The Angiosperm Advantage:

Evidence from carbon isotopes and the fossil record

GEOL 394H: Geology Honors Senior Thesis

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Abstract

Appearing in the fossil record only 130 Ma, angiosperms (flowering plants) quickly grew to dominance in the terrestrial realm, and remain the dominant group of land plants today. High productivity among angiosperms has been attributed to key innovations in the evolution of leaf veins, which transport water to the leaves. Leaf vein density has been shown to limit water availability in the leaf when evaporation rates are high. The hypothesis that high vein density in angiosperms provides a physiological advantage rests on the ability of angiosperms to fix carbon dioxide from the atmosphere faster than non-angiosperms under variable water availability. Carbon isotope abundance ratios ($\delta^{13}C$) measured from plant matter indicate the magnitude to which a plant discriminates against heavier $^{13}CO_2$ during photosynthesis. Initially for this study, it was hypothesized that plants that produce leaves with higher vein densities (i.e. angiosperms) would be more depleted in ^{13}C, compared against plants with lower leaf vein density (i.e. ferns). By comparing characters of modern angiosperms against some of the earliest angiosperms from the same family, this study is an attempt to observe the evolution of leaf vein density in angiosperms over time, and to observe differences in carbon isotope fractionation between modern and old plant material. The geology and fossil record of the Cloverly Formation (Late Aptian – Early Albian), especially the recent finding of the oldest known angiosperms from western North America, are discussed. For the present study, both modern and fossil leaf specimens were analyzed for leaf vein density and carbon isotope ratios. Modern leaves from plants of *Platanaceae occidentalis* (sycamore), *Osmundrastrum cinnamomeum* (cinnamon fern), and *Juniperus virginiana* (conifer) were collected from Seneca Creek State Park in Maryland. Fossil leaf specimens of early angiosperm leaves in the family Proteaceae, and fossil conifers of Taxodiaceaewere were collected from the Cloverly Formation (Early Cretaceous). Results from leaf vein density measurements demonstrate a distinct difference between the modern ferns and the modern and fossil angiosperm specimens. The smaller difference in leaf vein density between modern and fossil angiosperms could be due to an evolutionary trend in increasing leaf vein density in angiosperms since the Early Cretaceous, or lack of good preservation in the fossil material. Differences in $\delta^{13}C$ values between modern plant material from closed and open canopies demonstrate the effects of differences in carbon isotope fractionation due to soil respiration and canopy type, and indicate that differences in $\delta^{13}C$ due to differences in leaf vein density are generally obscured by environmental factors. Future work suggested includes studies that would better constrain leaf vein density measurements, for example comparing cleared and un-cleared modern leaves against fossil leaves, and comparing the new LEAF GUI software against the traditional measurements made with ImageJ. Results from this study support the traditional view of early angiosperms being highly productive plants, provide insight into the hypothesis that higher leaf vein density is coupled with greater carbon isotope fractionation, and suggest a method of calculating $\delta^{13}C_{atm}$ during the Early Cretaceous from $\delta^{13}C_{plant}$ measured from fossil carbon and pCO_2 values estimated for the Early Cretaceous.

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I. Introduction

The ecological importance of angiosperms (i.e. flowering plants) has been accredited to the ability of dominant clades to photosynthesize at high rates relative to other plant groups. The hypothesis that the earliest angiosperms produced leaves with lower photosynthetic rates (Feild et al. 2003; 2004) challenges the traditional concept that depicts early angiosperms as highly productive plants (e.g. Hickey & Doyle 1977; Wing & Boucher 1998). Recent studies show that leaf vein density is low in basal angiosperms and infer that early Cretaceous angiosperms also had low vein density (Brodribb & Feild 2010; Feild et al. 2011). Vein density limits water availability in leaf tissue and thus limits photosynthesis. The evolution of high vein density among derived angiosperms may explain high productivity among angiosperms. A more in-depth paleontological and geochemical examination of the fossil record is required to develop a more thorough understanding of early angiosperm evolution.

Leaf vein density can impact the maximum capacity of photosynthesis in a leaf (Sack & Frole 2006; Brodribb *et al.* 2007; Noblin *et al.* 2008; Boyce *et al.* 2009) and the magnitude of carbon isotope fractionation in plants. During photosynthesis, atmospheric carbon dioxide is reduced to organic carbon, which is preferentially enriched in the lighter ^{12}C isotope. In C3 plants, the magnitude of carbon isotope fractionation within a photosynthesizing leaf is in part a function of stomatal conductance (i.e. diffusion limitation) and in part by enzymatic fractionation by the enzyme RuBisCO (Farquhar et al. 1989). In a leaf, stomatal conductance and water loss via transpiration are controlled by the opening and closing of stomata, which are pore-like structures on leaf lamina. When water supply from leaf veins to the leaf tissue cannot keep up with evaporative demand, the stomata close, and as photosynthesis proceeds water use efficiency will be higher (Farquhar et al. 1991). This means that among plants with high vein density in environments with high water availability, the stomata of the leaves remain open more often, allowing for a free exchange with atmospheric CO_2. In turn, transpiration is high, and water use efficiency is low. These plants take in more carbon dioxide from the atmosphere and fix less ^{13}C.

A stable isotope approach to this investigation of leaf vein density from Early Cretaceous plants is warranted because stable isotope analysis of plant matter allows measurement of the degree of carbon isotope fractionation in a leaf during photosynthesis, which is expected to be driven by water availability. Under similar environmental conditions, plant type can serve as a strong predictive tool for carbon isotope abundance in a leaf. For example, there is an observed 2.2 – 2.7‰ $\delta^{13}C$ difference between deciduous angiosperms and evergreen conifers (e.g. Diefendorf et al. 2010). This study tests the extent to which this difference in $\delta^{13}C$ between plant types holds true in the past by comparing modern angiosperms and non-angiosperms and analogous plants from the Early Cretaceous. Measurements of leaf vein density can be used as a proxy for water availability in a leaf. Leaf vein density measurements are made to estimate differences in water use efficiency between modern and Early Cretaceous angiosperms and non-angiosperms.

II. Background

The diversification of angiosperms during the mid-Cretaceous resulted in a radical transformation of Mesozoic terrestrial ecosystems, leading to the angiosperm role as the dominant group of terrestrial land plants (Hochuli *et al.* 2006). Although they were the last major group of land plants to colonize the continents (Crane *et al.* 1995), angiosperms have quickly grown to ecological dominance (Wing *et al.* 1993; Feild *et al.* 2003).

Evidence from the fossil record documents the diversification of angiosperms between 130 – 90 Ma (Crane *et al.* 1995), during which time the relative diversity of many non-angiosperm groups declined (Figure 1). However, there is a period of delay between the appearance of angiosperms in the fossil record and the time when they dominate fossil assemblages (Wing & Boucher 1998).

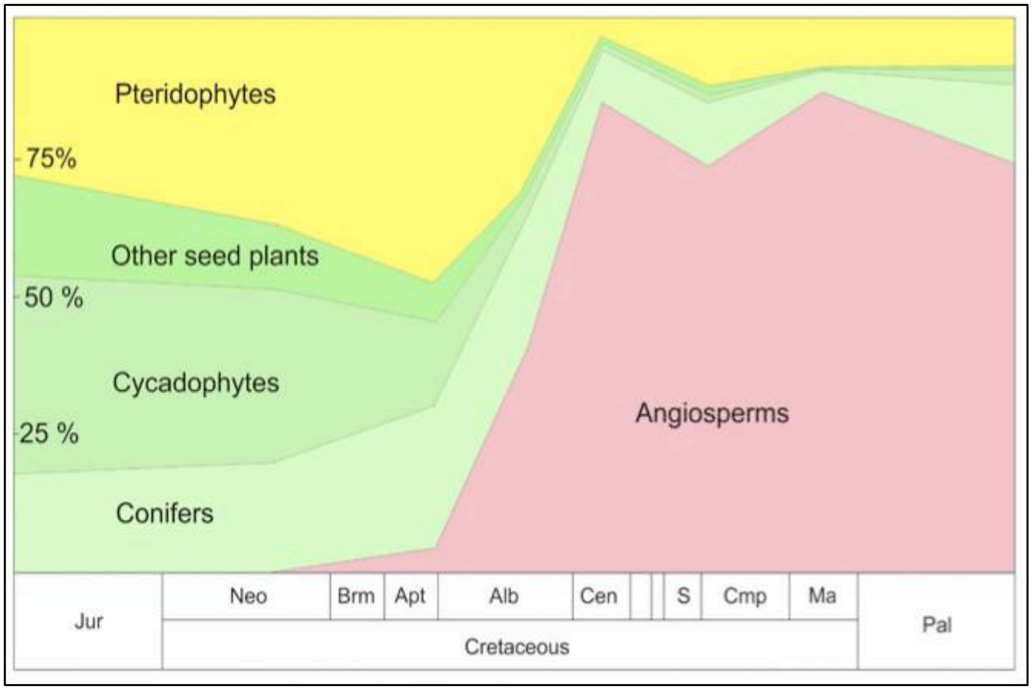

Figure 1. Change in the composition of fossil leaf floras from the Late Jurassic through Early Paleocene, showing the rapid radiation of angiosperms during the middle Cretaceous and the impact of angiosperm diversification on the relative density of other land plant groups. (Recreated from Lidgard & Crane 1988)

Photosynthetic capacity of angiosperms

The modern global dominance of angiosperms is often attributed to their high vegetative productivity, which is related to their ability to photosynthesize more efficiently than other plant groups (Brodribb & Feild 2010). The traditional concept of angiosperm evolution depicts early angiosperms as plants with high growth rates and fast generation times (Hickey & Doyle 1977; Wing & Boucher 1998), and suggests that early angiosperms had high productivity rates. However, recent studies suggesting that angiosperms originated as forest understory species with low photosynthetic capacity (Feild *et al.* 2004, 2009) radically challenge this classical view. This view implies that the photosynthetic capacity of angiosperms has increased after the initial radiation.

The maximum photosynthetic capacity of a leaf is regulated and limited chiefly by the supply of water available to the system. During oxygenic photosynthesis, enzymes within a leaf capture CO_2 from the atmosphere and then combine it with electrons from the splitting of H_2O, which reduces the carbon to carbohydrates and releases O_2 to the environment. To exchange these gases with the atmosphere, a leaf must open its pore-like stomata (Figure 2). Transpiration accompanies this gas exchange, leaking water vapor to the atmosphere through the open stomata. This stomatal conductance and water loss are related directly by the size of the stomatal aperture. To combat the lethal risk of dehydration, plants have evolved innovative features to increase water transport within a leaf during their evolutionary history (Sperry 2003). Of these traits, leaf vein density is most relevant to this study.

