FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

EVOLUTION AND BIOLOGICAL ACTIVITY OF CYCADOPHYTA

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Gustavo Matías De Angeli

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To: Dean Michael R. Heithaus
        College of Arts, Sciences and Education

This thesis, written by Gustavo Matías De Angeli, and entitled Evolution and Biological Activity of Cycadophyta, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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ABSTRACT OF THE DISSERTATION

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Worldwide, environment gradients effects on leaf physical and chemical defenses traits, were studied in very phylogenetically heterogeneous plants; leading to results that may be confounding. To address this, in this study, we focused on a single Order, Cycadales. We collected data for 8 key leaf traits from 80 Cycads species around the world (~27% of all cycad species known). The leaf traits included, foliar area, leaf mass area, thickness, leaf density, toughness, water, hinokiflavone and amentoflavone content, all of which are closely related to trade 'offs between plant defenses and leaf construction. We quantified the relative contributions of environment variables to leaf trait variation for all species comparing results to a recent phylogeny data base. Our results suggest that the climatic adaptation of leaves in this ancient group required a decoupling between traits associated with tissue investment and leaf thickness. Adaptation to more humid, warmer areas required an increase in chemical protection against herbivores, whereas drier environments with high temperature range, selected for thicker leaves better protected against excessive radiation. These findings contribute to the understanding of Cycads evolution characteristics across environmental gradients.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

Ecological traits are the features that describe the overall ecology of a taxon such as life history, geographical distribution, climatic tolerances, or edaphic preferences (H. John 2000).

In plants, traits represent biological adaptations to the environment and fundamental tradeoffs between individual species, as they direct the acquisition, processing, and investment of resources (Ulrike 2013).

Each species ecological traits display some variability in response to environmental factors that can be explained by   two complementary mechanisms: (1) evolutionary ancestry or genetic variability, which is the phenotypic variability between individual genotypes; and (2) phenotypic plasticity, which represents the potential of each genotype to produce different phenotypes under differing environmental conditions (Albert et al. 2011, Lambers et al. 1998, Schilchtin 2002; Violle et al. 2012).

In terms of plant defenses traits, plants have two types of herbivory defenses, chemical and morphological to protect their leaves (Prado et. al. 2014). Both are metabolically expensive and plants must balance trade-offs between different strategies (Coley et al., 1985; Kikuzawa and Lechowicz, 2011).

Worldwide, leaf traits of plants are classified according to the Leaf Economic Spectrum (LES), which links evolutionary life history of plants to leaf functional traits (Wright et. al. 2004). On one side of LES are species that produce long-lived leaves with high leaf mass per area (LMA), which reflects on high density, high thickness and that also represent bigger investment in structural and mechanical defenses (e.g., leaf toughness).   This leaves characteristics are mostly seen in shade-tolerant plant species

from tropical rainforest habitats, where herbivory pressure is higher and leaf toughness enhances resistance against it, because it is costly for an insect herbivore to eat tough leaves (Coley, 1983; Hochuli,1996 ; Lucas et al., 2000 ; Kitajima et al., 2012), increasing survival and offsetting the biomass allocation cost of producing tough leaves in shaded areas under the canopy. A contradiction that rises up is that greater cell wall thickness is necessary for leaf toughness and this reduces photosynthesis capacity. Cell walls that are thick, waxy and fibrous generally increase the leaf's internal resistance to CO_2 diffusion, leading to greater allocation of N for the construction of cell walls in the leaf, and thus a possible trade-off characterized by reduced photosynthesis (Hikosaka, 2004), a capacity that is already reduced in plants growing under the canopy of rainforests.

On the other side of LES are species that produce thinner and short-lived leaves with low LMA, high photosynthetic rate and low investment in mechanical and structural defenses, what leads to softer leaves. Therefore this type of leaves, must rely on other defenses traits, such as toxic secondary metabolites and trichomes (Coley and Aide, 1991 ; Kursar and Coley, 2003). The before mention patterns have been demonstrated at a global scale, making use of a wide variety of data sets encompassing species from a wide array of clades and families, giving a great understating of LES and plant defenses at a worldwide scale. However, researches and conclusion were made on hugely phylogenetically divergent plants and results may not be really representative of climatic adaptation but also influenced by phylogenetic variability.

That is why a new system with the possibility of studying leaf trait and trade-offs variation through environmental gradients in a large group of phylogenetically close related species that evolved to adapt to very divergent climatic environments and herbivory threats,

would be ideal to help elucidate the structural and physical adaptations that allowed species diversify in contrasting environments, without phylogenetical discrepancies.

Therefore, I propose studying these phenomena in the order Cycadales. Cycadales is a relatively small clade with one ancestor in common, phylogenetically controlled and with a huge environmental range (Mankga et. al. 2020). Order Cycadales encompasses two families, 10 genera, and around 300 species tree (Stevenson, 1992; Nagalingum et al., 2011). The group appeared and has been evolving on earth since Permian period, 300 million years ago (Hendricks, 1987; Gao and Thomas, 1989; Calonje et al., 2017).

The relatively small size of this order, large evolutionary history and exceptional ecological distribution range, makes them the ideal candidates for the study of leaf functional traits and defenses among the environmental gradients of their native habitats while keeping a close phylogenetic familiarity.

LITERATURE REVIEW

DESCRIPTION

Cycadophytes (cycads) are long-lived woody plants with frond-like leaves clustered at the stem tip, unlike other extant gymnosperms (Fig 1a). Cycads are plants with leaves and habits similar to palm trees, though some species are quite small. Cycads are dioecious, with most bearing large, colored female or male cones. Pollen grains contain motile spermatozoa, which is a primitive feature. They were once much more abundant and, following their appearance in the Permian, remained important members of the world's Mesozoic floras.

Their extinction was especially pronounced in the Late Cretaceous, when they were gradually displaced by angiosperm trees. The survivors are regarded as 'living fossils'. Order Cycadales include 10 genera and approximately 300 species. All are tropical or subtropical The Cycadaceae family is sister to the rest of the cycads in the family Zamiaceae. Cycadaceae includes only the genus Cycas and it's confined to the Southeastern Asia, the Pacific Islands, and a few species in eastern Africa and Madagascar. Family Zamiaceae includes four genera that are American: Zamia, Microcyas, Dioon, and Ceratozamia; five are Old World: Macrozamia and Bowenia (Australian), Stangeria and Encephalartos (south-eastern Africa only). Cycads are declining all over the world, with four species on the verge of extinction and seven species having fewer than 100 plants left in the wild. Two-thirds of Cycads are listed as threatened on the International Union for Conservation of Nature's Red list.

Cycads have a cylindrical trunk that rarely branches. Leaves sprout directly from the trunk and typically fall when the tree reaches maturity, leaving a crown of leaves at the top. The leaves form a rosette, with new foliage emerging from the crown's top and center. Because the trunk may be buried, the leaves appear to emerge from the ground, giving the plant the appearance of a basal rosette. The leaves are typically larger in proportion to the trunk size, and in some cases larger than the trunk itself.

The leaves are pinnate (shaped like bird feathers, pinnae, figure 1b), having a central leaf stalk from which parallel "ribs" (stalks that grow perpendicular to it from each side) emerge from each side. Usually, the leaves are compound (the leaf stalk has leaflets protruding from it as "ribs") or have edges (margins) that are so sharply cut (incised) that they give the impression of being compound. Some Asian Cycas species, including Cycas multipinnata, Cycas micholitzii, and Cycas debaoensis, as well as the Australian genus Bowenia, have bipinnate leaves, which means that the leaflets each have their own subleaflets that develop in the same way on the leaflet as the leaflets do on the stalk of the leaf (self-similar geometry).

Cycads are unique among gymnosperms because they are the only ones that can establish fresh mutualistic relationships with cyanobacteria (Adam et. al. 2013). All species of cycads form symbiotic relationships with cyanobacteria in specific structures known as coralloid roots (Figure 2).

Figure 1a. Cycas revoluta

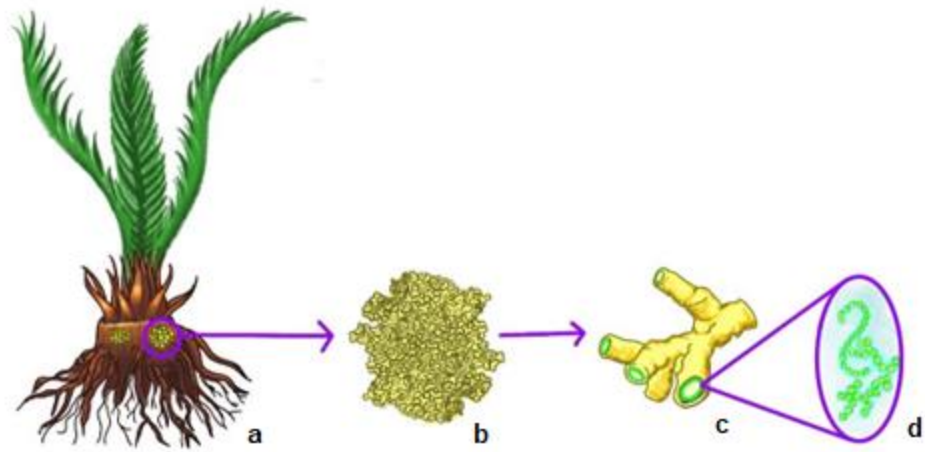Figure 1b. Diversity in leaf form for 15 of the 80 cycad species studied.

Figure 2. Zoom in from Cycad to cyanobacteria. a. bare roots'cycad b. coralloid roots c. transversal section of coralloid roots d. cyanobionts layer in the roots.

Origins

Cycads are a group of ancient plants that have been present on Earth for over 300 million years. The origin of cycads is rooted in the history of plant evolution, and the study of their origins has provided valuable insights into the evolution of plants over time.

Cycads are part of a larger group of plants known as gymnosperms, which are characterized by their lack of flowers and seeds that are not enclosed in a fruit. The earliest gymnosperms appeared during the late Devonian period, around 380 million years ago, and were likely similar to ferns in their form and function. These early gymnosperms developed seeds that were protected by a coating, allowing them to disperse more effectively and to survive harsh environmental conditions.

During the late Carboniferous period, around 300 million years ago, the first evidence of cycads appears in the fossil record. At this time, the Earth's climate was warm and humid, and forests of giant ferns and club mosses dominated the landscape. It is thought that cycads evolved from a group of seed ferns that were common at this time.

The emergence of cycads in the fossil record coincides with a period of great diversity and evolutionary innovation among seed plants. During this time, the first conifers and ginkgos appeared, and the gymnosperms began to dominate the landscape, with cycads becoming one of the most diverse and widespread groups.

The Mesozoic Era, which lasted from around 252 million years ago to 66 million years ago, saw the peak of cycad diversity. During this time, cycads were a dominant plant group in many parts of the world, including the supercontinent of Pangaea. They were widespread and diverse, with a variety of leaf shapes and reproductive structures. Some species grew to enormous sizes, with trunks up to 20 meters tall.

The extinction of dinosaurs and other plant and animal groups at the end of the Cretaceous period, 66 million years ago, had a major impact on the diversity of cycads. Despite this major extinction event, cycads persisted and continued to evolve into the modern era.

Today, there are around 300 species of cycads that are distributed across the globe. While many species are endangered due to habitat loss and over-collection, cycads remain a unique and important part of the Earth's biodiversity.

Distribution

Cycads are found throughout the world, with their distribution varying depending on the species. The global distribution of cycads reflects their ancient origin and their ability to adapt to a range of environmental conditions.

The majority of cycads are found in the tropics and subtropics, where they are often associated with arid or semi-arid habitats. The highest concentration of cycads is in the Americas, where there are over 150 species in the family Zamiaceae. These species are found in a range of habitats, from rainforests to deserts, but are most common in areas with rocky soils and low rainfall.

