Reconstructing past terrestrial vegetation structure relies in large part on the interpretation of chemical and morphological characteristics of fossilized leaves. Closed-canopy forests are characterized by gradients of penetrating light, altered relative humidity and varied concentrations of CO₂ below and within the canopy (Hollinger, 1989; Graham et al., 2014). Leaf traits (e.g., morphologic and isotopic signatures) vary in relation to the immediate atmospheric and light conditions experienced at the leaf site during growth (van der Merwe and Medina, 1991; Graham et al., 2014; Haworth and Raschi, 2014; Coble and Cavalieri, 2015; Dunn et al., 2015). Indeed, functional traits observed in leaves represent the product of functional optimization under the local growth conditions, albeit constrained by genetic predisposition. Therefore, analyses of the variation in leaf traits from fossil assemblages may enable inferences to be made as to the degree of canopy closure (Graham et al., 2014; Bush et al., 2017) and ecosystem productivity (Reich et al., 1997; Boyce and Zwieniecki, 2012). To achieve the most accurate reconstruction of ancient forests, we need to understand how functional traits behave within the coordinated multidimensional trait space of the leaf economic spectrum while acknowledging the role species may have in shaping this response within a modern closed-canopy forest.

The range in bulk carbon isotope composition (δ¹³C) of leaves is often used to determine the range in environmental conditions from which leaves are drawn (Farquhar et al., 1989; Graham et al., 2014). The so called “canopy effect” describes the characteristic decrease in δ¹³C of leaves beneath a closed canopy in a vertical gradient from the upper canopy to the understory (van der Merwe and Medina, 1991; Graham et al., 2014). Higher δ¹³C
values in the upper canopy are driven by increased rates of photosynthesis, and thereby limited discrimination against $\delta^{13}$C (van der Merwe and Medina, 1991; Graham et al., 2014; Coble and Cavaleri, 2015). In contrast, shaded leaves in the understory have lower rates of photosynthesis (due to reduced light) and refix high concentrations of respired CO$_2$, which is already depleted in $\delta^{13}$C and trapped in the lower canopy due to canopy closure (van der Merwe and Medina, 1991; Kürschner, 1997; Crowley et al., 2012; Royer et al., 2019). However, water limitation and nutrient availability may impact the $\delta^{13}$C signature of leaves of C$_3$ plants by altering plant stresses and photosynthetic activity (Cernusak et al., 2009); therefore, care must be taken in examining the context of observed difference in $\delta^{13}$C values.

Micromorphological features of leaves, such as epidermal cell area and the cell wall undulation (curvature) index also change with sun exposure (Hectors et al., 2010; Wagner-Cremer et al., 2010; Dunn et al., 2015; Wang et al., 2018). Sun leaves typically have smaller cells with less cell wall undulation (Kürschner, 1997). Shade leaves develop undulation because uneven plasticity in the outer cell wall causes deformation of the anticlinal cell wall during continued cell growth. However, species-specific responses in changing cell wall undulation, may result in altered inferences in the degree of canopy closure, dependent upon the species or morphospecies observed (Bush et al., 2017). Additionally, canopy closure is rarely consistent (Grove et al., 2000). Tree and branch fall gaps can introduce anomalous and significant changes in light penetration when contrasted to the average light gradient, yet these are likely regions of intense leaf turnover and therefore have a higher contribution to the fossil assemblage (Greenwood, 2005). Anomalous contributions to the fossil assemblage may also occur near waterbodies, where light penetration is higher and aquatic environments favor fossil preservation (Burnham et al., 1992).

Leaf mass per area (LMA), a measure of dry leaf mass per unit of light-intercepting leaf area, is highly responsive to light conditions (Watson, 1942; Turney et al., 2002; Poorter et al., 2009), and a key determinant and driver of the leaf economic spectrum (Wright et al., 2004; Poorter et al., 2009). Although not directly recorded by fossilized leaves, LMA has been inferred from correlations with petiole dimensions (Royer et al., 2007, 2010), cuticle thickness (Soh et al., 2017), and adaxial epidermal cell density (Haworth and Raschi, 2014). Leaf mass per area is a product of cell size, packing, and leaf thickness, and the acclimation in LMA is driven by a requirement to optimize photosynthetic function on a leaf-by-leaf basis (Markesteijn et al., 2007; McMurtrie and Dewar, 2011). As plants are sessile organisms, a single tree must be able to maximize the photosynthetic function of leaves with a minimal cost of growth (Markesteijn et al., 2007). Leaf mass per area is therefore reduced in shaded leaves because the smaller, more densely packed cells are exchanged for larger cells, and the increased blade area improves light capture where light availability is reduced (Poorter et al., 2009; Coble and Cavaleri, 2015).

Isotopic and morphological features of fossil leaves have the potential to record information on light environment and leaf function, but preservational, or taphonomic, biases need to be considered. Fossil assemblages do not derive leaves from all levels of the forest equally (Greenwood, 2005; Ellis and Johnson, 2013; Su and Croft, 2018). Leaf production in sun-exposed positions is higher compared to the understory (Burnham et al., 1992; Osada et al., 2001), leading to a greater proportion of sun- versus shade-morphotype leaves in the litter and leaf assemblage (Greenwood, 2005; Steart et al., 2005). In addition, tropical forests are prone to inconsistent degrees of canopy closure because of intense storm systems and variable regrowth strategies that create areas within the canopy that are exposed to high light environments (van der Meer et al., 1994). Lastly, forest gaps associated with waterbodies, where sediments accumulate, reinforce the bias in leaf fossil assemblages toward sunlit morphotypes (Roth-Nebelsick and Konrad, 2019).

