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COMMUNICATION

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Leaf nutrients of two *Cycas* L. species contrast among in situ and ex situ locations

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Abstract: An understanding of leaf nutrient relations is required for tree conservation and horticulture success. The study of cycad leaf nutrient dynamics has expanded in recent years, but direct comparisons among reports remains equivocal due to varying sampling protocols. We used *Cycas micronesica* K.D. Hill and *Cycas nongnoochiae* K.D. Hill trees to determine the influence on leaf nutrient concentrations of in situ versus ex situ locations and orientation of leaves within the tree canopy. Nitrogen, phosphorus, and potassium concentrations of leaves from ex situ plants exceeded those from in situ plants, and the differences were not explained by soil nutrient differences. Calcium concentrations of leaves varied among the site pairs, with differences primarily explained by soil calcium. Magnesium concentrations of leaves were not different among all location pairs even though soil magnesium concentrations varied among the sites more than any of the other elements. Differences in leaf macronutrient concentrations among four *C. micronesica* provenances were minimal when grown in a common garden. Lateral orientation of leaves did not influence any of the essential elements for either of the species. These findings indicate that the lateral orientation of cycad leaves does not influence leaf nutrient concentrations, leaf nutrient relations of cycad plants in managed ex situ settings do not align with leaf nutrient relations in habitat, and these differences are not explained by soil nutrition for most elements. We suggest that leaf nutrient concentrations should be determined in all niche habitats within the geographic range of a cycad species in order to fully understand the leaf physiology of each species.

Keywords: Cycad, *Cycas micronesica, Cycas nongnoochiae,* Guam, plant nutrients.

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Author details: Thomas Marler is a terrestrial ecologist who has conducted plant physiology research in Micronesia, Philippines, and Thailand for 29 years. Anders Lindström is curator of the Living Plant Collections at Nong Nooch Tropical Botanical Garden, member of IUCN Cycad Specialist Group, and cycad taxonomist.

Author contribution: Development of concepts for experimental methods was shared by both authors, TEM was responsible for all data collection, entry, and analysis. Both authors shared the authoring and editing role.

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INTRODUCTION

Cycads comprise a severely threatened plant group (Fragniere et al. 2015). The need for more applied research to inform cycad conservation and horticultural decisions has been recognized (Norstog & Nicholls 1997; Cascasan & Marler 2016). The literature on cycad leaf nutrient relations is insufficient, and a need to standardize sampling protocols among various studies and taxa has been discussed (Marler & Lindström 2018). Toward that end, we have recently shown that plant size (Marler & Krishnapillai 2018a), position of leaflet along the rachis (Marler & Krishnapillai 2019a), incident light, and leaf age (Marler & Krishnapillai 2019b) are plant traits that should be recorded to ensure repeatable methods in cycad leaf nutrient studies. Additionally, the nutrient status of the soils directly subtending a cycad plant differs from that of the bulk community soil (Marler & Krishnapillai 2018b; Marler & Calonje 2020), so sampling of soil directly beneath plants from which leaf samples are collected is needed to adequately interpret research results. Details on these influential plant traits and soil properties are missing from the methods of most published reports on cycad leaf nutrients (Grove et al. 1980; Watanabe et al. 2007; Álvarez-Yépiz et al. 2014; Marler & Ferreras 2015, 2017; Krieg et al. 2017; Zhang et al. 2015, 2017, 2018).

Several questions concerning cycad leaf nutrient relations remain unanswered. For example, the influence on leaf nutrients of lateral orientation of leaves within the canopy has not been studied. Similarly, we are not aware of any reports which include a comparison of leaf nutrients between cultivated plants and in situ plants. Therefore, the plasticity of intra-specific leaf nutrient relations among various growing conditions is not known.

Cycas micronesica is listed as Endangered (Marler et al. 2010) and *Cycas nongnoochiae* is listed as Vulnerable (Hill 2010) by the International Union for Conservation of Nature Red List of Threatened Species. Threats to *C. nongnoochiae* are more typical of global threats, and include plant collecting, loss of habitat, and fire damage. The acute threat to *C. micronesica* is damage from invasive non-native insect species. *Cycas micronesica* leaves persist for many years and the native range includes Palau, Yap, Guam, and Rota Islands (Hill 1994). *Cycas nongnoochiae* leaves are usually replaced annually and the endemic range includes two adjacent mountains in central Thailand (Hill & Yang 1999; Marler et al. 2018). Both species are arborescent.

