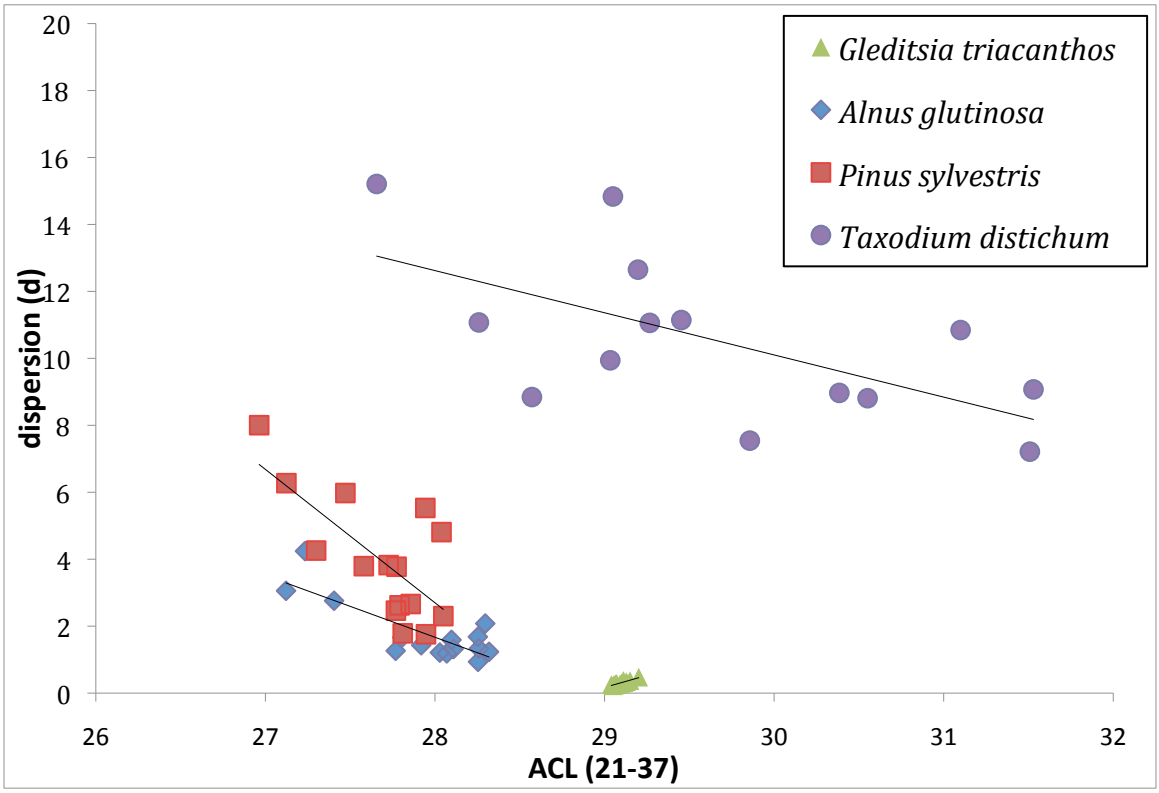


Figure 12. Average chain length compared to dispersion (d) for four species with replicate individuals sampled. Data includes all samples recorded, across season and light exposure. Lines represent linear regressions for each species.

Table 3. Equations, R^2 values, and p values of linear regressions for species shown in Figure 12.

Sample	Regression Equation	R^2 value	P value
Gleditsia triacanthos	d = 1.4405(ACL) – 41.603	0.8896	<0.0005
Alnus glutinosa	d = -1.8467(ACL) + 53.378	0.6498	<0.0005
Pinus sylvestris	d = -3.9843(ACL) + 114.26	0.5070	0.003
Taxodium distichum	d = -1.4876(ACL) + 54.78	0.3869	0.018

It was previously suggested that this correlation is due to genetic constraints within a species and/or environmental constraints on populations (Dodd and Poveda 2003). Because all the samples presented here were grown in the same environment, then this suggests that the environment does not directly drive this correlation. The species here came from diverse genetic backgrounds—*Pinus sylvestris* #1, for example, was collected as seed in Siberia, while *Pinus sylvestris* #2-5 were supplied by local nurseries (C. VanderMey, personal communication)—but were grown in a relatively controlled, regular environment. This fact

suggests that genetic factors play a larger role here than environmental factors. Therefore genetic and biosynthetic constraints are the likely cause, but the question of why this correlation exists, and why it seems to be so strong across a wide range of species life forms, remains unanswered. Alkane production and distribution may possibly be linked with production of other specific plant lipids (Maffei 1994).