While there is a need to compensate for the biases in production and preservation toward sunlit leaves, adequate sampling can ensure that a full range of the environmental conditions experienced by the forest are represented (Graham et al., 2014). Moreover, estimation of canopy closure and leaf function in the observed fossil assemblage can be achieved via careful calibration with extant species (Roth-Nebelsick and Konrad, 2019). While research on calibrating numerous fossil leaf traits in extant forests is beginning to emerge, few studies incorporate measurements beyond two traits within a single ecosystem (Kürschner, 1997; Turney et al., 2002; Graham et al., 2014; Dunn et al., 2015; Bush et al., 2017), and none, to our knowledge, have examined micro- and macromorphological features alongside carbon isotope signatures. Moreover, alternate and unquantified sources of trait variation, e.g., environmental stress and a disparity in the magnitude of responses between species (especially in the hyper-diverse tropics), represent a challenge in the use of fossil leaf traits to reconstruct environmental conditions and thereby the degree of canopy closure. The creation of robust metrics that use multiple fossil leaf traits within a single environment as well as a quantification of possible sources of error will increase confidence in palaeoecological reconstructions.

The aims of this study were to (1) examine the coordination of commonly used fossil leaf traits ($\delta^{13}$C, cell area, undulation index, and petiole metric) within the extant Daintree Rainforest in Queensland Australia to test the efficacy of a multi-variable metric to determine local light conditions within a tropical closed-canopy forest system and (2) examine whether fossil leaf traits can be used to predict the leaf functional trait of LMA within a diverse extant tropical forest.

**MATERIALS AND METHODS**

All leaf samples were obtained from the Daintree Rainforest Observatory (DRO) in Cape Tribulation (~16.117 N, 145.45 E), 140 km north of Cairns in Far North Queensland, Australia. The DRO, administered by James Cook University, includes two 1-ha permanent tree-census plots. One of these plots can be accessed by a 48.5 m high canopy-access crane (Leibherr Model 91EC, Adelaide, Australia). Forest at the DRO is described as a complex mesophyll vine forest Type 1a (Tracey, 1982) with a tall but irregular canopy varying in height from 25 to 33 m with indistinct stratification of the subcanopy. Within the crane plot are 85 canopy tree species (comprising 60 genera and 35 families) representing a basal area of ~33 m$^2$ ha$^{-1}$ (Tng et al., 2016). The Daintree Rainforest is considered one of the oldest continuously vegetated tropical systems on earth, and while not as speciose as some tropical closed forest systems, it represents a broad phylogenetic diversity, with many Gondwana relic species (Costion et al., 2015).

Initial leaf samples for canopy–understory comparisons were collected in 2014 from 89 species of plants found within the closed-forest canopy of the DRO—seven monocotyledonous, 81 dicotyledonous, and one cycad species *Bowenia spectabilis* (data available from the...
A range of growth forms, including trees and shrubs (collectively referred to here as trees) and lianas, were sampled. Samples were collected from both the upper canopy crown (using the canopy access crane) and the understory ~0.4 to 1.5 m, with an attempt to collect the same species from both environments. During this initial sampling, in-situ leaf physiological measurements (e.g., light-saturated photosynthesis \([\text{A}_\text{sat}}]\) and responses to \([\text{CO}_2]], [A–C]], and irradiance \([A–I]], \text{curves}\) were made on at least two leaves of 71 species collected, using a LI-COR 6400 Portable Photosynthesis machine (LI-COR, Lincoln, NE, USA). In all upper canopy crown samples, 12 leaves were harvested with nine analyzed for basic leaf functional traits and three leaves frozen for later micromorphological analysis. In the case of understory samples, only the two leaves used for physiological measurements were harvested and analyzed for leaf functional traits given typically limited leaf material.

In 2017, additional leaves were sampled across the light-gradient continuum found in the forest canopy. Five species (Argyroderondron peraltatum, Myristica globosa subsp. muelleri, Endiandra micro-neura, Cleistanthus myrianthus, and Rockinghamia angustifolia) were harvested due to their prevalence at all forest strata of the study site. Leaves were sampled from the upper canopy crown, canopy interior (i.e., partial cover by surrounding vegetation), the understory, and from within a significant tree fall gap. At each location, six leaves were harvested, with three analyzed for basic leaf functional/morphological traits and the remainder archived for micromorphological analysis. At each sampling location, leaf area index (LAI) was determined using a LAI-2200C Plant Canopy Analyzer (LI-COR). In addition, and to examine the impact of environmental stress upon the fidelity of leaf traits, parallel upper canopy crown and canopy interior samples were also collected from individuals impacted by a long-term (3-yr) ongoing throughfall exclusion (i.e., "drought") experiment within the DRO crane plot (Ting et al., 2018). The throughfall exclusion plot (0.4 ha), wherein rainfall/throughfall is intercepted and diverted off the plot has resulted in an area of impacted and diverted off the plot has resulted in an area of throughfall exclusion plot (0.4 ha), wherein rainfall/throughfall impacted by a long-term (3-yr) ongoing throughfall exclusion (i.e., stress upon the fidelity of leaf traits, parallel upper canopy crown (LI-COR). In addition, and to examine the impact of environmental morphological analysis. At each sampling location, leaf area index (LAI) was determined using a LAI-2200C Plant Canopy Analyzer (LI-COR). In addition, and to examine the impact of environmental stress upon the fidelity of leaf traits, parallel upper canopy crown and canopy interior samples were also collected from individuals impacted by a long-term (3-yr) ongoing throughfall exclusion (i.e., "drought") experiment within the DRO crane plot (Ting et al., 2018). The throughfall exclusion plot (0.4 ha), wherein rainfall/throughfall is intercepted and diverted off the plot has resulted in an area of elevated water stress within the crane-accessible area that includes replicated individuals of species in natural conditions.