In South Africa, there are around 39 species of cycads, many of which are endemic to the region. These species are often found in rocky areas, but some also grow in coastal dunes and forests. South African cycads are highly prized by collectors and are often illegally harvested for their ornamental value, leading to many species being threatened with extinction.

Australia has around 30 species of cycads, which are mostly found in the northern parts of the country. These species are adapted to a range of habitats, from rainforests to open woodlands, and are able to tolerate both wet and dry conditions. The Australian cycads are also popular as ornamental plants, with some species having been introduced to other parts of the world.

In Asia, cycads are found in a range of habitats, from lowland rainforests to high-altitude mountains. The most diverse area for cycads in Asia is in the Philippines, where there are around 65 species. These species are found in a range of habitats, including lowland and montane forests, and some are threatened by habitat loss and over-harvesting.

In Africa, outside of South Africa, cycads are found in a few isolated areas. The island of Madagascar has a high diversity of endemic species, with around 47 species recorded. These species are adapted to a range of habitats, from dry deciduous forests to wet rainforests.

Cycads are also found in the Pacific islands, with some of the most diverse areas being in Fiji, New Caledonia, and the Solomon Islands. In these regions, cycads are often found in forests, on cliffs, and on rocky outcrops.

Overall, the distribution of cycads is wide-ranging and diverse, reflecting their adaptability to different environments. (Figure 3. Nagalingum et. al. 2011, Science).
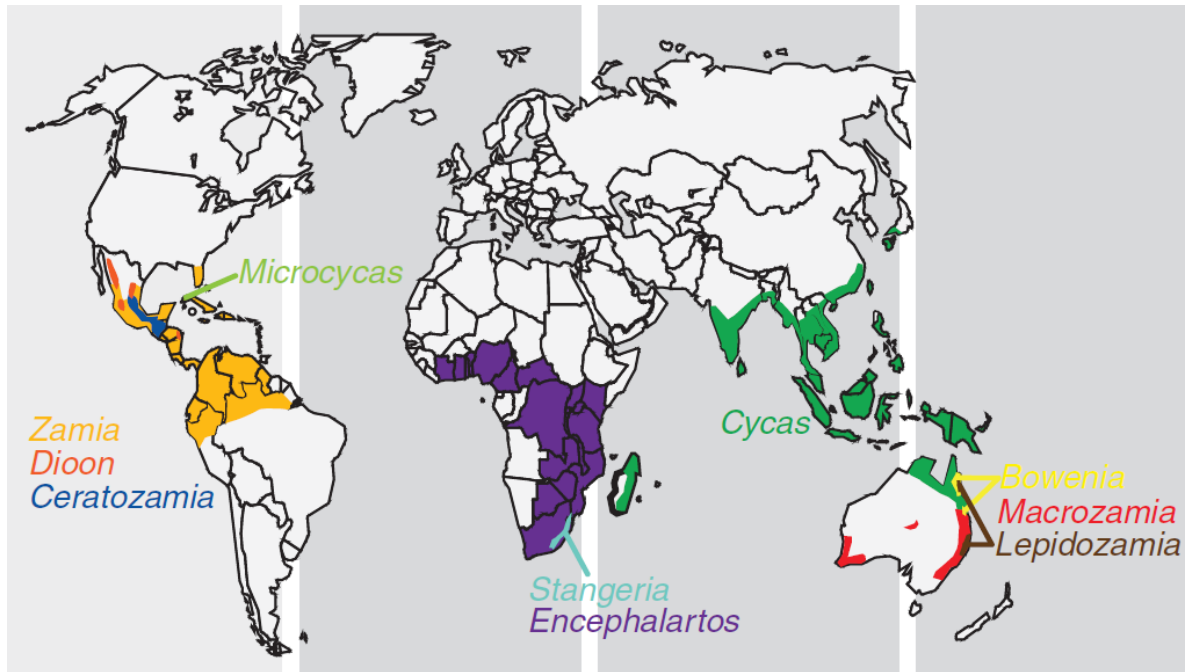
Figure 3. Cycads distribution (Recent Synchronous Radiation of a Living Fossil, Nagalingum et. al. 2011, Science)

Functional traits

Plant functional traits are defined as any morpho-, physio-, and phenological plant characteristics affecting overall plant fitness through their influence on survival, growth, and reproduction (Violle et al., 2007). These plant characteristics are biological adaptations to the environment and represent fundamental tradeoffs between individual species, as they direct the acquisition, processing, and investment of resources in plants (Chapin et al., 2000). Variations in functional traits impact ecosystem properties (e.g., biologic diversity, cycling of nutrients, soil characteristics) (Hooper et al., 2005) as species respond to and are filtered by climate, disturbance, and biotic interactions (Lavorel and Garnier, 2002)

Plant physical and chemical defenses

Plants have multiple strategies, both chemical and morphological, to protect their leaves.

Structural traits such as spines and thorns (spinescence), trichomes (pubescence), thickened, toughened or hardened leaves (sclerophylly), incorporation of granular minerals into plant tissues, and divaricated branching (shoots with wiry stems produced at wide axillary angles) play a leading role in plant protection against herbivory. Sclerophylly refers to the hardened leaves, and plays an active role in plant defense against herbivores by reducing the palatability and digestibility of the tissues, thereby, reducing the herbivore damage.

When it comes to protection, plants can also use flavonoids as chemical defenses against both biotic (such as herbivores and pathogens) and abiotic stresses like UV radiation and heat.

Flavonoids also have antioxidative properties that help to maintain a redox state in plant cells. They play a crucial role in protecting plants from herbivores and plant-feeding insects, as their presence can alter the taste and decrease the nutritive value of the plants, or even act as toxins. Additionally, flavonoids are essential for plant resistance against pathogenic bacteria and fungi. The antipathogenic properties of flavonoids are non-specific and can result from their antioxidative properties. (Harborne J.B.1992)

Mechanical or chemical defenses are metabolically expensive, and plants must balance trade-offs between different strategies and vary depending on multiple factors like environmental gradients and type of herbivore threat (Coley et al., 1985; Kikuzawa and Lechowicz, 2011).

METHODOLOGY

Data Set

The data set spans 80 cycads species from all genera in Cycadophyta at 57 sites (approximately 27% of all cycads known). We focused on eight key features of leaves that together inform us about cycads strategies in terms of leaf functional traits and defenses (table 1). Leaf mass per area (LMA) is a morphological trait widely used as a good indicator of plant functioning (i.e. photosynthetic and respiratory rates, chemical composition, resistance to herbivory, etc.). The LMA can be broken down into the leaf density (LD) and leaf volume to area ratio (TC or thickness), which in turn are determined by anatomical tissues and chemical composition.   Leaf toughness is an important functional trait in plant adaptation to herbivore pressures and physically stressful environmental factors like high winds. Furthermore, leaf toughness is linked to forest ecosystem function via the decomposition of leaf litter (Lucas et al., 2000; Onoda et al., 2011). Leaf thickness can be related to herbivory in different ways. Thicker leaves can make it more difficult for herbivores to access and consume the nutritious tissues of the plant, which can act as a physical defense mechanism. Additionally, thicker leaves can have higher concentrations of tough fibers or secondary compounds, which can make them less palatable or even toxic to herbivores (Coley, P. D.,1983). However, the relationship between leaf thickness and herbivory is not always straightforward. In some cases, thicker leaves may actually be more attractive to herbivores because they contain higher concentrations of nutrients or secondary compounds that the herbivore finds desirable. Additionally, some herbivores are better adapted to feeding on thick leaves, and may be able to consume them even more efficiently than thin leaves (Edara et. al. 2010).

Leaf Trait	Abbreviation	Unit	Functional role
Leaf Area	LA	cm^2	Respiration, evaporation, photosynthesis and light interception
Leaf mass Area	LMA	$gr\ m^{-2}$	Indicator of plant functioning and structure
Leaf density	LD	$gr\ cm^{-3}$	Correlated to plant strategies with morphology, physiology and biochemistry
Thickness	Tc	mm	Ability of plants to occupy dry, high irradiance environments
Toughness	Tg	$KgF\ cm^{-2}$	Resistance against herbivores pressure and environmental factors
Water content	% Hum	%	Drought and salinity tolerance, because water stress restricts transpiration including closure of stomata and water evaporation from leaf surface.
Amentoflavone	Am	$mg\ gr^{-1}$	UV- Ray protection properties
Hinokiflavone	Hi	$mg\ gr^{-1}$	Antibiotic, antifungal and insecticide action

Table 1. Description of the eight leaf traits studied

Leaf water content is a crucial factor in determining a plant's tolerance to drought and salinity, given that water stress affects transpiration, including stomatal closure and water evaporation from the surface of the leaf. Also, higher levels of water content have been related to higher photosynthetic rates (Zhou et. al. 2021).

Amentoflavone and hinokiflavone are secondary plant metabolites (Habermehl et al., 2008) that can act as physiological regulators, cell cycle inhibitors, pigments to attract pollinators and seed distributors, UV protection, pollen development, rhizobia attraction, antimicrobial and antifungal agents, chemical messengers, physiological regulators, physiological regulators, cell cycle inhibitors, feeding deterrents for plants, and cell cycle inhibitors to name a few of the functions (Fischer and Long, 1992; Galeotti et al., 2008; Jacobs and Rubery, 1988)

Leaf trait data collection

Leaf trait data (Table 1) were collected from Cycad foliar samples obtained from the Montgomery Botanical Center's (MBC) Cycad collection at Coral Gables, Miami, Florida, which includes one of the world's greatest collections of Cycads and Palms.

Eighty cycad species with at least three healthy and mature individuals suitable for physiological measurements were selected for the present study, with 22 from genera Cycas (family Cycadaceae), 10 from genera Ceratozamia, 5 from Dioon, 18 from Encephalartos, 1 from Lepidozamia, 4 from Macrozamia, 2 from genera Bowenia and Stangeria and 17 from Zamia (family Zamiaceae). Each specimen at MBC collection was brought directly from its native habitat, specifically, the individuals we used for this study

15

came from different places around Australia, Bahamas, Belize, China, Colombia, Democratic republic of Congo, Ecuador, Guam, Honduras, Indonesia, Jamaica. Kenya, Mozambique, South Africa, Thailand, Taiwan, Mexico, Zimbabwe, Uganda, Panama, Papua New Guinea, etc (supplementary table attached).

Leaf physical and chemical measurements

Three mature leaves from three different individuals per species were selected for the measurements. Leaflets were chosen as the fundamental unit for measurement and the middle section of the leaves was used for measuring hardness. Leaflets were used, as these are most analogous to angiosperm simple leaves or leaflets, typically measured in studies of functional traits. Leaflets were kept in sealed bags with wet tissues and were brought back to the laboratory within 30 min of sampling.

Leaf thickness (TC, mm) was averaged from values taken with vernier calipers at five places on each fresh leaflet. Leaf area was determined (Epson STD4800, Winfolia Software), The leaf hardness was average from values taken with Forge Aude model M3-50 Mark10 series 3, each leaf hardness was measured as KgF/cm^2 (Biasi et. al. 2016), then the leaves were oven-dried at 70°C to constant mass, weighed, and LMA was determined as leaf dry mass divided by leaf area (g/cm2). Leaf density (mass/volume) was determined as LMA divided by leaf thickness (Witkowski & Lamont, 1991). After taking all measurements, leaflets where grinded using Foss CT293 Cyclotec.

To determine amentoflavone and hinokiflavone content within the collected cycads leaves and to estimate the detectability by mass spectrometric methods, High Performance Liquid Chromatography (HPLC)-MS and MS/MS experiments were

performed on methanolic grinded leaf extracts (see protocol attached).

All previous results were then compared to a recent phylogeny data base (Ledile et. al. 2020) to determine the relative age of divergence for each of the species examined and corrected if necessary.

Climate Data

The 80 cycad species geographical distribution were obtained and 20 climatic variables were used (supplementary table 1). Bioclimatic variables of each distribution point were extracted from the NASA Center for Climate Simulation data base.

