

# Variations in angiosperm leaf vein density have implications for interpreting life form in the fossil record

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## ABSTRACT

**It is often difficult to interpret plant life form and position within a forest based on fossils of isolated plant organs. Here we propose leaf vein density as a new tool to interpret fossil angiosperm life form, and in particular to trace the emergence of angiosperms as members of the canopy. Angiosperm leaf vein density was analyzed in two tropical forests and one temperate forest. Comparisons of vein density between canopy and understory plants showed that vein density variation mainly reflected the position of the leaf in the canopy, independent of ecological strategy (shade tolerant versus sun demanding), phylogenetic position, and site (tropical versus temperate). Vein density values of a standing forest were reflected in its leaf litter, suggesting that fossil leaf assemblages are representative of past forest ecosystems. Comparison of vein density distributions of Cretaceous–Paleocene paleofloras (132.35–58.0 Ma) to those of a modern tropical leaf litter assemblage suggests that angiosperms emerged in forest canopies by at least 58 Ma.**

## INTRODUCTION

Fossil plants are usually found as isolated organs rather than entire organisms. Their morphology, anatomy, and taxonomy provide information on species diversity, plant evolution, paleogeography, paleoecology, and paleoclimate, yet they have been scarcely used to infer the structure of past ecosystems. Hence, key questions for the history of terrestrial biomes, such as when an angiosperm-dominated canopy first appeared, remain unsolved. In this study we present vein density ( $D_v$ , vein length per unit leaf area), a trait visible on fossil leaf compressions, as a tool to shed light on the emergence of angiosperms as forest canopy members.  $D_v$  correlates with transpiration capabilities (Sack et al., 2005; Sack and Frole, 2006) and maximum conductance to water vapor (Boyce et al., 2009). Increased  $D_v$  enhances leaf hydraulic conductance, which in turn increases photosynthetic rates (see Brodribb et al., 2007; McKown et al., 2010; Sack et al., 2013).  $D_v$  is also a tradeoff between the investments for efficient transpiration and the costs of building vascular tissues (e.g., McKown et al., 2010).

In some temperate and most neotropical forests, angiosperms inhabit a wide range of microhabitats, where they fulfill different resource needs by a variety of ecological strategies. Some of these strategies are reflected in their life form, morphology, and physiology. Leaf trait variations between trees living in different forest strata have been documented and tradi-

tionally attributed to the local light regime (e.g., Ellsworth and Reich, 1992; Sack and Scoffoni, 2013) or to differential hydraulic limitations at the top and bottom of the canopy (Zwieniecki et al., 2004; Boyce, 2009; Cavaleri et al., 2010). When access to other resources is not restricted, such as in neotropical rainforests, irradiance is an important limiting factor to angiosperm growth (Campanello et al., 2008). Low light transmittance has been documented in the understory of a tropical forest in Costa Rica (Coomes and Grubb, 2000) and in a temperate deciduous forest in Maryland, USA (Brown and Parker, 1994) (1.3% and <5%, respectively). In general, upper canopy leaves have higher stomatal density (Yáñez-Espinosa et al., 2003), allowing more efficient transpiration rates and carbon gain (Rijkers et al., 2000). Shade-tolerant and sun-demanding tropical forest species show differences in leaf hydraulic architecture, conductance, and  $D_v$  (Sack et al., 2005; Sack and Frole, 2006) associated with respectively lower (shade tolerant) and higher (sun demanding) whole-plant relative growth, carbon assimilation, and transpiration rate.

Although differences in  $D_v$  between sun and shade leaves have been reported (see Uhl and Mosbrugger, 1999; Sack and Scoffoni, 2013), our study is the first to analyze  $D_v$  variation at the ecosystem level (three forests, ~200 individuals, 115 species) and one of the first to sample from the uppermost layer of canopy leaves. We tested three hypotheses.

(1) In closed-canopy forests dominated by angiosperms, the differences between understory and canopy leaves are reflected in a vertical gradient of  $D_v$  values. To test this, we measured  $D_v$  variation among canopy and understory plants in two forests in Panama and one in Maryland.

(2)  $D_v$  variation within a forest is reflected in its leaf litter.  $D_v$  values measured in a tropical leaf litter assemblage were compared to those of the corresponding standing forest.