**Macromorphological leaf traits**

Leaf petiole dimensions including width (parallel to plane of lamina) and depth (perpendicular to plane of leaf lamina) were measured on individual leaves using a micrometer before removal and drying at 60°C for 72 h. Leaf area of fresh leaves was measured using a flatbed scanner (Canon CanoScan Flatbed Scanner LiDE120, Sydney, NSW, Australia), with area determined against a scaled calibration using Image J 1.52a Software (National Institutes of Health, Bethesda, MD, USA). Dry mass of individual leaf lamina was recorded and used to calculate LMA. In addition, we calculated the petiole metric (Eq. 1), originally developed as a proxy to estimate LMA from preserved traits of fossil specimens (Royer et al., 2007).

\[ \text{Petiole metric} = \frac{\text{Petiole-width}^2}{\text{Leaf area}} \]  

(1)

**Micromorphological characteristics**

Parallel canopy and understory leaves of 15 dicotyledonous species collected in 2014 and all samples collected in 2017 were shipped frozen to the University of Adelaide for clearing, staining, and determining epidermal cell area and undulation index. Subsections (38.5 mm²) from each of three leaves per sample were cleared using a 2:1 solution of 35% hydrogen peroxide (v/v) and 80% ethanol (v/v) at 75°C; cuticles were stained with aqueous crystal violet (0.05% w/v) before mounting in glycerin jelly. Dilute concentrations of hydrogen peroxide failed to yield adequate cuticle material for multiple samples, predominantly of the species Argyrodendron peraltatum, Cleistanthus myrianthus, and Myristica globosa subsp. muelleri. These samples were placed in a clearing solution of 20% chromic acid (v/v) for a minimum of 24 h but were not dyed, because chromic acid adequately stained cell structures. A UC50 camera (Olympus, Melbourne, VIC, Australia) mounted on an AX70 microscope (Olympus) with AnalySIS software (Soft Imaging System, Münster, Germany) was used to take three photographs per sample of the abaxial epidermis under 40× magnification. From each leaf, 15 cells were traced using a HP (Palo Alto, CA, USA) Pavilion 15 touchscreen and stylus and analyzed for cell area and perimeter using ImageJ. Each sample measurement is therefore calculated from an average of 45 cells. The measure of undulation index—a comparative measure of the perimeter of a cell compared to the perimeter of a circle with the same area—was calculated as (Kürschner, 1997): 

\[ \text{Undulation index} = \frac{C_e}{2\pir} \]  

(2)

where \(C_e (\mu m)\) is the circumference of the cell, \(C_o\) is the circumference of a circle with the same area as the cell, and \(A_e (\mu m^2)\) is the area of the cell. To determine the measurement error inherent with calculating the undulation index, we traced a single cell 15 times. For a cell with an average undulation index of 1.05, the measured range was 0.04.

**Isotopic analysis**

Samples collected in 2014 were ground and analyzed for total C and N and δ^13N and δ^13C, using a Costech Elemental Analyser coupled via a ConFloIV to a ThermoFinnigan Delta V PLUS Continuous-Flow Isotope Ratio Mass Spectrometer (ThermoFischer Scientific Australia, Scoresby, VIC, Australia). Samples collected in 2017 were prepared similarly and analyzed using an Euro Elemental Analyser (EuroVector, Pavia, Italy) coupled to a Nu Horizon Isotope Ratio Mass Spectrometer (Nu, Wrexham, UK) at the University of Adelaide.

**Statistical analyses**

Leaf macromorphological characteristics and the scaling between leaf area or mass and measured petiole dimensions across all dicotyledonous species sampled was analyzed using standardized major axis regression using the R package smatr (Warton et al., 2012). Monocotyledonous species were excluded from these analyses given the lack of true petioles. Furthermore, the utility of using the petiole metric to predict measured LMA within our data set was examined using linear regression with both petiole metric and canopy position (understory or canopy crown) as fixed factors. All analyses were conducted in R (R Core Team, 2017) with graphical representation made using ggplot2 (Wickham, 2016).
For those 15 species for which both macro- and micromorphological traits were collected in the upper canopy crown and understory, traits ($\delta^{13}$C, LMA, cell area, and undulation index) are graphically presented and analyzed for significant differences using a paired t-test. One species, *Argyrodendron peralatum*, was found to be unsuitable for clearing and analysis of micromorphological features. As such, it was removed from the analysis of cell area and undulation index. The impact of an imposed throughfall exclusion (drought simulation) on the measured leaf traits was examined in samples collected in 2017 using a linear mixed effects model. Samples from the upper and inner canopy crown were compared using canopy position and location (i.e., drought, non-drought) as fixed factors and species as a random factor. Due to a lack of significant differences in targeted leaf traits between drought and non-drought trees (see Results), all canopy leaf samples were pooled and used in subsequent analyses of LMA, $\delta^{13}$C, cell area, and undulation index. The direct role of LAI in influencing species’ leaf traits was explored using a general linear model containing both LAI and species.

Subsequently, the potential for reconstructing LAI from target leaf traits was tested using ordinary least-squares linear regression, excluding the consideration of species identity. A similar approach was used to examine the relationship between LMA and target leaf traits to establish their suitability for reconstructing LMA in fossil assemblages. Further to these pairwise analyses, multiple factor models for both LAI and LMA were tested using ordinary least-squares stepwise regression in the *olsrr* package (Hebbali, 2018), with model selection made using adjusted $R^2$ and $\Delta$AIC as criteria. Standard errors and 95% confidence intervals for the most parsimonious models were developed using nonparametric bootstrapping in the boot package (Canty and Ripley, 2019).

All data are available through the international open-access Dryad Digital Repository (https://doi.org/10.5061/dryad.3f8bg79ff).