3 groups of climatic variables were used for this study.

1) Temperature indicator group: beside mean annual temperature (MAT), variables related to seasonality and temperature variability were collected, mean diurnal range (MDR), isothermally (I), mean temperature seasonality (MTS), mean maximum temperature (MMT) of the warmest month, minimum temperature of the coldest month, annual temperature range, mean temperature of wettest, driest, warmest and coldest quarters.

2) Precipitation indicator group: in addition to mean annual precipitation (MAP), mean precipitation of the wettest and driest month were measured (PCW, PDM), as well as precipitation seasonality (PS), mean precipitation of the wettest, driest, warmest and coldest quarters (PWQ, PDQ, PWMQ, PCQ).

3) Elevation over the sea level.

The coverage of species and sites is around 2.4-fold greater than previous similar studies on cycads (Yong-Jiang Zhang et. al. 2015). The sites where Cycads were gathered from, extend to all biomes known to be native habitats of cycads, where mean annual temperature (MAT) ranges from 16 C to 28 C and mean annual precipitation ranges from 424 to 7360 mm per year. This covers all of the range of MAT-MAR space in which Cycads occur (Fig. 4 and 5). In relation to major biome types of the world, we can appreciate cycads native habitats range from arid desert, shrubland and woodland to temperate forest, tropical seasonal forest, temperate rainforest and tropical rainforest. The broad coverage of our data set allowed us to quantify the relationship of leaf functional traits to climate at a scale not previously done on cycads.
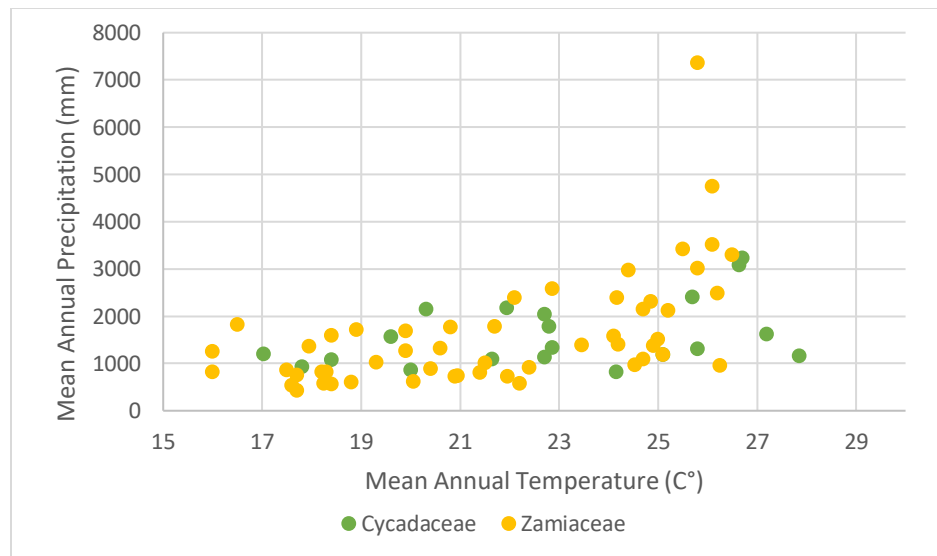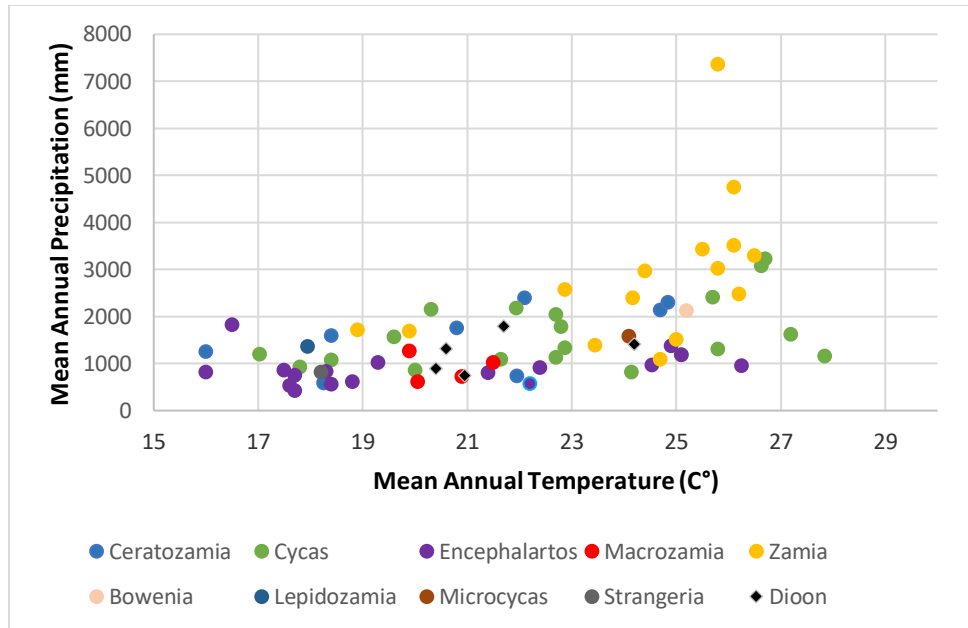

Figure 4a. Cycads environmental range labeled by family

Figure 4b. Cycads environmental range labeled by genera

Data Analysis

Even though 80 cycads species were sampled and analyzed for their physical traits, 57 were used for amentoflavone and hinokiflavone content analysis.

Given the large number of environmental factors involved and their collinearity, a principal component analysis (PCA) was performed to reduce the number of dimensions of less important environmental factors.

Redundancy data analysis (RDA) was conducted to analyze the 6 leaf functional traits of 80 cycad species using Vegan R package. (Package 'vegan' J Oksanen, FG Blanchet, R Kindt, P Legendre, PR Minchin, RB O'hara, .Community ecology package, version 2 (9), 1-295).

Relationship among leaf traits were analyzed using the RDA, PCA, ANOVA and pairwise functions. All analyses and figures were conducted in R 2022.07.01. The significance of differences in leaf traits among cycad genus and climatic variables were examined using one-way ANOVA with Tukey's post hoc tests in SPSS software v17 (SPSS Inc., Chicago, IL, United States).

Previous results were compared to a recent phylogeny data base (Dayana E. Salas-Leiva, Alan W. Meerow, Michael Calonje, M. Patrick Griffith, Javier Francisco-Ortega, Kyoko Nakamura, Dennis W. Stevenson, Carl E. Lewis, Sandra Namoff, Phylogeny of the cycads based on multiple single-copy nuclear genes: congruence of concatenated parsimony, likelihood and species tree inference methods, Annals of Botany, Volume 112, Issue 7, November 2013, Pages 1263–1278) to determine the relative age of divergence for each of the species examined and corrected if necessary.

Results

Genera leaf trait variations

The seven leaf traits showed large variability across genera (table 2). Foliar area (LA) showed the largest variation, varying from 3.9 to 378.62 cm^2 (more than 97 - fold). LMA varied more than 20 times, from 0.259 to 5.31 gr m^{-2}. Thickness (TC) ranged from 0.232 to 2.291 mm, almost 10-fold difference. Toughness (TG) varied from 0.740 to 15.191 KgF (more than 20-fold). Leaf density (LD) presented a minimum of 0.458 and a maximum of 7.805, more than 17-fold.

	LA	TC	LMA	LD	TG	Am	Hi
Ceratozamia							
mean	190.5	0.648	1.001	1.543	3.441	29.95	3.83
minimum	19.03	0.472	0.26	0.58	1.987	25.53	0.9467
maximum	378.6	0.873	2.238	3.246	5.627	34.78	11.6
Cycas							
mean	34.8	1.134	1.362	1.372	1.731	50.34	6.18
minimum	4.96	0.37	0.385	0.458	0.74	31.21	2.69
maximum	122.1	2.291	2.195	2.483	3.749	72.96	12.82
Bowenia							
mean	46.01	0.232	0.26	1.119	0.856	8.26	11.05
Dioon							
mean	5.81	0.61	2.68	4.45	7.47	25.34	2.7
minimum	3.9	0.5	2.408	4.203	6.606	23.27	0.81
maximum	7.41	0.708	2.959	4.817	8.959	27.4	4.58
Encephalartos							
mean	35.15	0.97	2.92	2.81	7.51	31.7	4.1
minimum	13.3	0.696	0.758	0.895	3.158	15.64	1.53
maximum	78.69	1.219	5.013	4.68	15.191	41.39	17.64
Lepidozamia							
mean	39.71	0.811	0.863	1.064	2.998	3.45	1.08
Microcycas							
mean	4.5	0.4	1.57	3.93	2.37	32.34	14.78
Macrozamia							
mean	22.92	0.742	2.67	3.73	2.439	22.69	1.29
minimum	12.84	0.673	1.076	1.598	2.467	5.57	0.57
maximum	33.06	0.84	5.316	7.805	5.278	25.8	1.91

Strangeria							
mean	185.9	0.361	0.494	1.369	4.008	13.86	15.73
Zamia							
mean	22.92	0.854	1.19	1.616	3.771	17.05	18.49
minimum	10.24	0.477	0.527	0.78	1.764	6.82	8.78
maximum	325.5	1.696	2.198	2.909	8.578	29	32.7

Table 2. Comparison of cycads physical traits among genus in this study. Foliar area (FA, cm2), thickness (TK, mm), Leaf mass per area (LMA, gr cm -2), Leaf density (LD, gr cm-3), toughness (TG, kgf). Data source: cycads, 80 species (present study), those genera not showing minimum and maximum values means only one species of that genus was sampled.
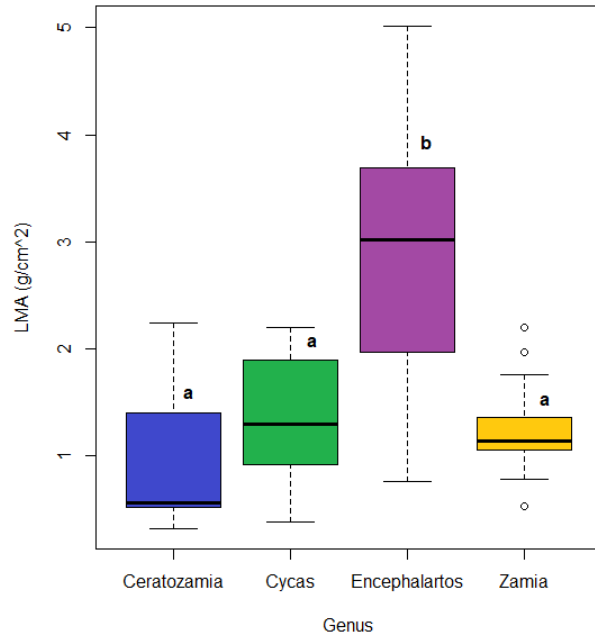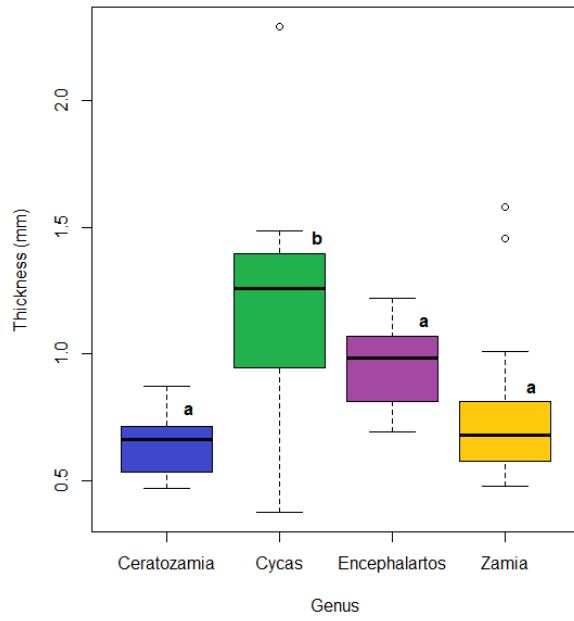
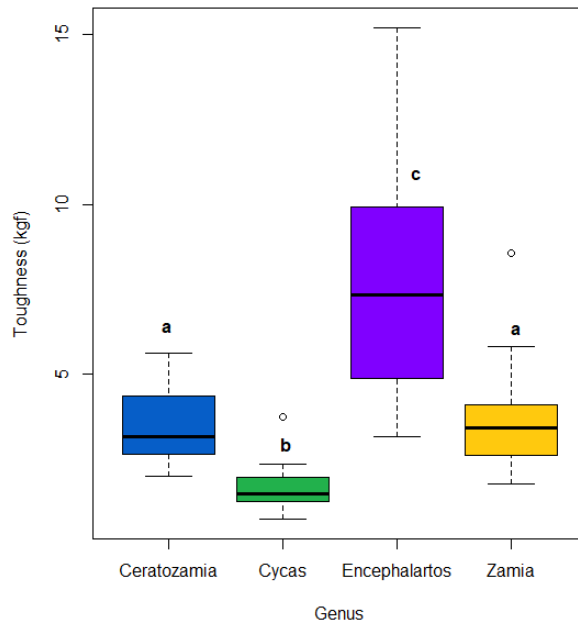

