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Exploring cycad foliage as an archive of the isotopic composition of atmospheric nitrogen

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Abstract

Molecular nitrogen (N_2) constitutes the majority of Earth's modern atmosphere, contributing ~0.79 bar of partial pressure (pN_2). However, fluctuations in pN_2 may have occurred on 10^7–10^9 year timescales in Earth's past, perhaps altering the isotopic composition of atmospheric nitrogen. Here, we explore an archive that may record the isotopic composition of atmospheric N_2 in deep time: the foliage of cycads. Cycads are ancient gymnosperms that host symbiotic N_2-fixing cyanobacteria in modified root structures known as coralloid roots. All extant species of cycads are known to host symbionts, suggesting that this N_2-fixing capacity is perhaps ancestral, reaching back to the early history of cycads in the late Paleozoic. Therefore, if the process of microbial N_2 fixation records the $\delta^{15}N$ value of atmospheric N_2 in cycad foliage, the fossil record of cycads may provide an archive of atmospheric $\delta^{15}N$ values. To explore this potential proxy, we conducted a survey of wild cycads growing in a range of modern environments to determine whether cycad foliage reliably records the isotopic composition of atmospheric N_2. We find that neither biological nor environmental factors significantly influence the $\delta^{15}N$ values of cycad foliage, suggesting that they provide a reasonably robust record of the $\delta^{15}N$ of atmospheric N_2. Application of this proxy to the record of carbonaceous cycad fossils may not only help to constrain changes in atmospheric nitrogen isotope ratios since the late Paleozoic, but also could shed light on the antiquity of the N_2-fixing symbiosis between cycads and cyanobacteria.

KEYWORDS

cyanobacteria, *Cycas*, *Lepidozamia*, *Macrozamia*, nitrogen fixation, nitrogen isotopes, symbiosis

1 | INTRODUCTION

Molecular nitrogen (N_2) is the most abundant gas in Earth's modern atmosphere, with a partial pressure (pN_2) of ~0.79 bar. However, pN_2 may have fluctuated over the course of Earth's ~4.5 billion-year history. Modeling work has demonstrated that large (>0.1 bar) pN_2 fluctuations are plausible on >10^7 year timescales under reasonable estimates of biogeochemical fluxes (Busigny, Cartigny, & Philippot, 2011; Goldblatt et al., 2009; Johnson & Goldblatt, 2018; Mallik, Li, & Wiedenbeck, 2018; Stüeken, Kipp, Koehler, Schwieterman, et al., 2016). Such large changes in pN_2 would carry implications for global climate due to the effect of pressure broadening on the absorption of infrared radiation by greenhouse gases in the troposphere (Goldblatt et al., 2009; Stüeken, Kipp, Koehler, Schwieterman, et al., 2016). For this reason, there has been considerable effort in recent years to constrain pN_2 using geological proxies that are sensitive to atmospheric pressure (e.g., Som, Catling, Harnmeijer, Polivka, & Buick, 2012; Som et al., 2016; Goosmann, Catling, Som, Altermann, & Buick, 2018). A less-explored consequence of pN_2 fluctuations is that the isotopic composition ($\delta^{15}N$) of atmospheric N_2 may have changed over time, perhaps leaving a trace in certain geological materials.

For instance, if past changes in pN_2 have been driven by the burial of nitrogen in biomass—which some models suggest is a likely mechanism (Johnson & Goldblatt, 2018; Stüeken, Kipp, Koehler, Schwieterman, et al., 2016)—then the isotopic composition of atmospheric N_2 would have shifted proportionally to the amount and isotopic composition of buried nitrogen via mass balance (similar to what is observed during glacial–interglacial changes in pCO_2; Leuenberger, Siegenthaler, & Langway, 1992; Marino, McElroy, Salawitch, & Spaulding, 1992). This may have driven the $\delta^{15}N$ of atmospheric N_2 toward lower values at times if isotopically heavy ($\delta^{15}N \gg 0$) biomass was being buried. If a suitable archive were to exist of the $\delta^{15}N$ of atmospheric N_2 in deep time, then it could perhaps be used to constrain the magnitude of pN_2 fluctuations if assumptions were made about the $\delta^{15}N$ of the buried or released nitrogen. Moreover, such an archive would also provide a critical test of the constancy of atmospheric $\delta^{15}N$ values on geological timescales, which is a foundational premise in deep-time studies of nitrogen isotope systematics (Ader et al., 2016; Stüeken, Kipp, Koehler, & Buick, 2016).