RESULTS

Understory versus upper canopy crown leaves

**Macromorphological characteristics across species**—Across the 81 dicotyledonous species examined from the Daintree Rainforest, representing 98 species-by-location occurrences, a comparison of petiole area as estimated from petiole-width$^2$ and petiole width $\times$ petiole depth using standardized major axis regression showed that while there was no significant difference between leaves as collected from different canopy positions (i.e., understory versus canopy; $LR = 1.97$, df = 1, $p = 0.16$) there was a significant discrepancy in the common slope from a 1:1 relationship (Appendices S1, S2, $LR = 41.57$, df = 2, $p < 0.0001$). The slope coefficient of all pooled data (understory and canopy leaves) of 0.77 (CI = 0.72 to 0.83) shows that the use of petiole-width$^2$ likely overestimates petiole cross-sectional area, especially in leaves with generally larger petioles. However, since petiole depth is often not preserved in the fossil record we continue to estimate cross-sectional area using petiole-width$^2$ but make both petiole width and depth available in the associated deposited data sets (data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.3f8bg79ff).

Examination of the log-log correlation between petiole cross-sectional area (as estimated by petiole-width$^2$) and leaf area (Fig. 1A) showed that both slope estimates (Appendix S2, $LR = 2.87$, df = 1, $p = 0.09$) and elevation (Wald statistic = 0.45, df = 1, $p = 0.505$), which is analogous to the intercept, were not significantly different between canopy and understory leaves and that the common slope was only marginally significantly different from 1 ($LR = 6.69$, df = 2, $p < 0.05$). In contrast, examination of the log-log correlation between petiole cross-sectional area (as estimated by petiole-width$^2$) and leaf mass (Fig. 1B) showed that, although the slope of the relationship was not significantly different between the two groups (Appendix S2, $LR = 0.48$, df = 1, $p = 0.49$), there was a significant difference in the elevation of the relationship (Wald statistic = 37.34, df = 1, $p < 0.001$).

The proportional scaling between the petiole metric (area-normalized petiole-width$^2$) and LMA is significant within both canopy and understory leaves (Fig. 1C). Both sets of leaves have a common slope (Appendix S2, $LR = 2.9$, df = 1, $p = 0.08$), but the elevation is shifted significantly (Wald statistic = 69.05, df = 1, $p < 0.001$) toward lower LMA values in understory leaves.

**Detailed examination of paired species**—Trait differences between canopy and understory were examined in those 15 species for which upper canopy crown and understory individuals could be compared directly (including both dicotyledonous, and monocotyledonous species). Paired t-tests showed a significant difference in LMA, cell area, undulation index, and $\delta^{13}$C (Table 1, Fig. 2). There was a consistent change in LMA and $\delta^{13}$C in all 15 species between the understory and upper canopy crown (Fig. 2A); average LMA across species was lower in the understory ($57 \pm 21$ g m$^{-2}$) and greater in the upper canopy crown ($121 \pm 35$ g m$^{-2}$), while $\delta^{13}$C (Fig. 2B) was more negative in the understory ($-35.5 \pm 1.7%$o) and less negative in the canopy crown ($-29.9 \pm 1.0%$o). Average cell area also changed significantly, showing a general increase in cell size in the understory compared to the upper canopy crown (Fig. 2C, Table 1) with one exception, *Endiandra leptodendron*, which showed an increase in epidermal cell size from understory to upper canopy crown. The dicotyledonous liana *Hypserpa decumbens* and monocotyledonous *Archontophoenix alexandrae* showed the greatest average decline in cell area of 468 and 464 μm$^2$, respectively (Fig. 2C). Undulation index (Fig. 2D) was shown to have a significant decline between understory and upper canopy crown ($t_{14} = -2.73$, $p < 0.05$), despite a number of species (e.g., *Myristica globosa* subsp. *muelleri*, *Cryptocarya murrayi*, *Brombya platynema*, and *Archontophoenix alexandrae*) essentially showing no trend with differences in average undulation index <0.05.

Leaves from across the light-gradient continuum

**Drought versus non-drought leaves**—Although canopy position was found to significantly impact LMA ($p < 0.05$) and $\delta^{13}$C ($p < 0.1$) in both drought and non-drought trees, none of the targeted leaf traits (LMA, $\delta^{13}$C, cell area, and undulation index) were found to differ significantly between drought and non-drought trees (Appendices S3 and S4). Interestingly, there was a highly significant impact upon $\delta^{15}$N, suggesting an impact of drought on N-cycling in this system. However, given a lack of distinction in the targeted “fossil” leaf traits between the drought and non-drought leaves, the results were pooled in the subsequent analyses.

**Influence of LAI upon species functional traits**—To move beyond the categorical comparison of upper canopy crown and understory
leaves, we can use leaf area index (LAI) as a direct metric for the degree of canopy openness experienced by a particular set of leaves to examine how that may impact leaf traits (Fig. 3). Specifically, we can see that measured LMA (Fig. 3A) is significantly impacted by both LAI ($F_{1, 20} = 82.2$, $p < 0.001$), and species identity ($F_{4, 20} = 19.5$, $p < 0.001$). Although only marginally significant, there is also an interaction between species and LAI ($F_{4, 20} = 2.3$, $p = 0.093$) on determining LMA, reflecting a differential impact of LAI on LMA among different species. This relationship is in contrast to $\delta^{13}$C (Fig. 3B), which also showed a significant impact of LAI ($F_{1, 20} = 29.2$, $p < 0.001$) with values becoming more negative closer to the understory, as well as a significant impact of species identity ($F_{4, 20} = 4.75$, $p = 0.007$), but with no significant interaction term ($F_{4, 20} = 0.98$, $p = 0.44$), indicating the response to LAI is invariant among species. Average cell area (Fig. 3C) also showed a significant impact from LAI ($F_{3, 16} = 35.9$, $p < 0.001$) and species identity ($F_{3, 16} = 9.2$, $p < 0.001$), with
TABLE 1. Comparison of average leaf traits in 15 extant species sampled within the closed-canopy Daintree Rainforest, Australia. Paired t-tests show significant differences as a result of sampling location either forest canopy or understory.

