

# Extending the generality of leaf economic design principles in the cycads, an ancient lineage

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## Summary

- Cycads are the most ancient lineage of living seed plants, but the design of their leaves has received little study. We tested whether cycad leaves are governed by the same fundamental design principles previously established for ferns, conifers and angiosperms, and characterized the uniqueness of this relict lineage in foliar trait relationships.
- Leaf structure, photosynthesis, hydraulics and nutrient composition were studied in 33 cycad species from nine genera and three families growing in two botanical gardens.
- Cycads varied greatly in leaf structure and physiology. Similarly to other lineages, light-saturated photosynthetic rate per mass ( $A_m$ ) was related negatively to leaf mass per area and positively to foliar concentrations of chlorophyll, nitrogen (N), phosphorus and iron, but unlike angiosperms, leaf photosynthetic rate was not associated with leaf hydraulic conductance. Cycads had lower photosynthetic N use efficiency and higher photosynthetic performance relative to hydraulic capacity compared with other lineages.
- These findings extend the relationships shown for foliar traits in angiosperms to the cycads. This functional convergence supports the modern synthetic understanding of leaf design, with common constraints operating across lineages, even as they highlight exceptional aspects of the biology of this key relict lineage.

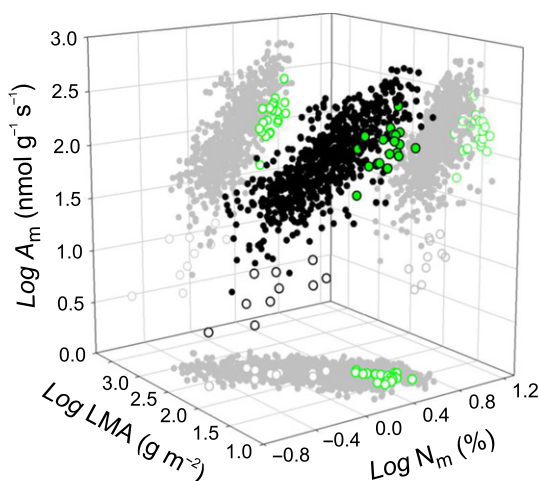
## Introduction

Cycads are the most ancient living seed plant lineage (Brenner *et al.*, 2003), considered as living fossils because of the morphological similarity between fossil and extant species. The oldest known cycad fossil dates to the Paleozoic (Mamay, 1969; Gao & Thomas, 1989), and the group reached dominance in the Mesozoic, the age of dinosaurs. Cycads show high diversity in morphology and structure, with leaves that are typically compound and possessing a thick cuticle (Norstog & Nicholls, 1997; White-lock, 2002). Cycads vary greatly in their leaf vein system; some have single midveins in leaflets, others have midveins with dichotomizing lateral veins, and yet others have multiple parallel veins as in ferns (Stevenson *et al.*, 1996). Leaf veins usually contain tracheids, but primitive vessels have also been found in some *Cycas* species (Huang & Zhang, 1999; Sack & Scoffoni, 2013). The leaf structural and physiological trait variation, and its association with light-saturated photosynthetic rate have not been

quantified for cycads although these have been well established for angiosperms of a wide range of ecosystems and taxonomic groups. Indeed, it is not well-known whether cycad leaves show strong variation in physiology, or whether that variation would be constrained by similar principles as in angiosperms. In contrast to typical angiosperms, cycads have no response in stomatal density to elevated  $CO_2$  (Haworth *et al.*, 2011) suggesting that they may have a relatively inflexible leaf design and physiology compared with angiosperms. The aim of this study was to assess for cycads the association of traits with photosynthetic rate, as expected based on studies of angiosperms, conifers, ferns and mosses (Fig. 1), including nutrient composition, leaf structure, stomatal conductance and leaf hydraulics.

The first trait tested for association with leaf photosynthetic rate was nutrient concentration. Nitrogen (N) is a crucial component of photosynthetic proteins, especially Rubisco (Field & Mooney, 1986). Total Rubisco activity and amount increase as leaf N increases (Sage *et al.*, 1987), and across angiosperm, conifer and fern species, leaf photosynthetic rate tends to correlate with leaf N on an area and/or mass basis (Fig. 1) (Field &

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**Fig. 1** Relationships among leaf nitrogen (N) concentration ( $N_m$ ), leaf mass per area (LMA, or canopy mass per area for mosses) and light-saturated photosynthetic rate per mass ( $A_m$ ). Closed black circles, Glopanet (mainly angiosperms but also with conifers and three cycads) (Wright *et al.*, 2004); closed green circles, mosses (Waite & Sack, 2010); open circles, ferns (Karst & Lechowicz, 2007).

Mooney, 1986; Reich *et al.*, 1997; Wright *et al.*, 2004; Karst & Lechowicz, 2007). Other nutrients such as phosphorus (P), potassium (K), iron (Fe), sulfur (S) and zinc (Zn), are also important components of the photosynthetic apparatus and reactions (e.g. Randall & Bouma, 1973; Spiller & Terry, 1980; Ares *et al.*, 2009). However, perhaps due to a bias based on the much greater abundance of N and P, to our knowledge no interspecific relationships between leaf photosynthetic rate and nutrients other than N and P have been tested in any lineage.

In angiosperms, the effect of leaf structure on photosynthetic rate is well recognized (Reich *et al.*, 1998; Niinemets & Sack, 2006). Across angiosperm and conifer species, light-saturated photosynthetic rate per leaf dry mass ( $A_m$ ) and leaf N concentration per mass ( $N_m$ ) correlate negatively with leaf mass per area (LMA) across a wide range of ecosystems (Fig. 1), typically independently of climate (Reich *et al.*, 1997; Wright *et al.*, 2004). These relationships, known as 'leaf economic spectrum' (LES) relationships, arise when photosynthetic rate per leaf area ( $A_a$ ) and leaf N per area ( $N_a$ ) are partially or fully independent of LMA. The conversion of  $A_a$  to  $A_m$  by dividing by LMA thus results in LES relationships by mathematical necessity (Lloyd *et al.*, 2013; Osnas *et al.*, 2013). Yet, these relationships do reflect a physical influence of leaf structure on photosynthetic rate per mass (Sack *et al.*, 2013; Westoby *et al.*, 2013). Plants with higher LMA, due to thicker and/or denser leaves, tend to have a longer diffusion path and greater diffusion resistance from stomata to chloroplasts. Additionally, greater mass 'dilution' of cytoplasmic and chloroplast components by nonmetabolic components such as cell walls and nonstructural carbohydrates (Parkhurst, 1994; Reich *et al.*, 1997, 1998; Roderick *et al.*, 1999a,b; Shipley *et al.*, 2006) would lead to a lower  $A_m$ . The negative correlation between LMA and  $N_m$  is also due to the same mass dilution effect (Wright *et al.*, 2004; Sack *et al.*, 2013; Westoby *et al.*, 2013). We expected that this mass dilution effect also exists for other nutrients and mediates the photosynthetic nutrient use

efficiency. Recently, a hypothesis that LMA and the LES relationships would be structurally determined by leaf vein density has been proposed (Blonder *et al.*, 2011, 2013, 2014). However, a recent test found that LMA was uncorrelated with leaf vein density across 275 phylogenetically diverse angiosperm species, and within eight of the nine individual families for which sufficient data were available (Sack *et al.*, 2013, 2014). Like conifers, which show LES relationships while having only a single central vein in their needles (Reich *et al.*, 1998), cycads provide a key system for determining whether LES relationships can arise in leaves that lack minor veins within their lamina.