We used these two cycad species to answer the following questions: (1) Do leaf macronutrient concentrations differ among ex situ versus in situ locations? (2) Does the provenance influence leaf macronutrient concentrations when grown in a common garden? (3) Does the lateral orientation of the large pinnately compound leaves of arborescent cycad plants influence leaf mineral and metal concentrations?

MATERIALS AND METHODS

Habitat relations study

An ex situ collection of Guam, Rota, and Yap *C. micronesica* genotypes was established in Angeles City, Philippines (15°09'N). The plants were grown in full sun and were maintained with no plant competition, but were not provided irrigation or fertilizer. An ex situ collection of Guam, Palau, Rota, and Yap *C. micronesica* genotypes and *C. nongnoochiae* genotypes was established at Nong Nooch Tropical Botanical Garden (NNTBG) in Chonburi, Thailand (12°46'N). The *C. micronesica* plants were grown under shade cloth with ≈50% sunlight transmission and received irrigation as needed, but no fertilization. The *C. nongnoochiae* plants we sampled were managed in a landscape setting with tree canopy cover. They were irrigated as needed, but did not receive fertilization.

We collected samples from two ex situ garden locations and four in situ locations to compare leaf nutrient concentrations for five *C. micronesica* and one *C. nongnoochiae* location pairs. *Cycas micronesica* provenances included Guam, Palau and Yap. We could not include the Rota provenance because there were no healthy trees for in situ Rota habitats due to non-native insect herbivore infestations. For each in situ locality we documented canopy cover with a spherical densiometer (Forest Densiometers, Bartlesville, OK, USA). The densiometer was positioned at the horizontal plane located at the tip of the tallest leaf of each plant for each determination. We limited the replications to plants close to full sun conditions to match the Philippine ex situ replications, and close to 50% openness to match the Thailand ex situ replications. We also recorded the height of each replication from the location that was sampled first for each paired site. These data were used to locate replications with similar heights from the second location for each pair of locations. The dates of sample collection for the two locations in each pair were restricted to less than one month apart to ensure no seasonal effects would complicate the findings. There were eight replications for the Guam and Yap site pairs, and six replications for the Palau site pair.

(1) In situ *C. nongnoochiae* leaves were sampled in Tak Fa, Thailand on 17 June 2013 (15°19'N), and the canopy openness ranged from 45% to 60%. Ex situ leaves were sampled at NNTBG on 24 June 2013, and the plants were selected to match the same canopy openness. (2) Ex situ leaves from Guam *C. micronesica* trees were sampled in Thailand on 11 August 2013 and Philippines on 30 August 2013. Matching in situ *C. micronesica* leaves were sampled in an east Guam habitat on 06 September 2013 (13°27'N). All unprotected in situ localities throughout Guam were severely threatened by several non-native insect pests, so we used a semi-managed plot in which imidacloprid was used to provide systemic tree protection. The imidacloprid applications began in 2007 and were repeated every 3–4 months. These in situ plants exhibited minimal infestations of the non-native insect herbivores. Moreover, they received no management protocols other than the pesticide applications. The densiometer was used to select appropriate trees with ≈50% sunlight for the Thailand samples and full sun for the Philippine samples. (3) In situ *C. micronesica* leaves were sampled in Ngellil Island, Palau on 20 May 2017 (7°20'N). The densiometer was used to select trees with ≈50% sunlight. Matching ex situ leaves from Palau *C. micronesica* trees were sampled in Thailand on 07 June 2017. There were no Palau genotypes in the Philippine ex situ collection. (4) Ex situ leaves from Yap *C. micronesica* trees were sampled in Thailand on 18 Jan 2018 and Philippines on 26 January 2018. Matching in situ *C. micronesica* leaves were sampled in Yap on 04 February 2018 (9°31'N).