(3) The first forests characterized by an angiosperm-dominated canopy had  $D_v$  values similar to those of modern forests. Modern data from a tropical forest litter assemblage were compared with Hauterivian–Paleocene (132.35–58 Ma) fossil data from Feild et al. (2011). Fossil and molecular data indicate that forests with an angiosperm-dominated canopy emerged either prior to the Cretaceous–Paleogene (K–Pg) boundary, in the Albian–Cenomanian (Davis et al., 2005; Wang et al., 2009), or in the Paleocene, set in motion by the K–Pg mass extinction (Burnham and Johnson, 2004; Wing et al., 2009).

We propose  $D_v$  as a new tool that can help define the timing of the emergence of angiosperms as canopy members.

## METHODS

We collected canopy (full sun) and understory (shade) leaf samples from 36 species in the Parque Nacional Metropolitan (Panama; PNM) semideciduous tropical dry forest (8°59'N, 79°33'W, mean annual precipitation 1865 mm, mean annual temperature 26.3 °C), 64 species in the Parque Nacional San Lorenzo (Panama; FTS) evergreen humid rainforest (9°17'N, 79°58'W, mean annual precipitation 3152 mm, mean annual temperature 25.8 °C), and 32 species in a Maryland temperate deciduous forest (Smithsonian Environmental Research Center, SERC; 38°53'20"N, 76°33'32"W, mean annual precipitation 1080 mm, mean annual temperature 13.2 °C). A canopy crane was used to collect leaves from the uppermost layer of the forest canopy for both PNM and FTS. A 2 cm<sup>2</sup> section of leaf lamina was excised from each sample, cleared in NaOH, stained with Safranin, and photographed at 40× for  $D_v$  measurements. All veins present in a known area ( $a = 4–10$  mm<sup>2</sup>) were traced manually, and their total length ( $L$ ) was measured using digital image analysis (Image J; National Institute of Health, Bethesda, Maryland). Then, individual leaf vein density ( $D_v$ ) was calculated as:

$$D_v = L/a. \quad (1)$$

A litter assemblage from PNM was sampled by collecting the upper 2 cm of a 1 m<sup>2</sup> area near

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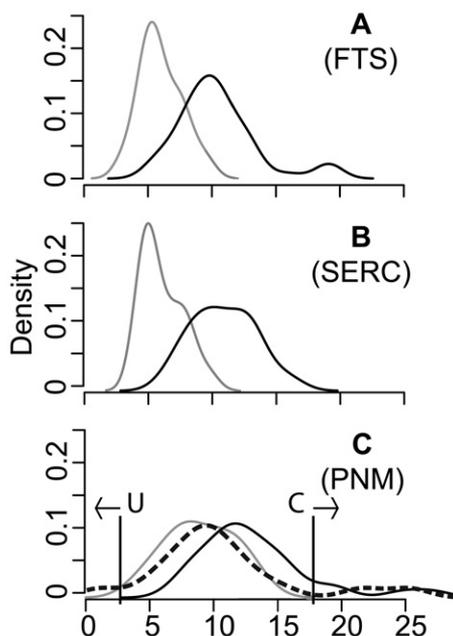
the PNM crane. From this assemblage, we analyzed 139 leaves belonging to 18 morphotypes, as described above. We provide a more detailed description of our leaf sampling, clearing, staining, and measuring protocol in the GSA Data Repository<sup>1</sup>, and all  $D_v$  measurements are provided in Table DR4.

Probability density functions of canopy and understory  $D_v$  at each site, as well as  $D_v$  of litter and fossil assemblages, were calculated using the R function “density” (R Core Team, 2013). Average  $D_v$  values of canopy and understory leaves were calculated from species/morphotype averages and compared among different sites, ecological strategies (sun demanding versus shade tolerant), and phylogenetic groups (early-diverging Magnoliid clade versus later diverging monocots and dicots, following the phylogeny of Stevens, 2012). The average  $D_v$  of the PNM standing forest was compared to the PNM leaf litter assemblage, and then  $D_v$  of the PNM litter was compared to fossil data selected from Feild et al. (2011). We compared  $D_v$  of the fossil data to a litter assemblage rather than standing forests in order to account for potential taphonomic biases (Burnham, 1997). Wilcoxon rank-sum tests conducted in R 3.0.2 (R Development Core Team, 2013) were used for comparisons of all averages because our data were not normally distributed (Shapiro-Wilk normality test). All life forms together (trees, shrubs, hemiepiphytes, lianas, and vines) were used in our analyses (for proportions of each life form, see the Data Repository). No statistical analysis of  $D_v$  versus life form could be performed because of the restricted sample size.