The third trait tested for association with photosynthetic rate was xylem water transport efficiency. Leaf or stem hydraulic conductance has been found to be closely associated with leaf transpiration rate and maximum photosynthetic rate within the same species under different environmental conditions, within several sets of angiosperm species, and across representatives of many lineages (Kuppers, 1984; Meinzer & Grantz, 1990; Sober, 1997; Mencuccini, 2003; Santiago *et al.*, 2004; Brodribb *et al.*, 2007; Campanello *et al.*, 2008; Zhang & Cao, 2009). These data, and manipulative experiments, have indicated that hydraulic conductance places a constraint on stomatal conductance ( $g_s$ ) and on photosynthetic gas exchange. The leaf hydraulic conductance ( $K_{leaf}$ ) plays a particularly important role because the leaf is a bottleneck in the whole-plant water transport pathway (Sack & Frolle, 2006; Brodribb *et al.*, 2007). Although  $K_{leaf}$  is well-recognized as a key determinant of  $A_a$  across species in very diverse species sets, the relationships of gas exchange with  $K_{leaf}$  may be climate- or habitat-specific, as they would tend to shift for optimality between habitats (Sack *et al.*, 2005; Feild *et al.*, 2011b; Sack & Scoffoni, 2013). Additionally, a negative relationship between mass-normalized  $K_{leaf}$  ( $K_{leaf-m}$ ) and LMA has been reported for angiosperms (Simonin *et al.*, 2012). Given the negative relationship of  $A_m$  to LMA, these relationships may result in a positive association of  $A_m$  and  $K_{leaf-m}$ .

All of these previous findings represent a modern synthetic understanding that leaf structure, nutrient composition, water flux and photosynthetic rates are all related (e.g. Wright *et al.*, 2003; Cramer *et al.*, 2009), and are subject to the general principles of leaf design. However, to confirm the generality of these principles of leaf design, trait relationships need to be evaluated across different ecosystems and taxonomic groups, including lineages other than angiosperms. Cycads represent an ideal model for such a test, given the diversity of extant species, and the earlier origin and previous global dominance of this lineage. We studied cycads in two botanical gardens with similar climates to determine trait differences arising from genetic rather than plastic expression across natural habitats (Monson, 1996). We tested the hypotheses that photosynthetic performance is related to hydraulics, nutrient composition and leaf structure in cycads, and the relationships among functional traits should be similar in cycads to those found for other seed plants. We aimed to extend the understanding and generality of fundamental leaf trait relationships by focusing on the modern representatives of this ancient lineage and to characterize the uniqueness of cycads in foliar trait relationships.

## Materials and Methods

### Study site and plant material

This research was carried out in the Cycad Garden at Xishuangbanna Tropical Botanical Garden (XTBG; 21°41'N, 101°25'E, elevation 570 m) in southern Yunnan Province and the National Cycad Germplasm Conservation Center at Fairylake Botanical Garden (FBG; 22°34'N, 114°10'E, elevation 100–130 m) in Shenzhen, Guangdong Province, southern China. Mean annual temperature at XTBG is 21.7°C, and mean annual precipitation is 1560 mm with 80–85% occurring in the May–October rainy season (40-yr average, 1959–1998, data from Xishuangbanna Station for Tropical Rain Forest Ecosystem Studies). The soil of the Cycad Garden at XTBG is sandy alluvium with pH of 5.0–6.0; its total nutrient composition is 1.12 mg g<sup>-1</sup> N, 0.51 mg g<sup>-1</sup> P, 8.49 mg g<sup>-1</sup> K, 8.33 mg g<sup>-1</sup> calcium (Ca), 2.89 mg g<sup>-1</sup> magnesium (Mg), 23.04 mg g<sup>-1</sup> Fe, 0.56 mg g<sup>-1</sup> manganese (Mn), 73.25 mg kg<sup>-1</sup> Zn, and 22.0 mg kg<sup>-1</sup> Cu (0–20-cm depth). Soil hydrolyzable N, available P and available K are 66.5, 13.4 and 85.8 mg kg<sup>-1</sup>, respectively. Mean annual temperature at FBG is 22.4°C, and mean annual precipitation is 1933 mm falling mostly in the May–October rainy season (30-yr average, 1971–2000, data from Shenzhen Weather Station). The soil of the National Cycad Germplasm Conservation Center at FBG is yellow soil with pH of 5.5–7.0, containing 1.25 mg g<sup>-1</sup> N, 0.52 mg g<sup>-1</sup> P, 16.1 mg g<sup>-1</sup> K, 1.20 mg g<sup>-1</sup> Ca, 2.82 mg g<sup>-1</sup> Mg, 30.13 mg g<sup>-1</sup> Fe, 0.42 mg g<sup>-1</sup> Mn, 51.4 mg kg<sup>-1</sup> Zn, and 9.43 mg kg<sup>-1</sup> Cu (0–20-cm depth) in total. Soil hydrolyzable N, available P and available K are 115, 132 and 141 mg kg<sup>-1</sup>, respectively. The physiological measurements at XTBG were performed in the rainy season 2009, and the measurements at FBG were performed in the rainy season 2011.

Thirty-three cycad species with at least three healthy and mature individuals suitable for physiological measurements were selected for the present study, with 10 from genus *Cycas* (family Cycadaceae), 19 from genera *Ceratozamia*, *Dioon*, *Encephalartos*, *Lepidozamia*, *Macrozamia* and *Zamia* (family Zamiaceae), and three from genera *Bowenia* and *Stangeria* (family Stangeriaceae) (Supporting Information Table S1). These species vary highly in native habitats (Table S1). Based on their native habitats, the species were classified into three classes: native to dry habitats, moist habitats, or both dry and moist habitats (for habitat information see Table S1). The genus *Cycas* is a sister lineage to all other cycads based on the phylogeny of cycads inferred from morphological characters or gene sequences (Brenner *et al.*, 2003; Chaw *et al.*, 2005; Zgurski *et al.*, 2008). A fossil-calibrated phylogeny dates the *Cycas* lineage to the Mesozoic, whereas the lineage leading to *Zamia* dates to the Cenozoic (Nagalingum *et al.*, 2011). *Zamia* has evenly spaced, longitudinally parallel, dichotomizing veins in leaflets whereas *Cycas* only has a single middle vein (Stevenson *et al.*, 1996). Leaf hydraulic conductance ( $K_{\text{leaf}}$ ) was measured for 23 species, given difficulties in removing the effects of mucilage on  $K_{\text{leaf}}$  measurement for the remaining 10 species. Sun-exposed individuals were used for physiological measurements except for three shade-tolerant species which were

cultivated under a tree overstorey (*B. spectabilis*, *B. serrulata*) or in a shade house (*S. eriopus*). Four species were studied in both XTBG and FBG (*Cycas szechuanensis*, *C. panzhihuaensis*, *Z. furfuracea*, *E. gratus*), and these four species were entered twice in the dataset. Six mature leaves from three to six individuals per species were selected for the measurements. Leaflets were chosen as the fundamental unit for measurement and the middle section of the leaves were used for physiological measurements such as gas exchange and nutrient compositions. Leaflets were used, as these are most analogous to angiosperm simple leaves or leaflets, typically measured in studies of functional traits.

### LMA, leaf thickness and density

Leaf thickness was averaged from values taken with vernier calipers at five places on each fresh leaflet. Leaf area was determined (LI-3000A area meter; Li-Cor, Lincoln, NE, USA), then the leaves were oven-dried at 70°C to constant mass, and weighed, and LMA was determined as leaf dry mass divided by leaf area (g m<sup>-2</sup>). Leaf density (mass/volume) was determined as LMA divided by leaf thickness (Witkowski & Lamont, 1991).