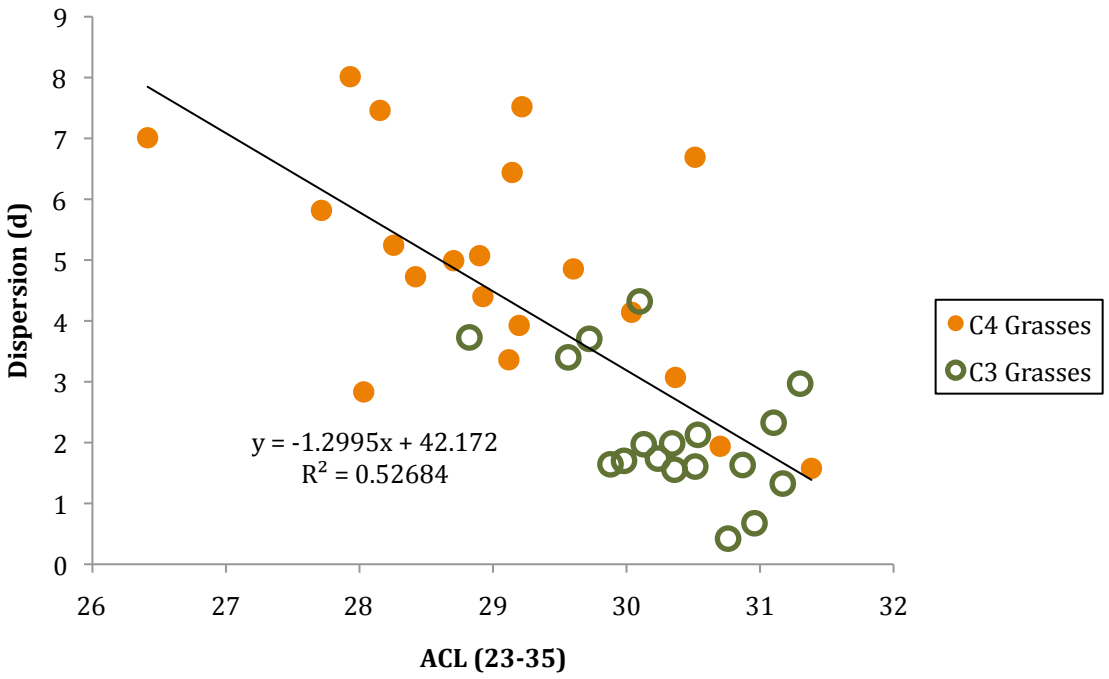


Figure 13. Correlation between ACL and dispersion for C_4 and C_3 grasses collected across the Great Plains area during 1996-1997 (F. Smith, unpublished data, Smith and Freeman 2006). The line represents a linear regression for all data, i.e. both types of grasses.

The correlation between ACL and dispersion also holds only within species, with the exception of grasses, and was not observed in any permutation of angiosperm/gymnosperm, broadleaf/conifer, evergreen/deciduous, or other groupings (Figure 14). The average dispersion of all angiosperm samples was 2.16, while that of all gymnosperm samples was 6.00, but these values were not significantly different from one another (paired Student's t-

test, p = 1.0000), even when excluding unusual outlier species (e.g. *Platanus orientalis*). This may suggest genetic constraints on alkane synthesis imposed by environmental conditions at the time of speciation (Dodd and Rafii 2000).

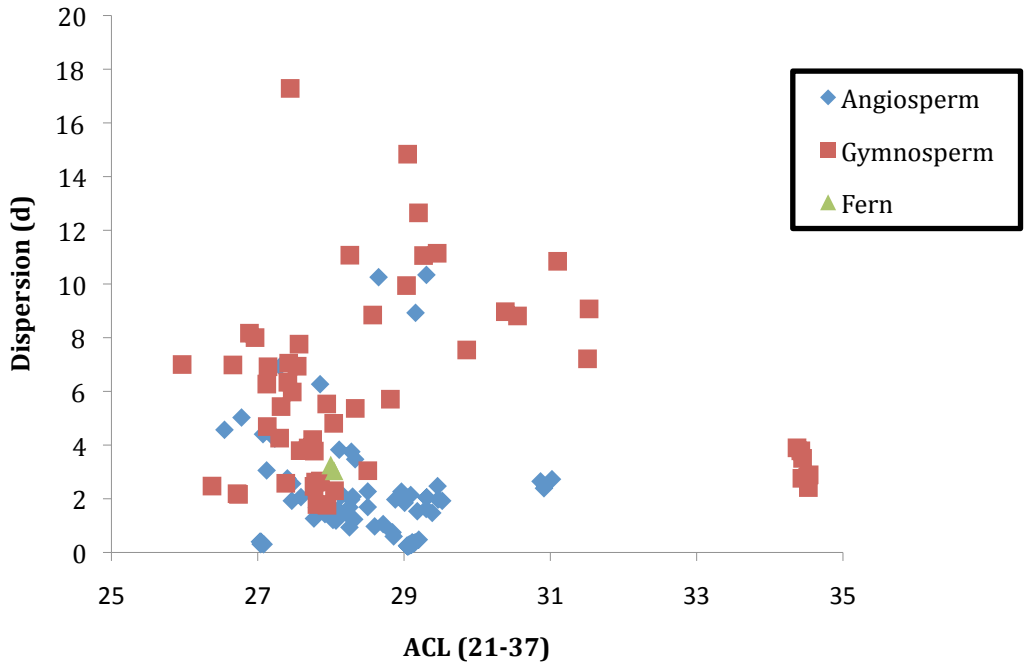


Figure 14. Average chain length versus dispersion for all samples, showing no distinction between groups.