a) LMA variation among genera
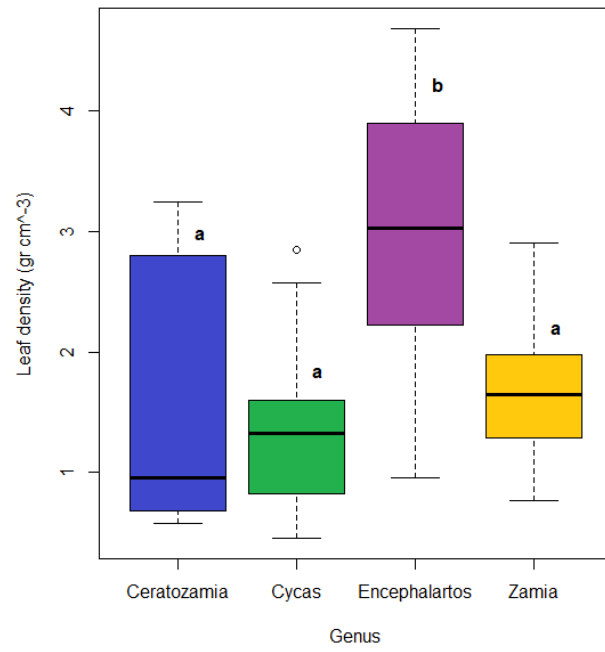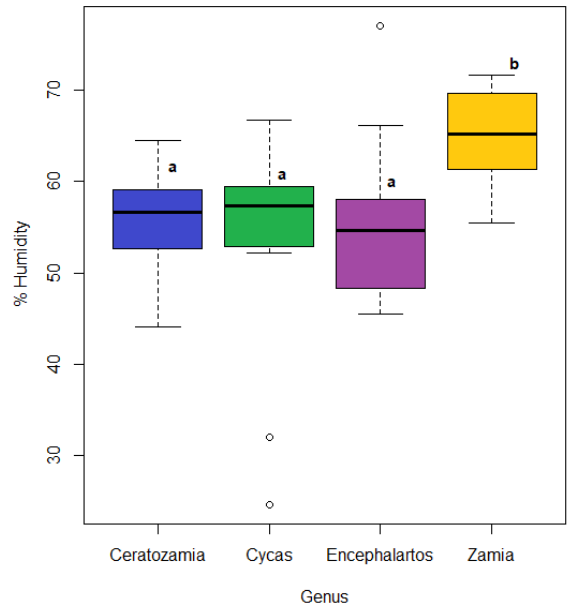
b) Leaf density variation among genera
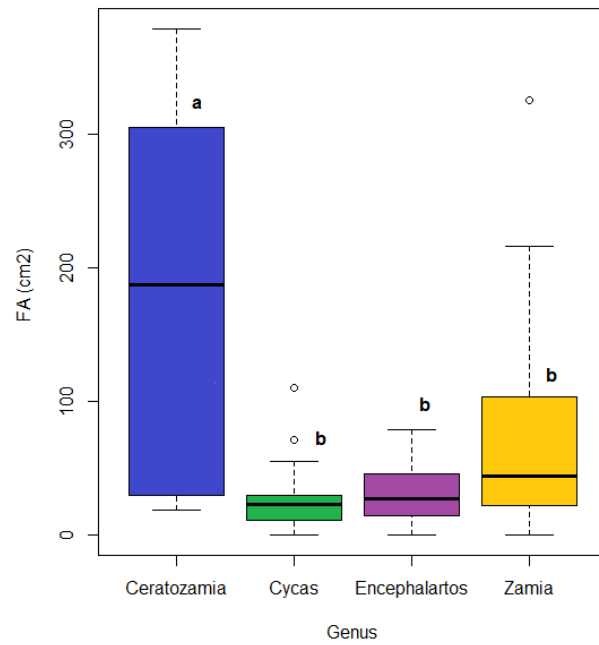
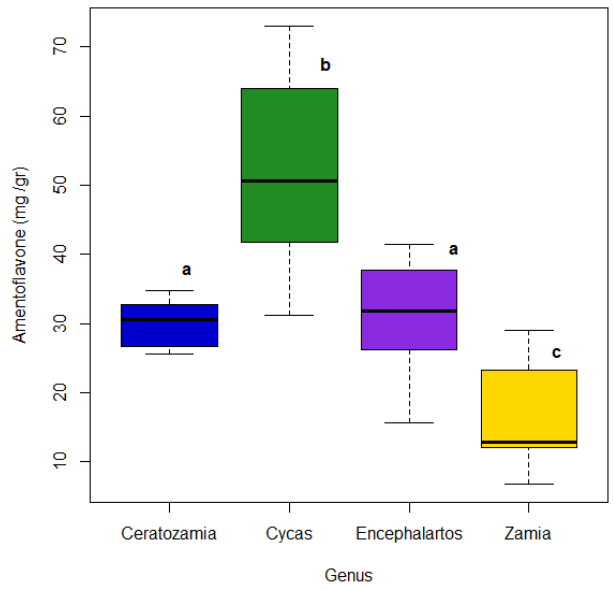c) Foliar area variation among genera
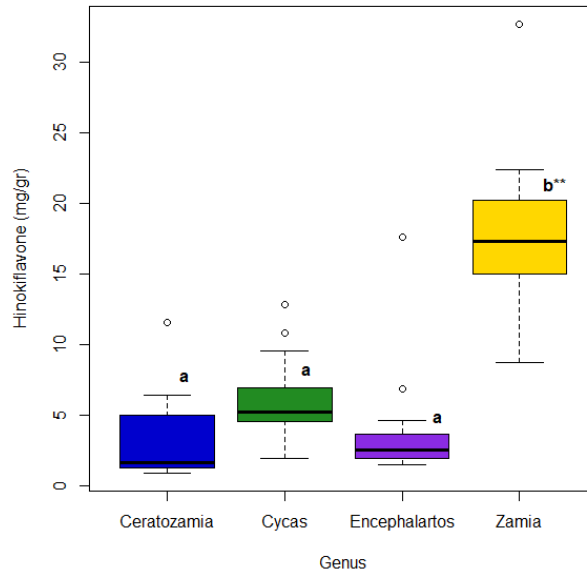
d) Thickness variation among genera

e) Toughness variation among genera

f) percentage of leaves humidity variation among genera

g) Amentoflavone content among genera

h) Hinokiflavone content among genera

Figure 5. Boxplots of leaf traits for all genus with sampled species n>5. The bottom and top of the boxplots indicate the first and third quartile, the two whiskers correspond to the 1.5 times of the outliers, and the solid dots within the boxes are the mean values. Different lowercase letters indicate significant differences between genera (*$p < 0.01$, **$p < 0.001$). (a) FA, foliar area; (b) LD, leaf density; (c) LMA, leaf mass area; (d) TC, thickness; (e) TG, toughness, (f) percentage of humidity in leaves, (g) amentoflavone content and (h) hinokiflavone content among genera.

Relationship between physical and chemical traits
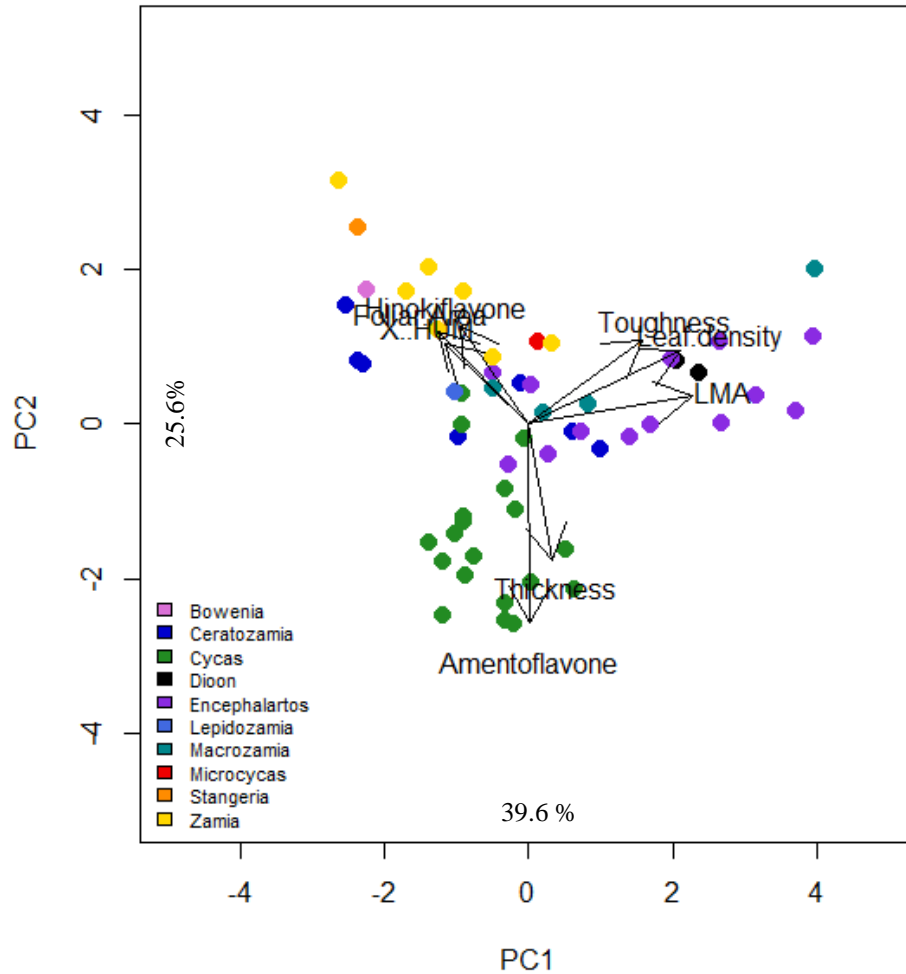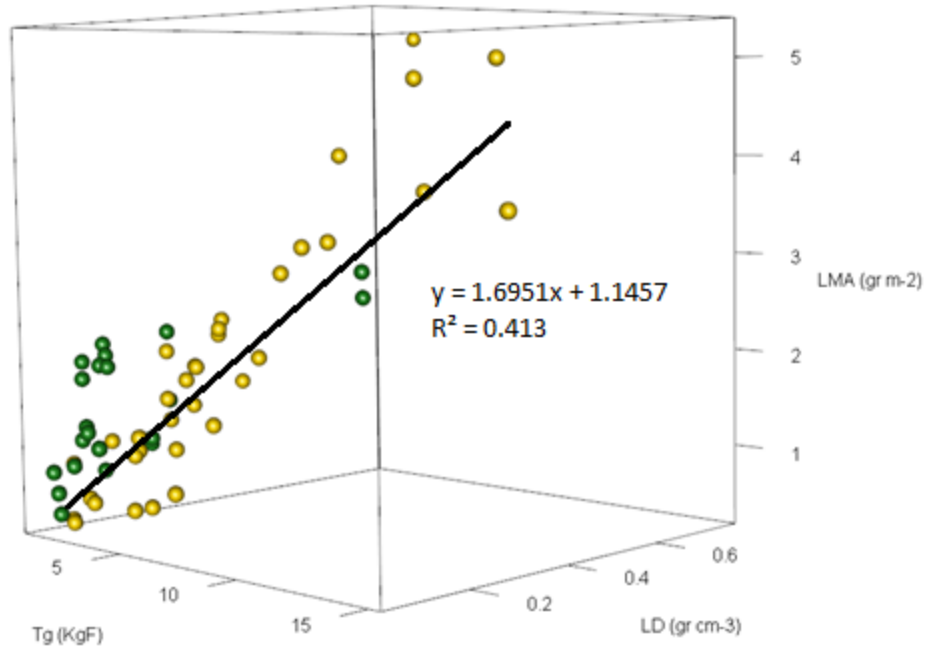

Figure 6.
PCA analysis labeled by genus with all physical and chemical traits involved for 57
cycad species.