### Gas exchange measurements

The light-saturated net CO<sub>2</sub> assimilation rate per area ( $A_a$ ;  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and stomatal conductance ( $g_s$ ;  $\text{mol m}^{-2} \text{s}^{-1}$ ) were measured using a portable photosynthetic system (LI-6400; Li-Cor). Leaves were measured on sunny days between 08:30 and 10:30 h solar time, at a photosynthetic photon flux density of 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and ambient temperature (28–35°C) and CO<sub>2</sub> concentration. Relative humidity ranged from 40% to 63%. Leaf temperatures ranged from 28 to 36°C at XTBG, and 27 to 32°C at FBG. Light-saturated net CO<sub>2</sub> assimilation rate per mass ( $A_m$ ;  $\text{nmol g}^{-1} \text{s}^{-1}$ ) was determined as  $A_a$  divided by LMA.  $A_a$  and  $g_s$  relative to hydraulic supply, representing leaf photosynthetic performance relative to investment in leaf hydraulic system ( $A_K$  and  $g_K$ ;  $\text{mmol MPa mol}^{-1}$  and  $\text{mol MPa mol}^{-1}$ , respectively), were calculated as  $A_a$  and  $g_s$ , respectively, divided by  $K_{\text{leaf-a}}$  ( $\text{mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$ ; see later).

### Nutrient and chlorophyll concentrations

Leaves with rachis removed were oven-dried at 70°C for 48 h and analyzed for C, N, P, K, Ca, Zn, Fe and S. Leaf C and N concentrations were determined using a Vario MAX CN auto element analyzer (Elementar Analysensysteme, Hanau, Germany). After the samples were digested with concentrated HNO<sub>3</sub>–HClO<sub>4</sub>, leaf P, K, Ca, Zn, Fe and S concentrations were measured using an inductively coupled plasma atomic-emission spectrometer (IRIS Advantage-ER; Thermo Jarrell Ash Corporation, Franklin, MA, USA). Nutrient concentrations per unit area were calculated by multiplying mass-based nutrient concentrations by LMA. Photosynthetic N use efficiency ( $A_N$ ) and P use efficiency ( $A_P$ ) were determined as  $A_m$  divided by mass-based concentrations of N and P ( $N_m$  and  $P_m$ ) respectively. Leaf chlorophyll concentration was measured with a chlorophyll meter (SPAD 502; Minolta



Corp., Osaka, Japan) for six sun-exposed mature leaves from three to six individuals per species. For each leaf, 10 measurements across the lamina were averaged ( $\text{Chl}_a$ ; in SPAD units, using a SPAD meter; SPAD-502; Minolta Corp.). Previous studies reported strong relationships between SPAD measurements and total extracted  $\text{Chl}_a + b$  per area when leaves varied in nutrient composition, thickness and surface features (Marquard & Tipton, 1987; Singha & Townsend, 1989; Fanizza *et al.*, 1991; Manetas *et al.*, 1998). Furthermore, when multiple species have been compared, the majority tended to have similar relationships (Marquard & Tipton, 1987; Gratani, 1992; Markwell *et al.*, 1995; Castelli *et al.*, 1996; Uddling *et al.*, 2007; Marenco *et al.*, 2009), suggesting that SPAD measurements can be considered as a proxy for extractable chlorophyll. Chlorophyll concentration per mass ( $\text{Chl}_m$ ) was determined as  $\text{Chl}_a$  divided by LMA.

### Leaf hydraulic conductance

Leaf hydraulic conductance ( $K_{\text{leaf}}$ ) was measured using the evaporative flux method (Sack *et al.*, 2002; Brodribb *et al.*, 2007; Sack & Scoffoni, 2012). Leaves were cut from the plants in the late afternoon, recut immediately under water and placed with cut ends underwater and covered with black plastic bags to rehydrate overnight. Measurements of  $K_{\text{leaf}}$  were made on central segments of leaves, excised underwater, including rachis and 4–18 leaflets rather than on individual leaflets because petioles of the leaflets were very short or nonexistent. The leaf segment was held horizontal in a frame strung with fishing line, above a fan, and under full sunlight to drive transpiration, with the cut end of the rachis connected to water-filled tubing downstream of a water pressure-drop flow meter with two calibrated pressure transducers (Melcher *et al.*, 2012) logging to a computer, and the transpiration flow rate ( $E$ ) was monitored. When a steady-state  $E$  was reached (i.e. a stable value maintained for at least 3 min), the leaf segment was removed into a ziplock bag that had been exhaled into, to prevent dehydration. Leaf water potential ( $\Psi_L$ ) was determined after at least 30 min equilibration, using a pressure chamber (SKPM 1400; Skye Instruments Ltd, Lladrindod Wells, UK), and leaf area was measured (LI-3000A area meter; Li-Cor). The leaf area-normalized leaf hydraulic conductance ( $K_{\text{leaf-a}}$ ;  $\text{mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$ ) was calculated as the steady-state  $E/\Psi_L$ , normalized by leaf area. Leaf temperature during measurement was monitored with a T-type thermal couple logging to a data logger (CR1000; Campbell Scientific, Logan, UT, USA), and  $K_{\text{leaf-a}}$  was then corrected for the effects of temperature on the viscosity of water by standardizing to 20°C (Sack *et al.*, 2002; Brodribb *et al.*, 2007). The  $K_{\text{leaf-a}}$  reported is the maximum, determined by plotting  $K_{\text{leaf-a}}$  against  $\Psi_L$  and extrapolating to  $\Psi_L$  of zero (Brodribb *et al.*, 2007). Maximum mass-normalized leaf hydraulic conductance ( $K_{\text{leaf-m}}$ , leaf water supply per dry mass;  $\text{mol s}^{-1} \text{g}^{-1} \text{MPa}^{-1}$ ) was determined as  $K_{\text{leaf-a}}$  divided by LMA.

### Data analyses

Statistical analyses were applied using SPSS V21 (IBM Corp., Armonk, NY, USA). To examine trait-trait linkages, we tested

relationships hypothesized *a priori* (see the Introduction section and Fig. 1). We analyzed the relationship between functional traits using linear or nonlinear regression according to which best approximated the structure of the relationship. Equal variances of the variables were tested and one-way ANOVAs were used to test trait differences among cycad genera. The differences in slope and intercept of bivariate relationships between cycads and plants from the global datasets (Wright *et al.*, 2004) or an angiosperm  $K_{\text{leaf}}$  dataset (Simonin *et al.*, 2012) were tested with standardized major axis tests using SMATR v2.0 (Warton *et al.*, 2006). The study site effect on leaf gas exchange characteristics ( $A_a$ ,  $g_s$  and  $A_m$ ) was tested by a two-way ANOVA with garden as the fixed factor and species as the random factor using four species studied in both XTBG and FBG (*C. szechuanensis*, *C. panzhihuaensis*, *Zamia furfuracea*, *Encephalartos gratus*). A principal components analysis (PCA), (Pearson, 1901) was performed to summarize the joint variation of the functional traits (see Table S2) for the 33 species, with mean trait values of each species used for the analyses.

## Results

### Variation of leaf traits among diverse cycad species

The cycad species varied strongly in leaf gross morphology and shape (Fig. 2), as well as in photosynthetic rate, and stomatal and hydraulic conductance (Tables 1, S3). Stomatal conductance ( $g_s$ ) varied 10-fold (from 0.03 to  $0.32 \text{ mol m}^{-2} \text{s}^{-1}$ ), whereas leaf hydraulic conductance per mass ( $K_{\text{leaf-m}}$ ) varied 13-fold (from 12.1 to  $157 \text{ mol s}^{-1} \text{g}^{-1} \text{MPa}^{-1}$ ; Table S3).

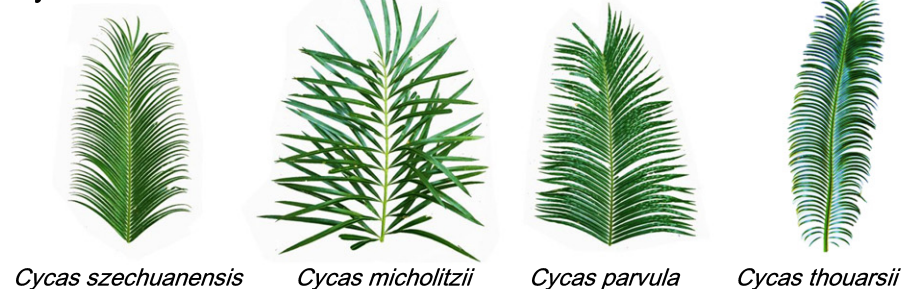
Cycad species also varied strongly in nutrient concentrations and LMA, leaf thickness and leaf density (Tables 1, S3).  $N_a$  and  $P_a$  varied four- to five-fold, and the area based concentrations of other nutrients varied four-fold ( $S_a$ ) to 20-fold ( $Ca_a$ ).  $N_m$  and  $P_m$  varied across species three- to four-fold, whereas mass based concentrations of other nutrients varied 1.1-fold ( $C_m$ ) to 20-fold ( $Fe_m$ ). LMA, leaf thickness and leaf density varied five- to seven-fold across the species (Tables 1, S3). Cycad species also varied strongly in photosynthetic use-efficiency in relation to nitrogen, stomatal conductance and hydraulic conductance (Table 1).