Leaflets from the youngest leaves on plants with no visible active leaf growth were sampled. Trees with no signs of recent reproductive events were selected. Leaflets were collected from basal, midpoint, and apical locations on each leaf, and one leaf from each cardinal direction was sampled per plant. All leaflets were homogenized into one sample per replicate.

The tissue was dried at 75 °C and milled to pass through 20-mesh screen. Total nitrogen was determined by dry combustion (FLASH EA1112 CHN Analyzer, Thermo Fisher, Waltham, Mass, U.S.A.) (Dumas 1831). Samples were also digested by a microwave system with nitric acid and peroxide, then phosphorus, potassium, calcium, and magnesium were quantified by inductively coupled plasma optical emission spectroscopy (Spectro Genesis; SPECTRO Analytical Instruments, Kleve, Germany) (Hou & Jones 2000).

Common garden study

We used *C. micronesica* plants growing in homogeneous conditions at NNTBG to determine the influence of provenance on leaf macronutrient concentrations. Provenances were Guam, Palau, Rota, and Yap. Sampling was conducted on 07 June 2017. The plants were growing in homogeneous constructed mineral soil medium in raised beds underneath shade cloth with ≈50% sunlight transmission. For each replicate, leaves from the youngest flush that were oriented north, east, south, and west were selected and leaflets were harvested from base, midpoint, and apex of each rachis. Leaflets from the three rachis locations and four cardinal directions were combined into one sample for each replice. Six homogeneous trees of each species were selected within the height range 1.0–1.6 m. Macronutrients were determined as previously described.

Leaf orientation study

The influence of leaf orientation within the canopy on essential element concentrations in leaf tissue of *C. micronesica* and *C. nongnoochiae* trees was determined at Nong Nooch Tropical Botanical Garden. We restricted the sampling to *C. micronesica* plants from Guam. Sampling was conducted on 18 January 2018. The plants were growing in homogeneous constructed mineral soil medium in raised beds underneath shade cloth with ≈50% sunlight transmission. For each replication, leaves from the youngest flush that were oriented north, east, south, and west were selected and leaflets were harvested from base, midpoint, and apex of each rachis. The three rachis locations were combined into one sample for each cardinal direction for each replication. Six homogeneous trees of each species were selected within the height range 1.0–1.3 m. Macronutrients were determined as previously described. In addition, the nutrients boron, copper, iron, manganese, sulfur, and zinc were digested and determined by spectroscopy as described for the macronutrients.

Soil analyses

A soil sample was collected beneath each sampled tree and combined into a composite sample for each location. The soil cores were 15cm in depth and were positioned at half the length of the longest leaves. There were four cores positioned in cardinal directions for each tree. The soil was combined and homogenized for one analysis per sampling date per location. Total nitrogen content was determined by dry combustion. Extractable essential nutrients other than phosphorus were quantified following digestion with diethylenetriaminepentaacetic acid (Berghage et al. 1987), and total metals were quantified following digestion with nitric acid (Zheljazkov et al. 2002). Analysis was by inductively coupled plasma optical emission spectrometry. Available P was determined by the Olsen method (Olsen et al. 1954) for every site except for the Yap site. A modified Truog method (Hue et al. 2000) was used for the acid Yap soils.

Statistics

Macronutrient concentrations from each of the location pairs were subjected to *t* test to compare *in situ* and *ex situ* locations. Macronutrients from plants in the common garden setting were subjected to a one-way ANOVA (PROC GLM, SAS Institute, Cary, Indiana) to compare provenances. The leaf orientation data were subjected to one-way ANOVA to determine the influence of lateral orientation on leaf traits. The two species were analyzed separately. Means separation was conducted with Tukey's HSD test for each response variable that was significant.

RESULTS

Habitat relations

Soil chemistry varied substantially among the in situ and ex situ locations (Table 1). Our two ex situ location differences were greatest for nitrogen and phosphorus and moderate for magnesium and zinc. Elements that exhibited the greatest range among the in situ locations were calcium, iron, manganese, phosphorus, and zinc. The mean of the in situ locations exhibited greater concentrations of every reported element than the mean of the ex situ locations.