n-*Alkane Carbon Isotope Ratios: PETM Plant Community Change*

The results of the compound-specific isotope analyses for eight species are shown in Table 4 and Figure 15. All species except the beech (with only one data point) and ginkgo, show a trend of decreasing $\delta^{13}C$ with increasing chain length. The results show a lack of distinction between deciduous gymnosperms and deciduous broadleaf angiosperms, as compared to Figure 2, which predicted a divergence in $\delta^{13}C$ values between the two groups with increasing chain length (Smith et al. 2007). Furthermore, the gymnosperms do not have

less negative $\delta^{13}C$ as a group than the angiosperms, contrary to expectations (Leavitt and Newberry 1992, Brooks et al. 1997). While the angiosperm trends are consistent with observations reported elsewhere (Chikaraishi et al. 2003, Bi et al. 2005, Collister et al. 1994), no compound-specific $\delta^{13}C$ data on deciduous gymnosperms have been previously reported. These data do not support a general distinction in $\delta^{13}C$ values between gymnosperms as a group and angiosperms, but suggest that the previously reported difference in $\delta^{13}C$ values between deciduous broadleaf trees and evergreen conifers may be due to leaf morphology or leaf habit rather than taxonomic group.

Table 4. $\delta^{13}C$ values (in ‰) for individual *n*-alkanes of selected species.

	n-Alkane Chain Length				
Species	25	27	29	31	33
Angiosperms					
Carpinus caroliniana		-32.2	-33.1		
Fagus sylvatica			-29.2		
Koelreuteria paniculata			-32.2	-33.7	-34.3
Pterocarya stenoptera			-29.5	-30.0	
Tilia cordata	-30.4	-31.0	-30.5	-31.8	
Gymnosperms					
Ginkgo biloba	-30.7	-30.5			
Metasequoia glyptostroboides	-30.8	-30.9	-31.2		
Taxodium distichum		-32.6	-32.0	-32.7	-33.5

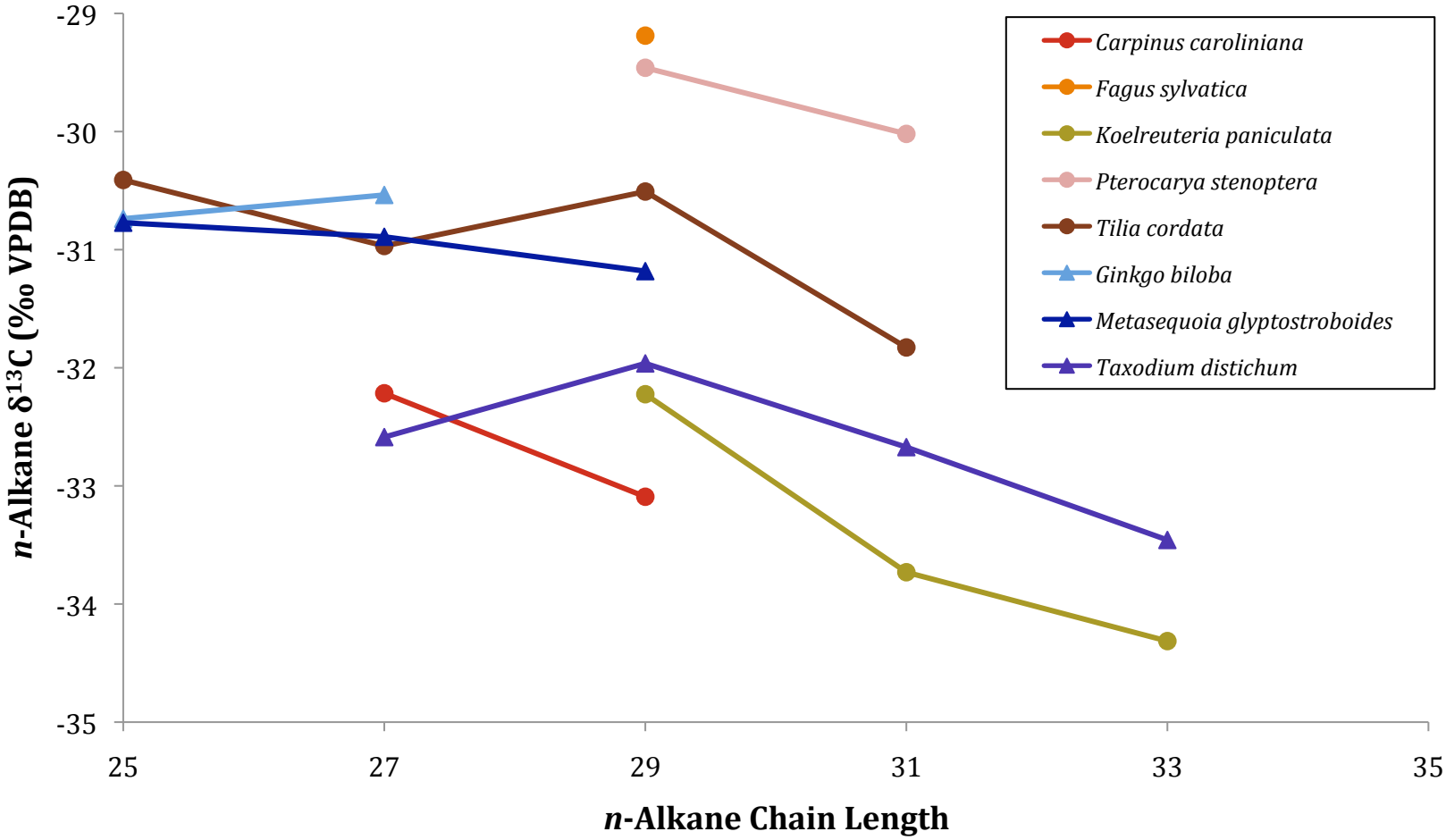


Figure 15. Carbon isotope ratios of *n*-alkanes of summer sun samples from selected species representing deciduous angiosperm and deciduous gymnosperm trees, showing an overall trend within both groups of decreasing $\delta^{13}C$ with increasing *n*-alkane chain length.