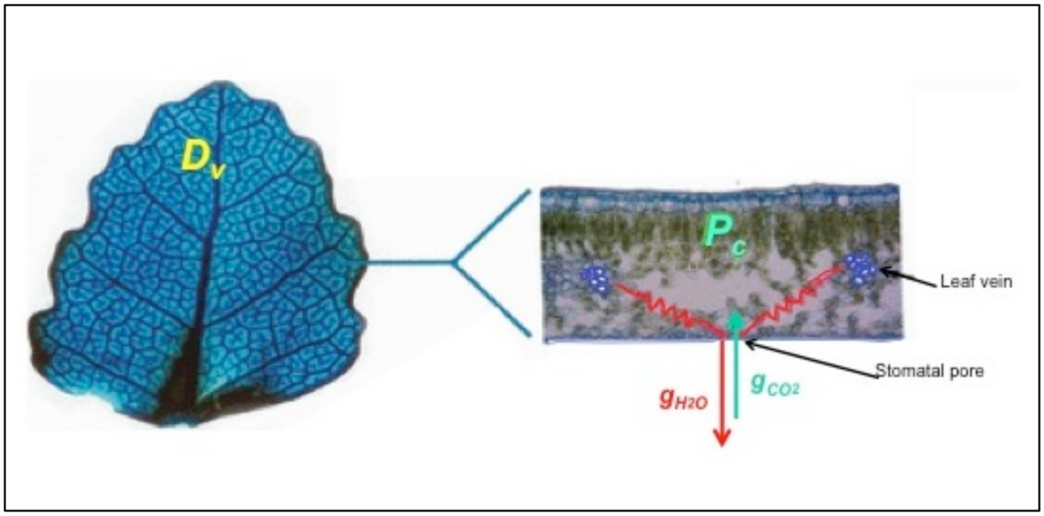

Figure 2. A cartoon depicting typical leaf vein density (D_v) of an angiosperm, and how photosynthetic capacity (P_c) is limited by water availability. While passing from the ends of the veins to the stomata, the transpiration stream (red arrows) encounters resistance flow, dependent upon hydraulic distance from vein endings to the stomata. Hydraulic efficiency is determined by D_v, and in turn limits photosynthetic capacity. The green arrow shows intake of CO_2 from the atmosphere (g_{CO2}). (Modified from Brodribb & Field 2010)

Leaf vein density

Leaf veins provide an efficient irrigation system within the vascular architecture of a leaf. There are two types of vascular tissue: xylem, which transport water and solutes from other parts of the plant to the leaf lamina, and phloem, which export the carbohydrates produced during photosynthesis (Roth-Nebelsick *et al.* 2001). The density of veins in the leaf lamina is correlated as a measure of maximum capacity for photosynthesis (Sack & Frole 2006; Brodribb *et al.* 2007; Noblin *et al.* 2008; Boyce *et al.* 2009), and are a possible limiting factor of water transport in leaves (Brodribb *et al.* 2007). Work completed by Boyce *et al.* (2009) further demonstrates the link between leaf veins and stomatal openings, suggesting that high leaf vein densities facilitate high transpiration rates by allowing the stomata to be open for longer periods of time without dehydration.

The density of leaf veins (mm mm^{-2}), defined as the length of veins branching in a given surface area of leaf lamina, varies between plant groups. Modern angiosperms exhibit a highly branched network of xylem (Figure 2), which leak water to the stomata (Zwienecki *et al.* 2002). Ferns use a more simply branched vascular system to irrigate fronds (Brodribb *et al.* 2005), and generally have a lower leaf vein density than angiosperms. In contrast, most conifers possess a simple vascular network with a single vein and narrow leaves. With a low vein length and low leaf area, it is difficult to compare conifers as having high or low leaf vein densities in comparison to angiosperm or fern plants. Flowering plants' high leaf vein density enables them to achieve unparalleled rates of transpiration (Boyce *et al.* 2009), allowing for higher rates of primary productivity. Phylogenetic evidence and the fossil record demonstrate that high leaf vein densities are unique to crown group angiosperms, which first appear in the fossil record of the Early Cretaceous (Boyce *et al.* 2009). This evolution of leaf vein density portrays a unique development in the nearly 400 million-year history of leaf evolution.

In the field of paleobotany, leaf architecture and vein density have been used traditionally as taxonomic characters (i.e. to identify and classify leaf fossils). More recent leaf vein density measurements of fossil plants have been used to provide insight into the impact these plants had upon their environments (Boyce *et al.* 2009). Although commonly used in systematics, vein density of leaves has been demonstrated to be a variable character in modern leaves, influenced by both climatic and environmental factors (Uhl & Mosbrugger 1999). This ability of angiosperms is an evolutionary innovation and advantage that no other land plants possess.

The diversification of angiosperms and the evolution of many modern orders of angiosperm clades occurred rapidly during the Aptian – Albian (late Early Cretaceous) transition. The rise of angiosperm productivity and increasing contribution to total plant biomass, however, is not well constrained temporally. Using ancestral state reconstruction and molecular phylogeny of modern angiosperms, Brodribb and Feild (2010) reconstructed the evolution of angiosperm vein density among key extant angiosperm phylogeny (Figure 3). This reconstruction suggests that the key innovation(s) allowing for high vein density ($>\sim 5$ mm mm^{-2}) in most angiosperms evolved with the origin of the Mesangiospermae (eudicots, magnoliids, and monocots) in the Late Barremian – Aptian, and that angiosperms had achieved widespread ecological dominance by the Albian – Cenomanian boundary. This hypothesis should be subject to explicit testing against the fossil record. To date, only one study has published Early Cretaceous angiosperm vein density data (Feild *et al.* 2011).

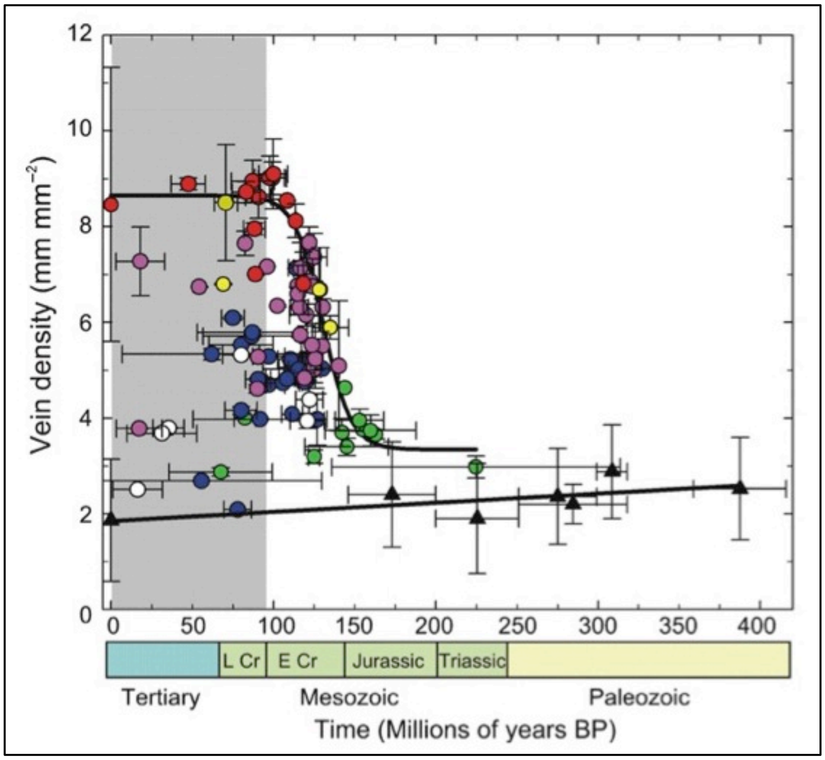

Figure 3. Projected surge of leaf vein density of angiosperms during the Cretaceous, reconstructed for key nodes (circles) of extant angiosperm phylogeny, compared against non-angiosperm vein density values (triangles). The nodes included in this reconstruction include: basal eudicots (blue circles), basal-most lineages (green circles), Chloranthales (white circles), core eudicots (red circles), magnoliids (pink circles), and monocots (yellow circles). (Brodribb & Feild 2010)

Carbon isotope discrimination in leaves

Atmospheric carbon dioxide is composed of the two natural stable isotopes of carbon, ^{12}C and ^{13}C, in different amounts. Lighter $^{12}CO_2$ molecules are considerably more abundant given that 98.89% of all carbon is in the ^{12}C isotope. Terrestrial plants contain less ^{13}C than the atmospheric carbon dioxide, and are thus "depleted" of ^{13}C relative to the atmosphere. This depletion occurs while the plant incorporates atmospheric CO_2 into the biomass during photosynthesis, and alters the ratio between the two stable carbon isotopes (Farquhar et al. 1989), commonly expressed as *R*,

$$R = \frac{^{13}C}{^{12}C}. \qquad \text{Eq. 1}$$

In geochemistry applications, *R* of the sample is commonly compared against *R* of the standard Vienna Pee Dee Belemnite (V-PDB), and expressed in terms of the isotope ratio $\delta^{13}C$,

$$\delta^{13}C = \left[\frac{R_{sample}}{R_{standard}} - \frac{R_{standard}}{R_{standard}}\right] * 1000\,. \qquad \text{Eq. 2}$$

$\delta^{13}C$ is expressed in per mille (‰), or parts per thousand. Organic matter is consistently depleted in ^{13}C compared to V-PDB, and according to Equation 2, when $R_{sample} < R_{standard}$, $\delta^{13}C$ will be a negative value. Accordingly, a less negative $\delta^{13}C$ measured from plant matter means that the sample is more enriched in ^{13}C, or 'heavier,' and closer to the $\delta^{13}C$ value of the standard, and hence the plant fractionated less during photosynthesis.

Geochemists interested in the natural variation of stable isotopes spurred the initial push for measuring plant isotopic composition. Early systematic measurements of carbon isotope abundances of plants (Wickman 1952; Craig 1953) yielded a $\delta^{13}C$ range of -15‰ to -33‰, which prompted future efforts to understand the processes that regulate fractionation during photosynthesis (Park & Epstein 1960). Measuring carbon isotope abundance in plants has since evolved into a powerful tool for botanical studies, and has become a standard means for distinguishing between the photosynthetic pathways used by different plants: the Calvin Cycle (C3), Hatch-Slack cycle (C4), and Crassulacean acid metabolism (CAM) pathways. Isotope discrimination varies among plants using different photosynthetic pathways, resulting in varied $\delta^{13}C$ values of plants using these different pathways (O'Leary 1981). However, lesser variations are observed among species using the same pathway. For example, $\delta^{13}C$ for C3 plants ranges between -30‰ to -22‰. The range in values in the C3 plants is the result of different physiological and environmental factors, which impact this study of modern and Early Cretaceous plants.

Photosynthetic fractionation of carbon isotopes in C3 plants

To fully understand why C3 plants are generally depleted in the heavier ^{13}C isotope compared to atmospheric CO_2, it is vital to consider the mechanisms that drive carbon isotope fractionation. Isotopes are variants of a given element that have different masses based on the count of neutrons in the nucleus. In the case of carbon, ^{13}C contains six protons and seven neutrons, whereas ^{12}C contains six protons and only six neutrons. Isotope effects are differences in the physiochemical properties of molecules caused by isotopes. Within plants, carbon fractionation occurs due to differences in rates of the processes and reactions in which each carbon isotope participates. This isotope effect, denoted as α, can be described as,

$$\boldsymbol{\alpha = \frac{R_{atm}}{R_{product}} = \frac{k^{12}}{k^{13}}}, \qquad \textbf{Eq. 3}$$

where the constants k^{12} and k^{13} refer to the reaction rates of ^{12}C and ^{13}C, respectively (O'Leary 1980; Farquhar *et al.* 1989).