| Trait                          | Understory | Canopy     | df | t    | p     |
|-------------------------------|------------|------------|----|------|-------|
| Leaf mass/area (LMA; g m⁻²)    | 57.3 ± 21  | 120.7 ± 35.3| 14 | 9.4  | <0.001|
| δ¹³C (%)                      | -35.5 ± 1.7| -29.9 ± 1.0 | 13 | 13.4 | <0.001|
| Cell area (μm²)               | 500 ± 203  | 262 ± 110  | 13 | -6.03| <0.001|
| Undulation index              | 1.37 ± 0.33| 1.24 ± 0.14 | 13 | -2.36| <0.05 |

 predicted LMA, stepwise OLS regression found the most parsimonious when examining which combination of measured traits best pre-

 Predicting LAI from leaf traits—Across the four species for which a full complement of fossil leaf-trait was determined in 2017, cell area, δ¹³C, and petiole metric were all shown to have a significant relationship with branch level LMA (Table 2). Cell area was found to be the most significant single predictor of LMA (Adj-R² = 0.39, p < 0.001, Table 2), closely followed by δ¹³C (Adj-R² = 0.38, p < 0.001, Table 2). To examine whether a combination of leaf traits could be used to better predict LAI, we developed a model to predict LAI using stepwise OLS regression. The full model, using the candidate terms (δ¹³C, cell area, undulation index, and petiole metric), was compared to those containing various combinations. Stepwise selection using ΔAIC and assessment of Adj-R² found that a model containing cell area, δ¹³C, and petiole metric was the most parsimonious with Adj-R² of 0.71 (Table 3; Appendix S5), with an estimate of the 95% CI of the Adj-R² (0.46 to 0.82) being derived via bootstrap resampling. This 3-factor model was found to be a significant improvement over even a 2-factor model containing just cell area and δ¹³C with Adj-R² = 0.64 (Table 3; Appendix S5).

 Predicting LMA from leaf traits—To determine which single trait best predicted the measured LMA for our dicotyledonous species, all leaf samples that had a complete suite of measured traits (i.e., 48 branch locations) across the forest stand were examined (Appendix S6). Epidermal cell area (Adj-R² = 0.42, p < 0.001), petiole metric (Adj-R² = 0.41, p < 0.001), and δ¹³C (Adj-R² = 0.17, p = 0.0024), all have a significant relationship with LMA (Appendix S6). Moreover, when examining which combination of measured traits best predicted LMA, stepwise OLS regression found the most parsimonious linear model to include cell area, petiole metric, and δ¹³C, with no significant improvement in the model when undulation index was also included (Table 4; Appendix S7). The combined 3-factor model (RMSE = 22.0, Adj-R² = 0.60, 95% CI = 0.40 to 0.74) was found to be significantly better than either the best single factor (cell area, RMSE = 25.7, Adj-R² = 0.42, CI = 0.25 to 0.55) or 2-factor models (petiole metric + cell area, RMSE = 22.0, Adj-R² = 0.57, CI = 0.36 to 0.71). Although the addition of δ¹³C provided marginal, but significant improvement to the model (ΔAIC = 2.13, Appendix S7), given the practical constraints on gathering parallel δ¹³C information from individuals within fossil assemblages, we also present parameter estimates for the 2-factor model (Table 4).

 Predictive power of LMA and LAI models—In summary, while certain traits all independently displayed significant correlations with measured LAI and LMA in the Daintree Rainforest, we used model selection to identify the most parsimonious model for predicting both LAI and LMA from among the leaf traits that we measured. Our analysis indicated that a model that included, as independent variables, petiole metric, cell area, and δ¹³C could explain 71 and 60% of variation in LAI and LMA respectively, with little to be gained from adding more parameters. The RMSE of the optimal 3-factor model was 1.16 m² m⁻² for LAI and 22.0 g m⁻² for LMA, and the bootstrap resampling estimates of the 95% CIs on reported Adj-R² were 0.46 to 0.82 in the LAI model and 0.40 to 0.74 in the LMA model.

 Relating leaf function to leaf traits

All extant species examined within the Daintree Rainforest demonstrated a clear linear relationship (F₁,27 = 65.1, p < 0.001, Adj-R² = 0.45) between LMA and net photosynthetic capacity as measured in a saturating light environment (Fig. 4; β = 0.0659 ± 0.0082). Therefore, as might be expected from consideration of the leaf economic spectrum, predicting key functional leaf traits such as LMA from fossil leaves has the potential to provide insights into leaf functioning in fossil flora.

 DISCUSSION

We examined which leaf traits were the most useful proxies for determining both LMA and canopy position of leaf fossils, thereby providing insight into plant function and the extent of forest canopy closure. Petiole metric, cell area, and leaf δ¹³C appeared to be the most consistent predictors of canopy position across species, whereas undulation index reflected canopy cover in some species, but not others. Similarly, in quantifying variation in LMA (leaf mass per area), a key trait in the leaf economic spectrum (Wright et al., 2004) and a good predictor of leaf function (Fig. 4), we found that measured LMA was closely linked to a leaf’s light environment and that petiole metric, cell area, and leaf δ¹³C, the same traits that were most useful for predicting canopy cover, also had the strongest relationship with LMA.