The two genera of shade-tolerant species in family Stangeriaceae (*Bowenia* and *Stangeria*) showed significantly lower  $g_s$ ,  $A_a$  and LMA than other cycad genera, whereas other genera did not differ significantly on average in any measured trait ( $P > 0.05$ , one-way ANOVA). Further, no significant difference was found between family Cycadaceae and family Zamiaceae in any measured trait (one-way ANOVA). No study site effects (difference between two gardens) on  $A_a$ ,  $g_s$  and  $A_m$  were detected ( $P > 0.05$ , two-way ANOVA).

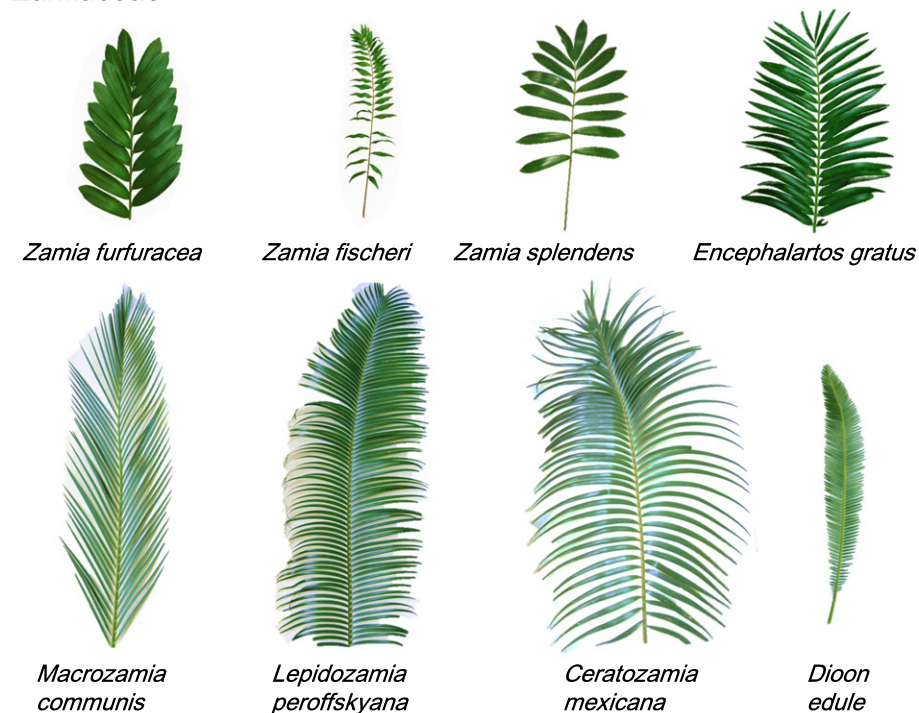
### Coordination of leaf traits among diverse cycad species

The leaf economic trait relationships that were hypothesized based on those established for angiosperms were also very strong across the cycad species, and there were stronger trait relationships for mass-based than area-based light-saturated  $\text{CO}_2$  assimilation rate. The  $A_a$  was positively related to  $g_s$  ( $r^2 = 0.74$ ,

## Cycadaceae



## Zamiaceae



## Stangeriaceae



**Fig. 2** Diversity in leaf form for 15 of the 32 cycad species studied.

$P < 0.001$ , relationship not shown). Additionally,  $A_m$  was negatively related to LMA, and positively related to  $Chl_m$ ,  $N_m$ ,  $P_m$ ,  $Fe_m$  (Fig. 3),  $K_m$  and  $S_m$  (relationships not shown). The relationship between  $A_m$  and  $Fe_m$  was found when data from each site were analyzed separately ( $P < 0.001$ ). These relationships were significant even without including the two *Zamia* species (*Z. fischeri*, *Z. vazquezii*) that had substantially higher  $A_m$  (and in the case of *Z. fischeri*, substantially higher  $Fe_m$ ) than other species (Table S3; dashed lines in Fig. 3).

In general, many cycad leaf traits were related to LMA, and particularly mass-based traits (Fig. 4). Photosynthetic N and P

use efficiencies ( $A_N$  and  $A_P$ ),  $N_m$ ,  $Chl_m$ ,  $P_m$ ,  $S_m$  and  $Fe_m$  were negatively related to LMA (Fig. 4). Although no statistical association was found between  $K_{leaf-a}$  and  $A_a$  (Fig. 5a), a significant relationship was found when they were expressed on a mass basis, that is, between  $K_{leaf-m}$  and  $A_m$  (Fig. 5b).

#### Comparison of trait ranges with those in previous studies of cycads and other lineages

Our data indicated a very strong physiological diversity among the cycads and similar trait relationships as previously reported

**Table 1** Comparison of cycads in this study with Glopnet (Wright *et al.*, 2004), data spanning plant lineages, mosses and ferns in the mean and range of light-saturated photosynthetic rate per area ( $A_a$ ), light-saturated photosynthetic rate per mass ( $A_m$ ), photosynthetic nitrogen (N) use efficiency ( $A_N$ ), light-saturated photosynthetic rate over leaf hydraulic conductance ( $A_K$ ), N concentration per area ( $N_a$ ), N concentration per mass ( $N_m$ ), phosphorus (P) concentration per area ( $P_a$ ), P concentration per mass ( $P_m$ ), area based leaf hydraulic conductance ( $K_{leaf-a}$ ), and leaf mass per area (LMA) values

	$A_a$	$A_m$	$A_N$	$A_K$	$N_a$	$N_m$	$P_a$	$P_m$	$K_{leaf-a}$	LMA
Cycads										
Mean	9.34	59.5	2.95	2.07	3.38	2.00	0.20	0.12	5.44	178
Minimum	3.15	23.6	1.42	0.61	1.52	1.22	0.08	0.07	2.50	50
Maximum	14.1	179	6.06	3.98	6.57	3.01	0.39	0.24	10.1	323
Glopnet										
Mean	11.5	128	6.31		1.94	1.94	0.13	0.11		128
Minimum	1.00	4.80	0.63		0.26	0.25	0.02	0.01		14
Maximum	42.0	662	25.5		9.14	6.36	0.88	0.60		1510
Sampling across plant lineages										
Mean	8.16			1.12					8.11	
Minimum	0.89			0.35					0.50	
Maximum	19.0			3.03					21.1	
Mosses										
Mean	0.93	8.11	0.01		0.08	0.80				13
Minimum	0.59	3.31	0.01		0.03	0.24				6
Maximum	1.41	14.0	0.03		0.12	1.51				31
Ferns										
Mean	4.50	67.5	2.88	1.30	1.64	1.75	0.08	0.09	2.93	90
Minimum	1.10	9.40	0.86	0.67	0.53	0.80	0.03	0.04	0.50	37
Maximum	12.3	115	5.29	2.20	3.54	3.29	0.20	0.13	5.23	158

Data source: cycads, 33 species (present study); Glopnet, 2548 species mainly angiosperms but also including conifers and including three cycads (Wright *et al.*, 2004); data spanning plant lineages, 12 angiosperms, 17 gymnosperms, six ferns, four bryophytes, five lycophytes (Brodrribb *et al.*, 2007); Mosses, 10 species (Waite & Sack, 2010); ferns, 19 species (Wright *et al.*, 2004; Brodrribb *et al.*, 2007).

for other lineages. The wide range of values for  $K_{leaf-a}$  and  $A_a$  for the 33 cycad species studied encompassed the full range of values previously reported for conifers (Fig. 5a). However, the  $A_a$  of cycads were at the high end of the  $A_a$  range of conifers and had a higher median (Fig. 5c), whereas  $K_{leaf-a}$  of cycads showed similar range and median relative to conifers (Fig. 5d). This helps explain the high  $A_K$  values of cycads (Table 1). The  $A_a$  of two cycad species (*C. cairnsiana* and *C. media*) previously measured by Brodrribb *et al.* (2007) were close to the mean values of the cycads in the present study, whereas  $K_{leaf-a}$  of those two species were close to the highest value found in this study.