The similarity in $\delta^{13}C$ values across *n*-alkane chain length between angiosperms and deciduous gymnosperms does not support the explanation proposed by Smith et al. (2007) for the difference in the terrestrial and marine carbon isotope excursions observed in various sediment records during the PETM (Zachos et al. 2001, Smith et al. 2007). The data here must be expanded upon in order to fully support or refute the conclusion, but it may be that the division in $\delta^{13}C$ values drawn between gymnosperms and angiosperms (Leavitt and Newberry 1992, Chikaraishi et al. 2003) is not a taxonomic but rather a morphological distinction. These data suggest that a re-examination of the PETM terrestrial CIE may be needed.

Conclusions

In summary, while the patterns of *n*-alkane distributions in the leaf waxes of the plants presented here vary widely, consistent trends are observed at the individual and species level. With the exception of one species, *Taxodium distichum*, alkane distribution does not change significantly within a tree from sun to shaded leaves or in mature leaves from summer to fall. Why a single species does not follow the trend displayed by twenty-one other species, including one morphologically similar and in the same family (*Metasequoia glyptostroboides*), is a subject for further investigation. Not only are *n*-alkane distributions conservative within a species across a single growing season and across the range of a species, but they appear to be conservative in some species on the scale of millions of years as well (Lockheart et al. 2000). This finding emphasizes the potential for *n*-alkanes to serve as species-specific biomarkers for ecosystems from across deep time. Studies

directed at sampling all modern species within genera of interest (e.g. *Platanus* or *Fagus*) would explore this potential.

The strong correlation between ACL and dispersion (d) within species also deserves further inspection. The correlation is likely associated with genetic constraints on *n*-alkane production and genetic polymorphisms within populations and may also be driven by environmental forcing on leaf wax composition (Dodd and Rafii 2003). However, the latter suggestion is less likely considering the lack of effect of light exposure or seasonality on ACL and the strength of the trend from specimens grown in a homogeneous environment at the Chicago Botanic Garden. Environmental effects on the relationship between ACL and dispersion may be more definitively ruled out with an experiment in the extremely controlled environment of a greenhouse. Furthermore, the reason for the correlation holding only within species and not families, except in the case of grasses, is unclear, but may be linked to physiological differences between monocots and dicots. Importantly, these results present somewhat of a paradox when considered together with the evidence of Sachse et al. (2006) that ACL is correlated to environment. ACL and dispersion appear to correlate regardless of environment, but the environment also appears to be the driver of ACL in plant communities regardless of species, as seen in the investigation of the PETM change in ACL. Further elucidation of the role of *n*-alkanes in leaf wax and why there is such variation in *n*-alkane distribution among plant species could help explain some of the observations here.

As it stands now, the different trends in $\delta^{13}C$ previously identified as being between angiosperms and gymnosperms (Smith et al. 2007) does not withstand the addition of new data, and may be related to differences in leaf habit instead. The PETM plant community change hypothesis, based on the taxonomic division, therefore may be in need of a

reevaluation and modification, although the data here are from a very small number of samples and thus far from conclusive. In order to confirm the suggestions presented here, a broader range of plant groups must be tested, drawn from differing taxonomic groups, leaf morphologies, and leaf habits. A closer examination of evergreen angiosperms as well as deciduous gymnosperms would test the new hypothesis that the trend of diverging $\delta^{13}C$ values with increasing chain length is based on leaf habit instead of taxonomic group.

Future Research

The molecular and isotopic composition of *n*-alkanes has proven to be highly informative on for plant species and groups, confirming the promise for the use of *n*-alkanes as biomarkers for vascular plants. All of the samples collected for this study will be analyzed for both bulk tissue and *n*-alkane $\delta^{13}C$ values, completing the goals outlined at the beginning of the thesis research, and the expanded data set will provide firmer evidence for the support or refutation of the PETM plant community change hypothesis. They will also allow for a fuller investigation of carbon isotope trends across species and plant groups, as well as within trees, and it is likely that groups not distinguished by *n*-alkane composition will be separated by carbon isotope composition.