There has been limited work constraining the $\delta^{15}N$ of atmospheric N_2 in deep time. Marty, Zimmermann, Pujol, Burgess, and Philippot (2013) used N_2/Ar ratios and $\delta^{15}N$ values of fluid inclusions in ~3.5 Ga hydrothermal quartz to calculate that pN_2 was between 0.5 and 1.1 bar and that the $\delta^{15}N$ of atmospheric N_2 was within ~3‰ of the modern value. They used these data to speculate that pN_2 has not markedly changed across Earth's history. However, fluid inclusions containing trapped air are scarce in the geologic record and present a considerable analytical and interpretative challenge, making it difficult to extend this technique to large sample sets on broader timescales. More recently, Silverman, Kopf, Bebout, Gordon, and Som (2019) showed that the nitrogen isotopic fractionation during N_2 fixation changes as a function of pN_2 in some species of cyanobacteria. As microbial N_2 fixation is thought to have arisen early in Earth's history (Stüeken, Buick, Guy, & Koehler, 2015; Weiss et al., 2016) and cyanobacteria may have dominated primary productivity through much of the Precambrian (Brocks et al., 2017; Gueneli et al., 2018), they could perhaps provide a long-term record of both pN_2 and the $\delta^{15}N$ of atmospheric N_2. However, because $\delta^{15}N$ values of bulk marine sediments can be altered by a few permil during diagenesis (Robinson et al., 2012) and represent a mixture of biomass from N_2-fixing and non-N_2-fixing organisms, this would require the nitrogen isotopic analysis of discrete cyanobacterial fossils instead of bulk marine sediments. While such fossilized remains have been reported throughout much of the Proterozoic (Awramik & Barghoorn, 1977; Golubic, Sergeev, & Knoll, 1995; Pang et al., 2018), the preservation of sufficient organic material in these minute fossils would make analyses challenging, and again temporally limited.

Here, we investigate a possible macroscopic archive of the $\delta^{15}N$ value of atmospheric N_2: the foliage of plants with symbiotic N_2-fixing bacteria. Several plant lineages are known to form symbioses with bacteria that fix atmospheric N_2 into a bioavailable form and supply it to their host (Vessey, Pawlowski, & Bergman, 2005); most notable are the legumes, which host N_2-fixing bacteria (rhizobia) in their root nodules. The use of foliar $\delta^{15}N$ values for recognizing N_2 fixation in these plant-microbe symbioses has long been established (e.g., Handley & Raven, 1992; Shearer & Kohl, 1986), and in many cases, $\delta^{15}N$ values in the foliage of plants with N_2-fixing symbioses have been shown to closely approximate those of atmospheric N_2 (Pate & Unkovich, 1999; Shearer et al., 1983; Yoneyama, Muraoka, Murakami, & Boonkerd, 1993). This suggests that the measurement of $\delta^{15}N$ in fossilized leaves of such plants could perhaps provide an archive of atmospheric $\delta^{15}N$ values. However, most plants with N_2-fixing symbionts do not have a long enough fossil record to encompass a sufficient stretch of geologic time to detect possible fluctuations in pN_2 (and therefore in $\delta^{15}N$ of atmospheric N_2).

The cycads (Division: *Cycadophyta*) are an exceptional case: These gymnosperms have been deemed "living fossils" due to their morphological similarity to their ancestors, which originated in the late Paleozoic (Zhifeng & Thomas, 1989) and reached substantial diversity by the Mesozoic (Taylor, Taylor, & Krings, 2009). All extant species of cycads are known to host symbiotic, N_2-fixing cyanobacteria (primarily of the genera *Nostoc* and *Calothrix*; Rasmussen & Nilsson, 2002) in modified, subaerial to shallow subterranean, apogeotropic roots known as coralloid roots (Costa & Lindblad, 2002). The occurrence of active symbionts in all extant cycad species is consistent with an ancestral nature of N_2 fixation in this lineage (Raven, 2002), meaning that fossilized cycad foliage may indeed provide an archive of atmospheric $\delta^{15}N$ values. As cycads were quite abundant in the past—particularly during the Mesozoic—it may be possible to leverage their organic remains (in the form of carbonaceous compression fossils) as a comprehensive record of foliar $\delta^{15}N$ values across the last 200–300 Myrs.

While all of these factors make cycads compelling study organisms for recording atmospheric $\delta^{15}N$ values in deep time, a validation

of this proxy is first needed in modern ecosystems. This is particularly important because there are multiple confounding factors that can interfere with a plant's ability to record the $\delta^{15}N$ value of atmospheric N_2, even in the presence of active symbiotic N_2-fixing bacteria. These include isotopic fractionation during microbial N_2 fixation, isotopic fractionation during nitrogen transport within plant tissues, and facultative uptake of soil nitrogen pools with variable $\delta^{15}N$ (Boddey, Peoples, Palmer, & Dart, 2000; Chalk, Inácio, Balieiro, & Rouws, 2016; Handley & Raven, 1992; Unkovich & Pate, 2000). Furthermore, while such processes have been studied in detail in other plants with N_2-fixing symbioses, such as legumes, nitrogen isotopic studies of the cycad–cyanobacteria symbiosis are comparatively rare and often restricted to individual sites (e.g., Álvarez-Yépiz, Cueva, Dovčiak, Teece, & Yepez, 2014; Pate & Unkovich, 1999).