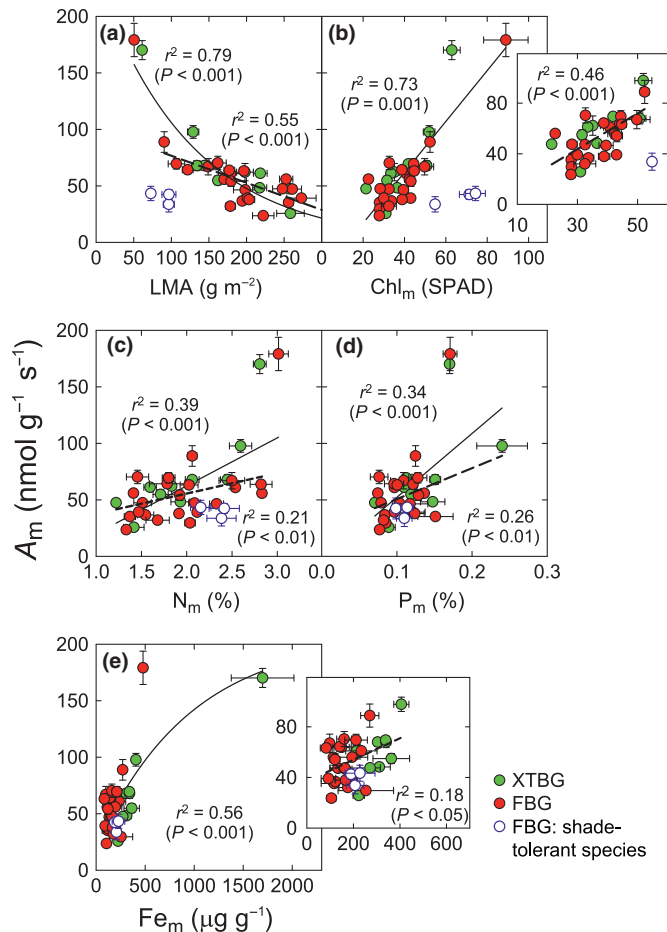
By contrast, in several features, cycads showed lower maximum physiological capacities than angiosperms. Thus, the maximum  $A_a$ ,  $A_m$  and  $A_N$  in cycads were much lower than those in a global database consisting mainly of angiosperm species (Glopnet; Table 1). The mean  $A_a$  of cycads was very close to the Glopnet mean and slightly higher than the mean for a dataset of representatives from several plant lineages (angiosperms, conifers, ferns, bryophytes and lycophytes), and much higher than values compiled for mosses and ferns (Table 1). The mean LMA, and mass- and area-based concentrations of N and P for cycads were higher than the Glopnet mean, and also higher than those compiled for mosses or ferns. Consequently, the mean  $A_m$  and  $A_N$  values of cycads were much lower than the Glopnet mean (Wright *et al.*, 2004), slightly higher than that of ferns, and much higher than that of mosses. The mean  $K_{leaf-a}$  of cycads was low relative to other plant lineages, but, the  $A_K$  of cycads was higher than the mean for a dataset with representatives spanning all lineages, and higher than that compiled for ferns.

The bivariate relationships among LMA,  $A_m$  and  $N_m$  in cycads were generally consistent with the relationships for the species in Glopnet. No statistical differences in slope were found between the two datasets, although the relationships differed significantly in their intercepts (Fig. 6). Cycads tended to have a higher  $N_m$  at a given LMA, and a lower  $A_m$  at a given  $N_m$ , indicating lower photosynthetic N use efficiency compared with the global mean (Fig. 6b,c).

The relationship between  $K_{leaf}$  and LMA in cycads generally followed the relationship for species in a combined dataset mainly for angiosperms (Simonin *et al.*, 2012). No relationship was found between  $K_{leaf-a}$  and LMA (Fig. 7a), whereas  $K_{leaf-m}$  was significantly negatively related to LMA (Fig. 7b). No difference in slope was found between the two datasets, although the intercepts differed significantly ( $P < 0.01$ ; Fig. 7b). The intercepts for  $\log(K_{leaf-m})$  vs  $\log(LMA)$  of cycads and angiosperms were 5.31 and 4.84  $\text{mol s}^{-1} \text{g}^{-1} \text{MPa}^{-1}$ , respectively.

In a PCA analysis, the first axis, which explained 32% of the total variation, represented the variation in investment in leaf mass structure, and thus with leaf composition and physiology. The first PCA axis was positively related to LMA, leaf thickness,  $C_m$  and  $Chl_a$ , and negatively correlated with  $A_m$ ,  $Chl_m$ ,  $A_N$ ,  $A_p$ ,  $K_{leaf-m}$ ,  $N_m$ ,  $S_m$ ,  $Fe_m$ ,  $P_m$ ,  $Zn_m$ ,  $K_m$  and  $K_{leaf-a}$  (Fig. 8a). The second axis, which explained 21% of the total variation, represented the variation in stomatal opening for gas exchange and water use efficiency; this axis was positively correlated with  $g_s$ ,  $A_a$ ,  $K_{leaf-a}$ ,  $g_K$  and  $A_K$ , and negatively correlated with water use efficiency ( $AI/g$ ). *Zamia fischeri* and *Z. vazequezii* were separated far from other species along the first axis, whereas shade-tolerant species were