Investigations into the diversity of *n*-alkane distributions and the relationship between ACL and dispersion stand to cast light on the physioecology of modern plants, which is a field still in its adolescence. In the field of plant paleoecology, the comparisons made here can be used in the reconstruction of ecosystem dynamics from other time periods than the PETM, for example the late Cretaceous during the diversification of angiosperms (Wing et al. 1993, Wing and Boucher, 1998). Global angiosperm species diversity increased

tremendously during the Cretaceous, while cycad and pteridophyte diversity simultaneously fell drastically (Wing and Boucher 1998). Further exploration into the molecular and isotopic differences between these plant groups could help us understand ecosystems dynamics at an especially exciting time in plant evolution. The methods of analysis in this study may also be combined with other methods in the search for trends and differences for use in paleoecology, e.g. stomatal density and index (Kouwenberg et al. 2007, Royer et al. 2001) or hydrogen isotope composition (δD) of paleosol *n*-alkanes for use in paleohydrology (Smith et al. 2007, Smith and Freeman 2006). Present knowledge of the molecular and isotopic variation in *n*-alkanes is based on relatively few, scattered studies based on typically arbitrarily chosen species from anthropogenic settings. The potential is great for further exploration of chemotaxonomic dynamics in natural ecosystems both extant and extinct.

Appendix A: Detailed sample preparation and analysis procedure

Notes:

- All materials labeled 'solvent-rinsed' were cleaned three times with methanol, then three times with dichloromethane (DCM), and finally three times with hexane (using 500 mL Teflon, solvent-rinsed squeeze bottles, Nalgene brand) and allowed to dry completely before use.

- All materials labeled 'ashed' were placed in a Thermolyne Furnace (F30420C-80; Type 30400, Thermo Scientific) to be heated to 420°C. Method: Temperature ramp over 2.5 hours to 420°C; dwell at 420°C for 1 hour; and end, allowing the furnace to cool slowly to room temperature over 24-36 hours. (This slow cooling allows glass materials to shrink slowly and avoid stress fracturing.)

- Silica gel (grade 62) was cleaned for column chromatography by placing approximately 200 mL of dry gel into a 2 L beaker with 400 mL of 1:1 DCM:methanol and sonicated for 20 minutes. The solvent was then removed, either by decanting or with a large burette, and the process repeated two more times, for a total of three. 400 mL of hexane was then added and the process repeated two more times. The total number of all washes is six: three with DCM:methanol and then three with hexane. The gel was then allowed to dry completely, and poured into an ashed jar to be activated in an oven at 70°C for a minimum of 24 hours.

Herbarium Mounting and Storage:

1. Specimens were collected and pressed between sheets of blotter paper, newspaper, and cardboard for several weeks. Once completely dried, the pressed specimens were transferred to a large folder to await mounting. All specimens were frozen for a minimum of three days, in the freezers at the Chicago Botanic Garden, to kill any potential pests. Specimens, still in labeled (numbered) newspaper, were placed in a plastic box to protect from condensation.

2. Each dry, pressed sample was placed on a single archival quality mounting sheet (100% cotton rag, unbuffered UC-Type, caliper 0.015, 11½" by 16½"; Herbarium Supply Co.) and positioned in a "portrait" style, and orienting the specimen to best represent the natural habit of the plant, with room available for a label in the bottom right corner as well as a fragment folder. The specimen was then strapped to the sheet with wetted, gummed linen tape (Pacific Papers), cut to the appropriate size. The linen tape dries quickly, and so it is important to have already placed the specimen appropriately and to have cut the tape to the required length and width.

3. Small twigs and leaves were sewn to the sheet with linen thread. No glue was used in mounting the specimen, so that later tissue analysis remains entirely viable and so that no glue can interfere with the molecular preservation of the tissues. Large samples were clipped and separated between two archival sheets. A gummed paper label (Figure 3; Pacific Papers) was affixed to the lower right hand corner of each sheet, and a fragment folder (100% cotton rag, acid-free, medium size; Pacific Papers) was also glued to each sheet. Any loose specimen fragments were placed in the fragment folder.

4. After being allowed to dry fully, the strapped and labeled specimens were each placed in a separate species folder (acid free; Herbarium Supply Co.) Large fruits and cones were stored in origami boxes made from a single sheet of mounting paper cut to one 8" × 8" square (for the top half) and one 7 ½ " × 7 ½" square (for the bottom half), making boxes with internal dimensions approximately 2" × 2" × 1 ½". Two 2" × 2" cards (using scrap mounting paper) were labeled with species name, family, item description, collector name and number, and date. One card was glued to the inside of the bottom box half, and one card was glued to the outside (top) of the top box half, so that each part of the total box is labeled identically.

5. The species folders and large fruit boxes were stored in drop-front herbarium boxes (University Products), labeled "Angiosperms," "Gymnosperms," "Ferns," and "Large Fruits and Cones." These boxes were in turn stored, along with all remaining herbarium materials (for use on later specimens), in a large two-shelf steel cabinet (HON), dubbed the "Isotope Paleoecology Laboratory Herbarium." Herbarium boxes will be re-labeled and species folders re-organized as the herbarium expands in the future.

Grinding and Storage:
1. Leaf samples were removed from the brown paper bags (grocery and lunch bags) in which they were stored, using a staple remover and solvent-rinsed tweezers. The twig and all loose leaves were placed on a fresh sheet of aluminum foil (dull side up). Broad (deciduous) leaves, including petiole, were then individually transferred to a solvent-rinsed ceramic mortar and pestle (CoorsTek brand) using solvent-rinsed tweezers or forceps.