7a) LMA and leaf density were both significantly and positively correlated to toughness (p<0.01, r= 0.66, p<0.01, r=0.47, respectively)
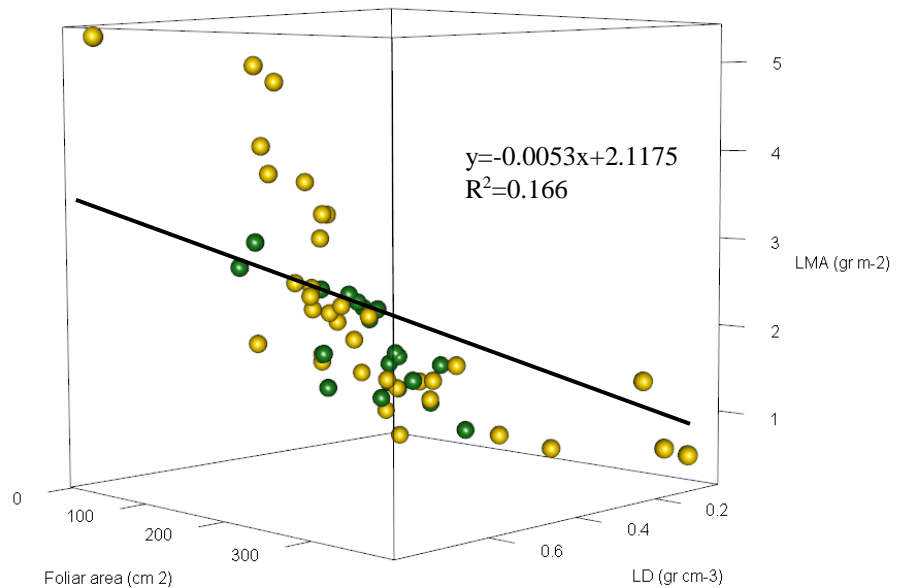
7b) Both LMA and leaf density were found negatively related to foliar area (p<0.01, r= -0.36; p<0.01, r= -0.35, respectively)
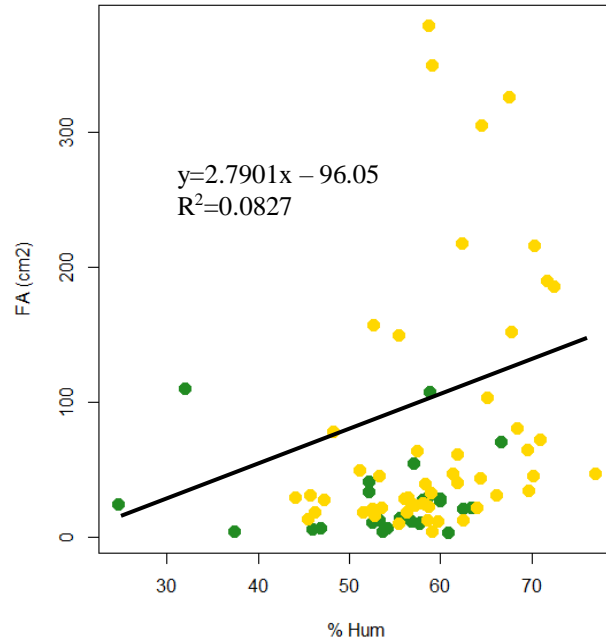
7c) The percentage of leaf humidity was positively related to foliar area (r= 0.29, p<0.05)
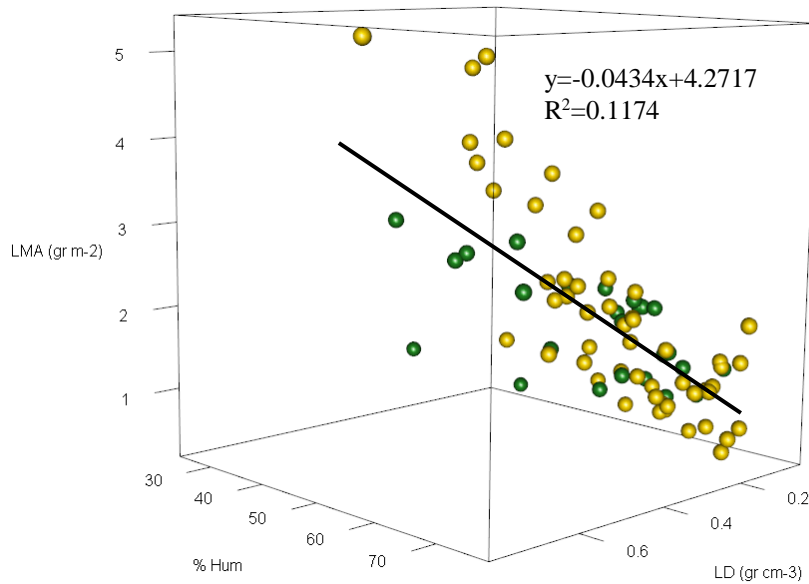
7d) Percentage of humidity was found negatively related to LMA (r=-0.34 p<0.01), and leaf density (r= -0.41, p<0.01)

Figure 7 a, b, c, and d.
Pairwise graph of significant relationship among physical traits.
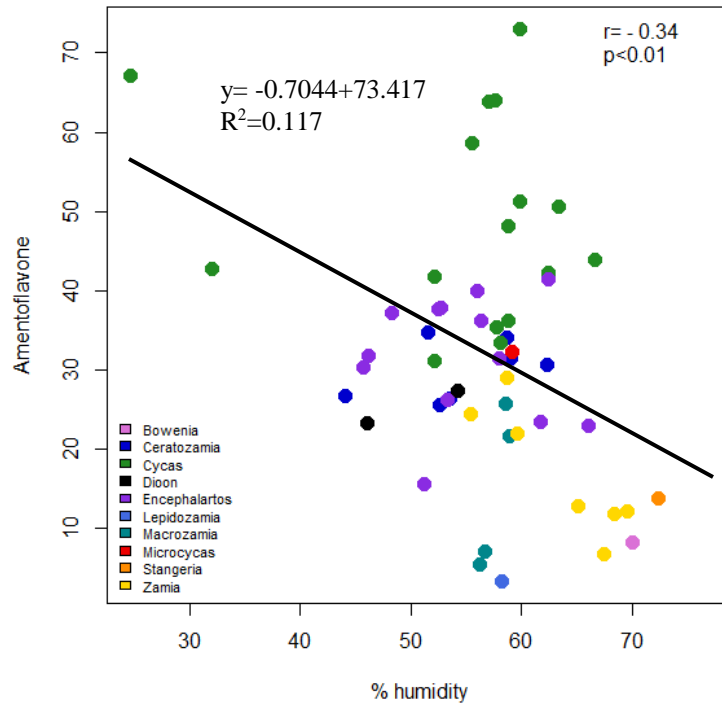
7e) Amentoflavone content was found to vary negatively related to percentage of humidity in leaves (r= -0.34, p<0.01)
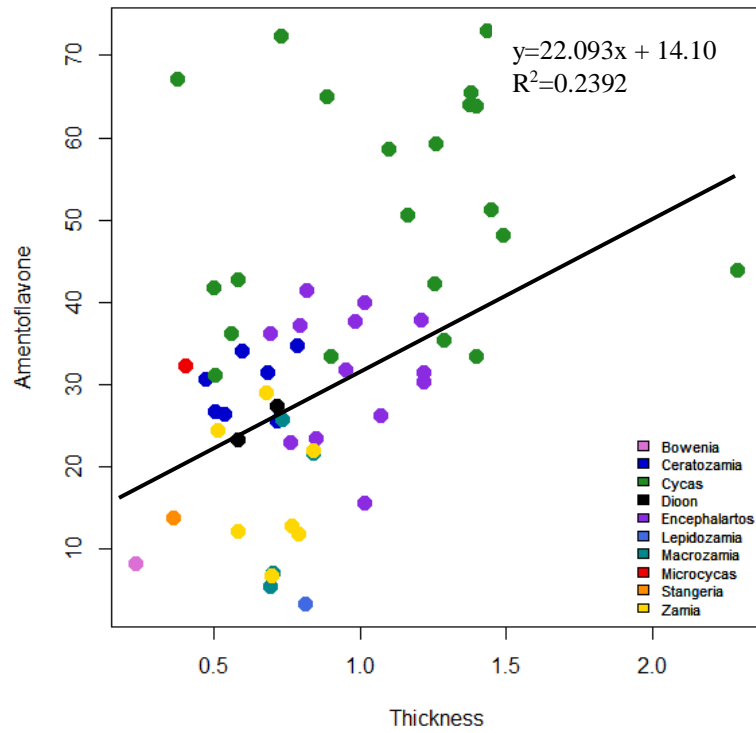
7f) Amentoflavone presented a significant increase correlative with thickness (r=0.45, p<0.01)
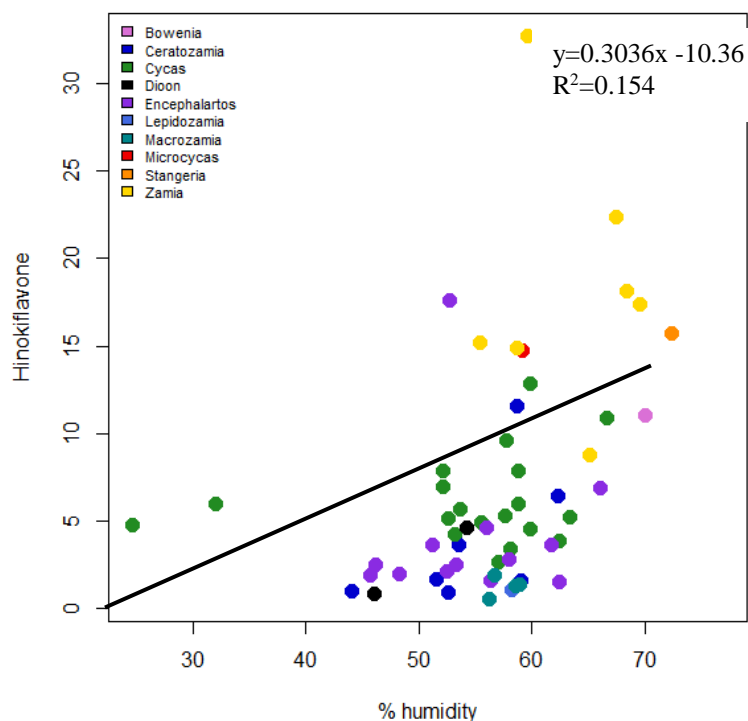
7g) Hinokiflavone was found to vary according to percentage of humidity in leaves (r=0.39, p<0.01)

Figure 7 e, f, g.
Significant relationship among physical traits, chemical traits and bioclimatic variables for pairwise comparisons.