The petiole metric has previously been used to reconstruct an average LMA of ecosystems (Royer et al., 2007) and the ecology and function of plants found in fossil leaf assemblages (Royer et al., 2010; Peppe et al., 2014, 2018). Our observations of LMA were within a range seen across rainforests globally (e.g., 50–120 gm⁻²; Poorter et al., 2009). However, we found a marked difference in the relationship between petiole metric and LMA for canopy crown versus understory leaves (Fig. 1C). The petiole metric, which is an expression of petiole cross-sectional area per unit leaf surface area, on no significant interaction term. For the undulation index (Fig. 3D), we see that species identity has the greatest influence (F₁,16 = 22.0, p < 0.001), yet LAI is also significant (F₁,16 = 6.15, p = 0.02), with a general increase in undulation index closer to the understory. It is worth noting that the petiole metric (Eq. 1), often used to predict LMA, showed a similar trend to that observed with measured LMA (Fig. 3E), specifically that the petiole metric was predicted by both LAI (F₁,20 = 22.0, p < 0.001) and species identity (F₁,20 = 11.1, p < 0.001), with a marginally significant interaction (F₄,20 = 2.7, p = 0.06).

Leaf micromorphological features determined from 14 species.

#### TABLE 1.

| Trait                          | Understory | Canopy     | df | t    | p     |
|-------------------------------|------------|------------|----|------|-------|
| Leaf mass/area (LMA; g m⁻²)    | 57.3 ± 21  | 120.7 ± 35.3| 14 | 9.4  | <0.001|
| δ¹³C (%)                      | -35.5 ± 1.7| -29.9 ± 1.0 | 13 | 13.4 | <0.001|
| Cell area (μm²)               | 500 ± 203  | 262 ± 110  | 13 | -6.03| <0.001|
| Undulation index              | 1.37 ± 0.33| 1.24 ± 0.14 | 13 | -2.36| <0.05 |

- Leaf micromorphological features determined from 14 species.
relates to both the mechanical support for a leaf and the hydraulic connection between the leaf and the branch (Yamada et al., 1999; Ray and Jones, 2018). A constant petiole metric would suggest that the hydraulic role is dominant in scaling petiole size with leaf size; that is, as leaf area increases there is a proportional increase in the cross-sectional area of the petiole to accommodate the additional xylem conduits needed to deliver water. However, the positive relationship between LMA and petiole metric previously observed (Royer et al., 2007, 2010) suggests a substantive role and requirement for mechanical support, as for a given leaf surface area, as leaf mass increases the cross-sectional area of petiole per unit leaf surface area also increases.

We found that understory leaves have on average a larger petiole metric for a given LMA than canopy leaves (Fig. 1C). The scaling relationship between petiole area and leaf area (Fig. 1A) is similar for understory and canopy leaves, suggesting the requirement for hydraulic coupling between petiole size and evaporative surface area is common to both sunlit and shaded environments. On the other hand, the scaling between petiole area and leaf mass is different between understory and canopy leaves (Fig. 1B). This divergence in mass-based allometric scaling constants between understory and canopy leaves accounts for our observed variance in the petiole metric to LMA relationship across groups (Fig. 1C) and may also account for variance previously seen in both herbaceous dicots (Royer et al., 2010) and ferns (Peppe et al., 2014). See Appendix S8 for formulation and further discussion. In the shaded understory, a similar amount of petiole area is required to support a given amount of leaf area as in the canopy. However, because light is scarce in the understory, plants spread their leaves thinly to capture as much light as possible and to have less leaf mass to maintain metabolically per unit leaf area, thus giving them a smaller LMA. Our data show that even though they have a smaller LMA, they still require a similar petiole cross-sectional area to support a given amount of leaf area as they would in a high-light environment. It may be that the existence of sun flecks in the understory means that the evaporative demand experienced by an understory leaf can at times be similar to that in the canopy, even if this only occurs very occasionally (Way and Pearcy, 2012).

The petiole metric may be most useful in predicting LMA across broad environmental gradients (Royer et al., 2007, 2010; Peppe et al., 2018), or when restricted to samples verified as being from the upper canopy crown. The petiole metric does not perform well as a singular predictor of LMA across the full gradient of an intact forest canopy. At the same time, increased variance in the petiole metric of a particular morphotype observed within a given fossil assemblage may be indicative of a complex canopy (containing a breadth of LAI) and thereby itself be indicative of canopy closure.

One of the strongest single predictors of canopy position was bulk leaf δ¹³C with the observed range (−37 to −26‰) similar to ranges reported across vertical gradients in tropical forests in

![FIGURE 2. Comparison of leaf traits in 15 extant species from the closed-canopy Daintree Rainforest, Australia. (A) Leaf mass per unit area, (B) δ¹³C of bulk leaf tissue, (C) average area of abaxial epidermal cells, (D) undulation index of abaxial epidermal cells. Species-level comparison of understory (US) and upper canopy crown (CC) samples highlight possible differences in the magnitude of trait plasticity between species.](image-url)
Central America (Graham et al., 2014), Eastern Amazon (Ometto et al., 2006), and Borneo (Kenzo et al., 2015). Leaf δ13C reflects canopy position due to two mechanisms. One is that the ratio of intercellular to ambient CO2 concentrations ($\frac{C_i}{C_a}$) likely increases from canopy crown to understory because there is less sunlight to drive photosynthesis with increasing canopy depth and, in a stratified forest and in the absence of turbulent mixing, ambient CO2 concentration increases in the understory. The second is that the δ13C of source CO2 can be more negative in the understory than in the canopy crown due to the influence of respired CO2 from the soil and plants in the understory (Sternberg et al., 1989; Buchmann et al., 1997; Buchmann et al., 2002; Apgaua et al., 2017). Respired CO2 typically has a δ13C in the order of −25‰, whereas CO2 in the free troposphere above the canopy has a δ13C close to −8‰. The extent to which the CO2 in the understory air is influenced by respired CO2 depends on the amount of turbulent mixing between the under-canopy air space and the free troposphere above the canopy.