Farquhar and Richards (1984) propose the use of $\Delta^{13}C$ as the measure of carbon isotope discrimination, or fractionation, by the plant,

$$\boldsymbol{\Delta^{13}C = (\alpha - 1) * 1000 = \left(\frac{R_{atmosphere}}{R_{product}} - 1\right) * 1000}. \qquad \textbf{Eq. 4}$$

Unlike $\delta^{13}C$, $\Delta^{13}C$ is not measured against a standard. During carbon fractionation in plants, the lighter isotopic species reacts more rapidly than the heavier species, resulting in a positive fractionation ($\Delta^{13}C$) value. The value of $\Delta^{13}C$ will be more positive with greater discrimination against ^{13}C. The values for $\Delta^{13}C$ and $\delta^{13}C$ are related as,

$$\boldsymbol{\Delta^{13}C = \frac{\delta^{13}C_{atm} - \delta^{13}C_{plant}}{1 + \delta^{13}C_{plant}}}. \qquad \textbf{Eq. 5}$$

Fractionation of CO_2 within plants occurs during two major steps: diffusion of CO_2 through the air and discrimination during enzymatic fractionation. Figure 4 illustrates these two main fractionation effects within a plant. The slower motion of the heavier ^{13}C-bearing CO_2 molecules results in an isotope effect quantified as $\alpha_{diffusion}$ = 1.044 (Craig 1954). Although diffusion through air is a contributing fractionating factor, enzymatic discrimination of ^{13}C accounts for the majority of isotope fractionation within a leaf. The carboxylating enzyme ribulose-1,5-biphosphate carboxylase oxygenase (RuBisCO), involved in the first major step of carbon fixation within a plant, reacts more readily with $^{12}CO_2$ than it does with $^{13}CO_2$. This discrimination effect is quantified as $\alpha_{fixation}$ = 1.027 (Farquhar & Richards 1984). Various other fractionation effects occur, but diffusion and enzyme discrimination account for most ^{13}C discrimination within a leaf.

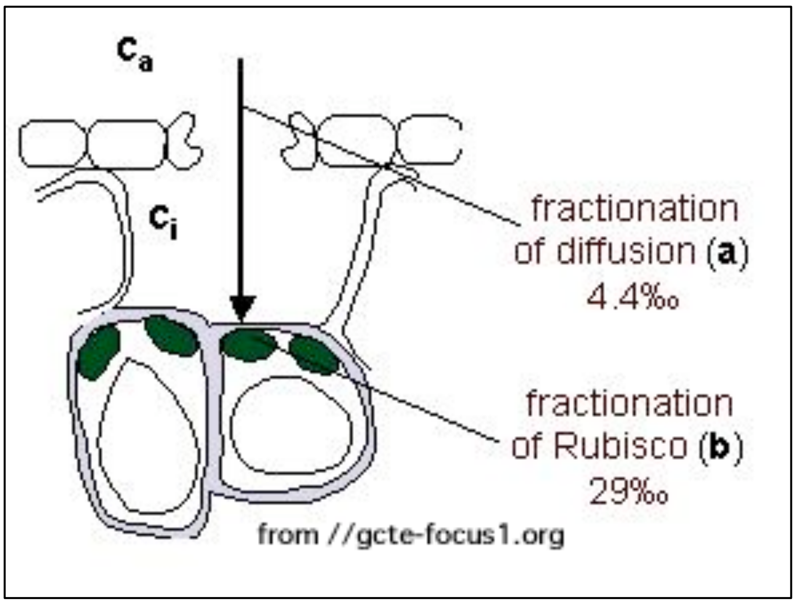

Figure 4. Cartoon diagram depicting a cross-section of the stomata of a C3 plant, illustrating the two main fractionation effects of CO_2. (Source *biology.duke.edu*)

Multiple equations have been developed to incorporate the effects of fractionation during C3 photosynthesis. Farquhar *et al.* (1982) formulate the effects as:

$$\boldsymbol{\Delta^{13}C = a\left[\frac{\rho_a - \rho_i}{\rho_a}\right] + b\frac{\rho_i}{\rho_a} = a + (b-a)\frac{\rho_i}{\rho_a}}. \qquad \textbf{Eq. 6}$$

where a is the constant for fractionation due to diffusion through air, b is the constant caused by the RuBisCO enzyme, and ρ_a and ρ_i (also commonly given as C_a and C_i) represent the atmospheric and intercellular partial pressures of CO_2, respectively, given in parts per million (ppm).

Factors controlling carbon isotope abundances in leaves

The magnitude of carbon isotope fractionation by a plant can vary due to physiological processes that modify $\delta^{13}C$ and environmental conditions that control water-use efficiency and the source of atmospheric carbon dioxide to the plant. According to Equation 6, any factors controlling the atmospheric or intercellular partial pressure of CO_2 will impact the magnitude of fractionation.

Physiological processes

Stomatal conductance is related to isotopic fractionation within a leaf through the Farquhar *et al.* equation (Equation 6). When stomatal conductance is low (i.e. the stomata are more closed), ρ_i is low. This drives the extent of enzymatic fractionation to near zero, causing $\Delta^{13}C$ to tend toward *a*, or 4.4‰. Alternatively, when stomatal conductance is high, ρ_i approaches ρ_a, and $\Delta^{13}C$ approaches the larger value of *b*, or 27‰ (Farquhar & Sharkey 1982; Farquhar et al. 1989).

The carbon isotopic composition of a plant can be expected to be driven partially by the density of veins throughout the leaf. With a greater density of leaf veins, the water supply is closer to the sites of transpiration and water loss (stomata), allowing for a greater stomatal conductance (Farquhar et al. 1991). As discussed above, a leaf with open stomata is able to exchange CO_2 more freely with the atmosphere, resulting in a greater $\Delta^{13}C$. The increased opportunity for discrimination results in a greater depletion of ^{13}C and a more negative $\delta^{13}C$ value for the leaf tissue, whereas low stomatal conductance will result in a less negative $\delta^{13}C$ value. Because vein density serves as the limiting factor for water supply to the photosynthetic tissue, a leaf with higher vein density (e.g. an angiosperm) is expected to have a more isotopically light composition than a leaf with lower vein density (e.g. a fern).

Water use efficiency

Carbon isotope abundances in plants have also been demonstrated as a function of water-use efficiency of a plant. From testing desert plants, Ehleringer and Cooper (1988) discovered that carbon-13 enriched plants are found in water-stressed environments, indicating that water-use efficiency increases as soil water availability decreases. A water-stressed environment, such as an open canopy, is expected to produce ^{13}C-enriched plants compared to a closed canopy. A study by Cerling *et al.* (2004) confirms variation in $\delta^{13}C$ values of plants as a function of canopy position: -29.0±1.7‰ (canopy top), -30.4±0.9‰ (gaps within the canopy), and -34.0±1.5‰ (subcanopy).

Source of carbon dioxide

The composition of the carbon dioxide used by plants for photosynthesis impacts the magnitude of carbon isotope fractionation. Due to low $\delta^{13}C$ values of litter being decomposed, CO_2 originating from soil respiration is depleted in ^{13}C. If this CO_2 is fixed by the flora before it mixes with the atmospheric CO_2, organic matter produced by these enclosed flora are expected to have lower $\delta^{13}C$ values than upper canopy trees with greater carbon dioxide mixing (Medina & Minchin 1980). Initial studies exploring the effect of atmospheric carbon dioxide examined closed-canopy forests and show that foliar $\delta^{13}C$ increases with canopy height (Marshall *et al.* 2007).

Atmospheric CO_2 over time

To reconstruct paleoclimate data from carbon isotope abundances from fossil plant carbon, changes in atmospheric carbon dioxide over time must be considered. Over the past century, atmospheric concentration of CO_2 has increased by 30% (Marshall *et al.* 2007). Because the atmosphere is so well mixed, global averages can often be used to infer $\delta^{13}C_{atm}$. As of October 4, 2011, atmospheric CO_2 is measured at 388.92 ppm (Mauna Loa Observatory, Tans & Keeling). Although less precise and not as well constrained as modern measurements, multiple methods are used to estimate atmospheric CO_2 during the Cretaceous. However, there is little understanding of past $\delta^{13}C_{atm}$ values. Estimated pCO_2 values during the Cretaceous are given in Table 1.

Table 1. Estimated values of pCO_2 during the Cretaceous.

Age (Ma)	pCO_2 estimate (ppm)	Method/Technique	Source
~115	~900	Fossil soils	Royer 2010
~114	~700	Stomatal index	Royer 2010
~107	~700	Stomatal index	Royer 2010
~105	~600	Fossil soils	Royer 2010
~100	~1,130	$\delta^{13}C$ analyses of bryophyte fossils, theoretical modeling	Fletcher *et al.* 2008
85.8	~661	Stomatal index	ChuanBiao *et al*. 2011
83.5	~560	Stomatal index	ChuanBiao *et al*. 2011

Geologic Setting

When examining the early history of angiosperms, it is crucial to consider geographic differences between the Early Cretaceous and the present. By the middle Cretaceous, continental rifting and seafloor spreading that began during the Late Triassic created a nearly equatorial mid-continent seaway (Figure 5). The Tethys Sea separated the continents of Laurasia and Gondwana (cf. Raven & Axelrod 1974; Hickey & Doyle 1977). During the Early Cretaceous, the Sevier Orogeny in western North America caused uplift in the region currently known as western Wyoming (Love et al. 1947). The rocks of the Cloverly Formation record the deposition of nonmarine sediments from the Sevier orogeny in the Cordilleran forebasin in northern Wyoming and southern Montana (Figure 5) (Wilborn 2008), which occurred during the earlier stages of uplift of the Rocky Mountains (Zaleha 2006).

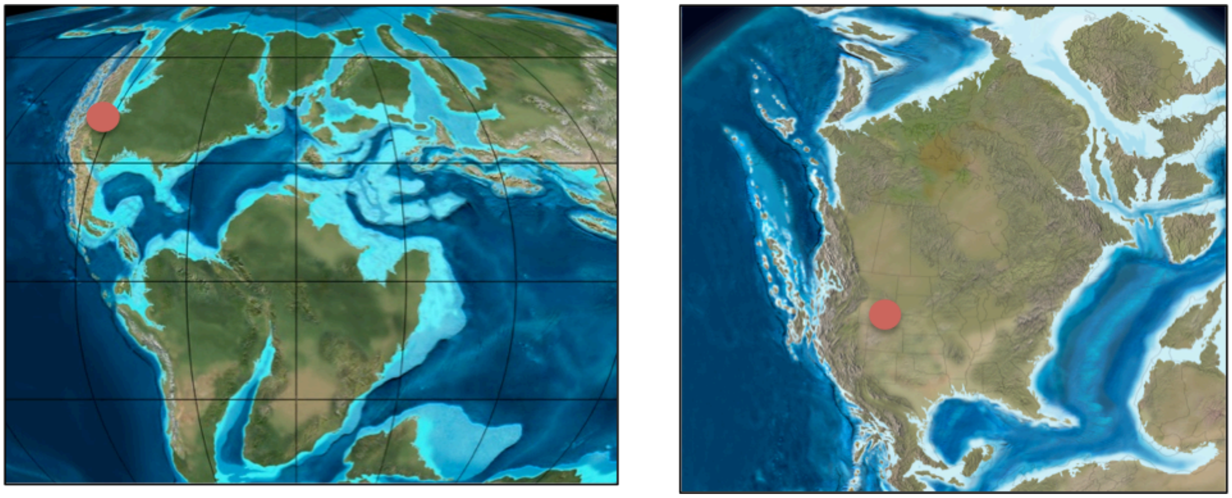

Figure 5. *Left:* Paleomap reconstruction showing spatial relationship of paleocontinents Gondwana and Laurasia to the Tethys Sea during the Early Cretaceous (~120 Ma). *Right:* Paleomap reconstruction of Laurasian paleocontinent during the Early Cretaceous (~130 Ma). Red circles indicate approximate location of the Cloverly Formation. (Source *Ron Blakey, NAU Geology)*

Multiple stratigraphic models attempt to organize the sediments of the Cloverly Formation (Wilborn 2008). The sediments of the Cloverly Formation range in thickness between 20-80 meters, which have been subdivided by different workers (Moberly 1960; Ostrom 1970; Zaleha 2006) into different intervals or members. Moberly (1960) recorded the Pryor Conglomerate Member at the base of the formation in most regions, and identified the "Lower Little Sheep Mudstone" as mudstones with volcanic debris below a large conglomerate unit in the southern Bighorn Basin. Overlying the Little Sheep Mudstone is the Himes Member, a mix of red-rellow-brown mudstones and sandstone channel fills (Moberly 1960). Ostrom (1970) subdivides the Cloverly Formation into intervals. Interval IV is the Pryor Conglomerate, V is the Little Sheep Mudstone, VI is the lower Himes Member, and VII is the upper Himes Member. Zaleha (2006) reports interval A as a conglomerate layer near the base of the formation (i.e. the Pryor Conglomerate) and the lower half of the Little Sheep Mudstone. Zaleha's interval B is the top of the Little Sheep Mudstone, and C is the upper part of the Himes Member.