For principal component analysis of the 8 traits measured, we found that LMA and leaf density were both significantly and positively correlated to toughness (p<0.01, r= 0.66, p<0.01, r=0.47, respectively fig. **7a**). and both LMA and leaf density were found negatively related to foliar area (p<0.01, r= -0.36; p<0.01, r= -0.35, respectively, **7b**). The percentage of leaf humidity was positively related to foliar area and hinokiflavone content (r= 0.29 fig. **7c**, r= 0.39 fig. **7g** respectively, p<0.01) and negatively to amentoflavone content (r= - 0.34, p<0.01 fig. **7e**), and negatively to LMA (r=-0.34 p<0.01,), and leaf density (r= -0.41 fig. **7d**, p<0.01).

Thickness was found to relate significantly to amentoflavone content (r= 0.49, p<0.01 fig. **7f**) and higher in Cycadaceae compared to Zamiaceae .

For pairwise comparison of physical traits, 80 cycad species were used. For chemical traits, 57 species (results attached).

Pairwise comparison between thickness and toughness was found non-significant and leaf thickness did not present significant relations to other physical traits.

Influence of climate on leaf physical and chemical traits
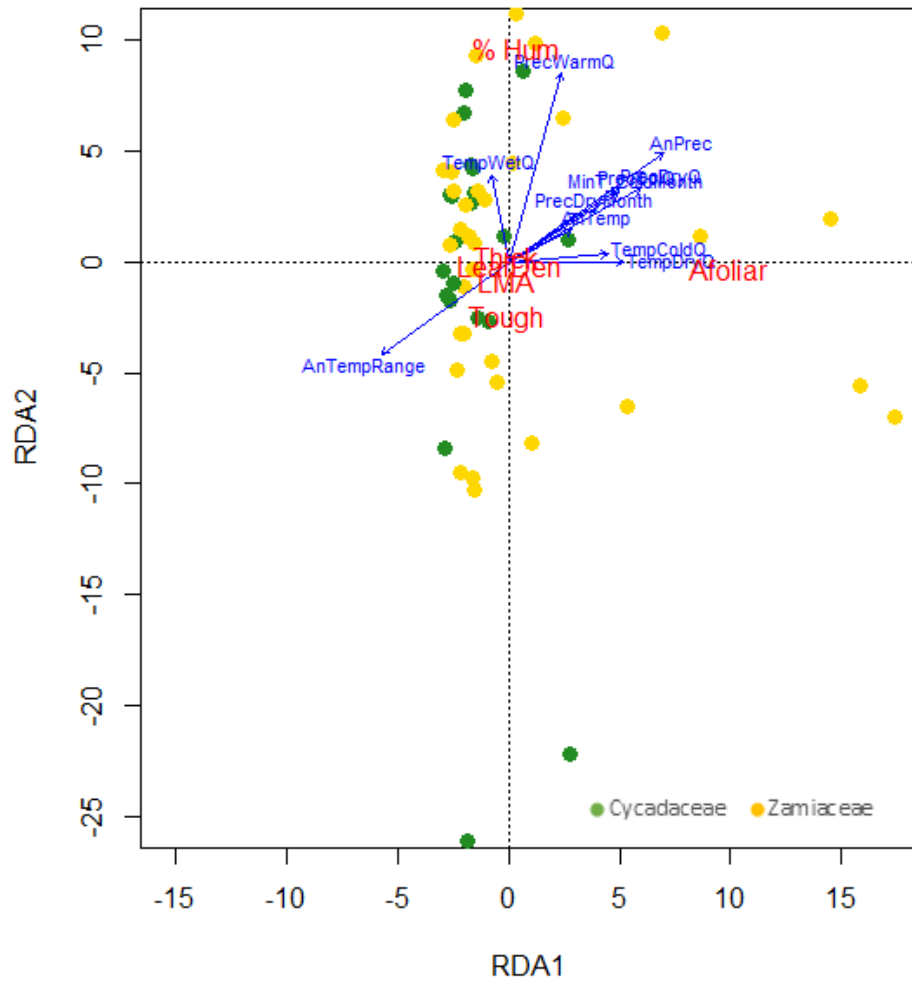
Figure 8.
Climate-related trait dimensions from redundancy analysis (RDA).
The climate variables (shown by blue arrows) are annual mean temperature, annual temperature range, temperature in wet quarter, temperature in cold quarter, temperature in dry quarter., annual mean precipitation, precipitation in warm quarter, precipitation in dry month. The traits (shown by red arrows) are leaf mass per area *(LMA)*, thickness, toughness, leaf density, leaf area and percentage of humidity in leaves.

All climate variables together accounted for 60% of the physical traits variation as shown in the RDA (p<0.01). Annual precipitation, precipitation in cold and dry quarter, and temperature in cold and dry quarter were found the most significant (p<0.01). RDA was found non-significant for amentoflavone and hinokiflavone content variation.

For pairwise comparison, 6 bioclimatic variables were found highly correlated to physical traits in our results (p<0.01): from the temperature indicator group: isothermality, temperature seasonality, annual temperature range and temperature in wet quarter. From precipitation indicator group: annual precipitation, precipitation in wet and warm months and quarters were found significant with physical traits.

For pairwise comparisons, 4 bioclimatic variables were found highly correlated to chemical traits in our results (p<0.01), from temperature indicator group: diurnal range, isothermality, annual temperature range. From precipitation indicator group: precipitation seasonality.

Elevation was found not significant with all of the traits measured.

r= 0.42
p<0.01

y=5.0719x + 1326.4
R²=0.143

r= - 0.37
p<0.01

y=-0.0194x+ 19.50
R²=0.0882

Annual precipitation

Annual temperature range

FA
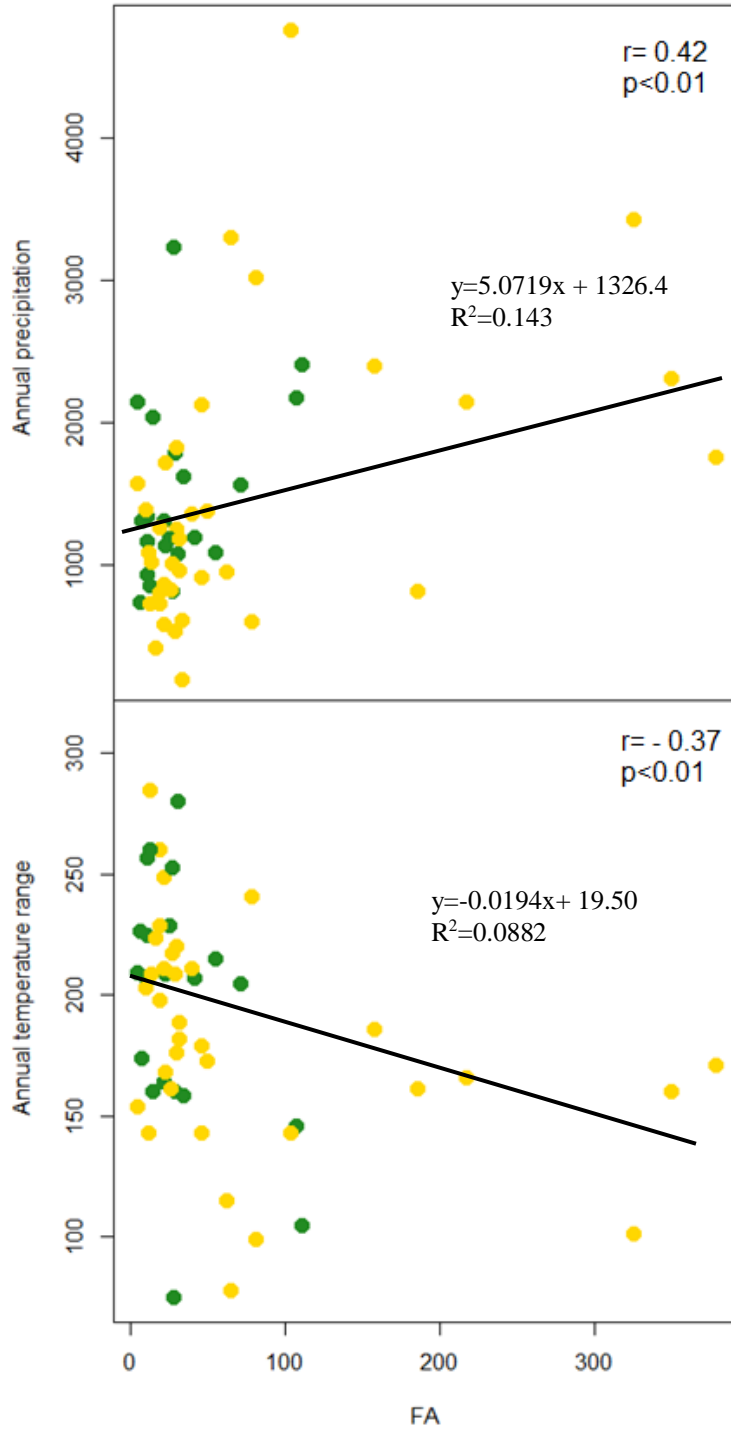
a) Foliar area was found positively related to annual precipitation and negatively to annual temperature range

35
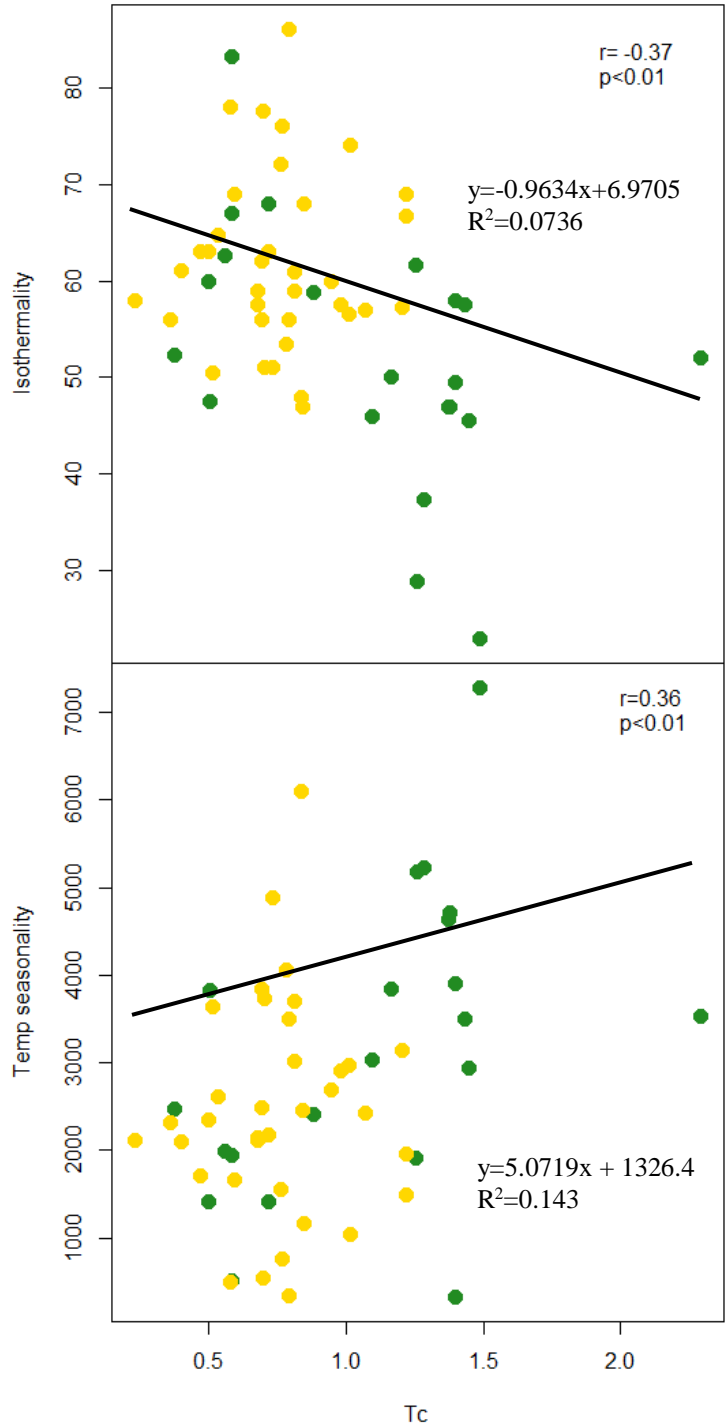
b) Thickness of leaves was found positively related to temperature seasonality and negatively to isothermality.