## RESULTS

Vein density was significantly higher in the canopy than in the understory in FTS (average  $D_v = 10.4 \text{ mm/mm}^2$  versus  $5.98 \text{ mm/mm}^2$ ), PNM (average  $D_v = 13.28 \text{ mm/mm}^2$  versus  $8.98 \text{ mm/mm}^2$ ), and SERC (average  $D_v = 10.87 \text{ mm/mm}^2$  versus  $6.07 \text{ mm/mm}^2$ ) forests ( $p < 0.01$ ; Fig. 1). However, the three sites presented some differences. First, canopy  $D_v$  was not significantly different between FTS and SERC ( $p = 0.47$ ), but it was higher in PNM compared to both FTS ( $p < 0.01$ ) and SERC (although the difference is not significant,  $p = 0.08$ ). Second, understory  $D_v$  was not significantly different between FTS and SERC ( $p = 0.94$ ), but it was significantly higher in PNM compared to the other two sites ( $p <$

<sup>1</sup>GSA Data Repository item 2014322, protocol for leaf sampling, clearing, staining and measuring (with Tables DR1–DR3); Figure DR1 (bootstrap analysis of vein density,  $D_v$ , of the forest litter); Figure DR2 (bar graph of  $D_v$  of sun-demanding and shade-tolerant species, and two-way ANOVA); Figure DR3 (phylogeny and  $D_v$ ); and Table DR4 (raw data), is available online at [www.geosociety.org/pubs/ft2014.htm](http://www.geosociety.org/pubs/ft2014.htm), or on request from [editing@geosociety.org](mailto:editing@geosociety.org) or Documents Secretary, GSA, P.O. Box 9140, Boulder, CO 80301, USA.



**Figure 1. Vein density ( $D_v$ ) probability curves showing significant differences (Wilcoxon rank-sum tests,  $p < 0.01$ ) between canopy (black) and understory (gray). A: Parque Nacional San Lorenzo (Panama, FTS). B: Temperate deciduous forest in Maryland, USA (SERC, Smithsonian Environmental Research Center). C: Parque Nacional Metropolitan (Panama, PNM). Curves represent smooth functions describing the relative likelihood for the continuous value  $D_v$  to plot within particular region of histogram (not shown here for legibility reasons). Black dotted line in C represents PNM litter, and black vertical lines mark  $D_v$  values higher and lower than the maximum understory (U) and minimum canopy (C) values encountered in PNM.**

$0.05$ ). Combining all three sites, sun-demanding and shade-tolerant species had similar  $D_v$  values in both the canopy ( $11.9 \text{ mm/mm}^2$  versus  $10.6 \text{ mm/mm}^2$ ,  $p = 0.529$ ) and the understory ( $5.8 \text{ mm/mm}^2$  versus  $6.6 \text{ mm/mm}^2$ ,  $p = 0.64$ ; Fig. DR2 in the Data Repository). The average  $D_v$  of late-diverging angiosperms was slightly but not significantly higher than that of early-diverging angiosperms ( $11.42 \text{ mm/mm}^2$  versus  $9.9 \text{ mm/mm}^2$  for canopy, and  $6.73 \text{ mm/mm}^2$  versus  $5.87 \text{ mm/mm}^2$  understory), as confirmed by randomization analyses (Fig. DR3). A two-way ANOVA (see Fig. DR2) shows that the stratum the leaf came from (canopy versus understory) was the main factor determining  $D_v$  ( $p < 0.01$ ). The effect of the ecological type on  $D_v$  was only slightly significant ( $p = 0.046$ ). No interaction occurred between ecological type and strata ( $p = 0.18$ ), whereas there was some interaction between ecological type and site ( $p = 0.03$ ).