**TABLE 2.** Linear regression of single leaf traits against leaf area index from which branches were collected. Samples consisted of six branches each from four tropical tree species collected within the intact-canopy Daintree Rainforest, Australia.

| Model | Intercept ± SE | Slope coefficient ± SE | Adj-$R^2$ | p       |
|-------|----------------|------------------------|-----------|---------|
| Cell area (µm²) | −0.80 ± 1.0     | 0.014 ± 0.004          | 0.39      | <0.001  |
| δ13C (%) | −15.8 ± 4.3     | −0.58 ± 0.13           | 0.38      | <0.001  |
| Petiole metric | 5.37 ± 0.92     | −3003 ± 1032           | 0.25      | <0.05   |
| Undulation index | −2.26 ± 4.17    | 4.14 ± 3.17            | 0.024     | 0.225   |

**TABLE 3.** Parameter estimates of linear regression model predicting leaf area index (LAI) of six branch locations from four species collected within the closed-canopy Daintree Rainforest, Australia. The 3-factor model on the left represents the most parsimonious model (df = 20) based upon stepwise OLS and assessment of Adj-$R^2$ (= 0.71) and ΔAIC of all models containing four candidate factors (Appendix S5). The RMSE of the 3-factor model was 1.16 m² m⁻², and the 95% CI of the Adj-$R^2$ was 0.43–0.81 as estimated via bootstrap resampling using 1000 replicates. The 2-factor model on the right (df = 21) represents a significantly poorer predictor of LAI (Adj-$R^2$ = 0.64, RMSE = 1.20, 95% CI of the Adj-$R^2$ (0.34–0.80); both models represent a significant improvement over even the best single-factor model presented in Table 2 (ΔAIC = 11.7).

| Model | Coefficient ± SE | t | p       | Coefficient ± SE | t | p       |
|-------|------------------|---|---------|------------------|---|---------|
| (Intercept) | −11.90 ± 3.24    | −3.67 | 0.002  | −14.23 ± 0.430   | −4.15 | <0.001  |
| Cell area (µm²) | 0.009 ± 0.003    | 3.47 | 0.002  | 0.012 ± 0.003    | 4.33 | <0.001  |
| δ13C (%) | −0.427 ± 0.098   | 4.37 | <0.001 | −0.435 ± 0.108   | −4.02 | <0.001  |
| Petiole metric | −1698 ± 703     | −2.42 | 0.025  | −1700 ± 1032     | −2.42 | 0.025   |

**FIGURE 3.** Influence of leaf area index (LAI) in determining leaf traits in five extant species within the closed-canopy Daintree Rainforest, Australia. (A) Leaf mass per unit area, (B) δ13C of bulk leaf tissue, (C) average area of abaxial epidermal cells, (D) undulation index of abaxial epidermal cells. (E) Petiole metric. Data represent branch average values with species-specific linear regression trendlines plotted to aid interpretation.
TABLE 4. Parameter estimates of linear regression model predicting leaf mass per area (LMA) from functional traits gathered across 48 branch locations within the closed-canopy Daintree Rainforest, Australia. The 3-factor model on the left (df = 43) represents the most parsimonious model based upon stepwise OLS and assessment of Adj-\(R^2\) (=0.60) and ΔAIC (Appendix S7). The RMSE was 22.0 g m\(^{-2}\), and the 95% CI of the Adj-\(R^2\) was 0.40–0.74, as estimated via bootstrap resampling using 1000 replications. The 2-factor model on the right (df = 44) is marginally better but significantly poorer (Adj-\(R^2\) = 0.57, RMSE = 22.0, CI = 0.35–0.71) at predicting LMA; however, it does not require the determination of δ\(^{13}\)C of fossil assemblages.

| Model                   | Coefficient ± SE | t      | p    | Coefficient ± SE | t      | p    |
|-------------------------|------------------|--------|------|------------------|--------|------|
| ( Intercept)            | 169.4 ± 41.2     | 4.11   | <0.001 | 91.9 ± 136       | 6.732  | <0.001 |
| Cell area (μm\(^2\))    | −0.092 ± 0.026   | −3.53  | 0.001 | −0.108 ± 0.026   | −4.23  | <0.001 |
| Petiole metric          | 38.593 ± 9279    | 4.16   | <0.001 | 39.650 ± 9571    | 4.14   | <0.001 |
| δ\(^{13}\)C (%o)        | 2.51 ± 1.26      | 1.99   | 0.053 |                  |        |      |

previous measurements at the Daintree Rainforest, we have found that during the daytime the δ\(^{13}\)C of CO\(_2\) in the understory could be as much as 2‰ more negative than in the upper canopy (Apgaua et al., 2017). Thus, to a first approximation, we can infer that somewhat less than half the 5‰ average difference in δ\(^{13}\)C between canopy crown and understory relates to the δ\(^{13}\)C of source CO\(_2\), and the remainder to leaf internal physiological properties. For example, in addition to the higher \(\Delta t\) driven by lower photosynthesis in typically low light of the understory, it has also been suggested that mesophyll conductance increases with canopy depth, tracking the decrease in LMA (Duursma and Marshall, 2006). Therefore, the ratio of chloroplastic to intercellular CO\(_2\) concentrations may also increase with canopy depth, allowing for a larger discrimination against \(^{13}\)C in the understory.