2. Broad leaves were ground in the mortar, being careful to keep all plant material within the bowl of the mortar. Plant material was ground until it reached a relatively coarse texture (<1 cm). Any especially large or tough petioles or leaves were first clipped and then transferred to the mortar for grinding. Once ground, the total leaf material of each sample was poured into an ashed jar (VWR brand, 4 oz. or 120 ml, tall straight sided jar, clear, with pulp/tinfoil lined cap.) Each jar and cap was labeled with initials and date, species name and/or code, and sample information (e.g. "Ag1 sun fall" for *Alnus glutinosa* #1 sun sample, collected in fall.) For species with very large leaves, only a single leaf was ground for analysis: *Artocarpus altilis, Matteuccia pensylvanica, Osmunda regalis, Rhus typhina.* For species with large leaves, two to four leaves were ground together: *Annona muricata, Asimina triloba, Ceratonia siliqua, Koelreuteria paniculata, Platanus orientalis*. For species with smaller leaves, 12-25 leaves were ground together: *Alnus glutinosa, Carpinus caroliniana, Celtis occidentalis, Fagus sylvatica, Ginkgo biloba, Gleditsia triacanthos, Lindera benzoin, Populus deltoides, Pterocarya stenoptera, Salix alba, Tamarindus indica, Tilia cordata.* For the species *Metasequoia glyptostroboides* and *Taxodium distichum*, with leaves of especially small mass, as many as 50 leaves were ground together.

3. Needled (coniferous) samples were treated differently, and were not ground in a mortar. Instead, once removed from the paper storage bag and placed on clean aluminum foil, groups of differently aged needles (age cohorts) were identified, with the youngest needles at the tips of the twig and oldest needles at the base. Solvent-rinsed tweezers were used to separate needles of different age cohorts from one another and place each cohort in a separate ashed

jar. Thus, a single conifer sample is stored in multiple jars, labeled—in addition to label information listed above—with "1st year," "2nd year," and so forth. "1st year" denotes needles grown in the same growing season in which the twig was collected (in this case, 2007); "2nd year" denotes needles from the previous growing season (2006); etc. Samples with deciduous needles (e.g. *Larix* sp.) were not separated by age, but transferred as a whole using tweezers to storage jars. The number of needles used for extraction varied by species: roughly 10 needles for *Pinus sylvestris*, 30-50 needles for *Larix decidua* and *Picea abies*. For *Thuja occidentalis*, with very small, scale leaves appressed to the stem, the entire twig was stored whole in a jar, and clipped intact for extraction.

4. Labeled jars were alphabetized by species and returned to their cardboard boxes, where they can remain indefinitely.

5. Extremely large leaves (*Cyathea cooperi, Cycas circinalis,* and *Washingtonia robusta*) were kept in paper bags, and a measure of sample was clipped from the leaves for extraction. In order to control for variation within the leaves, multiple samples (minimum 2) were taken across the length of the leaf, from the base of the leaf blade to the tip.

Lipid Extraction:
1. Small sub-samples of leaf material (0.4 g ±0.01g) were transferred via solvent-rinsed scupulas or tweezers to solvent rinsed Teflon MARS Xpress vessels. A 13 mm long,Teflon-coated, solvent-rinsed stir bar was also added. After the addition of 30 mL of 9:1 DCM:methanol, the vessel was capped with solvent-rinsed stopper and cap. One vessel for each batch of leaf samples analyzed was used as a blank, and filled with 0.4 g of ashed silica gel and 30 ml of solvent mixture. All filled Xpress vessels were placed into a carousel and loaded into the MARS-X (Microwave-Accelerated Solvent Extraction System 'Xpress' or "MARS-X," CEM Corporation 2007, Version 194A05) and run with the following method: ramp to 100°C for 5 minutes, hold at 100°C for 15 minutes, and cool down for 30 minutes.

2. Once vessels are cooled, they can be safely uncapped in a fume hood. The lipid extract, now suspended in the solvent mixture, was pipetted from the vessels into ashed, numbered test tubes, separating the liquid from the solid sediment, which was subsequently disposed of. The test tubes were placed in a rack and set in a TurboVap LV (Caliper Life Sciences), into a water bath heated to 35°C, where they were jetted with compressed nitrogen (zero grade) until they were evaporated to the point that the height of the remaining extract in the tube was approximately 1 cm.

3. The concentrated lipid/solvent mix was then pipetted with ashed Pasteur pipettes from the tube into two ashed 4 mL vials for each sample. Each amount of liquid drawn into the pipette was used to 'rinse' the inner walls of the test tube in order to collect any lipids that had precipitated during the course of evaporation. All 4 mL vials were capped with solvent-rinsed caps. One vial was numbered only, to be further processed, and one vial was labeled 'TLE archive' (for 'Total Lipid Extract') and stored in a refrigerator in labeled vial files (Wheaton, part #228780).

Silica Gel Column Chromatography

1. Post-extraction samples, in 4 mL vials, were placed into an N-Evap (112, Organomation Associates) with solvent-rinsed Luer needles, and evaporated using a jet of nitrogen (zero grade) until they were totally dry. Samples were then re-suspended in a small amount of hexane.