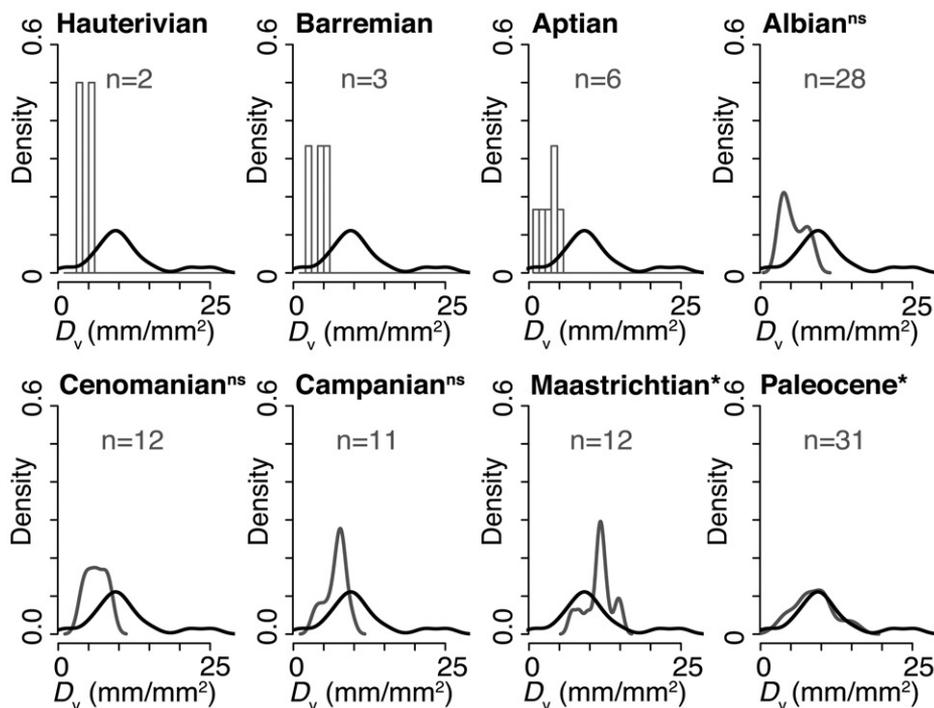
The  $D_v$  density distribution of the PNM litter assemblage (average  $D_v = 10.66 \text{ mm/mm}^2$ ) was slightly more similar to the PNM understory ( $p = 0.057$ ) than to the PNM canopy ( $p < 0.05$ ; Fig.

1C). The litter included  $D_v$  values that were both below the lowest PNM canopy value and above the highest PNM understory value, indicating that both canopy and understory leaves were present in the litter. Because it was not possible to establish a priori if a given leaf in the litter came from the canopy or the understory, we resampled the PNM standing forest to determine average  $D_v$  values for a wide variety of canopy to understory proportions, and compared these results to the average  $D_v$  of the PNM leaf litter (Fig. DR1). The average  $D_v$  of the leaf litter was comparable to that of a subsample of the standing forest composed of  $\sim 38\%$  canopy leaves (95% confidence interval of  $\pm 66.1$ ). This result must be viewed with caution, and it is uncertain if it can be applied to other ecosystems. The large confidence interval was likely due to the sizeable overlap in canopy and understory  $D_v$  values in our standing PNM forest sample. The PNM litter  $D_v$  was significantly different ( $p < 0.05$ ) from all fossil assemblages except for the Paleocene and Maastrichtian (Fig. 2).

## DISCUSSION

The significant difference in  $D_v$  between canopy and understory leaves, regardless of ecological strategy, suggests that the location of a leaf within the forest is a key factor controlling  $D_v$ . A light gradient through forest strata probably modulates  $D_v$ , as it does other leaf traits (Rijkers et al., 2000), which may explain the similarity between sun-demanding and shade-tolerant species from the same stratum in our sample (Table 1). However, this trend is in contrast with previous studies (Sack et al., 2013), and must be confirmed with more exhaustive sampling. Alternatively,  $D_v$  plasticity within trees, previously interpreted as a strategy for leaf acclimation between shade and sun environments (e.g., Carins Murphy et al., 2012), may be controlled by interplaying factors such as tree height, relative humidity, temperature, and  $\text{CO}_2$  concentration (Sack et al., 2006), which also affect transpirative demand. For example, Zwieniecki et al. (2004) observed the development of shade leaf morphologies even though leaf expansion in the understory took place under full light conditions before the establishment of a shading canopy. Our experimental design does not allow us to discriminate between different factors determining  $D_v$ , and further studies are needed across other forests and taking into account multiple variables to better explain the observed pattern. Nonetheless, the joint influence of different factors does not affect our hypothesis that  $D_v$  is higher in the canopy than in the understory due to differential transpiration, metabolic needs, and photosynthetic rates.