The Pryor Conglomerate Member and some basal conglomerates of the Little Sheep Mudstone Member are characterized as a conglomerate-bearing alluvial system, interpreted as lacustrine and flood deposits. The remainder of the Little Sheep Mudstone Member is characterized as a fluvial system, dominated by sandstones and conglomerates. Mudstones, diamictites, and wackes generally comprise the top interval of the Cloverly Formation, which corresponds to the uppermost portion of the Little Sheep Mudstone Member and the lowermost part of the Himes Member in the Bighorn Basin in Wyoming (Moberly 1960; Ostrom 1970; Zaleha 2006).

The general stratigraphy of the Cloverly Formation is relatively straightforward, but the exact placement of the unconformity with the underlying Morrison Formation has long been a point of contention. Ostrom (1970) cites paleontological data to assert that the Cloverly Formation is Late Aptian to Early Albian in age. Since Ostrom's early publication, radiometric dating has helped to constrain the ages of the Cloverly Formation (Figure 6), and the duration of the unconformity.

The Cloverly Formation is well known for its diverse vertebrate fossil record, but megaflora and palynoflora fossils from the Cloverly are poorly explored and little understood (Wilborn 2008). Recent discoveries by Jud (2010) indicate the presence of angiosperm macrofossils within the Cloverly Formation (Figure 6) from 115-106 Ma, which are from the lag period between the appearance of angiosperms in the fossil record and the time when they dominate fossil assemblages (Wing & Boucher 1998). These spectacular fossils (Figure 6) represent the oldest known angiosperm macrofossils from western North America. Because they are from this lag period in angiosperm diversification, it might be suspected that these fossils would have low leaf vein densities compared to their modern analogs.

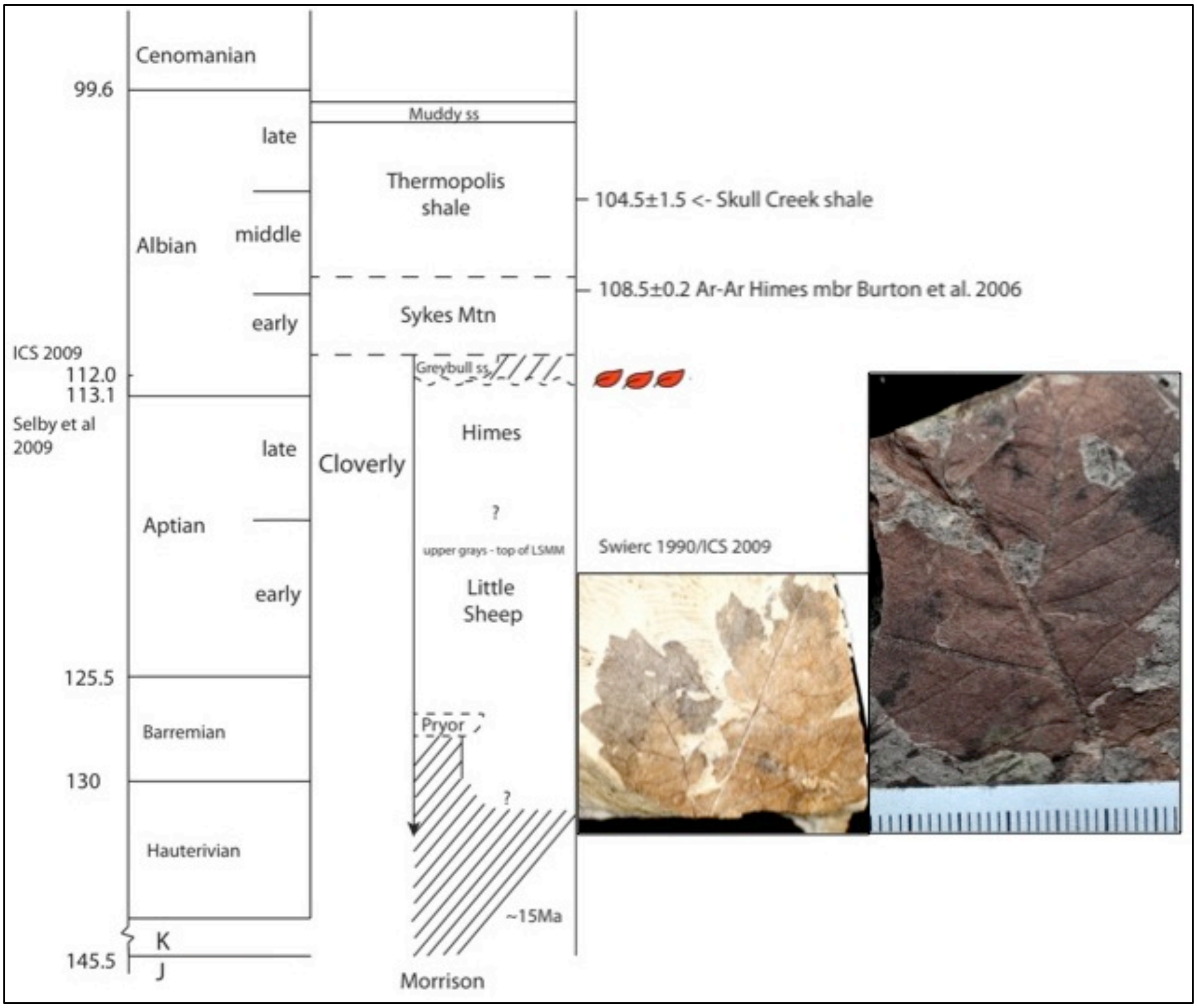

Figure 6. Stratigraphic column of the Cloverly Formation, with known age constraints, and photographs of angiosperm macrofossils recovered from the Cloverly Formation. Red cartoon leaves denote stratigraphic horizon identified with plant megafossils during Jud 2010 field season. (Source: Jud, unpublished)

III. Materials and methods

Specimen collection

Modern study site

Modern leaves were collected from Seneca Creek State Park located in Gaithersburg, Maryland in Montgomery County. Clopper Lake, a 90-acre impoundment on Long Drought Branch maintained by a dam, is a central attraction at the park. Leaves for this study were collected during over different days from trees growing along trails leading around the edge of Clopper Lake.

Leaves from the first period, April 2011, were collected and tested to gather data to demonstrate feasibility for this proposed study. Fiddleheads of the fern *Osmundrastum cinnamomeum*, fully-developed needles of conifer *Juniperus virginiana*, and buds of the angiosperm *Platanus occidentalis* (sycamore) were collected for carbon isotope analysis.

During the second collection trip at the end of August 2011, leaves of the fern *Osmundrastum cinnamomeum*, conifer *Juniperus virginiana*, and the angiosperm *Platanus occidentalis* were collected, with a focus on comparing ferns and conifers against sycamores growing in the same environment type. Leaves were collected from the sycamore tree at a height of ~1.5 meters from the ground. The environmental controls focused primarily on water access to the plant, so leaves were collected only from plants that were close to the lakeshore.

The final collection was undertaken in mid-October to create an environmental reconstruction, observing the variation of $\delta^{13}C$ of *Osmundrastum cinnamomeum* and *Platanaceae occidentalis* across a variety of niches. Parameters recorded during collection include: age of tree (diameter at ~1 meter height), proximity to lake margin, elevation above lake level, proximity to stream, open or closed canopy. Specimens were collected from plants under different canopy conditions: open canopy, gap canopy, and closed canopy.

Fossil collection site

The geology of the Lower Cretaceous, as reflected in the sediments deposited in the state of Wyoming, is particularly pertinent to this study. Because the sediments of the Cloverly Formation have been recognized to contain the oldest known angiosperm macrofossils known from western North America (Jud 2010), leaf vein density and carbon isotope abundance measurements for this study were made from fossil plants recovered from this formation during the Summer 2010 and 2011 field collection seasons. The angiosperm macrofossils are from the Himes Member of the Cloverly Formation (Figures 7 & 8), and are estimated to be approximately 115-106 million years old. These leaves belong to the order Proteales, basal Mesangiospermae/eudicots, which includes the modern sycamore plant. Fossilized conifer needles were also collected from the family Taxodiaceae, *Sphenolepsis* sp. or *Sphenolepsis* cf. *condita*.

Figure 7. Outcrop of the Himes Member of the Cloverly Formation (outlined with yellow stipples), from which fossil specimens were collected. (Source: Spencer Schecht)

Plant fossils were collected from a well-lithified, medium gray siltstone of the Himes Member (Figure 7). The rocks are small-scale irregular and cross-laminated with interlaminae of light gray tuffaceous siltstone. Plant debris occurs throughout the layer, with identifiable but incomplete leaves, suggesting some minimal transportation. Hundreds of plant fossils were collected, and after sifting through the collection at the Smithsonian Institution National Museum of Natural History (NMNH), only a few specimens were determined to be appropriate for this study. Four fossil leaves were identified that retain excellent preservation of high- order leaf vein density, but do not preserve organic carbon. Seven fossil angiosperm and five fossil conifer specimens were identified for carbon isotope testing.