r= -0.37
p<0.01

y=-343.06x + 2245.3
R^2=0.1253
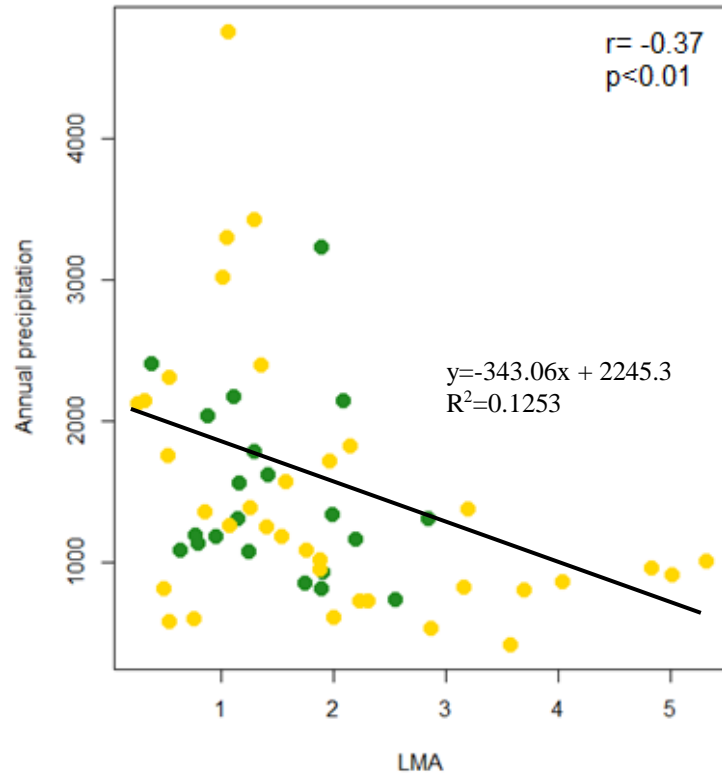
c) Annual precipitation was found negatively related to LMA.

r= -0.38
p<0.01

y=-2832.9x + 2250.8
R^2=0.1229
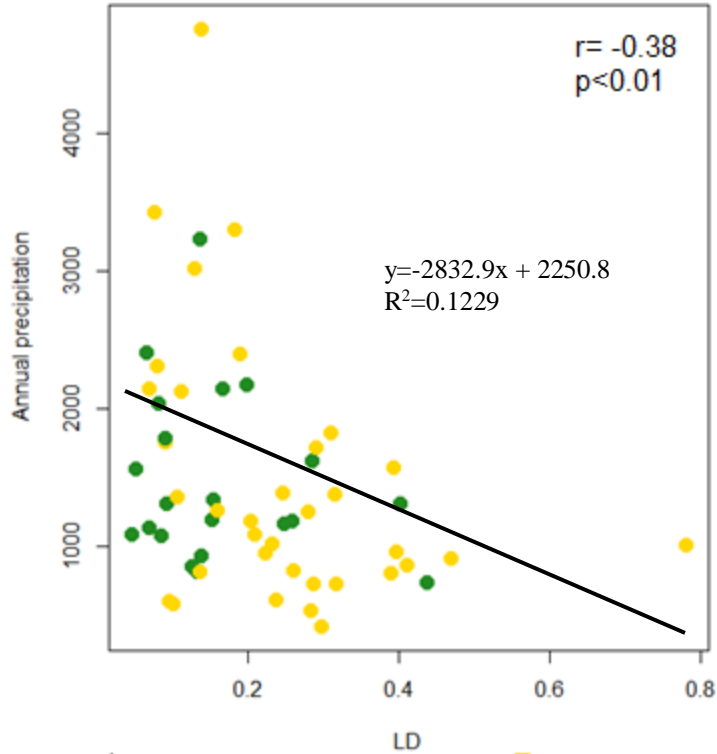
d) Leaf density was found to vary negatively with annual precipitation
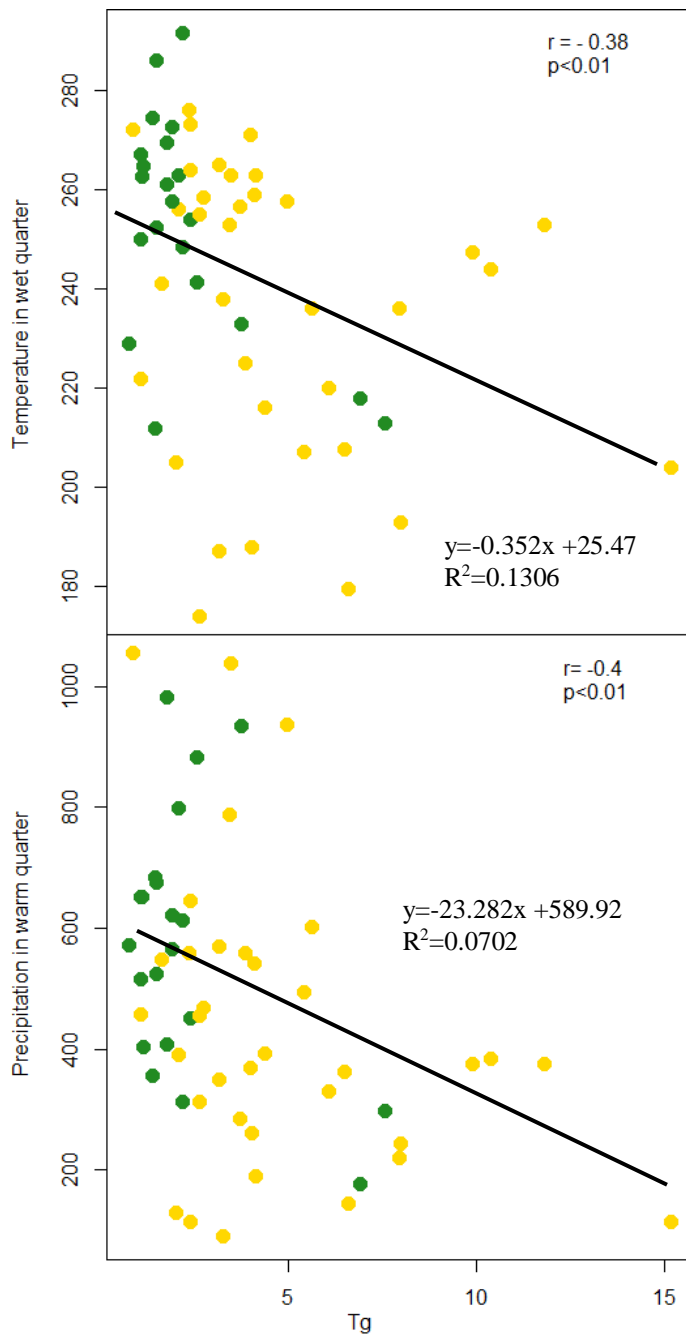
e) Leaf toughness was found to vary negatively with temperatures in wet quarter and precipitations in warm quart

Figure 9 a, b, c, d & e.
Significant relationship among physical traits and bioclimatic variables

Trait	interaction w/ other traits	vary with
↑*Foliar Area*	↑ % Hum ↓LMA, LD	↑ Annual precipitation ↓ Annual T range
↑*LMA*	↑ LD, Toughness ↓FA, %hum	↑ - ↓ Annual precipitation, prec. Warm quarter
↑*Leaf Density*	↑LMA, Toughness ↓FA, %Hum	↑ - ↓ Annual precipitation, prec. Warm quarter
↑*% Humidity*	↑FA, ↓LMA, LD	↑ Annual precipitation ↓ -
↑*Thickness*	↑ - ↓ LD	↑Temperature seasonality ↓ Isothermality
↑*Toughness*	↑LMA, LD ↓	↑- ↓Temperature wet quarter, prec. warm quarter
↑*Amentoflavone*	↑ Thickness ↓ % Hum	↑Prec. Seasonality ↓Isothermality
↑ *Hinokiflavone*	↑% Hum ↓ -	↑- ↓Prec. Seasonality, annual T range, diurnal range

Table 3. Summary of chemical and physical traits interaction and variation with climatic variables

Discussion

As seen in table 3, our data suggests that for Cycads, species with bigger leaves seem to present higher content of water, characteristics that increase with higher levels of annual precipitations and smaller annual temperature range, representative of tropical rainforest environment. This characteristic may not be of surprise. It has been shown that in environments where sun light is scant like under the canopy of tropical forests, plants develop bigger leaves to provide them a greater surface for photosynthesis (Wright et. al. 2017), also, since water resource is constant and sun exposure is slight, evaporation is highly diminished, explaining the higher humidity content of the leaves.

In tropical rainy forest, cycads species with low LMA and leaf density, meaning softer leaves, herbivory resistance may be explained by the presence of biflavonoids compounds known for their antibiotic, antifungal and insecticide function (Mierziak 2014) and which are known to be rich and diverse in cycads (Moawad et, al. 2013), in this case Hinokiflavone, a biflavonoid known for its cytotoxic as well as antioxidants properties appears to be higher under these conditions.

Toughness showed a very significant correlation to LMA and leaf density, suggesting bigger concentrations of fiber, cellulose and/or waxes content. Contrary to previous finding on other plant clades, where toughness was shown to be higher in rainy tropical forest kind of environment as a way to deal with higher herbivory pressure (Coley, 1983; Hochuli,1996; Lucas et al., 2000; Kitajima et al., 2012), this trait seems to be higher in cycads from dry and arid environments, manly found in native habitats of Encephalartos species from Africa.

For our dataset, leaves thickness was found not related to any of the other physical traits measured, meaning it is not determined by water content nor dry matter. For cycads, it seems that leaf thickness helps them withstand high temperature changes during the year, showing thicker leaves as their native habitat moves further from the equator, where seasonal changes are more severe. This would also explain the higher amentoflavone content, a biflavonoid known from its UV- protection capacities, a property highly valuable for plants growing under direct sunlight and with big annual temperature range stressful conditions.

Overall, it seems that 3 different evolution patterns can be appreciated on cycads:

- family Cycadaceae presents thicker and richer in amentoflavone leaves, properties that allow species in this family to survive in sunnier conditions with wider temperature range compared to the rest of their sisters in family Zamiaceae.

- In rainy tropical forest, with higher annual precipitation and humidity, meaning greater quantities of bacterial, fungi and insects' threats, cycads present bigger leaves but weaker in physical defenses, and seem to have develop a higher content of hinokiflavone, a biflavonoid known for its cytotoxic properties. This strategy was mainly seen in cycads from genus Zamia and Ceratozamia.

- On the other hand, for the third strategy, cycads present higher LMA and leaf density that derivers in tougher leaves. This characteristic is mainly shown in cycads from genus Encephalartos. A possible explanation for a bigger investment in physical than chemical defenses could be explained phylogenetically or by differences in the diversity of herbivory threats in their native habitats.

Limitations

A fact that needs further analysis and consideration is thickness measurement in Cycadaceae.