Green leaf nitrogen concentration was significantly greater in the ex situ locations than the in situ locations for all six habitat pairs (Table 2). The paired comparison that exhibited the greatest difference was the Palau *C. micronesica* genotype, with nitrogen in leaves from the in situ site exhibiting a 44% increase above that from the ex situ site. Green leaf phosphorus concentration was also greater in the ex situ locations than the in situ locations for all six habitat pairs (Table 3). The location differences for *C. nongnoochiae* leaf phosphorus exceeded the location differences for all *C. micronesica* site pairs. The Palau *C. micronesica* plants exhibited the greatest difference between the two locations for the five *C. micronesica* site pairs, with the ex situ site exhibiting leaf phosphorus that was double that of the in situ site. The patterns for green leaf potassium concentration were similar to those for leaf phosphorus (Table 4). The in situ *C. nongnoochiae* leaf potassium concentration was one-fourth that of the ex situ leaf concentration. The Palau *C. micronesica* plants again exhibited the greatest difference between the two locations, with the ex situ plants exhibiting a 75% increase above that of the in situ plants.

Green leaf calcium concentration was significantly different for all six location pairs (Table 5). In contrast to nitrogen, phosphorus, and potassium, the in situ locations exhibited greater leaf calcium concentration than the ex situ locations for *C. nongnoochiae* and the Guam and Palau genotypes of *C. micronesica*. The Yap *C. micronesica* trees, however, exhibited greater leaf calcium concentration in the in situ locations for both site pairs. Green leaf magnesium concentration was similar for each of the six location pairs (Table 6). The leaf magnesium concentration of *C. nongnoochiae* trees was less than that of the five *C. micronesica* location pairs. The plasticity of magnesium concentration

Table 1. Chemical elements of soils subtending *Cycas micronesica* or *Cycas nongnoochiae* plants in various locations.

	Off-site	**Off-site**	**in situ**	**in situ**	**in situ**	**in situ**
Substrate property	Philippines	Thailand	Yap	Guam	Palau	Thailand
Nitrogen (mg·g^{-1})	1.3	4.3	5.2	10.2	13.4	4.9
Phosphorus (µg·g^{-1})	92.7	9.5	14.2	50.1	62.5	45.8
Potassium (µg·g^{-1})	76.7	64.4	99.5	406.6	511.2	273.8
Calcium (mg·g^{-1})	0.9	1.1	2.1	11.9	12.9	10.1
Magnesium (µg·g^{-1})	96.3	141.6	1292.2	543.4	1112.7	1021.2
Manganese (µg·g^{-1})	19.1	18.7	14.3	143.2	56.1	15.5
Iron (µg·g^{-1})	8.4	11.5	328.7	15.7	20.7	7.3
Copper (µg·g^{-1})	1.2	1.8	3.9	1.5	2.2	0.9
Zinc (µg·g^{-1})	9.9	5.5	8.8	39.6	8.7	2.8

Table 2. Green leaf nitrogen concentration (mg·g^{-1}) of *Cycas micronesica* and *Cycas nongnoochiae* plants in various locations. Ex situ sites included Chonburi, Thailand (curated by Nong Nooch Tropical Botanical Garden) and Angeles City, Philippines (curated by University of Guam).

Cycas Genotype	Site	Ex situ	In situ	t	p
C. nongnoochiae	Thailand	25.63±1.22	29.88±1.52	2.224	0.043
Guam *C. micronesica*	Philippines	16.89±2.11	23.15±2.56	4.569	<0.001
Guam *C. micronesica*	Thailand	18.95±1.99	25.14±3.02	3.435	0.004
Palau *C. micronesica*	Thailand	20.46±2.04	29.51±2.99	8.320	<0.001
Yap *C. micronesica*	Philippines	21.12±2.14	26.89±2.01	3.849	0.002
Yap *C. micronesica*	Thailand	24.26±2.24	30.23±2.35	5.407	<0.001

Table 3. Green leaf phosphorus concentration (mg·g^{-1}) of *Cycas micronesica* and *Cycas nongnoochiae* plants in various locations. Ex situ sites included Chonburi, Thailand (curated by Nong Nooch Tropical Botanical Garden) and Angeles City, Philippines (curated by University of Guam).