The difference in  $D_v$  range among sites was probably the effect of extra-environmental factors. Small sample size may have produced the high understory  $D_v$  at PNM, but it cannot



**Figure 2.** Vein density ( $D_v$ ) probability curves of eight paleofloras (in gray) compared to  $D_v$  density curve of the Parque Nacional Metropolitan (Panama, PNM) litter (in black). Abbreviation  $n$  indicates number of fossil leaf morphotypes. For small sample sizes ( $n < 11$ ), histogram bars are shown instead of density curves. Asterisk and ns indicate significant and not significant Wilcoxon rank-sum tests, respectively. The PNM litter has 18 morphotypes. Fossil  $D_v$  data were selected from Feild et al. (2011) and include only morphotypes from a single formation in each time period (Hauterivian—Chengzihe Formation, near Jixi, China; Barremian—Las Hoyas locality, La Huérguina Formation, Iberian Mountains, Spain; Aptian—Crato Formation, Brazil; Albian—Hoisington III, Dakota Formation, Kansas, USA; Cenomanian—Courtland, Dakota Formation, Minnesota, USA; Campanian—Grubach Formation, Austria; Maastrichtian—Guadas Formation, Colombia; Paleocene—Cerrejón Formation, Colombia).

**TABLE 1.** AVERAGE VEIN DENSITY ( $D_v$ ) VALUES OF SUN-DEMANDING AND SHADE-TOLERANT SPECIES IN BOTH CANOPY AND UNDERSTORY STRATA

Site	Average $D_v$ , sun demanding		$p$ value	Average $D_v$ , shade tolerant		$p$ value	$n$
	canopy	understory		canopy	understory		
PNM	13.81 $\pm$ 3.85 (6)	8.67 (1)	NA	10.49 $\pm$ 1.55 (3)	11.37 (1)	NA	11
FTS	12.09 $\pm$ 4.21 (10)	8.18 (1)	NA	10.33 $\pm$ 2.52 (15)	5.81 $\pm$ 2.07 (7)	<0.001	33
SERC	10.03 $\pm$ 2.71 (7)	5.09 $\pm$ 0.72 (7)	<0.001	11.46 $\pm$ 2.34 (7)	6.77 $\pm$ 1.83 (10)	<0.001	31
Total	11.91 $\pm$ 3.84 (23)	5.83 $\pm$ 1.6 (9)	<0.001	10.67 $\pm$ 2.35 (25)	6.65 $\pm$ 2.21 (18)	<0.001	75

Note: PNM—Parque Nacional Metropolitan, Panama; FTS—Parque Nacional San Lorenzo, Panama; SERC—Smithsonian Environmental Research Center, a temperate deciduous forest in Maryland, USA. Total is all forests together. Only species with known ecological strategy were included. Canopy versus understory values were compared using Wilcoxon rank-sum tests, and  $p$  values were indicated as NA when the sample size (number of species, in parentheses) was too small to allow any statistical test.  $N$  is number of samples.

explain the higher canopy values at PNM. PNM is located in the middle of Panama City, and its flora and fauna are likely affected by human disturbances (pathways and logging) that create forest gaps and allow more light to penetrate in the forest, resulting in an overall higher  $D_v$  (Fig. 1). All samples included both early successional (usually sun demanding) and late successional species (usually shade tolerant); a bias in functional type composition in the PNM forest is unlikely to be the cause of the higher  $D_v$ .