Here, we explore the nitrogen isotope systematics of the cycad–cyanobacteria symbiosis on a larger geographic scale. We present new data from a nitrogen isotopic survey of cycad foliage growing in three different environments across Australia. We further compile published data from the literature in order to evaluate the significance of the observed trends in a global context. Using this combined dataset, we consider whether factors such as mean annual temperature (MAT), mean annual precipitation (MAP), leaf age, plant sex, micro-habitat, and taxonomic affinity correlate with nitrogen isotopic variability in cycads. We also investigate the nitrogen isotopic composition of cyanobacteria isolated from the coralloid roots of wild cycads and isotopic variability across individual cycad leaves in order to quantify the isotopic effect of N_2 fixation and its preservation at multiple levels within this symbiosis. We use organic carbon concentrations and isotope ratios alongside nitrogen in order to aid in interpretations of isotopic variability, since these parameters can help track changes in leaf stoichiometry. We consider all of these datasets together and suggest that they jointly support the idea that cycad foliage reliably records atmospheric $\delta^{15}N$ values on the modern Earth.

1.1 | Nitrogen isotope systematics: Global N cycling to intra-plant fractionation

The use of nitrogen isotopes as a biogeochemical proxy in modern and ancient systems has been reviewed elsewhere (e.g., Ader et al., 2016; Hogberg, 1997; Stüeken, Kipp, Koehler, & Buick, 2016); a brief summary is given here as it relates to the development of this proxy. The isotopic composition of atmospheric N_2 on the modern Earth is globally homogenous and has come to be used as the international reference material for nitrogen isotopic analyses (Mariotti, 1983). Modern atmospheric N_2 therefore has a defined $\delta^{15}N$ value of 0‰.

The dominant flux of nitrogen into the biosphere is the fixation of atmospheric N_2 into biomass by prokaryotes (Dos Santos, Fang, Mason, Setubal, & Dixon, 2012). This process is typically characterized by a small isotopic fractionation (−2 to +1‰) when the most common nitrogenase enzyme (which contains molybdenum in the catalytic site) is utilized (Carpenter, Harvey, Fry, & Capone, 1997; Minagawa & Wada, 1986; Zerkle, Junium, Canfield, & House, 2008). The release of organic-bound nitrogen during remineralization of biomass accounts for the dominant flux of nitrogen to the rest of the biosphere. Nitrogen is liberated as ammonium (NH_4^+), but in the presence of oxygen, this ammonium is typically rapidly converted to nitrate (NO_3^-) in the microbially mediated process of nitrification. In both the marine and terrestrial biospheres, the uptake of these dissolved, bioavailable nitrogen pools (ammonium and nitrate) constitutes the dominant influx of nitrogen into biomass (with nitrate dominating over ammonium in typical marine and terrestrial environments on the well-oxygenated modern Earth). The process of remineralization is typically associated with small nitrogen isotopic effects (Freudenthal, Wagner, Wenzhöfer, Zabel, & Wefer, 2001), and nitrification—although capable of generating large kinetic isotopic effects in culture (e.g., Casciotti, 2009)—is known to proceed rapidly even at very low dissolved oxygen levels (Kalvelage et al., 2011) and therefore is thought to be essentially complete in most natural environments, causing no expressed isotopic fractionation (Brandes & Devol, 2002; Devol, 2015). Thus, the initial supply of bioavailable nitrogen to the biosphere has an isotopic composition similar to that of atmospheric N_2.

In both the marine and terrestrial biospheres, the process that exerts the dominant control on the isotopic composition of the dissolved nitrogen pool is the removal of isotopically light nitrogen in the gas phase in low-oxygen environments (Amundson et al., 2003; Devol, 2015). This can occur either through the process of "canonical" denitrification, wherein nitrate is progressively reduced to N_2 gas through a series of microbially mediated reactions, or through anaerobic ammonium oxidation (anammox) by planctomycete bacteria where ammonium is reacted with nitrite to form N_2 gas. In both cases, kinetic isotopic effects cause preferential removal of ^{14}N in the gas phase (Brunner et al., 2013; Devol, 2015; Kritee et al., 2012), leaving the residual pool of dissolved nitrogen enriched in ^{15}N (i.e., with positive $\delta^{15}N$ values). In the modern ocean, this gives dissolved nitrate an average $\delta^{15}N$ value of +5‰ (Brandes & Devol, 2002; Sigman, Altabet, McCorkle, Francois, & Fischer, 2000); on land, this causes bulk soil $\delta^{15}N$ values in most regions to be more positive than atmospheric N_2 (Amundson et al., 2003; Craine et al., 2015)—though ammonia volatilization can also contribute to ^{15}N enrichment in some soils (Evans, 2007).