Cycas Genotype	Site	In situ	Ex situ	T	P
C. nongnoochiae	Thailand	1.31±0.06	3.44±0.41	11.997	<0.001
Guam *C. micronesica*	Philippines	1.77±0.13	2.04±0.21	2.152	0.048
Guam *C. micronesica*	Thailand	1.91±0.14	2.34±0.21	2.114	0.026
Palau *C. micronesica*	Thailand	1.45±0.16	2.94±0.18	15.395	<0.001
Yap *C. micronesica*	Philippines	1.61±0.21	2.39±0.22	3.394	0.004
Yap *C. micronesica*	Thailand	1.68±0.24	2.47±0.25	3.989	0.001

Table 4. Green leaf potassium concentration (mg·g^{-1}) of *Cycas micronesica* and *Cycas nongnoochiae* plants in various locations. Ex situ sites included Chonburi, Thailand (curated by Nong Nooch Tropical Botanical Garden) and Angeles City, Philippines (curated by University of Guam).

Cycas Genotype	Site	In situ	Ex situ	t	p
C. nongnoochiae	Thailand	4.41±0.39	18.19±2.19	12.227	<0.001
Guam *C. micronesica*	Philippines	11.79±0.55	16.14±1.62	5.413	<0.001
Guam *C. micronesica*	Thailand	12.57±0.66	18.02±1.88	6.382	<0.001
Palau *C. micronesica*	Thailand	10.45±1.35	18.29±1.38	9.128	<0.001
Yap *C. micronesica*	Philippines	12.49±2.12	16.88±2.05	4.710	<0.001
Yap *C. micronesica*	Thailand	14.92±2.63	18.86±2.11	3.719	0.002

appeared to be highly constrained with a homeostasis among numerous settings.

The behavior of the macronutrients separated into three general groups with regard to our paired site approach. The first group was comprised of nitrogen, phosphorus, and potassium where the ex situ plants universally exhibited greater leaf concentrations than the in situ plants and the differences could not be explained by differences in soil chemistry. The second group was comprised of the single element calcium where the soil calcium concentrations appeared to control of leaf calcium concentrations within the context of our methods. The third group was comprised of the single element magnesium where constrained variability caused no differences in leaf concentrations among all site pairs despite extreme differences in soil magnesium concentrations.

The influence of provenance

Differences in leaf macronutrient concentrations among the four *C. micronesica* provenances were not different for nitrogen (*P*=0.372), phosphorus (*P*=0.656), potassium (*P*=0.551), or calcium (*P*=0.654) when they were grown in a common garden setting (Figure 1). In contrast, leaf magnesium concentration was greater for the Guam, Rota, and Palau provenances than for the Yap provenance (*P*=0.037, Figure 1).

Table 5. Green leaf calcium concentration (mg·g^{-1}) of *Cycas micronesica* and *Cycas nongnoochiae* plants in various locations. Ex situ sites included Chonburi, Thailand (curated by Nong Nooch Tropical Botanical Garden) and Angeles City, Philippines (curated by University of Guam).

Cycas **Genotype**	**Site**	**In situ**	**Ex situ**	**t**	**P**
C. nongnoochiae	Thailand	7.02±0.76	3.24±0.44	4.425	<0.001
Guam *C. micronesica*	Philippines	18.48±2.01	6.85±0.77	5.103	<0.001
Guam *C. micronesica*	Thailand	15.98±1.45	6.11±0.72	5.339	<0.001
Palau *C. micronesica*	Thailand	19.94±2.33	6.96±0.92	12.287	<0.001
Yap *C. micronesica*	Philippines	3.32±1.16	6.22±1.29	2.567	0.022
Yap *C. micronesica*	Thailand	3.12±1.01	5.91±1.22	2.290	0.038

Table 6. Green leaf magnesium concentration (mg·g^{-1}) of *Cycas micronesica* and *Cycas nongnoochiae* plants in various locations. Ex situ sites included Chonburi, Thailand (curated by Nong Nooch Tropical Botanical Garden) and Angeles City, Philippines (curated by University of Guam).