Micromorphological features appeared to be generally correlated to LAI, with some significant exceptions. Epidermal cell area was generally larger in the understory compared to the upper canopy crown samples (Fig. 2C), with *Endiandra leptodendron* displaying an opposite trend. This overall pattern was consistent with more detailed examination across a range in LAI (Fig. 3C) and previous studies, where cell area is reduced with increasing light exposure (Rahim and Fordham, 1991). In contrast, undulation index was not only variable among species in its magnitude of change across LAI, but a large number of species displayed no response at all (Figs. 2D, 3D). For example, *C. australis* displayed the highest undulation index in the understory and a strong negative trend toward the canopy crown, while *M. globosa* had the lowest undulation index and no response to canopy position. These results support the conclusion that undulation index is not only species-specific (Bush et al., 2017), but also requires cautious calibration to extend to relative if it is to be used as a sole indicator of LAI within fossil leaf assemblages (Wilf, 2000). It should be noted that undulation index was calculated from the abaxial cells for consistency with previous studies (Bush et al., 2017). However, adaxial (outgrowth, light-capturing surface) epidermal tissue has in some cases been found to be more sensitive to environmental change, but this response could also be a species-specific mechanical response to irradiance (Xiao et al., 2011). Although adaxial epidermal cells were not used in this study, *M. globosa* had a noticeable difference between the two leaf surfaces. While there was no response in abaxial cells, undulation was prominent in adaxial cells (Appendix S9). Future studies which quantify differences between adaxial and abaxial leaf tissues will therefore be beneficial for calibrating proxies based on leaf cuticles.

Where cuticular preservation has taken place, such that petiole metric, cell area, and δ\(^{13}\)C can be measured, our model offers an opportunity to reconstruct both fossil LAI and LMA with reasonable confidence (Table 4). It is also worth noting that while including δ\(^{13}\)C did significantly improve the model for predicting LMA from fossil leaf traits, the improvement in RMSE between the actual and predicted values was marginal. Given the practical constraints associated with obtaining concurrent micromorphological and isotopic data, the two-factor model (Table 4) seems to represent the most serviceable tool for predicting LMA in fossil leaves. Modern measurements from additional species and sites will of course be useful in improving the parameterization and validity of the model's application to fossil assemblages. However, our current model already has the potential to predict both leaf function (via LMA) and the degree of canopy closure via LAI because a small range of low LAI values would indicate a high proportion of sunlit regions, while a large range would indicate the presence of a distinct canopy structure and a more closed canopy.

To compensate for taphonomic biases in production and preservation of fossil leaves, any sampling strategy of the fossil flora would require an adequate sample size, ideally inferred through sequential sampling until variation in leaf traits has plateaued. This sampling strategy follows on the approach of Graham et al. (2014), who used leaf δ\(^{13}\)C to characterize canopy closure and used re-sampling to estimate that approximately 100 leaves are required to capture the rarer understory leaves from tropical rainforest in Panama. With large sample size requirements, the most practical model for reconstructing LAI would be based on leaf δ\(^{13}\)C and epidermal cell area because these traits can be determined from fossil leaf fragments.
Thus, the models presented here provide a means of determining the range of reconstructed light environments of individual leaves to provide insight into the density of canopy cover in ancient forests.

The observed relationships between leaf traits and both LAI and LMA can also be used to examine habitat and growth strategy of individual morphotypes within a fossil flora. Carbon isotope ratios and undulation index of fossil leaves have been used in combination to try to characterize individual taxa from fossil floras (e.g., Bush et al. 2017), but with only limited success because δ13C and undulation index did not provide consistent information about canopy position. Although responsiveness of cell wall undulation to light environment varies significantly among species, it may still provide supporting ancillary information in the case of highly responsive taxa. However, cell area provides a promising alternative measure related to LMA and LAI. Combining cell area, δ13C, and petiole metric, where possible, would enable robust reconstruction of LMA and light environment across different taxa within a fossil assemblage.

CONCLUSIONS

This study examined multiple leaf traits from a range of canopy positions and species from the Daintree Rainforest, Australia, to evaluate their utility for reconstructing local light conditions, i.e., leaf area index (LAI), and leaf function, i.e., leaf mass per area (LMA), from fossil floras. All traits (undulation index, cell area, δ13C, and petiole metric) measured for 15 extant taxa varied significantly between the upper canopy crown and understory. Although responsiveness of cell wall undulation to light environment varies significantly among species, it may still provide supporting ancillary information in the case of highly responsive taxa. However, cell area provides a promising alternative measure related to LMA and LAI. Combining cell area, δ13C, and petiole metric, where possible, would enable robust reconstruction of LMA and light environment across different taxa within a fossil assemblage.

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AUTHOR CONTRIBUTIONS

F.A.M., A.W.C., and L.A.C. conceived and developed the experimental design, F.A.M., A.W.C. and H.D. collected samples and A.W.C., H.D., and K.H. determined leaf-level functional traits, H.D. and A.W.C. analyzed the data and developed a first draft. All authors contributed to writing the paper.

DATA AVAILABILITY STATEMENT

All data used in this publication are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.3fbbg79ff (Cheesman et al., 2020).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Estimates of petiole cross-sectional area.

APPENDIX S2. Coefficient estimates and CI of standardized major axis regression of leaf macromorphological characteristics.

APPENDIX S3. Comparison of leaf-level functional traits of five tropical tree species from drought and control trees.

APPENDIX S4. Results of GLM examining the impact of canopy position and location upon leaf traits.

APPENDIX S5. Linear regression models of leaf traits to predict leaf area index (LAI).

APPENDIX S6. Linear regression models of single leaf traits to predict leaf mass per area (LMA).

APPENDIX S7. Linear regression models of multiple leaf traits to predict leaf mass per area (LMA).

APPENDIX S8. Discussion on scaling of leaf mass index (LMA) with petiole metric.

APPENDIX S9. Example images of epidermal cells.

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