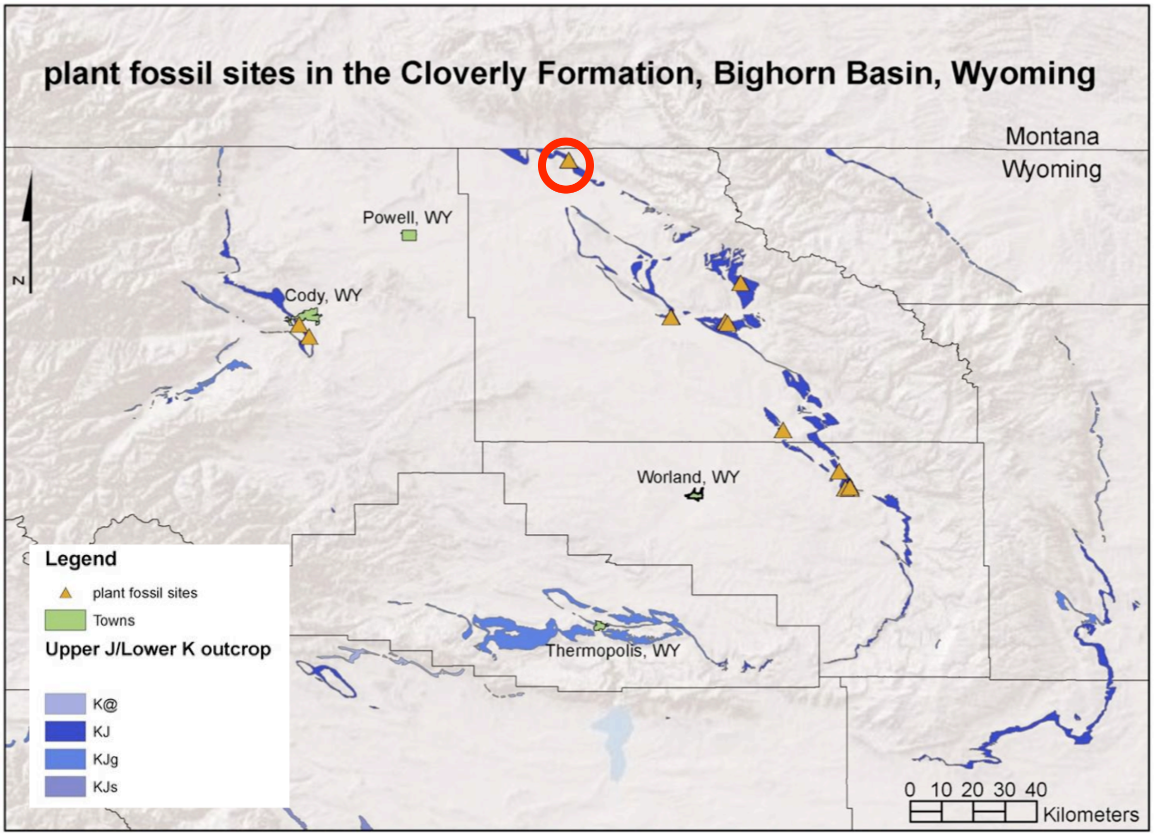

Figure 8. Map of Upper Jurassic and Lower Cretaceous outcrops in the Bighorn Basin, WY. Plant fossil collection sites of Jud 2010 field season marked as brown triangles, with red circle to the North indicating the general outcrop area from which the fossils for this study were recovered. (Source: Nathan Jud)

Leaf vein density measurements

Leaf vein density (D_v) is defined as the length of veins (mm) divided by the surface area of leaf lamina (mm^2). Photographs were taken of modern leaves collected in August and of appropriate fossil leaf specimens with a digital camera connected to a high-powered microscope and computer. D_v was measured from these digital images with ImageJ (NIH Image, Bethesda, MD, USA). ImageJ is an image-processing program developed at the National Institutes of Health and provided free of charge, and is commonly used for vein density measurements. D_v was measured from multiple 4 mm^2 boxes of the leaf lamina from images of both modern and fossil leaf specimens.

Carbon isotope measurements

Sample preparation

To prepare fossil specimens for carbon isotope analysis, preserved organic carbon was separated from the carbonaceous compression in the rock with clean steel needles. This carbon extraction was completed in the Department of Paleobiology at the Smithsonian Institution NMNH. Scraped carbon was collected into envelopes folded from weighing paper or plastic centrifuge tubes for transportation to the stable isotope geochemistry laboratory at the University of Maryland (UMD). Approximately 50 µg of each fossil sample were weighed into a tin cup, which was crushed and folded into a sphere and placed into a loading tray.

Plant matter of modern leaves required different steps for preparation for carbon isotope testing. Tissue from modern leaves was placed in plastic centrifuge tubes, covered in vented aluminum foil, and stored in the utility oven (80ºF, ~26ºC) located in the stable isotope laboratory at UMD for 2-3 days time to remove excess water from the samples. Once dried, the modern leaves were crushed with a mortar and pestle into a homogeneous powder. Results from testing $\delta^{13}C$ of modern leaves for the feasibility study indicate that measuring ~40 µg of each sample produces an acceptable signal and peak height for a reliable measurement. The powders were measured into tin cups, which were crushed and folded into a sphere and placed into a loading tray.

Testing samples with the mass spectrometer

Carbon isotope values of fossil and modern leaf samples were measured by combusting the powdered plant fragments in a EuroVector® elemental analyzer (EA) coupled with an Elementar IsoPrime® continuous flow (CF) gas source mass spectrometer. The CF mass spectrometer must be tuned and calibrated before an analytical session. Reference CO_2 is introduced into the system to determine the ideal position of the ion beam, and the accelerating voltage is adjusted to produce a stable signal. A pressure test must be run to ensure no gas leaks are present in the combustion system of the EA. Also vital is a reference gas stability test, which determines the consistency of the flow rate of the gas and ensures that the reference gas box and Faraday collector cups are functioning properly, and that consistent peak heights are being output to the computer.

After tuning and calibration, the crushed tin sample packets are placed into the loading carousel, which drops each sample individually into the combustion column of the EA. The combustion column is a 45 cm glass column packed with quartz wool, silica chips, and chromium oxide. In this column, the sample combines with a pulse of O_2 and flash-combusts at 1040ºC, converting all carbon into carbon dioxide. A stream of non-reactive pure helium gas carries the combustion products through the reduction chamber (650ºC) at a rate of approximately 100 mL/minute, where the products are carried through the reduction column. This is another glass column layered with quartz wool, silica chips, and fine copper wires. The helium gas carries the combustion products from the reduction chamber through a water trap containing $Mg(ClO_4)_2$ (magnesium perchlorate) and into the gas chromatograph. The gas chromatograph separates the CO_2 from other combustion products. The EA outputs the CO_2, along with the helium carrier gas, into the source of the continuous flow mass spectrometer.

Upon entering the mass spectrometer, the carbon dioxide gas passes an ion source (an electrically heated thorium filament), which bombards the CO_2 molecules by a stream of electrons. This bombardment generally knocks one electron out of the sample particles, forming singly positive ions (CO_2^+). The ions are repelled by a positively charged metal plate, and thus pass into the flight tube. Charged plates accelerate the ionized molecules along the flight tube, where a strong magnetic field bends the path of each ion, deflecting the lighter ions more than the heavier ions. These paths lead the ions into separate Faraday collecting cups, and, as each ion collides with the collector cup, the integrated computer records it as an electrical pulse. The integrated computer compiles the data in a Microsoft® Excel file, calculates the abundances of carbon isotopes, and reports them relative to the standard Vienna Pee Dee Belemnite (V-PDB) using standard δ-notation:

$$\boldsymbol{\delta^{13}C} = \left[\frac{\left(\frac{^{13}\boldsymbol{C}}{^{12}\boldsymbol{C}}\right)_{sample}}{\left(\frac{^{13}\boldsymbol{C}}{^{12}\boldsymbol{C}}\right)_{standard}} - \frac{\left(\frac{^{13}\boldsymbol{C}}{^{12}\boldsymbol{C}}\right)_{standard}}{\left(\frac{^{13}\boldsymbol{C}}{^{12}\boldsymbol{C}}\right)_{standard}}\right] * \mathbf{1000}\,. \qquad \text{Eq. 7}$$

Analysis of uncertainty

Error analysis for vein density measurements

To calculate the variance associated with leaf vein density measurements, each square was measured four different times, with the digital image rotated 90º to the right before each iterative measurement. The average value of these four iterations and the associated standard deviation was calculated using Microsoft® Excel. This calculated variance captures the error involved with tracing the leaf veins using the computer mouse, and also reflects on the distinguishability of the leaf veins within each image.

Other sources of error associated with leaf vein density measurements include inconsistent leaf vein preservation in fossils and the inability to distinguish veins on digital images captured. The ability to accurately measure leaf vein density across different forms of leaf preservation (i.e. a leaf bleached and cleared to distinctly show the veins, an unbleached modern leaf, and a fossil leaf impression) could also introduce underestimation of D_v. A discussion of a suggested method for quantifying this source of uncertainty is included in Section VI.

Uncertainty for carbon isotope measurements

To determine the uncertainty and offset of isotope ratios in each analytical session with the EA and the CF mass spectrometer, the plant matter is run in comparison to the NIST standard (NBS 912a) Urea, the established $\delta^{13}C$ for which is -29.39‰ The Urea standard is ideal for correcting isotopic values for modern and ancient leaf material because the samples have $\delta^{13}C$ values very similar to that of the standard. The standard is measured in amounts of ~90 µg into tin cups, crushed and placed into a loading tray with the organic material samples. The batch of samples is preceded by four to six Urea standards, followed by groups of ten samples and two standards, and thus repeated until the end of the run, concluded with four to six Urea standards.

Once the raw data are collected, they are corrected based on the outcomes of the analyses of the standards. The average and standard deviation of the isotopic composition of all standard measurements are calculated. The difference between the calculated average isotopic composition and the established isotopic abundance value ($\delta^{13}C$) for Urea (-29.39‰) is calculated, which is the offset correction factor for the isotopic abundance values measured for each sample. This offset factor is added to each raw isotopic abundance value, producing the corrected value. The calculated standard deviation of isotopic abundance from the standard values provides the 1σ uncertainty of the respective measurements for each sample. Appendix B contains an example of a calculation done for correcting carbon isotope abundance data. All values were calculated with Microsoft® Excel.

Peak heights

The mass spectrometer records the peak height of ions measured for each specimen. Multiple samples of the standard Urea were prepared at different weights to produce peak heights with the known Urea standard that are similar to those associated with the specimens that often produce low peak heights (i.e. fossil samples), in order to observe the precision of $\delta^{13}C$ measurements associated with these low peak heights (Figure 9). Based on the results of these measurements, only samples with peak height >0.5 nA are reliable.

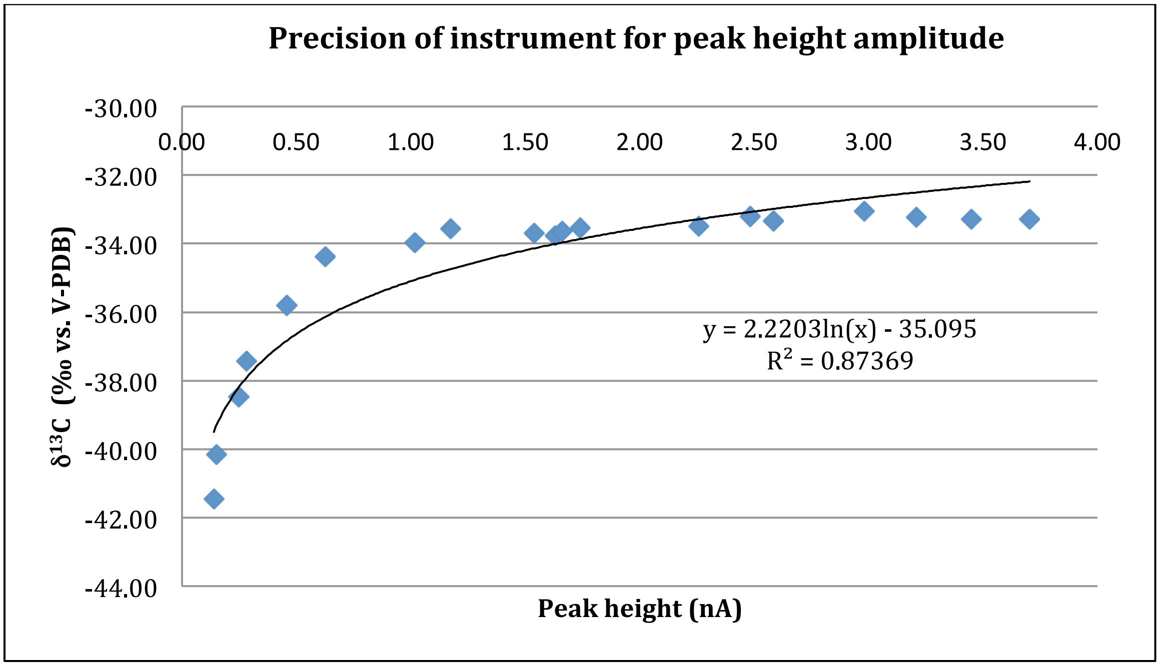

Figure 9. Precision of CF mass spectrometer when measuring samples with varying peak heights, indicating that the accuracy of the mass spectrometer decreases logarithmically at peak heights > 0.5 nA, and are not reliable.