Cycas **Genotype**	**Site**	**In situ**	**Ex situ**	**t**	**P**
C. nongnoochiae	Thailand	2.56±0.22	2.42±0.26	0.571	0.289
Guam *C. micronesica*	Philippines	4.46±0.53	5.22±0.87	0.858	0.202
Guam *C. micronesica*	Thailand	4.52±0.55	5.32±0.89	0.764	0.457
Palau *C. micronesica*	Thailand	6.95±1.85	5.48±1.68	1.123	0.288
Yap *C. micronesica*	Philippines	3.22±0.46	3.08±0.21	0.721	0.483
Yap *C. micronesica*	Thailand	3.66±0.78	3.41±0.69	0.555	0.587

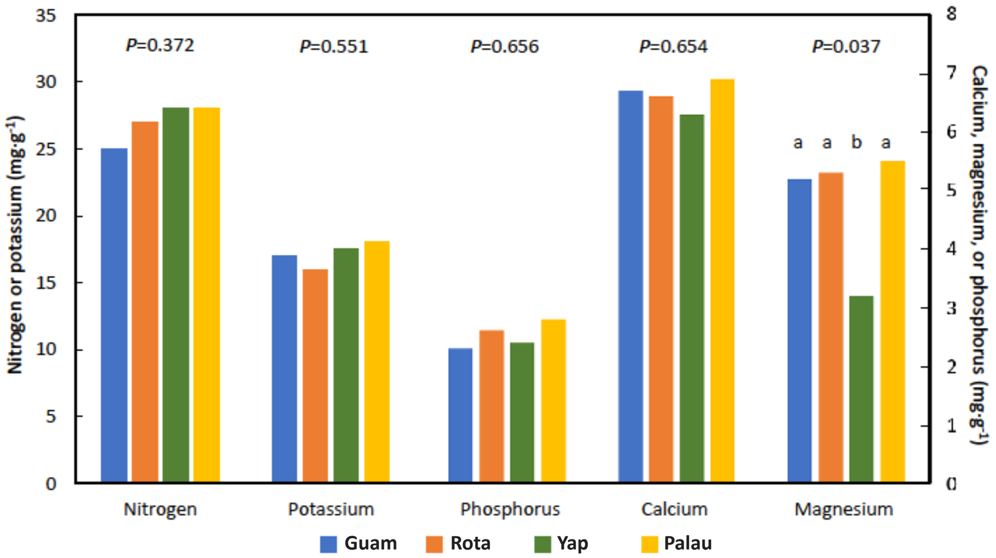

Figure 1. The influence of provenance on *Cycas micronesica* leaf tissue macronutrient concentrations when grown in homogeneous conditions at Nong Nooch Tropical Botanical Garden, Chonburi, Thailand. Columns for magnesium with same letter are not different according to Tukey's HSD test (P<0.05).

The influence of leaf orientation

Differences among the *C. micronesica* leaves that were oriented north, east, south, or west were not significant for any of the measured nutrient concentrations. These Guam-sourced trees produced leaves with nutrients in the following order of concentration: N (25.29 mg·g^{-1}) > K (18.09 mg·g^{-1}) > Ca (5.85 mg·g^{-1}) > Mg (4.22 mg·g^{-1}) > P (2.34 mg·g^{-1}) > S (1.12 mg·g^{-1}) > Fe (71.44 µg·g^{-1}) > B (43.39 µg·g^{-1}) > Mn (36.55 µg·g^{-1}) > Zn (32.49 µg·g^{-1}) > Cu (7.66 µg·g^{-1}). The differences among the *C. nongnoochiae* leaves that were oriented north, east, south, or west were not significant for any of the measured nutrient concentrations. This Thailand endemic species produced leaves with nutrients in the following order of concentration: N (29.98 mg·g^{-1}) > K (18.29 mg·g^{-1}) > P (3.36 mg·g^{-1}) > Ca (3.15 mg·g^{-1}) > Mg (2.49 mg·g^{-1}) > S (1.35 mg·g^{-1}) > Fe (76.42 µg·g^{-1}) > Mn (68.58 µg·g^{-1}) > Zn (28.03 µg·g^{-1}) > B (25.64 µg·g^{-1}) > Cu (9.69 µg·g^{-1}).