Importantly, plants with N_2-fixing symbioses are able to circumvent the soil nitrogen pool and obtain nitrogen directly from atmospheric N_2 via their microbial symbionts. This means that even in the presence of ^{15}N-enriched soil nitrogen, plants with N_2-fixing symbioses can have foliar $\delta^{15}N$ values that fall near 0‰ (Shearer & Kohl, 1986), thereby making them faithful recorders of atmospheric nitrogen isotope ratios. However, several processes can obscure this signature.

First, it is known that the isotopic effect of microbial N_2 fixation can vary under certain conditions. Culture work has shown that even molybdenum nitrogenase is capable of generating more ^{15}N-depleted biomass (down to −4‰; Zerkle et al., 2008) under iron-replete conditions. Moreover, "alternative" nitrogenases that

TABLE 1 Location and climate data of each sample site

Site	Species (No. of leaves analyzed)	Latitude	Mean annual temperature (°C)	Mean annual precipitation (mm)
D'Aguilar National Park, Queensland	*Lepidozamia peroffskyana* (*n* = 3), *Macrozamia lucida* (*n* = 6), *Macrozamia macleayi* (*n* = 3)	27°S	19.8	1,130
UWA Jandakot Reserve, Western Australia	*Macrozamia riedlei* (*n* = 41)	32°S	18.2	791
Tilba Tilba, New South Wales	*Macrozamia communis* (*n* = 47)	36°S	15.7	997

utilize vanadium or iron co-factors are known to yield biomass that is ^{15}N-depleted by several permil in vitro (Rowell, James, Smith, Handley, & Scrimgeour, 1998; Zhang, Sigman, Morel, & Kraepiel, 2014), and this is thought to be relevant in some terrestrial environments (Bellenger, Xu, Zhang, Morel, & Kraepiel, 2014). Thus, for a plant–microbe symbiosis to make a good archive of the δ^{15}N of atmospheric N_2, it must be known whether alternative nitrogenases are significantly expressed in the symbionts.

Second, the transport of nitrogen within plants can cause certain tissues to become enriched or depleted in ^{15}N relative to bulk biomass (Werner & Schmidt, 2002). For instance, it has been shown that actively N_2-fixing legume root nodules tend to become enriched in ^{15}N, causing an isotopic offset to develop between roots and shoots (Wanek & Arndt, 2002). It is therefore imperative that subsampling of a plant be conducted to determine which tissues yield suitable estimates of the whole-plant nitrogen isotopic composition.

Lastly, it is known that many N_2-fixing symbioses are opportunistic and only persist when nitrogen is not readily available in the soil (Vessey et al., 2005). This means that the simple identification of N_2-fixing potential in a plant–microbe symbiosis is not sufficient to demonstrate that N_2 fixation is indeed accounting for the plant's entire nitrogen demand; rather, plants may obtain their nitrogen through a mixture of soil and atmospheric sources. The balance between these supply pathways can be influenced by local environmental conditions (e.g., soil moisture, temperature, nutrient availability) or potentially even biological factors (e.g., leaf or plant age, sex). With this being the case, studies that aim to generate robust, quantitative estimates of a plant's reliance on microbial N_2 fixation typically measure the δ^{15}N of foliage in non-N_2-fixing "reference plants" growing in the same habitat (Shearer & Kohl, 1986). These plants serve as integrators of the δ^{15}N of bioavailable nitrogen in soil and can provide added assurance that an N_2-fixing plant is indeed receiving most of its nitrogen through symbiotic N_2 fixation rather than from the soil.

We consider each of these potential complications below. To address the isotopic effect of microbial N_2 fixation, we cultured isolated cyanobacteria from the coralloid roots of wild cycad populations and observed their isotopic fractionation in vitro. We also conducted a sampling transect of a single leaf in order to observe the intra-plant isotopic partitioning of nitrogen in cycad foliage. We then turn to our larger dataset and consider whether any environmental or biological factors influence the nitrogen isotopic composition of cycad foliage. We close by considering cycad foliage in comparison to non-N_2-fixing reference plants, and we discuss problems and prospects for employing this proxy in deep time through the isotopic study of carbonaceous cycad fossils.