IV. Results

Leaf vein density

D_v measured from *Platanus occidentalis* (sycamore), *Osmundrastrum cinnamomeum* (fern), and fossil angiosperms are presented in Figure (10). Standard deviation varies for measurements of each plant type: ±0.29 for modern sycamores, ±0.45 for fossil angiosperms, and ±0.06 for modern ferns (Figure 10). D_v determined from modern sycamores has a wide range from ~4.5 to ~7 mm mm^{-2}, but does not demonstrate a significant difference from D_v measured from fossil angiosperms. Ferns demonstrate significantly lower D_v than either of these angiosperms.

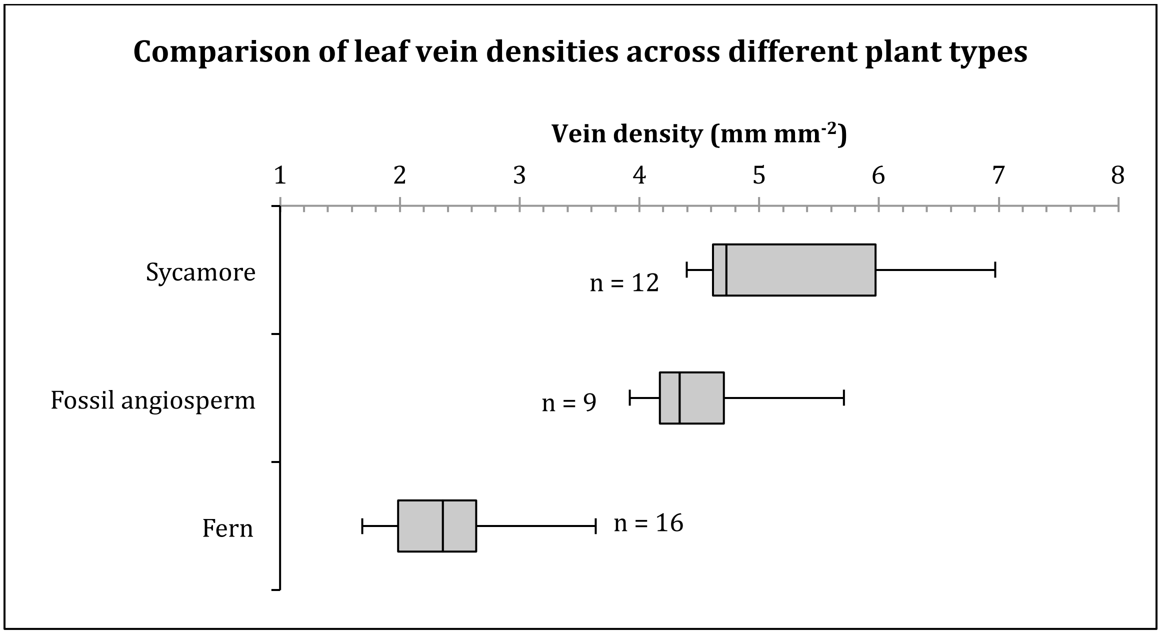

Figure 10. Box-and-whisker plot of average leaf vein densities measured of modern sycamores, fossil angiosperms, and modern ferns. The left-most whisker indicates the minimum value, the left box indicates the lower quartile, the dark line in the middle is the median value of the sample set, the right box indicates the upper quartile, and the right-most whisker is the maximum value. *n* indicates number of sample squares measured. Standard deviation (1σ) for modern sycamores is ±0.29, for fossil angiosperms ±0.45, and ±0.06 for modern ferns.

Carbon isotope abundances

The analytical precision for all carbon isotope abundance measurements is ±0.1‰ (1σ). Appendix B demonstrates calculation of uncertainty and correction of raw carbon isotope data with the standard Urea.

The canopy of the modern plant specimens was observed and determined in the field, and $\delta^{13}C$ values can be divided into these ranges (Figure 11). There is a statistically significant difference between the median $\delta^{13}C$ of the closed vs. open canopies for both sycamores (~1.5‰; two-tailed P value = 0.0026) and ferns (~5‰; two-tailed P value = 0.0183). This trend indicates that sycamore and ferns from plants in open canopies are generally less depleted in ^{13}C than sycamores and ferns collected from plants in closed canopies.

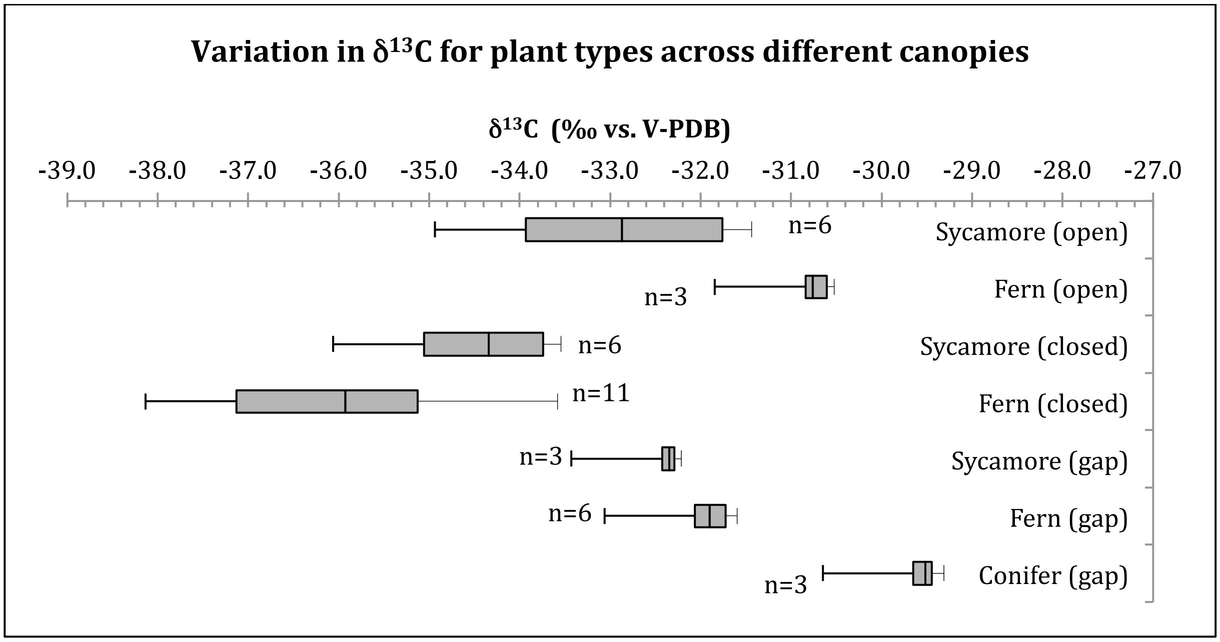

Figure 11. Box-and-whisker plot of $\delta^{13}C$ values from modern plant material, grouped by canopy type. The left-most whisker indicates the minimum value, the left box indicates the lower quartile, the dark line in the middle is the median value of the sample set, the right box indicates the upper quartile, and the right-most whisker is the maximum value of the sample set. *n* indicates number of specimens tested.

Because modern plant specimens were collected at three different times for this study, it is possible to track the ontogenetic change in $\delta^{13}C$ of leaves from the same plants across a growing season (Figure 12). Day 0 (first collection) is mid-April, day 140 (second collection) is late August, and Day 180 (third collection) is mid-October.

When collected and tested as a bud in mid-April, the $\delta^{13}C$ value of the sycamore is identical to the value for the conifer. Collected during late August, $\delta^{13}C$ of the developed sycamore leaf is still nearly identical to that of a conifer needle. Sycamore values measured from mid-October are more negative, with a change of ~2‰. The changes in $\delta^{13}C$ between the two ferns sampled demonstrate a similar increase in $\delta^{13}C$ between the first two collection periods. The $\delta^{13}C$ values of the conifer needle remain constant over growth.

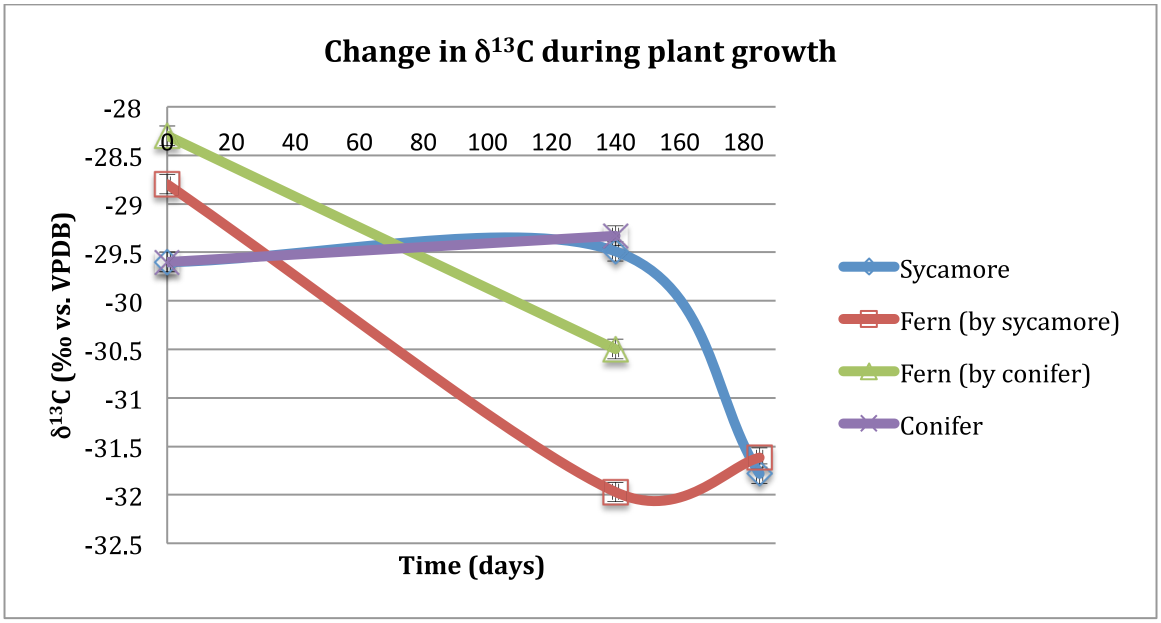

Figure 12. Comparison of average $\delta^{13}C$ values for leaves collected from the same plants over time, indicating change in $\delta^{13}C$ during plant growth. Error bars indicate standard deviation of $\delta^{13}C$ measurements ±0.1 (1σ).

When comparing $\delta^{13}C$ values measured from leaves of all different plants for this study, there are differences between the modern and fossil specimens (Figure 13). Between modern sycamores and fossil angiosperms, the difference in $\delta^{13}C$ ranges between ~3.5‰ to ~10‰. For conifers, this difference ranges from ~0‰ to ~4.5‰. The range of values between modern sycamores and modern conifers overlaps in a way similar to how the range of values between the fossil angiosperms and the fossil conifers overlap (Figure 13).

The carbon isotope ratios of the ferns are not distinct from those of the sycamores, but are different from the conifers (Figure 13). $\delta^{13}C$ values of plant matter collected solely from the sycamore veins are more positive than $\delta^{13}C$ values collected from entire plant matter of the same sycamore leaf (1.9‰ difference). There is no distinguishable difference between the ranges of $\delta^{13}C$ values for different modern sycamores and ferns, but the median $\delta^{13}C$ for modern sycamore plants is more positive than the median $\delta^{13}C$ for modern ferns.