DISCUSSION

We have used several approaches to examine *Cycas* leaf macronutrient plasticity, and our results indicate that plasticity of *C. micronesica* and *C. nongnoochiae* leaf concentrations of nitrogen, phosphorus, potassium, and calcium is largely determined by the growing environment. For nitrogen, phosphorus and potassium, the benign growing conditions of a managed garden versus the competitive conditions of a biodiverse forest community appeared to be a mitigating factor. For calcium, soil content variation appeared to be the mitigating factor. In contrast, leaf concentrations of magnesium were primarily under genetic control and were relatively unresponsive to variation in the growing environment.

Variability in leaf macronutrient concentrations among the various ex situ plants was generally less than that among the matched in situ plants. These observations support the interpretation that environmental variables of the growing site were more important for determining green leaf nutrient relations than genetic differences among provenances of *C. micronesica*. The same phenomenon was reported for *Quercus variabilis* Blume where differences in tissue macronutrient concentrations among various provenances disappeared when plants from each of the provenances were grown in a common garden (Lei et al. 2013).

We are aware of only three other reports in which cycad leaf nutrients were studied in more than one location. Marler & Ferreras (2015) determined leaf nutrient relations of *Cycas nitida* K.D. Hill & A. Lindstr. plants from four Philippine in situ localities with contrasting soil chemistry. The green leaf nitrogen relations were similar to our results with minimal differences among the localities, but the phosphorus concentrations varied 1.7-fold and the potassium concentrations varied 2.6-fold among the localities. Leaf nutrient relations of several cycad species were studied in two managed botanic gardens in China. In the first report from this work (Zhang et al. 2015), there were four species that were included from both gardens. In the second report from this work (Zhang et al. 2017), no information was provided concerning leaf nutrient concentrations of individual species, so a comparison of species between the two sites was not possible. Tissue sampling of the two garden sites was separated by two to three years in these studies, so a direct comparison with our methods which minimized the time separation effects is difficult because we ensured that each pair of sites were sampled on dates that were separated by less than one month. Despite these limitations, the four species that were studied in both gardens exhibited inconsistent leaf nutrient concentrations with regard to corresponding soil nutrients (Zhang et al. 2015), a result that did not corroborate our findings for calcium. Leaf calcium concentration in three of the four species was greater in the garden site with less soil calcium concentration. A contrast in soil sampling methods may explain the differences, in that we obtained our soil samples directly beneath the sampled trees while Zhang et al. (2015) examined general soil samples from each garden. Thus our soil data were from the substrates in which the plants we examined were growing, an approach that is required to ensure accuracy (Marler & Krishnapillai 2018b; Marler & Calonje 2020). Our results and other reports indicate much is left to be learned about site-to-site differences in cycad leaf nutrient relations.

The Thailand garden exhibited greater leaf concentrations than the Philippine garden for most macronutrients. We did not collect samples for the purpose of comparing these two garden settings, however future research may be guided by two influential factors that differed between these gardens. First, the Thailand garden plants received irrigation as needed, but the Philippine garden plants were rain-fed and received no supplemental irrigation after they had become established. Leaf water relations may exert a profound effect on leaf physiology for various cycad species (Zhang et al. 2018), and the relatively greater water stress in the Philippine garden may explain the generally lower leaf nutrient concentrations. Second, the Thailand garden plants were cultured under 50% shade cloth and the Philippine garden plants were cultured in full sun. Incident light influences leaf nutrient relations for *C. micronesica* (Marler & Krishnapillai 2019b), and the generally lower leaf nutrient concentrations in the Philippine garden may have been explained by the full sun growing conditions.