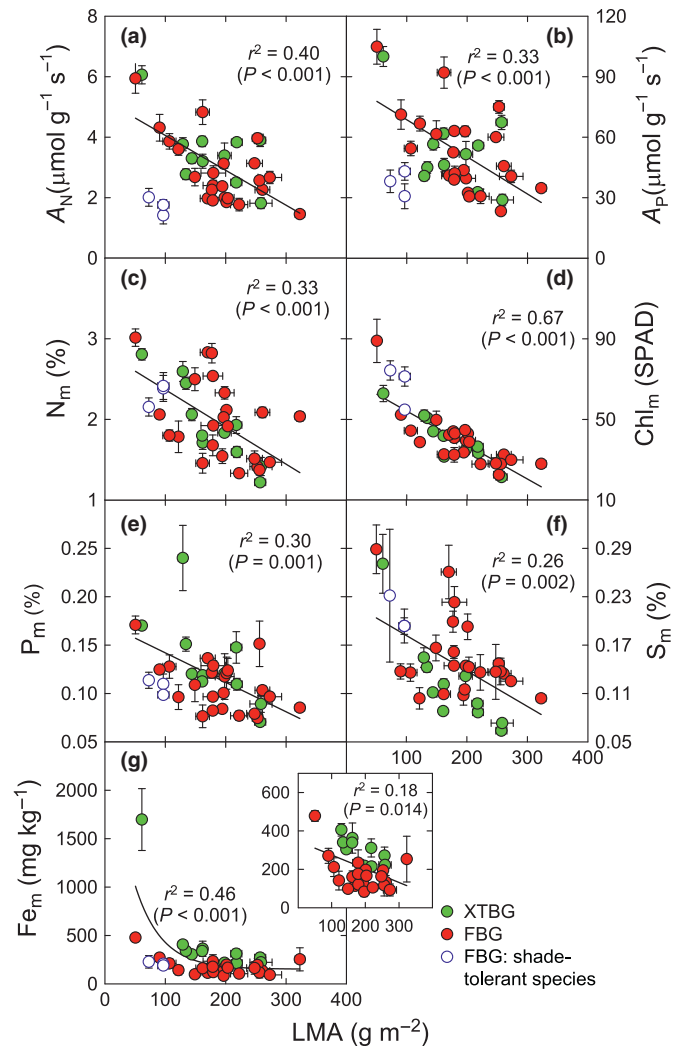


**Fig. 3** Light-saturated photosynthetic rate per mass ( $A_m$ ) in relation to leaf mass per area (LMA), chlorophyll concentration per mass (Chl<sub>m</sub>) and leaf nutrient concentrations per mass ( $N_m$ ,  $P_m$ ,  $Fe_m$ ) across cycads in Xishuangbanna Tropical Botanical Garden (XTBG, green circles) and Fairylake Botanical Garden (FBG, red circles). Points are means ± SE. Exponential (a, e) or linear (b–d) regressions were fitted to the data. The regressions do not include shade-tolerant species (open circles). The linear regressions indicated by the broken lines do not include *Zamia fischeri* and *Z. vazquezii*.

separated from other species along the second axis (Fig. 8b). The first and second axes could not separate cycads from FBG and XTBG, but most plants in XTBG fell into the upper two quadrants (Fig. 8b). Species native in dry habitats were not separated from those native in moist habitats or those found in both dry and moist habitats (Fig. 8c).

## Discussion

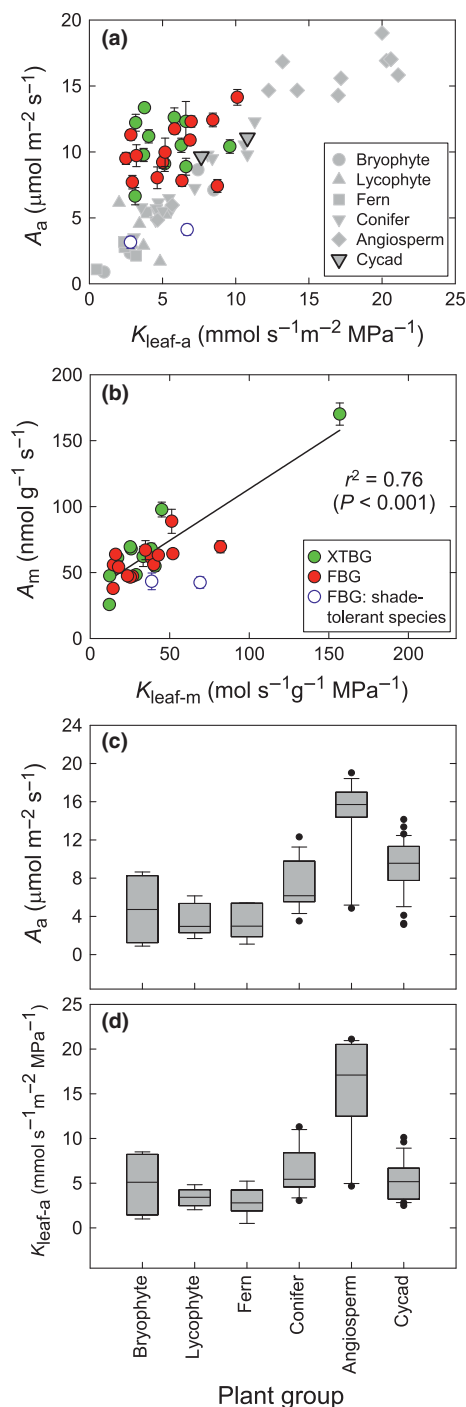
Cycads have previously been described as highly diverse in their overall morphology (Whitelock, 2002). One major novel finding of this study is a correspondingly strong variation among cycad species in leaf physiology and structure. The values for leaf traits, and the coordination of traits showed considerable contrasts with other plant lineages. Most impressively, the cycads showed similar nutrient values, but lower gas exchange for given nutrient values, and no apparent coordination of hydraulics and gas exchange on a



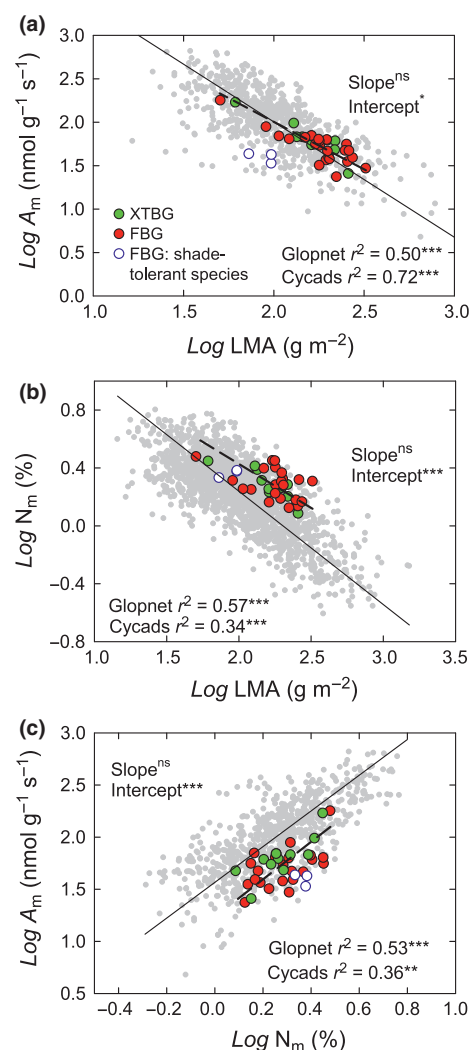
**Fig. 4** Leaf photosynthetic nitrogen and phosphorus use efficiency ( $A_N$  and  $A_P$ , respectively), nutrient concentrations per mass ( $N_m$ ,  $P_m$ ,  $Fe_m$ ,  $S_m$ ,  $Zn_m$ ,  $Cu_m$ ) and chlorophyll concentration per mass (Chl<sub>m</sub>) in relation to leaf mass per area (LMA) across cycad species in Xishuangbanna Tropical Botanical Garden (XTBG, green circles) and Fairylake Botanical Garden (FBG, red circles). Points are means ± SE. Linear (a–f) or exponential regressions (g) were fitted to the data with shade-tolerant species (open circles) not included.

leaf area basis across species. However, the cycads were convergent with other lineages in their leaf economic spectrum relationships. A recent molecular dating study suggested that the extant cycads are not older than *c.* 12 Myr (Nagalingum *et al.*, 2011). However, we hypothesize that these trait relationships first evolved in the ancient cycads and remain in modern cycads because first, cycads show relative morphological, and potentially, physiological stability in the face of long-term climate change (Haworth *et al.*, 2011), and second, fossil and extant cycads have strong morphological similarities.

Just as studies have shown these relationships to hold within and across extant biomes (Reich *et al.*, 1997; Wright *et al.*, 2004), our study extends the existence of these relationships to a lineage dating back 250 Myr, suggesting that they potentially existed for



**Fig. 5** The relationship between light-saturated photosynthetic rate and maximum leaf hydraulic conductance expressed on an area basis ( $A_a$  and  $K_{\text{leaf-a}}$ ; a), and on a mass basis ( $A_m$  and  $K_{\text{leaf-m}}$ ; b) and box plots of  $A_a$  (c) and  $K_{\text{leaf-a}}$  (d) of cycads compared with other plant groups. Green circles, Xishuangbanna Tropical Botanical Garden (XTBG) cycads; red circles, Fairylake Botanical Garden (FBG) cycads; Blue open circles, shade-tolerant species. (a, b) Error bars indicate  $\pm$  SE. The solid line is a linear regression fitted to the data with shade-tolerant species (blue open circles) not included. The grey points are data for different taxonomic plant groups from Brodribb *et al.* (2007). (c, d) Boundaries of the boxes indicate the 25<sup>th</sup> and 75<sup>th</sup> percentiles, whiskers denote the 90<sup>th</sup> and 10<sup>th</sup> percentiles, points represent observations beyond these percentiles and internal lines denote the medians.