2 | MATERIALS AND METHODS

2.1 | Collection of plant samples

Cycad leaves were collected in spring and early summer (late October–January) from natural populations growing at three different localities in Australia: University of Western Australia Jandakot Reserve (32.17°S, 115.83°E), Tilba Tilba, New South Wales (36.32°S, 150.04°E), and D'Aguilar National Park, Queensland (27.44°S, 152.83°E; 27.44°S, 152.82°E; 27.28°S, 152.76°E). Samples from New South Wales and Western Australia were classified as "young," "old," or "dead" according to their maturity, where "young" leaves were recently sprouted from the shoot apical meristem on mature plants, "old" leaves were at the periphery on their respective plants, and "dead" leaves were found on the ground beneath living plants. The Western Australia samples were further subdivided into male and female specimens to look for sex-specific isotopic differences. Non-cycad leaves were also collected at the New South Wales and Queensland sample sites to assess the nitrogen isotopic composition of non-N_2-fixing plants. All plant samples were freeze-dried for 2 days to ensure that all biomass was entirely devoid of water. Leaf samples were then ground to a homogenous powder in a metal ball mill (WIG-L-BUG®) that was cleaned with methanol between samples. The resulting powders were analyzed for carbon and nitrogen concentrations and isotopic ratios.

Climate data for each site were obtained from stations operated by the Australian Bureau of Meteorology (Table 1). For each locality, the MAP (mm) and MAT (°C) were compiled from the stations nearest to the sample site.

2.2 | Collection of cyanobacterial samples

Studies of cycads and their symbionts have often focused on plant specimens from greenhouses and botanical gardens, which may reflect a mixture of endogenous and locally acquired symbionts. Additionally, isotopic studies based on such samples may be unintentionally affected by increased external fixed nitrogen availability.

The cyanobacteria–cycad symbionts analyzed in this study were previously isolated and characterized from coralloid roots of cycads growing in their natural habitats, without influence of agricultural fertilization (Gehringer et al., 2010). The eight isolates used in this study (Table 2) were kept in a nitrogen-free medium from isolation onward to ensure the maintenance of their N_2-fixing abilities. Stationary-phase liquid cultures were inoculated in duplicate into 120 ml N-free, BG110 medium in T175 (Sarstedt) culture flasks (Gehringer et al., 2010). Cultures were grown at 24°C with a 16:8-hr day:night cycle at 60 µmols photons m^{-2} s^{-1} under a daylight plant fluorescent growth lamp. One-month-old cultures in late stationary phase were harvested by centrifugation in weighed, sterile 50-ml reagent tubes (Sarstedt), and washed twice with sterile distilled water. The pellets were freeze-dried, and the dry weight was determined prior to isotopic analysis of the biomass.

2.3 | Isotopic analyses

Isotopic analyses ($\delta^{15}N$, $\delta^{13}C$) of plant material were carried out following published protocols with a Costech™ ECS 4010 Elemental Analyzer (EA) coupled with a Conflo III to a ThermoFinnigan™ MAT253 isotope-ratio mass spectrometer (IRMS). Combustion was carried out with 10 ml O_2 at 1,000°C, and the resulting gases were then passed through a reduced copper column to consume excess O_2 and to reduce NO_x species to N_2. A magnesium perchlorate trap was used to remove water from the gas stream. Isotopic measurements were calibrated against three in-house standards (two glutamic acids "GA1" and "GA2," and dried salmon "SA") that have been calibrated to international reference materials USGS-40 and USGS-41. All isotopic data are reported in delta notation relative to air for nitrogen and Vienna Pee Dee Belemnite (V-PDB) for carbon.

Analytical blanks were monitored and subtracted from nitrogen data; blanks were negligible for carbon measurements. Average analytical accuracy of $\delta^{15}N$ among individual runs was −0.02 ± 0.04‰; accuracy of $\delta^{13}C$ measurements was −0.02 ± 0.10‰. The average analytical precision (1σ) among all runs was 0.3‰ for $\delta^{15}N$ and 0.4‰ for $\delta^{13}C$. Most cycad samples were analyzed at least twice, with an average standard deviation between sample replicates of 0.2‰ for $\delta^{15}N$ and 0.1‰ for $\delta^{13}C$.

Dry biomass from cultures of isolated cyanobacteria was analyzed by flash combustion with an Isolink EA coupled with a Conflo IV to a MAT253 IRMS (ThermoFinnigan™). The combustion reactor was packed with tungstic oxide as an additional combustion aid, followed by copper wire to consume excess O_2 and to convert NO_x species to N_2. The temperature was set to 1,020°C, and pure O_2 gas was injected at a flow rate of 250 ml/min for 5 s from the drop of the sample. Water generated during the combustion was trapped with magnesium perchlorate at room temperature. The measured isotopic ratios were calibrated to the air scale with USGS-40 and USGS-41, which were analyzed four times during the run. Analytical accuracy was monitored with the international reference material SGR-1 (untreated), for which a value of 17.4 ± 0.3‰ was obtained for $\delta^{15}N$ in good agreement with previous studies (Dennen, Johnson, Otter, Silva, & Wandless, 2006).