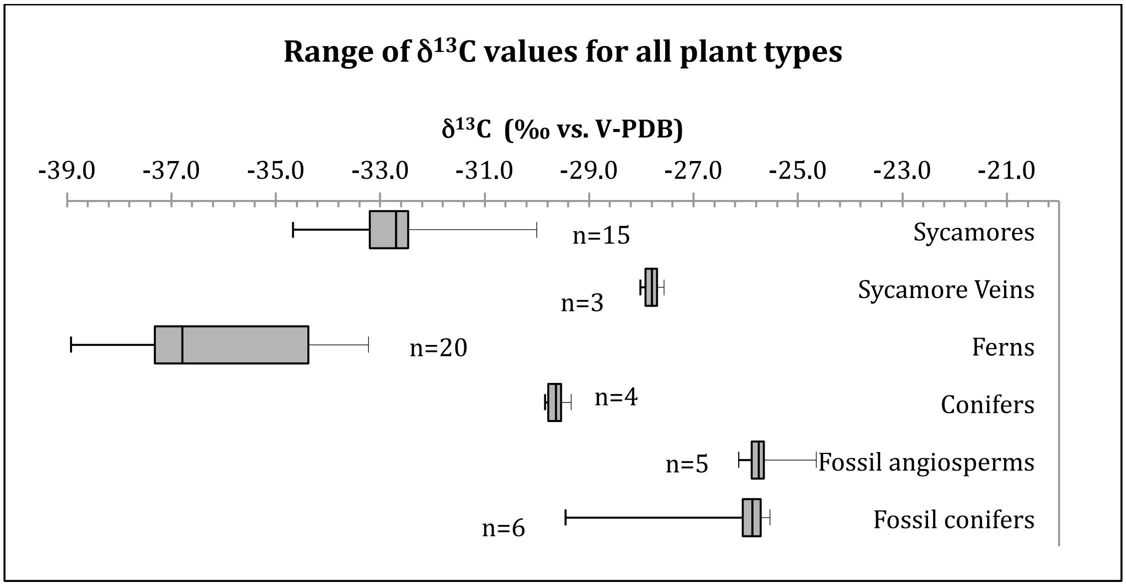

Figure 13. Box-and-whisker plot of average $\delta^{13}C$ measured for plant matter tested (exclusive only of sycamore buds and fern fiddleheads). The left-most whisker indicates the minimum value, the left box indicates the lower quartile, the dark line in the middle is the median value of the sample set, the right box indicates the upper quartile, and the right-most whisker is the maximum value. *n* indicates number of specimens tested. Standard deviation of all $\delta^{13}C$ measurements is ±0.1 (1σ).

Leaf vein density and carbon isotope abundance

Correlating leaf vein density with respective carbon isotope ratio measurements illuminates two different trends. Between the two fern plants sampled, the plant with a greater average leaf vein density has a more negative $\delta^{13}C$. However, when comparing measurements from leaves of all tested plant types, the plants with higher leaf vein densities have lower $\delta^{13}C$ values than plants with lower leaf vein densities.

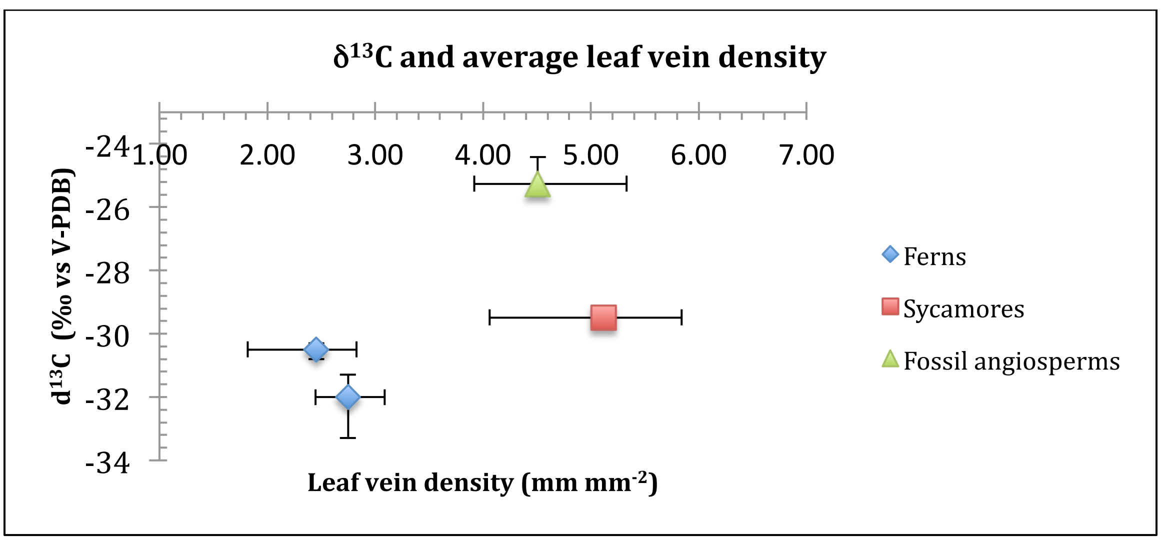

Figure 14. Leaf vein density plotted against $\delta^{13}C$ measurements for leaves from different plants. Points indicate average values for both D_v and $\delta^{13}C$. Whiskers show extent of variation of measurements of both

vein density and carbon isotope ratios. Standard deviation for all $\delta^{13}C$ measurements is ±0.1 (1σ). Standard deviation (1σ) for D_v measurements: ±0.06 for ferns, ±0.29 for sycamores, and ±0.45 for fossil angiosperms.

Peak heights

Peak heights for multiple fossil specimens are low (e.g. 0.25–0.35 nA, see Appendix C) compared against peak heights for the standards run in conjunction with the specimens (e.g. ~4–6 nA). According to (Figure 9) in the Methods section, samples that produce peak heights lower than ~0.5 nA in the mass spectrometer are not reliably accurate. Samples tested with peak heights below this value are not included in the data presented in this study.

V. Discussion of results

Leaf vein density

As expected, modern ferns exhibit a markedly lower leaf vein density than both modern and fossil angiosperms. The fossil angiosperms measured have a vein density slightly lower than, but still comparable to, its modern counterpart (Figure 10). The lower D_v could be due in part to poor preservation of leaf veins on the fossil, and thus a vein density underestimation. This difference could also be due to an actual evolutionary trend, resulting in an increased leaf vein density for more evolved/modern specimens in Mesangiospermae, as predicted by Brodribb & Feild (2010) (Figure 3).

Carbon isotope abundances

Open vs. closed canopy

When taking into account the different canopy types for each type of plant, there is a marked difference between the $\delta^{13}C$ of the closed vs. open canopies (Figure 11), indicating that the leaves from plants in closed canopies are more depleted in ^{13}C than leaves from plants in open canopies. These findings support the work of Cerling *et al.* (2004), who measured less negative $\delta^{13}C$ values from leaves of plants in the open canopy position. Also supporting this finding is that CO_2 from respiring soil is ^{13}C-depleted (Medina & Minchin 1980). In the case of a closed canopy, this ^{13}C-depleted CO_2 is not as easily able to mix with atmospheric CO_2 as in an open canopy. Hence, plants in a closed canopy will incorporate more ^{13}C-depleted CO_2 than plants in an open canopy, resulting in an overall more negative $\delta^{13}C$ value, as affirmed by this study (Figure 11).

Ontogenetic changes

The variation of average $\delta^{13}C$ values from leaves over time, or plant growth (Figure 12), illuminates some interesting patterns. A basic idea that must be kept in mind when considering these trends is that each value measured from a collection, although from the same plant, comes from a different leaf than the measurements from the other collection sets. The trends of $\delta^{13}C$ of the fern plants between the first two collection periods follows the growth of the leaves from young fiddleheads to fully developed leaves. This trend demonstrates that fractionation increases as the fern plants develop and as the leaves increase to their full leaf vein density potential. This supports the hypothesis that leaves with higher leaf vein density will be more depleted in ^{13}C.

In comparison to the ferns, the behavior of the sycamore $\delta^{13}C$ values is more variable. During the first two collection periods, the $\delta^{13}C$ of the sycamore remains almost constant, resembling that of the conifer (Figure 12). Between these collection periods, the plant has developed from producing buds to full leaves, but the fractionation of carbon isotopes does not increase with leaf development over time, contrary to what is expected. This behavior could be explained by limited sampling from the sycamore tree; perhaps the sycamore bud was picked from a different branch than the fully developed leaf, and carbon isotope fractionation varies greatly across the same height of a plant. More extensive sampling and testing could illuminate the reason behind this unexpected behavior. In the time between the second and third collection periods, $\delta^{13}C$ becomes more negative (Figure 12), indicating greater depletion of ^{13}C. From my initial hypothesis, this behavior is also expected from a leaf with greater leaf vein density.

Unlike the fern and sycamore plants, the conifer is an evergreen tree. It is expected that the $\delta^{13}C$ values measured from the conifer should remain fairly constant with time (Figure 12). The plant matter collected during the initial collection was a fully developed needle and did not develop over time (i.e. it did not change in leaf vein density) like the sycamore and fern plants did.

Different plant types and fossil specimens

The $\delta^{13}C$ values of the fossil specimens are distinctly more positive than those of the modern specimens (Figure 13), a finding that could result from a combination of confounding factors. The difference in these values could be due to differences in the magnitude of carbon fractionation of these plants while they were living. According to Equation 6, influencing internal (p_i) or atmospheric (p_a) partial pressure of CO_2 will impact the magnitude of carbon fractionation by a plant. A different p_i would be the result of physiological changes of the plant over time. Changes in p_a would be the result of different pCO_2 levels in the Early Cretaceous versus the present day.

The difference in the $\delta^{13}C$ values could also be completely unrelated to differences in carbon isotope fractionation, and instead be caused by diagenetic processes altering the composition of the carbon preserved in the fossil compressions. The effects of diagenesis are unpredictable, and could mask the carbon isotope fractionation values expected from photosynthesis. Any combination of these potential influences could be responsible for the differences in $\delta^{13}C$ values measured in this study.

Another factor that might explain the differences between $\delta^{13}C$ values of the modern sycamores and conifers and the fossil counterparts is the composition of the plant tissue analyzed. Compression fossils preserve organic carbon best from the veins of the plant; hence organic carbon was collected primarily from the veins of the fossils. The comparison of $\delta^{13}C$ values for whole plant matter vs. veins only from sycamores results in a 1.9‰ difference (Figure 13), with the $\delta^{13}C$ from the veins being more positive. This could account for some portion of the more positive $\delta^{13}C$ values of the fossil specimens. Further sampling would shed more light on the magnitude of the difference in $\delta^{13}C$ values from different parts of plant tissue.

The average $\delta^{13}C$ measured from the conifer samples is distinctively more positive than the values measured from both modern ferns and sycamores (Figure 13). According to the hypothesis that leaf vein density would be a controlling factor in $\delta^{13}C$, this is an expected trend, because leaf vein density for conifers is expected to be high (there is one leaf vein that runs the entire length of the thin needle). An alternative explanation to this could be due to the difference between the anatomy of scale-leaf conifers and the vein density in broad-leaved plants.

The median $\delta^{13}C$ of modern sycamores is more positive than the median $\delta^{13}C$ for modern ferns (Figure 13), indicating that carbon isotope fractionation is greater in these ferns than these sycamores. This is not accordance with the hypothesis of increased leaf vein density being correlated with greater carbon isotope fractionation.