Our results suggest that high  $D_v$  is a trait present in emergent canopy trees and lianas, and

therefore  $D_v$  can be applied to the fossil record to distinguish where and when angiosperms were a dominant canopy component. While average  $D_v$  values of early- and late-diverging angiosperms were similar, early-diverging angiosperms showed a smaller range of  $D_v$  variation. Based on the relatively high  $D_v$  values ( $>10$ ) within the magnoliids (also reported by Brodribb and Feild, 2010), we speculate that the ability to produce high  $D_v$  evolved early in angiosperm evolution, but was fully exploited only when angiosperms reached the canopy, around the time of the K-Pg boundary.

### Vein Density and the Fossil Record

The PNM litter assemblage does not significantly differ from the assemblage collected from living trees (Fig. 1C). The wide range in litter  $D_v$  values suggests that it is composed of both canopy and understory leaves. However, the considerable overlap in canopy and understory values makes any estimation of their proportional representation in the assemblage a difficult task (see Fig. DR1). Kitajima et al. (2005) suggested that many ( $>30\%$ ) leaves within the crown of a late successional tree exist below 10% of the photon flux density available at full exposure. Thus, if light conditions resembling our sampled understory exist below a relatively thin layer in the upper crown, a large population of leaves within the tree crown would be subject to understory-like light conditions and may contribute to the low  $D_v$  trend observed in the litter sample. Nonetheless, the presence of a wide range of  $D_v$  values reflects the occurrence of a stratified ecosystem with a closed canopy. Furthermore, this result suggests that paleofloras are good representations of whole ecosystems, enabling meaningful reconstructions of  $D_v$  variations in the fossil record. The presence of the same  $D_v$  trend in both tropical and temperate regions suggests that this trait varies independent of climate and therefore is applicable to ancient time periods for which climate is poorly constrained.

Although our results show no differences between ecological categories, other studies reported high  $D_v$  in pioneer vegetation (Feild et al., 2011) and sun-demanding trees (Sack and Frole, 2006). This could potentially lead to taphonomic biases in a fossil flora, with an overrepresentation of high  $D_v$  morphotypes near places of leaf burial (lake and stream margins). Individual leaves can glide over a distance of 30 m (Burnham et al., 1993), and mature forest elements may reach the forest margins this way. In situ preservation or litter transport by water could improve the representation of mature forest taxa even further. Because our methodology is based solely on species' presence, a well-sampled fossil locality (e.g., Wing et al., 2009) should provide a less-biased picture of representative elements within the forest.

$D_v$  of the PNM litter assemblage is comparable only to those of the Paleocene and Maastrichtian paleofloras (Fig. 2). Despite our small sample size, we know from the literature that earlier floras do not show  $D_v$  values high enough to be comparable to Maastrichtian, Paleocene, or modern values (Feild et al., 2011). The  $D_v$  curve of the Maastrichtian encompasses a smaller range than the modern litter assemblage (7.33–15.42 mm/mm<sup>2</sup> versus 1.11–25.3 mm/mm<sup>2</sup>, respectively). Additional data are needed to verify whether this observation is the result of the small Maastrichtian sample size (11 morphospecies), or if it reflects a different type of

ecosystem in which angiosperms had not yet attained all forest layers. We therefore conservatively suggest that the emergence of angiosperms as a dominant canopy component dates at least to the Paleocene, when  $D_v$  distribution was already similar to that of extant forests (Fig. 2). We expect that the prevalence of high  $D_v$  in angiosperm canopy trees permits the optimization of resources and photosynthetic capability and that angiosperms were only able to dominate the canopy level late in their evolutionary history. Angiosperms may have taken advantage of ecosystem upheaval caused by the K-Pg mass extinction event, exploiting their higher transpirational capabilities ( $D_v$ ) and faster growth rates, in order to be first to reach the canopy during the Paleocene ecosystem recovery.

## CONCLUSION

$D_v$  variation in modern angiosperm-dominated forests is mainly driven by the forest stratum in which a leaf is located. Available light and tree height are likely the major factors modulating  $D_v$  variation through forest strata, although other variables may also contribute. The similarity between the PNM litter and standing forest, and the evidence that both canopy and understory leaves occur in leaf litter, indicates that fossil floras preserve  $D_v$  variations within the standing forest. Paleocene floras have  $D_v$  curves similar to those of modern tropical forest litter, suggesting that angiosperms were canopy dominants at least since the Paleocene.

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