2.4 | Statistical analyses

Paired comparisons between sample groups were performed using either *t* tests (when data adhered to a normal distribution as determined by $p > .05$ in a Shapiro–Wilk normality test) or the Mann–Whitney test. Levene's test was used to assess the difference in variance of $\delta^{15}N$ between cycads and non-cycads growing in the same habitats. Linear regression and logarithmic regression were used to explore correlations between variables. All statistical analyses were conducted in the R Statistical Computing Environment (R Core Team, 2013).

3 | RESULTS

We considered leaves individually for statistical analyses instead of averaging $\delta^{15}N$ values for whole plants (except where noted otherwise) because this captures more isotopic variability and therefore should be more conservative in our attempt to demonstrate the isotopic consistency of cycad foliage. Furthermore, this approach is a more directly applicable calibration for analysis of fossilized cycad foliage, which will rely on subsampling of disarticulated leaves or the component leaflets (which may be sparsely preserved) and where whole-plant averages are unlikely to be obtainable. In any case, we found that this treatment does not influence the observed statistical relationships; all statistical tests yield the same inference when binning data per plant versus per leaf.

The average $\delta^{15}N$ value of all cycad leaves analyzed in this study is −0.9 ± 0.8‰ ($n = 128$; here and throughout the text, mean values are reported at ±1σ). The mean $\delta^{15}N$ value of all living cycad foliage (i.e., excluding dead leaves) is −0.8 ± 0.8‰ ($n = 105$), with a range from −2.2‰ to +0.9‰. These values (which average all analyzed leaves, including multiple analyses per plant) are both nearly identical to the average $\delta^{15}N$ value using per-plant averages, which is −0.8 ± 0.7‰ ($n = 51$). The mean $\delta^{15}N$ value of living foliage from New South Wales (−1.1 ± 0.7‰, $n = 34$) is statistically indistinguishable from that of Queensland specimens (−1.0 ± 0.8‰, $n = 30$), but both localities are slightly lighter than the specimens from Western Australia (−0.5 ± 0.8‰, $n = 51$) ($p = .01$, $p = 6e{-}5$, two-tailed *t* tests). The average $\delta^{15}N$ values of all foliage from individual species are as follows: *Macrozamia communis* (−1.1 ± 0.7‰, $n = 47$), *Macrozamia riedlei* (−0.5 ± 0.8‰, $n = 51$), *Macrozamia macleayi* (−0.4 ± 0.4‰, $n = 10$), *Macrozamia lucida* (−2.0 ± 0.3‰, $n = 10$), and *Lepidozamia peroffskyana* (−0.7 ± 0.5‰, $n = 10$). In the Western Australia samples, male plants have an average $\delta^{15}N$ value of −0.6 ± 0.7‰ ($n = 35$), which is statistically indistinguishable ($p = .2$, two-tailed *t* test) from female counterparts with a mean of −0.3 ± 0.9‰ ($n = 16$).

Intra-plant $\delta^{15}N$ values from a single subsampled leaf range from −2.2‰ to 0.1‰ ($n = 19$; Figure 1). Nitrogen isotope ratios within

TABLE 2 Diazotrophic cyanobacterial symbiont strains isolated from coralloid roots of their respective host cycad species, taken from Gehringer et al. (2010)[a]

Cyanobacterial species	No. of replicate cultures	Cycad host species	Location	Voucher No.
Nostoc sp. 40.5	2	*Macrozamia communis*	Currambene State Forest	MZ40
Calothrix sp. 61.4	2	*Macrozamia parcifolia*	Seaview Range	PIF13107
Nostoc sp. 62.1	2	*Macrozamia mountperriensis*	Brooweena	PIF9343
Nostoc sp. 65.1	2	*Macrozamia riedlei*	NW Narrogin	PIF30395
Nostoc sp. 73.1	3	*Macrozamia serpentina*	Mt. Slopeway	PIF12273A
Nostoc sp. 74.5	1	*Macrozamia macleayi*	Mt. Colosseum	PIF12248
Nostoc sp. B1.3	2	*Bowenia serrulata*	Byfield	PIF32324
Nostoc sp. C1.8	1	*Cycas media* subsp. *media*	Royal Botanic Gardens	C1