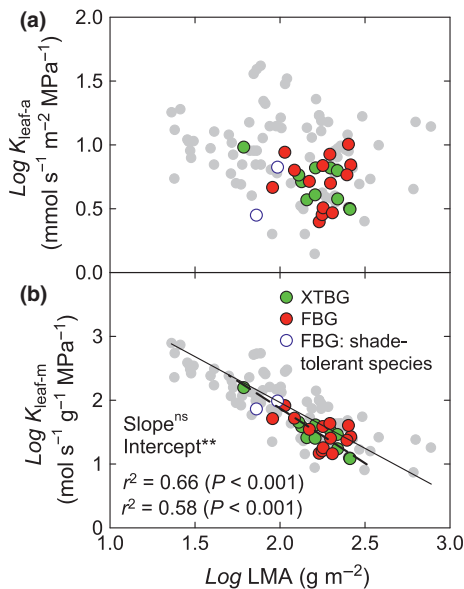
**Fig. 6** Relationships between leaf mass per area (LMA) and mass based light-saturated photosynthetic rate ( $A_m$ ), between LMA and leaf nitrogen concentration ( $N_m$ ), and between  $N_m$  and  $A_m$  across cycads in Xishuangbanna Tropical Botanical Garden (XTBG, green circles) and Fairylake Botanical Garden (FBG, red circles) and the global dataset (Glopnet). The grey points are global dataset of Wright *et al.* (2004). All variables were log-transformed. Solid lines are standardized major axis (SMA) lines fitted to the global dataset, whereas break lines SMA lines fitted to the cycad species with shade-tolerant species (blue open circles) not included. ns, no significant difference ( $P > 0.05$ ); \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

the species that dominated in those now extinct ecosystems. Further, the presence of these relationships among cycads just as previously found for angiosperms and conifers indicates that these trait relationships appeared multiple times as leaves evolved distinctly in different major seed plant lineages.

#### High variation in leaf functional traits and comparisons with other lineages

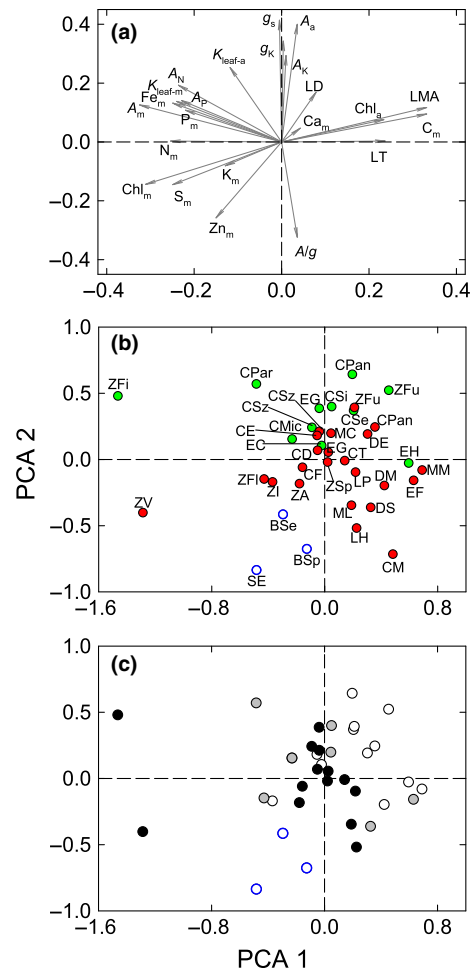
The high variation in leaf structural and physiological traits found among the cycad species within common gardens points to genetic divergences across species native to contrasting





**Fig. 7** Relationships between maximum leaf hydraulic conductance expressed on an area basis ( $K_{\text{leaf-a}}$ ; a) and leaf mass per area (LMA), and between  $K_{\text{leaf}}$  on a mass basis ( $K_{\text{leaf-m}}$ ; b) and LMA across cycad species in Xishuangbanna Tropical Botanical Garden (XTBG, green circles) and Fairylake Botanical Garden (FBG, red circles). The grey points are a combined dataset mainly for angiosperms from Simonin *et al.* (2012). The solid line is a standardized major axis (SMA) line fitted to the combined angiosperm dataset, whereas the break line a SMA line fitted to the cycad species with shade-tolerant species (blue open circles) not included. ns, no significant difference ( $P > 0.05$ ); \*\*,  $P < 0.01$ .

environments (Monson, 1996). Although the dominance of cycads in terrestrial ecosystems has declined since the late Mesozoic, cycads still grow successfully in a wide range of habitats from very moist tropical rainforest to dry lands and high elevations (Norskog & Nicholls, 1997; Whitelock, 2002). The native habitats of the cycad species used in this study also varied greatly in their native water regimes. The cycads varied especially strongly in traits known to shift across habitats differing in water availability, such as LMA, photosynthetic traits and leaf hydraulic conductance ( $K_{\text{leaf}}$ ). Indeed, the range of values for cycads extended across the whole range of the values previously reported for other gymnosperms. Notably, cycads possessed higher  $K_{\text{leaf-a}}$  and  $A_a$  than more primitive plant groups including bryophytes, lycophytes and ferns (Brodrick *et al.*, 2007; Waite & Sack, 2010), which contributes to higher relative growth rates for a given level of leaf allocation (Sack *et al.*, 2005; Zhang & Cao, 2009). Recently the higher maximum values of  $K_{\text{leaf-a}}$  and  $A_a$  of angiosperms has been proposed to have contributed to, or driven their dominance over earlier groups since the Late Cretaceous (Brodrick & Feild, 2010; Feild *et al.*, 2011a). Similarly, the higher values for cycads than modern members of more primitive lineages (bryophytes, mosses and ferns), together with their high diversification in leaf structure and function, provide a potential physiological explanation for the dominance of cycads across a variety of terrestrial habitats in the Mesozoic, assuming that this range of trait values also characterized ancestral cycads, as discussed earlier.



**Fig. 8** Principal component analyses (PCA) of mean values of leaf traits for 33 cycad species. Trait (a) and species (b, c) loadings on the first and second axes. Trait symbols are in Supporting Information Table S2 and species codes in Table S3. (b) Green circles, cycads in Xishuangbanna Tropical Botanical Garden (XTBG); red circles, cycads in Fairylake Botanical Garden (FBG). (c) White circles, species from dry habitats; black circles, species from moist habitats; grey circles, species found both in dry and moist habitats; blue open circles, shade-tolerant species.

Compared with angiosperms, however, cycads, like other gymnosperms, exhibited lower maximum and/or mean values for  $K_{\text{leaf-a}}$ ,  $A_a$  and  $A_m$  (Table 1). These traits would be related to low relative growth rates and lead to a major disadvantage for gymnosperms compared with angiosperms (Bond, 1989; Berendse & Scheffer, 2009). The lower  $K_{\text{leaf-a}}$  of the cycads compared with angiosperms may relate to their low leaf vein density, which limits development of high transpiration and photosynthetic rates and imposed a major disadvantage on cycads competing with angiosperms (Boyce *et al.*, 2009). Indeed, cycads had lower  $A_m$  for a given  $N_m$  than the mean for seed plants (Fig. 6c), that is, lower photosynthetic N use efficiencies ( $A_N$ ). This difference may have arisen due to greater partitioning of N to nonphotosynthetic structures (Hikosaka, 2004). Although information on N partitioning of cycads is unavailable, cycads have high concentrations of lignin, leaf mucilage and other secondary compounds (Norskog & Nicholls, 1997; Brenner *et al.*, 2003), requiring substantial

investment of N and other nutrients. The higher LMA of cycads compared with the Glopnet mean agrees with their high investments in leaf toughness and herbivory defense (Clark & Clark, 1988; Prado *et al.*, 2014) and potentially high leaf longevity compared with other seed plant lineages (up to 106 months for *M. riedlei*; Wright *et al.*, 2004). The lower  $K_{\text{leaf-a}}$ ,  $A_a$ ,  $A_m$  and  $A_N$  of cycads than angiosperms would impose disadvantages for competing with later seed plants, and by hypothesis, would have contributed to the decline in dominance of cycads in late Mesozoic (Cretaceous), when angiosperms began to diversify and establish dominance (Crane *et al.*, 1995; Berendse & Scheffer, 2009).