Why would the managed gardens produce plants with greater leaf macronutrient concentrations than the in situ plants when the soil nutrient status was not an explanatory factor and the plants in our two gardens received no supplemental fertilizer? We suggest the greater nitrogen, phosphorus, and potassium concentrations in the garden plants resulted from the profound inter-specific competition of the typical species rich cycad habitat versus the lack of inter-specific competition due to weed control in the garden settings. Manipulative studies have shown that greater plant species richness leads to decreased leaf macronutrient concentrations, indicating more efficient use of the leaf nutrients in the biodiverse settings (Lü et al. 2019). *Cycas* plants are responsive to containerized competition studies (Marler 2013; Marler et al. 2016). Species richness studies using sympatric species from the habitats of each model cycad species may answer these questions about greater leaf macronutrient concentrations in managed garden settings.

One of the factors that governs global leaf nitrogen and phosphorus variation is latitude. Both of these leaf nutrients are found in greater concentrations with greater latitude (Reich & Oleksyn 2004; Han et al. 2005). Our range of 7°20'N (Palau) to 14°07'N (Rota) for the *C. micronesica* provenances revealed no observable influence of latitude on leaf nitrogen or phosphorus concentration.

The collective results and observations indicate that the study of cycad leaf nutrient relations is a field of study that is in its infancy. The addition of more relevant reports is important for improving terrestrial plant conservation because cycads are one of the most threatened groups of plants worldwide (Fragniere et al. 2015). That reports are accumulating in the literature is encouraging, but appropriate sampling methods must be used to gather useful information. From the information known to date, such methods must assess plant size, position of leaflet along the rachis, incident light, and leaf age or description of the sequence of leaf flushes sampled (Marler & Krishnapillai 2018a, 2019a,b). Herein we have shown that the lateral direction of a *Cycas* leaf within the canopy did not influence the 11 minerals and metals measured, and our findings indicate the omission of this sampling information from many past reports on cycad leaf tissue analyses may be acceptable.

What are some of the areas of study that are needed? More multi-species studies are needed from robust botanic garden collections to more fully understand the genetic controls over cycad leaf nutrient status and whether these leaf physiology traits are correlated with phylogeny. To our knowledge, ours is the first provenance study for any cycad species, so more provenance studies are needed on indigenous species with wide geographic ranges and multiple niche areas of occupancy. The influence of season on cycad leaf nutrient status has not been studied to our knowledge, and this needs to be corrected. The single study that revealed leaflet location along the rachis strongly influenced leaf nutrient status was conducted with a species with ≈2 m mature leaf lengths (Marler & Krishnapillai 2019a). The range in mature length of the cycad pinnately compound leaf is immense among the described species (Norstog & Nicholls 1997). Future research should exploit this range in mature leaf length to determine if the influence of position along the rachis is an allometric phenomenon such that differences along the rachis are restricted to species that produce large leaves. The mobilization and resorption of leaf elements during the senescence process is an important plant behavior. We are aware of only three reports that describe nutrient resorption traits for cycads, and all three reports used *Cycas* species (Marler & Ferreras 2015, 2017; Marler & Krishnapillai 2018a). Most botanic gardens manicure their plants such that old leaves are removed prior to becoming unsightly during senescence, so studying nutrient resorption dynamics may be difficult in most ex situ settings. Curators may want to reconsider the use of this practice for cycad plants that are not positioned in the public areas as a means of enabling more nutrient resorption research in ex situ locations.

In summary, the paucity of cycad research is a limitation for conservation of this threatened plant group. The recent reports on leaf nutrient content have been conducted without sufficient sampling conformity. We have shown that the orientation of leaves on two arborescent cycad species did not influence leaf nutrient concentrations, so the omission of this information from past reports may be acceptable. We are the first to report that a representative cycad species expresses heterogeneous leaf macronutrient relations among in situ versus ex situ locations, and the differences in soil macronutrient concentrations did not explain most of this heterogeneity. We are also the first to report leaf nutrient concentrations of cycad plants derived from multiple provenances and grown in a common garden setting. The controls over nitrogen, phosphorus, potassium, and calcium concentrations appear to be influenced primarily by environmental factors whereas the controls over magnesium concentration appear to be primarily influenced by genetic factors. We suggest that leaf nutrient concentrations should be determined in all niche habitats within the geographic range of a cycad species in order to fully understand the leaf physiology of each species.

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