[a]The vouchers, in this case a leaf and reproductive materials of the sampled plant, merely indicate that there is a formal record of which plant was used. The PIF vouchers are stored in the Queensland Herbarium. The remaining vouchers were collected under the Scientific License S11788 issued by the National Parks and Wildlife Service, NSW, Australia, and stored in the Royal Botanic Gardens, Sydney.

this leaf inversely correlate with $\delta^{13}C$ values (p = 8e−6, R^2 = 0.69, linear regression) and C/N ratios (p = 5e−7, R^2 = 0.77, linear regression). A sampling transect of a single leaflet on the same leaf revealed a strong correlation between distance from the stem and $\delta^{15}N$ (p = 9e−6, R^2 = 0.86, logarithmic regression), $\delta^{13}C$ (p = 5e−9, R^2 = 0.97, logarithmic regression), C/N (p = 9e−7, R^2 = 0.91, logarithmic regression), and TN (p = 7e−7, R^2 = 0.92, logarithmic regression) (Figure 2).

Across the Western Australia and New South Wales samples, young leaves have an average $\delta^{15}N$ value of −0.5 ± 0.8‰ (n = 30), which is slightly heavier than old leaves (−0.9 ± 0.6‰, n = 45) and dead leaves (−1.0 ± 0.9‰, n = 23) (p = .03, two-tailed t test and Mann–Whitney test, respectively). In young leaves, $\delta^{15}N$ values do not correlate with C/N ratios (p = .1, R^2 = 0.05), whereas old leaves (p = 2e−6, R^2 = 0.42) and dead leaves (p = .03, R^2 = 0.19) show weak, negative correlations (Figure 3). Among the Australian sites, foliar $\delta^{15}N$ values do not correlate with MAT (p = .2) or MAP (p = .6) (Figure 4).

The average $\delta^{13}C$ value of all cycad leaves analyzed in this study is −26.3 ± 1.8‰ (n = 128). The mean $\delta^{13}C$ values of living foliage at each site are −25.2 ± 1.1‰, −26.0 ± 0.9‰, and −28.6 ± 1.6‰ for New South Wales, Western Australia, and Queensland, respectively. Among individual species, $\delta^{13}C$ values are as follows: *M. communis* (−25.2 ± 1.1‰, n = 47), *M. riedlei* (−26.0 ± 0.9‰, n = 51), *M. macleayi* (−28.9 ± 1.7‰, n = 10), *M. lucida* (−29.2 ± 1.8‰, n = 10), and *L. peroffskyana* (−27.9 ± 1.0‰, n = 10). Male (−26.1 ± 0.9‰, n = 35) and female (−25.9 ± 0.8‰, n = 16) plants from Western Australia (*M. riedlei*) do not significantly differ in $\delta^{13}C$ values (p = .2, Mann–Whitney test). The mean $\delta^{13}C$ value of old leaves from Western Australia and New South Wales (−26.1 ± 0.9‰; n = 45) is slightly lighter than that of young leaves (−25.2 ± 1.1‰, n = 30) and dead leaves (−25.3 ± 1.0‰, n = 23) (p = .003, Mann–Whitney test). C/N ratios do not correlate with leaf maturity, with young (C/N = 38.0 ± 6.6, n = 30), old (C/N = 38.6 ± 9.5, n = 45), and dead (C/N = 37.8 ± 8.3, n = 23) leaves all statistically indistinguishable from each other (p = .5, Mann–Whitney test).

The average $\delta^{15}N$ value of all cultures of isolated cyanobacteria in this study is −0.8 ± 1.5‰ (n = 15), with no significant difference (p = .6) between *Nostoc* (−0.8 ± 1.6‰, n = 13) and *Calothrix* (−0.5 ± 0.5‰, n = 2) symbionts. The mean $\delta^{15}N$ values of reference plant leaves at individual sites are −0.9 ± 2.6‰ (n = 40, range −4.9‰ to +5.3‰) in New South Wales and −0.8 ± 1.8‰ (n = 63, range −4.3‰ to +3.3‰) in Queensland. At both sites, the mean $\delta^{15}N$ value of cycads does not significantly differ from non-cycads (p = .4, p = .5, Mann–Whitney tests). However, the $\delta^{15}N$ values of non-cycads show a larger variance than those of cycads at both New South Wales (p = 2e−7; Levene's test) and Queensland (p = .002, Levene's test).

4 | DISCUSSION

4.1 | Isotopic effect of N_2 fixation by symbiotic cyanobacteria

We analyzed isolated symbiotic cyanobacteria from various species of natural cycad populations (Table 2). These isolated cyanobionts were cultured in nitrogen-free media to observe the isotopic fractionation during N_2 fixation. The cultures had a mean $\delta^{15}N$ value of −0.8 ± 1.5‰ (n = 15), which is best explained by reliance on molybdenum nitrogenase for N_2 fixation by the cyanobionts (Zhang et al., 2014); growth in nitrogen-free media should have enabled the expression of alternative nitrogenases as N_2 fixation proceeded if they were functional.