For the results shown, this trait is much higher in this family than in Zamiaceae, but we should consider that Cycadaceae leaves present a central vein not present in Zamiaceae, which for sure influences leaves thickness measurement. Possibly another way to measure thickness in this particular family would be more accurate and ideal for future studies.

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APPENDIX

List of cycads used in this experiment

# CODE	NAME	COUNTRY	REGION	FAMILY
9367	Bowenia spectabilis	Australia	Queensland	ZAMIACEAE
58752	Lepidozamia peroffskyana	Australia	New South Wales	ZAMIACEAE
59691	Macrozamia lucida	Australia	Queensland	ZAMIACEAE
95926	Macrozamia macdonnellii	Australia	Northern Territory	ZAMIACEAE
96275	Macrozamia macdonnellii	Australia	Northern Territory	ZAMIACEAE
96290	Cycas armstrongii	Australia	Northern Territory	CYCADACEAE
97948	Cycas maconochiei ssp. lanata	Australia	Northern Territory	CYCADACEAE
97949	Cycas maconochiei ssp. lanata	Australia	Northern Territory	CYCADACEAE
97953	Cycas maconochiei ssp. lanata	Australia	Northern Territory	CYCADACEAE
20030060	Macrozamia moorei	Australia	Queensland	ZAMIACEAE
20040270	Cycas cairnsiana	Australia	Queensland	CYCADACEAE
20040275	Cycas cairnsiana	Australia	Queensland	CYCADACEAE
20040277	Cycas cairnsiana	Australia	Queensland	CYCADACEAE
20080090	Cycas media	Australia	Queensland	CYCADACEAE
20110152	Zamia angustifolia	Bahamas	Eleuthera	ZAMIACEAE
20010924	Zamia decumbens	Belize	Toledo District	ZAMIACEAE
9511	Cycas bifida	China	Guangxi Zhuang Aut. Reg.	CYCADACEAE
9598	Cycas guizhouensis	China	Yunnan Province	CYCADACEAE
92371	Cycas panzhihuaensis	China	Sichuan Province	CYCADACEAE
93245	Cycas hainanensis	China	Hainan Province	CYCADACEAE
94153	Cycas hongheensis	China	Sichuan Province	CYCADACEAE
94710	Cycas tanqingii	China	Yunnan Province	CYCADACEAE
94717	Cycas bifida	China	Yunnan Province	CYCADACEAE
94719	Cycas bifida	China	Yunnan Province	CYCADACEAE
95100	Cycas diannanensis	China	Yunnan Province	CYCADACEAE
951560	Cycas sexseminifera	China	Guangxi Zhuang Aut. Reg.	CYCADACEAE
971593	Zamia encephalartoides	Colombia	Dpto. de Santander	ZAMIACEAE
20030801	Zamia roezlii	Colombia	Dpto. de Valle de Cauca	ZAMIACEAE
96259	Encephalartos laurentianus	Congo	Zaire	ZAMIACEAE

20070832	Zamia fairchildiana	Costa Rica	Puntarenas Province	ZAMIACEAE
20001000	Zamia lindenii	Ecuador	Esmeraldas	ZAMIACEAE
20070430	Cycas micronesica	Guam		CYCADACEAE
20030887	Zamia onan-reyesii	Honduras	Department of Cortes	ZAMIACEAE
981790	Cycas rumphii	Indonesia	Jawa Barat	CYCADACEAE
20080215	Zamia aff. portoricensis	Jamaica	Trelawny Parish	ZAMIACEAE
64575	Encephalartos hildebrandtii	Kenya		ZAMIACEAE
20060183	Encephalartos tegulaneus	Kenya		ZAMIACEAE
93920	Ceratozamia decumbens	Mexico	Veracruz	ZAMIACEAE
98618	Ceratozamia brevifrons	Mexico		ZAMIACEAE
931193	Ceratozamia kuesteriana	Mexico		ZAMIACEAE
931196	Ceratozamia kuesteriana	Mexico		ZAMIACEAE
931201	Ceratozamia kuesteriana	Mexico		ZAMIACEAE
20010165	Ceratozamia brevifrons	Mexico	Veracruz	ZAMIACEAE
20010168	Ceratozamia fuscoviridis	Mexico	Hidalgo	ZAMIACEAE
20010169	Ceratozamia fuscoviridis	Mexico	Hidalgo	ZAMIACEAE
20010173	Ceratozamia chamberlainii	Mexico	San Luis Potosi	ZAMIACEAE
20010174	Ceratozamia chamberlainii	Mexico	San Luis Potosi	ZAMIACEAE
20010226	Ceratozamia miqueliana	Mexico	Veracruz	ZAMIACEAE
20010227	Ceratozamia miqueliana	Mexico	Veracruz	ZAMIACEAE
20010235	Ceratozamia miqueliana	Mexico	Veracruz	ZAMIACEAE
20011257	Dioon argenteum	Mexico	Oaxaca	ZAMIACEAE
20011259	Dioon argenteum	Mexico	Oaxaca	ZAMIACEAE
20011262	Dioon purpusii	Mexico	Oaxaca	ZAMIACEAE
20011263	Dioon purpusii	Mexico	Oaxaca	ZAMIACEAE
20011293	Zamia soconuscensis	Mexico	Chiapas	ZAMIACEAE
20011362	Ceratozamia zoquorum	Mexico	Chiapas	ZAMIACEAE
20011363	Ceratozamia zoquorum	Mexico	Chiapas	ZAMIACEAE
20011364	Ceratozamia zoquorum	Mexico	Chiapas	ZAMIACEAE
20020073	Ceratozamia chimalapensis	Mexico	Oaxaca	ZAMIACEAE
20020252	Ceratozamia mirandae	Mexico	Chiapas	ZAMIACEAE
20020827	Ceratozamia robusta	Mexico	Chiapas	ZAMIACEAE
20040474	Dioon sonorense	Mexico		ZAMIACEAE
20040504	Dioon holmgrenii	Mexico		ZAMIACEAE
20040506	Dioon salas-moralesiae	Mexico		ZAMIACEAE
614	Encephalartos ferox	Mozambique	District of Gaza	ZAMIACEAE
20060196	Encephalartos manikensis	Mozambique		ZAMIACEAE
20060201	Encephalartos turneri	Mozambique	Nampula	ZAMIACEAE
2000278	Zamia ipetiensis	Panama	Prov. de Panamá	ZAMIACEAE
2000351	Zamia stevensonii	Panama	Prov. de Panamá	ZAMIACEAE
2000401	Zamia imperialis	Panama	Prov. de Coclé	ZAMIACEAE
2000775	Zamia elegantissima	Panama	Prov. de Colón	ZAMIACEAE

20010119	Zamia nesophila	Panama	Prov. de Bocas Del Toro	ZAMIACEAE
20010123	Zamia nesophila	Panama	Prov. de Bocas Del Toro	ZAMIACEAE
20010794	Zamia skinneri	Panama	Prov. de Bocas Del Toro	ZAMIACEAE
20040867	Zamia hamannii	Panama	Chiriqui Province	ZAMIACEAE
20110525	Zamia skinneri	Panama	Bocas Del Toro	ZAMIACEAE
RM1448	Cycas bougainvilleana	Papua New Guinea		CYCADACEAE
20030290	Zamia erosa	Puerto Rico		ZAMIACEAE
9165	Encephalartos aemulans	South Africa	KwaZulu-Natal	ZAMIACEAE
9751	Encephalartos horridus	South Africa	Eastern Cape	ZAMIACEAE
9842	Encephalartos lebomboensis	South Africa	Mpumalanga	ZAMIACEAE
64544	Encephalartos transvenosus	South Africa		ZAMIACEAE
97811	Encephalartos cerinus	South Africa	KwaZulu-Natal	ZAMIACEAE
97982	Encephalartos altensteinii	South Africa	Eastern Cape	ZAMIACEAE
2000262	Encephalartos natalensis	South Africa	KwaZulu-Natal	ZAMIACEAE
RM1433	Encephalartos longifolius	South Africa		ZAMIACEAE
77225	Cycas taitungensis	Taiwan		CYCADACEAE
95217	Cycas clivicola	Thailand	Kanchanaburi Province	CYCADACEAE
95222	Cycas edentata	Thailand	Narathiwat Province	CYCADACEAE
95453	Cycas divyadarshanii	Thailand	Kanchanaburi Province	CYCADACEAE
95455	Cycas siamensis	Thailand	Kanchanaburi Province	CYCADACEAE
941192	Cycas chamaoensis	Thailand		CYCADACEAE
941464	Cycas clivicola	Thailand		CYCADACEAE
981855	Cycas petraea	Thailand	Loei	CYCADACEAE
981917	Encephalartos macrostrobilus	Uganda		ZAMIACEAE
200069	Encephalartos concinnus	Zimbabwe		ZAMIACEAE

-Cycads species used in this study and native place where they were collected

Bioclimate variables from NASA database

VARIABLES	DESCRIPTION
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3	Isothermality
BIO4	Temperature Seasonality (standard deviation *100)
BIO5	Max Temperature of Warmest Month
BIO6	Min Temperature of Coldest Month
BIO7	Temperature Annual Range
BIO8	Mean Temperature of Wettest Quarter
BIO9	Mean Temperature of Driest Quarter
BIO10	Mean Temperature of Warmest Quarter
BIO11	Mean Temperature of Coldest Quarter
BIO12	Annual Precipitation
BIO13	Precipitation of Wettest Month
BIO14	Precipitation of Driest Month
BIO15	Precipitation Seasonality (Coefficient of Variation)
BIO16	Precipitation of Wettest Quarter
BIO17	Precipitation of Driest Quarter
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter

-Climate variables used in this study

Biflavonoid Protocol used in this experiment

1. From each plant sample to be analyzed, weight 100 mg of leaf powder into 2.0 screw-cap vial.

2. Add 2 ceramic beads to each vial & put the cap on.

3. Add 1.5 ml of 100% methanol to each tube. Close caps securely.

4. Place samples in the homogenizer and run them for 2 cycles of 2 minutes @ 6 m/s, and then through 2 cycles of 0.8 m/s for 3.0 min; dwell time = 2 min.

5. Spin tubes for 7 min @ max speed in centrifuge.

6. Transfer 1.0 ml of the supernatant to new, properly labeled 2.0 snap-cap vials.

7. Prepare SpeedVac

a. Turn on refrigerator

b. Turn on Concentrator

c. Place all vials with open caps into the speedvac in a balanced configuration.

d. Mount vials with cap lid facing the top.

e. Use the hexagonal rotor that looks like a bowl.

f. Change the settings

i. Dry extractions for 15 min

ii. Temperature @ 70˚C

iii. Run time & Heat Time are the same = 15 min.

g. Click run

8. Once finished, inspect the tubes. The goal here is to reduce the solvent ~100-200 ul. If the extractions are coming out completely dry, reduce the seedvac time for the next run.

9. [OPTIONAL RECONSTITUTION STEP] If the samples were over dried in the SpeedVac, then add 150 ul of MeOH. Warm the extractions for 5 min @ 45˚C, and vortex them for another 15 min. Spin the vials one more time.

10. Add 1.25 ml of cold HPLC water.

11. Vortex for 5 min at max speed in IKA Vortex4 digital machine.

12. Centrifuge tubes for 7 min @ max speed once more. After you will see a small brown pellet formed in the bottom of the tube, this pellet contains the b-flavonoids.

13. Transfer supernatant to new and properly labeled tubes. This is for storage. This fraction contains all neurotoxins (axoyglycosides). This can be placed in the freezer.

14. Re-dissolve the pellet with 300 ul of 100% MeOH.

15. Vortex for 5 min at max speed in IKA Vortex4 digital machine.

16. Add 150 ul of HPLC water and vortex for 5 min at max speed.

17. Filter 450 ul of extract with Spin-x centrifuge tube spin filters. Centrifuge at max speed for 1 min.

18. Transfer filtered extract to Autosampler vials with insert. Samples are ready to go through the HPLC.