2. An ashed Pasteur pipette was erected vertically, using a clamp and bench stand, with a folded Kim Wipe as a 'bib' around the midsection of the pipette. This was the gravity column. The pipette was plugged with a small piece of ashed glass wool tamped into place with a second pipette, and approximately 1 g of activated silica gel in hexane slurry (the stationary phase) was packed into the pipette, leaving a head space of ~2 cm for sample and solvent addition.

3. The sample, in hexane, was added, and then 4 mL of hexane pipetted on top (the eluent). The hexane fraction of the sample was eluted into an ashed 4 mL collection vial. Directly after the hexane, 4 mL of 1:1 DCM:methanol was slowly pipetted into the column and eluted into a second 4mL collection vial. Once complete, the first vial was capped and labeled the non-polar fraction (F1); the second vial was capped and labeled the polar fraction (F2). The non-polar fraction should be clear and colorless, while the polar fraction contains the pigments of the sample.

4. The F2 vials were archived in a separate vial file in a refrigerator. The F1 vials were evaporated in the N-Evap until dry. A very small amount of hexane was then added to the dry sample and used to rinse the vial before transferring with a short Pasteur pipette to an ashed 2 mL autosampler vial. This was repeated three more times. The 2 mL vial was then capped with a clean Teflon autosampler cap and labeled.

5. The 2 mL vial was evaporated a final time in the N-Evap until dry. Then hexane was added to dilute the sample to appropriate amount for mass spectrometry: approximately 1mL for angiosperms and 250 µl for gymnosperms, although this is a very rough approximate and sample volumes varied widely.

Gas Chromatograph/Mass Spectrometer

1. Each group of samples, in 2 mL autosampler vials, included a blank filled with clean hexane, and a standard of C_{21} through C_{40} *n*-alkanes in toluene (40mg/L each alkane; Fluka, Sigma-Aldrich). The hexane blank was run both at the beginning and end of the sample analysis to ensure consistency.

2. 1 µl of each sample was injected into a gas chromatograph/mass spectrometer (GC/MS) with Triplus Autosampler and Xcalibur software (ThermoFisher). Samples passed first through the gas chromatograph for separation and identification (Thermo Finnigan Trace GC Ultra, with 15m, 0.25mm ID Thermo TR-5ms SQC column) and then to a quadrapole mass spectrometer with a flame ionization detector (FID) for assessment of relative abundance (Thermo Finnigan DSQ). The GC/MS method was as follows: initial temperature at 100°C, hold for 2 minutes, then ramp at 11°C/minute for 20 minutes to 320°C, and hold at 320°C for 5 minutes. The retention time of each *n*-alkane was then known to 0.01 min from the

standard and used to identify sample *n*-alkanes. *n*-Alkanes were also identified using the gas chromatograph spectra and comparison with a molecular library.

3. Samples with volumes less than 200 µl were transferred by Pasteur pipet, including rinsing three times with hexane, evaporating the sample between each transfer, to vial inserts for sampling with the autosampler.

4. An FID integration report was created using Xcalibur software for each sample, calculating the peak area for all peaks within the retention time range for plant *n*-alkanes, as determined by the alkane standard.

Gas Chromatograph/Isotope Ratio Mass Spectrometer

1. After running in the GC/MS, samples were run through a gas chromatograph/isotope ratio mass spectrometer (GC/IRMS, ThermoFisher Trace GC Ultra and Delta V with Triplus Autosampler and Isodat). 1-2 µl of sample in hexane was injected into the GC (7m, 0.32mm ID Thermo RTX-5 column) via a split/splitless (SSL) injector in splitless mode with a base temperature of 220°C. Split flow was 20 ml/min (set at 28 ml/min). The GC method was as follows: initial temperature at 80°C, hold for 1 minute, ramp at 20°C/min to 320°C, hold for 5 minutes. Samples then passed through an interface (GC II-III Interface, ThermoFisher) to the IRMS.