Correlating leaf vein density and carbon isotopes

Correlating leaf vein density with carbon isotope ratio measurements from this study shows two different trends. First, the correlated values between the two fern plants indicate that there is greater fractionation in the plant with greater D_v (Figure 14). However, the difference between leaf vein densities of the two ferns is not great, and this could be a misleading trend, which could be better explained with more rigorous measurements. The second trend shows the opposite as what was expected, with carbon isotope fractionation tending to decrease in plants with higher leaf vein density (Figure 14).

VI. Suggestions for future work

Measuring leaf vein density

The use of leaf vein density as a character specific to plant type is a budding field. The ability to measure a consistent D_v is still uncertain, however, and is dependent upon different factors.

Different modes of leaf "preservation"

A factor to consider when using leaf vein density as a correlative character is the type of leaf from which D_v is being measured: cleared leaves, un-cleared modern leaves, and fossil leaf impressions. Cleared leaves are bleached and stained with a red dye that attaches to the veins, making this method show what is close to the "true vein density" of a leaf. Photographs taken of un-cleared modern leaves and fossil leaf impressions, however, generally demonstrate less clarity and greater uncertainty in leaf vein density measurements.

A simple study could explore and quantify the deviation of D_v measurements of modern leaves and fossil leaf impressions from the "true vein density" determined from cleared leaves. After collecting multiple leaves of different plants with a wide variety of leaf vein densities, the D_v will be measured from the un-bleached leaf. Half of the leaf samples from any one plant will then be cleared and dyed, whereas the other half will be subjected to a retting process. Retting a leaf involves soaking it in water and/or between layers of loam to soften it and separate the fibers. The process of retting a leaf simulates, to some extent, the burial process of leaves that become fossilized. D_v is measured from each of these leaves in their "new state," and the difference in D_v measurements for each different leaf type can be correlated and quantified.

Different methods for measuring leaf vein density

Data from this and other studies demonstrate that intraspecific D_v varies considerably as a function of the region measured on leaf lamina (e.g. Uhl & Mosbrugger 1999). The newly developed LEAF GUI software (Price *et al.* 2010) allows the user to input a digital image of an entire leaf and analyze the leaf vein density, among other characters. While using this tool would help to avoid the inconsistency of D_v measurements across the same leaf and help to better constrain intraspecific variation, it might be problematic when attempting to measure D_v from incomplete fossil leaf impressions. A comparison of vein density measurements calculated with this new LEAF GUI against measurements made with the more traditional method of measuring vein length from designated squares of leaf lamina with ImageJ (NIH) is advisable.

VII. Conclusions

The traditional idea that early angiosperms were highly developed plants (Hickey & Doyle 1977; Wing & Boucher 1998) has been challenged by studies suggesting that angiosperms originated as forest understory species with low photosynthetic capacity (Feild *et al.* 2004, 2009). Assuming that leaf vein density can be correlated with photosynthetic capacity of a plant, results from this study indicate that Proteales, a basal eudicot, from the Early Cretaceous had photosynthetic capacity approximately equal to their modern counterparts, the modern sycamores. This could suggest that the traditional view of highly productive early angiosperms holds, at least for eudicots.

The trend observed of greater carbon isotope fractionation in leaves from the fern and sycamore plants over time and plant growth (Figure 12) supports the initial hypothesis that plants producing leaves with higher leaf vein densities (i.e. fully developed leaves) are more depleted in ^{13}C than plant matter with lower leaf vein densities (i.e. fiddleheads or buds). However, the trend of lower carbon isotope fractionation in plants with higher leaf vein density (Figure 14) is not in compliance with this hypothesis. The variation in $\delta^{13}C$ due to environmental factors (Figure 11) (e.g. canopy type, water availability, source of CO_2) tends to overprint differences in $\delta^{13}C$ that might be expected from differences in leaf vein density. These results imply that a greater sampling size with more tightly constrained collection and measurement practices could further support or refute this hypothesis.

Building on the discussion of the carbon isotope ratios for different plant types and fossil specimens from Section V, the conifer specimens in this study are expected to generally retain physiological characters between the Early Cretaceous and today. Assuming this is true, it might be inferred that a fractionation-driven difference in $\delta^{13}C$ of modern vs. fossil conifer specimens does not result from changes in plant physiology, and thus changes in p_i. Because the overlap in $\delta^{13}C$ values between the modern specimens is similar to the overlap between the fossil specimens (Figure 13), this might signify that fractionation-driven differences in $\delta^{13}C$ of the modern and fossil angiosperms is minimally impacted by changes in plant physiology. Eliminating changes in p_i as the driving factor for changes in carbon isotope fractionation in these plants over time, the remaining factor influencing carbon isotope fractionation is changes in pCO_2 over time.

Assuming that the differences between the average $\delta^{13}C_{plant}$ values of the modern and fossil conifers and angiosperms reflects a difference in pCO_2 between the present and the Early Cretaceous, and not a change due to diagenesis or a major difference in carbon fractionation by the plants, these measured $\delta^{13}C_{plant}$ values can theoretically be used to calculate $\Delta^{13}C$ from Equation 6, and $\delta^{13}C_{atm}$ from Equation 5. With the assumption of a constant p_a and an elevated and p_i during the Cretaceous (see Table 1, compared against modern pCO_2 value of 388.92 ppm as of October 2011, as reported in Section II) it can be estimated from Equation 6 that plants of the Early Cretaceous had a smaller $\Delta^{13}C$, and fractionated less carbon-13 than present day analogous plants. This estimate is supported by the markedly more positive $\delta^{13}C$ values measured from fossil conifers and angiosperms than their modern counterparts (Figure 13).

Rearranging Equation 5, we get:

$$\boldsymbol{\delta^{13}C_{atm} = \Delta^{13}C\left(1 + \delta^{13}C_{plant}\right) + \delta^{13}C_{plant}}\ . \qquad \textbf{Eq. 8}$$

By plugging in values of $\delta^{13}C_{plant}$ and a calculated $\Delta^{13}C$ into Equation 8, values of $\delta^{13}C_{atm}$ during the Early Cretaceous can be estimated. For example, with a lower $\Delta^{13}C$ and a more positive $\delta^{13}C_{plant}$, $\delta^{13}C_{atm}$ during the Early Cretaceous would be lower than modern $\delta^{13}C_{atm}$. Being able to make these kinds of calculations about paleoclimate data helps us to understand how aspects of our Earth have evolved over time.

VIII. Acknowledgements

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X. Appendices

A. University of Maryland Honor Code

I pledge on my honor that I have neither given nor received any unauthorized assistance or plagiarized on this assignment.

Amanda L.D. Bender Date

B. Uncertainty analysis

Table 2. Uncertainty analysis of Urea standard.

Sample #	Name	$\delta^{13}C$ raw (‰)	
1	10242011_STD-1	-33.0	
2	10242011_STD-2	-33.2	
3	10242011_STD-3	-33.2	
15	10242011_STD-4	-33.1	
16	10242011_STD-5	-33.0	
29	10242011_STD-6	-32.9	
30	10242011_STD-7	-33.0	
43	10242011_STD-8	-33.1	
44	10242011_STD-9	-32.8	
57	10242011_STD-10	-32.8	
58	10242011_STD-11	-33.0	
65	10242011_STD-12	-33.1	
66	10242011_STD-13	-33.0	
67	10242011_STD-14	-32.9	
68	10242011_STD-15	-33.0	
	Urea standard: -29.39‰	**-33.0**	Average
		± 0.1	StDev
		3.6	Offset

C. Plant isotope abundance values

Table 3. Plant matter $\delta^{13}C$ values. Red highlighting indicates samples with peak heights too low (i.e. <0.5 nA) to be measured accurately by the mass spectrometer.

Taxon/Designation	Canopy (open/gap/closed) and/or Condition	Peak height (nA)	$\delta^{13}C$ (‰ vs. V-PDB) (±0.1)
Fossil conifer		4.23	-25.2
Fossil conifer		3.18	-25.6
Fossil conifer		4.04	-25.3
Fossil conifer		0.25	-27.1
Fossil conifer		0.32	-26.5
Fossil conifer		0.64	-25.5
Fossil conifer		3.15	-29.1
Fossil conifer		1.25	-25.8
Fossil angiosperm		0.35	-25.3
Fossil angiosperm		0.72	-24.4
Fossil angiosperm		1.25	-25.7
Fossil angiosperm		0.18	-27.0
Fossil angiosperm		1.13	-25.6
Fossil angiosperm		5.05	-29.4
Fossil angiosperm		3.19	-25.9
Platanus occidentalis	Bud, open canopy	4.87	-29.4
Platanus occidentalis	Veins only	5.34	-27.8
Platanus occidentalis	Veins only	7.08	-27.6
Platanus occidentalis	Veins only	4.90	-27.3
Platanus occidentalis	Open	8.93	-29.5
Platanus occidentalis	Open	5.82	-29.2
Platanus occidentalis	Open	8.23	-29.5
Platanus occidentalis	Open	6.61	-31.8
Platanus occidentalis	Open	7.07	-31.9
Platanus occidentalis	Open	6.69	-31.7
Platanus occidentalis	Gap	9.72	-32.3
Platanus occidentalis	Gap	5.66	-32.1
Platanus occidentalis	Gap	6.34	-32.2
Platanus occidentalis	Closed	7.99	-32.5
Platanus occidentalis	Closed	6.97	-32.2
Platanus occidentalis	Closed	5.98	-32.4
Platanus occidentalis	Closed	6.92	-33.9
Platanus occidentalis	Closed	6.82	-33.7
Platanus occidentalis	Closed	7.16	-33.7
Osmundrastrum cinnamomeum	Fiddlehead, open canopy	8.12	-28.3
Osmundrastrum cinnamomeum	Fiddlehead, gap canopy	6.34	-28.8
Osmundrastrum cinnamomeum	Open	5.03	-30.3
Osmundrastrum cinnamomeum	Open	7.07	-30.4
Osmundrastrum cinnamomeum	Open	8.85	-30.8
Osmundrastrum cinnamomeum	Gap	8.35	-31.4
Osmundrastrum cinnamomeum	Gap	16.03	-33.3
Osmundrastrum cinnamomeum	Gap	5.84	-31.3

Taxon/Designation	**Canopy (open/gap/closed) and/or Condition**	**Peak height (nA)**	**δ^{13}C (‰ vs. V-PDB)** (±0.1)
Osmundrastrum cinnamomeum	Gap	5.54	-31.5
Osmundrastrum cinnamomeum	Gap	7.86	-31.8
Osmundrastrum cinnamomeum	Closed	4.87	-34.3
Osmundrastrum cinnamomeum	Closed	5.40	-34.2
Osmundrastrum cinnamomeum	Closed	8.72	-34.3
Osmundrastrum cinnamomeum	Closed	5.47	-31.6
Osmundrastrum cinnamomeum	Closed	9.47	-32.1
Osmundrastrum cinnamomeum	Closed	6.98	-31.9
Osmundrastrum cinnamomeum	Closed	5.24	-35.8
Osmundrastrum cinnamomeum	Closed	6.95	-36.0
Osmundrastrum cinnamomeum	Closed	7.64	-35.5
Osmundrastrum cinnamomeum	Closed	6.18	-34.5
Osmundrastrum cinnamomeum	Closed	6.61	-34.7
Juniperus virginiana	Gap	7.01	-29.5
Juniperus virginiana	Gap	5.32	-29.1
Juniperus virginiana	Gap	6.80	-29.4
Juniperus virginiana	Gap	8.15	-29.6