### Relationship between leaf hydraulic conductance and leaf photosynthetic capacity in cycads

A positive relationship between area-based  $K_{\text{leaf-a}}$  and  $A_a$  previously has been shown for plants across a variety of taxonomic groups including bryophytes, lycopods, ferns, gymnosperms and angiosperms (Brodribb *et al.*, 2007). As described in the Introduction, the close relationship between leaf hydraulics and photosynthesis has been generally explained by the importance of efficient leaf water supply for maintaining relatively high leaf water potential during transpiration to allow stomata to remain open. However, no association was found between  $K_{\text{leaf-a}}$  and  $A_a$  across the cycad species studied. The absence of a relationship between  $K_{\text{leaf-a}}$  and  $A_a$  may be due to the fact that the cycad species originate in habitats with a wide range of water availabilities (e.g. soil water potential and vapor pressure deficit). Plants of a given life form and from habitats with similar water availabilities converge in a relatively narrow range of  $\Psi_L$  during peak transpiration, and therefore  $g_s$  could be associated with  $K$  (hydraulic conductance) as  $g_s = K \times (\Psi_L - \Psi_{\text{soil}})/VPD$ . Species from habitats with different water availabilities may operate at different  $\Psi_L$ , decoupling the coordination between leaf hydraulic conductance and gas exchange properties (Sack *et al.*, 2005; Field *et al.*, 2011b; Sack & Scoffoni, 2013). However, no coordination between leaf water transport and photosynthetic rate was detected even when the relationship was tested in species native to habitats with similar water availabilities (data not shown). Alternatively, decoupling between leaf water transport and photosynthetic rate could have arisen due to the influence of other factors such as leaf water storage, especially as cycad leaves possess hypodermis and transfusion tissues (Hu & Yao, 1981), which might allow stomata to remain open despite low water transport across the lamina. Indeed, cycads tend to have higher  $A_a$  despite similar  $K_{\text{leaf-a}}$  to conifers (Table 1; Fig. 5), representing a higher photosynthetic performance relative to hydraulic investment. Another potential factor contributing to the water and carbon decoupling is that some cycad species could be Crassulacean acid metabolism (CAM)-facultative plants (e.g. *D. edule*) (Vovides *et al.*, 2002), which would result in relatively high photosynthetic water use efficiencies. The relationship between  $K_{\text{leaf-m}}$  and  $A_m$  would arise from a negative relationship between  $K_{\text{leaf-m}}$  and LMA (Fig. 7b). Indeed, the negative relationship between  $K_{\text{leaf-m}}$  and LMA in cycads generally agreed with the relationship reported

for angiosperms (Simonin *et al.*, 2012), suggesting a strong generality in this relationship. The mathematical source of this negative relationship is that  $K_{\text{leaf-m}}$  is calculated as  $K_{\text{leaf-a}}$  divided by LMA, and that  $K_{\text{leaf-a}}$  is independent of LMA. The significance of the trend is a trade-off between mass investment in leaf hydraulics and in leaf longevity (Simonin *et al.*, 2012). The lower  $K_{\text{leaf-m}}$  at a given LMA in cycads than for angiosperms suggests a relatively low resource investment in leaf hydraulic system.

### Trait relationships in cycads with respect to the global leaf economic spectrum

The relationships of fundamental leaf functional traits for cycads generally agreed with previously reported trait relationships in the global leaf economic spectrum (Field & Mooney, 1986; Reich *et al.*, 1997; Wright *et al.*, 2004). Recent studies have also revealed that ferns and mosses follow the global leaf economic spectrum in general, though LMA was replaced by canopy mass per area for mosses (Karst & Lechowicz, 2007; Waite & Sack, 2010). Therefore, the fundamental ecological and physiological trade-offs constraining leaf functional traits apply not only to late-derived seed plants, but also to relatively primitive living plants such as mosses, ferns and cycads, confirming the generality of the principal trade-offs. Notably, *Z. fischeri* showed much lower LMA and leaf thickness, and much higher  $A_m$ ,  $K_{\text{leaf-m}}$  and nutrient concentrations than other cycad species. However, the leaf traits of even this 'outlier' species followed most of the functional trait relationships among other species, indicating tightly bounded domains constraining leaf traits (Reich *et al.*, 1997; Meinzer, 2003; Shipley *et al.*, 2006).

Another novel aspect of our study is the expanded set of nutrient relationships important in the leaf economic spectrum. In addition to the relationships among LMA,  $A_m$ ,  $N_m$  and  $P_m$ , we found relationships of LMA and  $A_m$  with  $Fe_m$  and  $S_m$ , as well as between LMA and photosynthetic nutrient use efficiencies ( $A_N$  and  $A_P$ ). Indeed,  $Fe_m$  was more strongly related to  $A_m$  than  $N_m$ , which agrees with the Chinese traditional knowledge that cycads show strong preference for Fe and soil addition of Fe may increase growth rates of some cycads. Fe is related to numerous electron transfer reactions and biochemical processes in photosynthesis and is required for biosynthesis of chlorophyll (e.g. Spiller & Terry, 1980). The concentration of Fe in the nontoxic range has been positively related to the amount of photosynthetic machinery and consequently to photosynthetic capacity (Spiller & Terry, 1980). The generality of these relationships needs to be tested in other taxonomic groups. Notably, the nutrient concentrations of cycads were close to the mean values for dominant angiosperm species across geographical locations in China (Hou, 1982), whereas  $N_m$  and  $P_m$  of cycads were even slightly higher than the Glopnet mean. Although cycads are often able to fix N via symbiotic cyanobacteria in roots, the strong relationship between nutrient concentrations and  $A_m$  suggests a conserved proportion of nutrients invested in photosynthetic machinery and thus to  $A_m$ . A limitation imposed by the amount of photosynthetic machinery on  $A_m$  was also indicated by the strong

relationship between  $Chl_m$  and  $A_m$ . Apparently due to a lack of data of nutrient concentrations other than N and P in the global dataset, interspecific relationships among concentrations of nutrients other than N and P with  $A_m$  and LMA have not been established across other taxonomic groups.

In conclusion, the relationships among cycad leaf functional traits generally agreed with the global economic spectrum, confirming the generality of the fundamental trade-offs, and potentially extending these principles of leaf design to the ancient past. High leaf hydraulic conductance and photosynthetic rate in cycads compared with bryophytes, lycophytes and ferns, as well as high diversification in leaf structure and function would have facilitated their dominance in a variety of ecosystems in the Mesozoic. Low leaf water supply and photosynthetic nutrient use efficiencies in cycads relative to angiosperms could be a potential factor in limiting their development of higher growth rates. Nutrients other than N (e.g. Fe and S) explained a significant amount of variation in  $A_m$  of cycads as well, suggesting the importance of introducing nutrients other than N and P into a higher resolution understanding of the leaf economic spectrum. Interestingly, no coordination between leaf hydraulic conductance and photosynthesis was found among cycads but overall they tended to have higher photosynthetic performance relative to hydraulic capacity compared with other plant lineages.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Table S1** Cycad species studied in Xishuangbanna Tropical Botanical Garden (XTBG) and Fairylake Botanical Garden (FBG), origin and native habitats

**Table S2** Traits, symbols and units

**Table S3** Means and SE for each trait by species

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