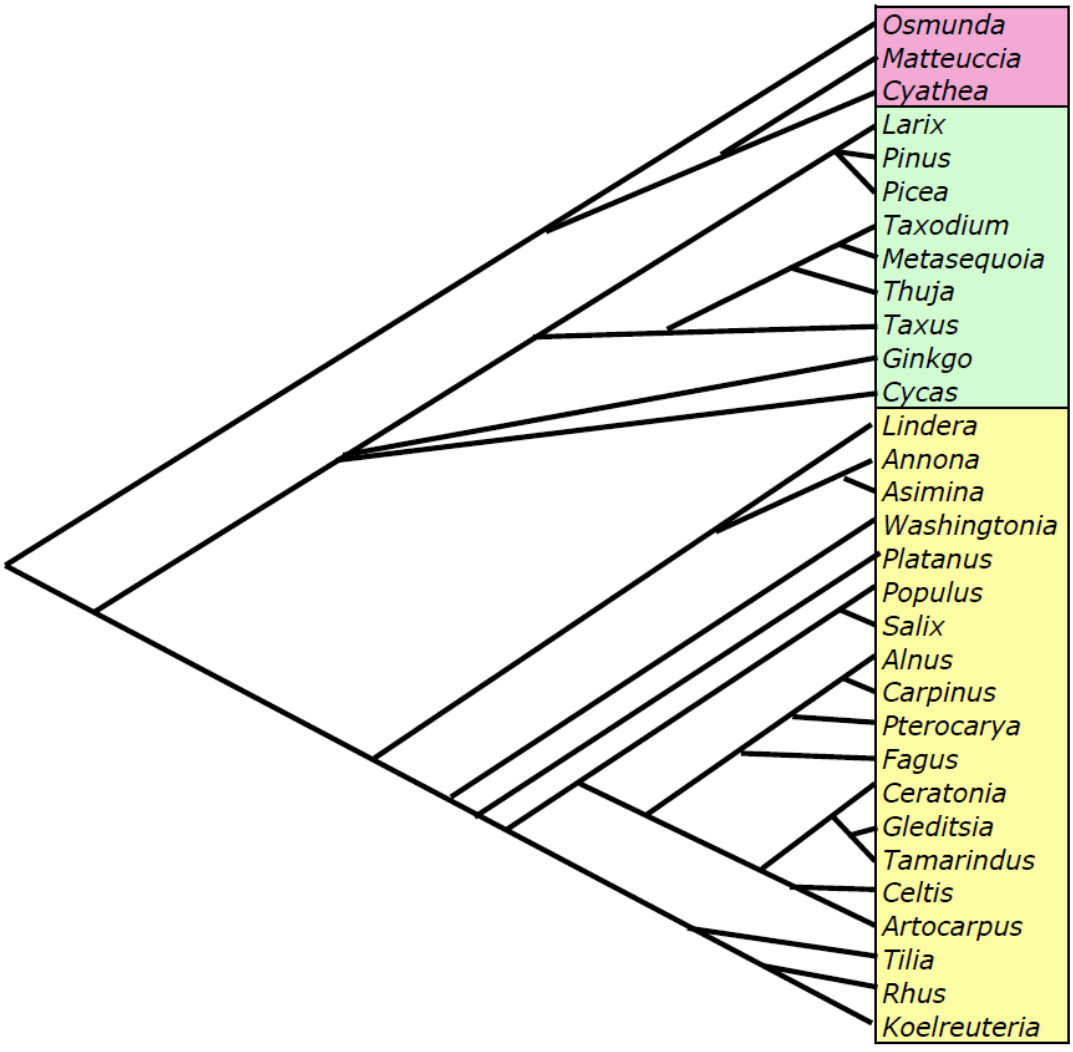
2. Table 5, Time Events for GC II-III Interface Method:

Time (s)	Split	Oxidize	Reference	Backflush
0.0	Out	Off	Off	On
30.0			On	
50.0			Off	
130.0			On	
150.0			Off	
230.0			On	
250.0			Off	
400.0	In			Off
1200.0	Out			On
1230.0			On	
1250.0			Off	
1330.0			On	
1350.0			Off	
1430.0			On	
1450.0			Off	
Acquisition Time: 1500.0				

2. Isotope ratios were compared to a CO_2 reference gas, calibrated to known standards (Mix A, Arndt Schimelmann). All samples were bracketed by a minimum of two alkane standards both before and after all sample runs in a single day.

3. Results were calculated with Isodat software and compared with GC/MS results for *n*-alkane identification, and samples were run at varying concentrations in order to evaluate alkane peaks of comparable size to reference peaks.

Appendix B: Figure 16, Table 6. The phylogenetic relationship of sampled species. Ferns are marked in red, gymnosperms in green, and angiosperms in yellow. Distances on the tree do not necessarily represent distance in time. Phylogenies from the Angiosperm Phylogeny Webiste (Stevens 2001), Judd et al. (2007), and Herendeen et al. (2003).



Genus	Family	Order
Osmunda	Osmundaceae	Osmundales
Matteuccia	Onocleaceae	Polypodiales
Cyathea	Cyatheaceae	Cyatheales
Larix	Pinaceae	Pinales
Pinus	Pinaceae	Pinales
Picea	Pinaceae	Pinales
Taxodium	Cupressaceae (Taxodiaceae)	Pinales
Metasequoia	Cupressaceae (Taxodiaceae)	Pinales
Thuja	Curessaceae	Pinales
Taxus	Taxaceae	Pinales
Ginkgo	Ginkgoaceae	Ginkgoales
Cycas	Cycadaceae	Cycadales
Lindera	Lauraceae	Laurales
Annona	Annonaceae	Magnoliales
Asimina	Annonaceae	Magnoliales
Washingtonia	Arecaceae	Arecales
Platanus	Platanaceae	Proteales
Populus	Salicaceae	Malpighiales
Salix	Salicaceae	Malpighiales
Alnus	Betulaceae	Fagales
Carpinus	Betulaceae	Fagales
Pterocarya	Juglandaceae	Fagales
Fagus	Fagaceae	Fagales
Ceratonia	Fabaceae (*Umtiza* tribe)	Fabales
Gleditsia	Fabaceae (*Umtiza* tribe)	Fabales
Tamarindus	Fabaceae (Detarieae tribe)	Fabales
Celtis	Cannabaceae (Ulmaceae)	Rosales
Artocarpus	Moraceae	Rosales
Tilia	Malvaceae (Tiliaceae)	Malvales
Rhus	Anacardiaceae	Sapindales
Koelreuteria	Sapindaceae	Sapindales

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