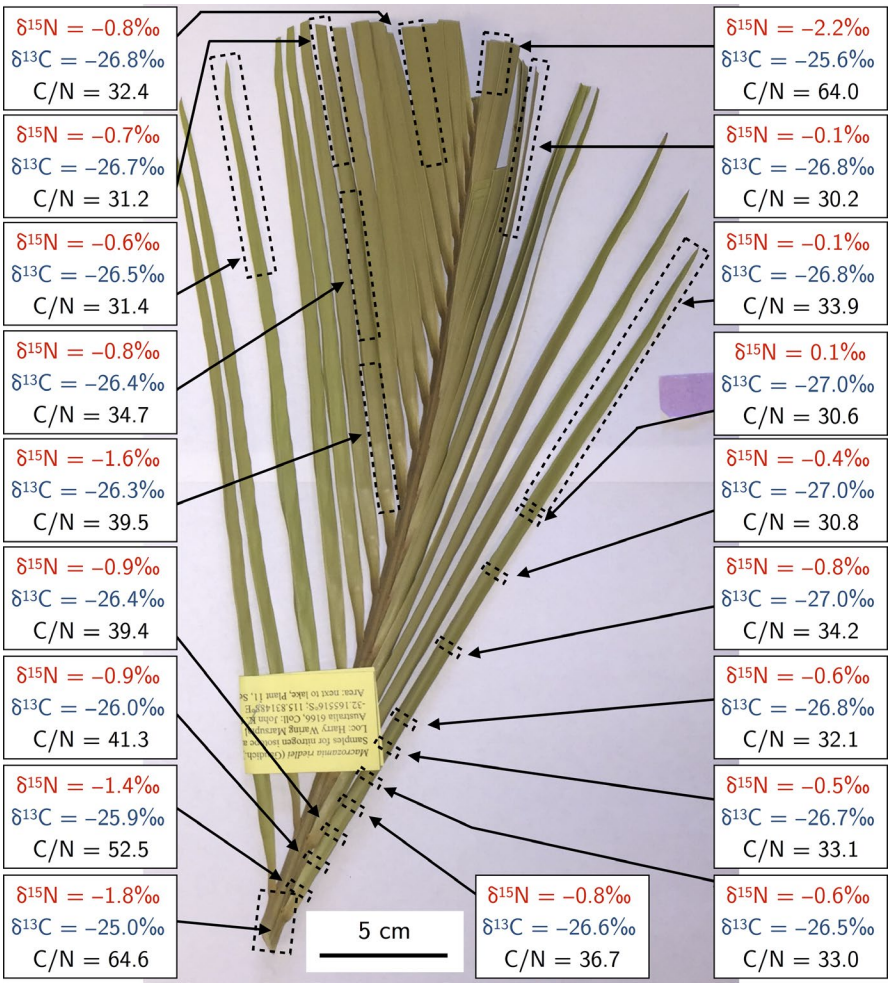

FIGURE 1 Intra-plant variability in $\delta^{15}N$, $\delta^{13}C$, and C/N ratios. Relative to leaves, stems are slightly isotopically depleted in carbon and nitrogen and have higher C/N ratios. However, all point samples on the leaf have $\delta^{15}N$ values within the range observed during N_2 fixation by isolated cyanobionts, suggesting minor isotopic fractionation during transport of nitrogen within the plant

We take these data to indicate that alternative nitrogenases do not play a significant role in N_2 fixation by cyanobionts in cycads. While a more detailed characterization of the molecular mechanism of N_2 fixation by cyanobionts in coralloid roots in the field would support this inference, for the purposes of this investigation we are mainly concerned with the net isotopic effect of symbiotic N_2 fixation. By measuring this value in vitro and observing a similar range of $\delta^{15}N$ values to that seen in other laboratory studies of N_2-fixing cyanobacteria using molybdenum nitrogenase (e.g., Carpenter et al., 1997; Zerkle et al., 2008; Zhang et al., 2014), we show that the supply of nitrogen from symbiont to host is likely within 1–2‰ of the atmospheric $\delta^{15}N$ value. This range thus imposes a limit on the precision of reconstructions of atmospheric $\delta^{15}N$ in deep time using cycad foliage, as changes of <1‰ in the $\delta^{15}N$ value of atmospheric N_2 would be difficult to resolve.

4.2 | Isotopic fractionation during intra-plant nitrogen transport

Next, we consider the detailed subsampling of a mature leaf on a *M. riedlei* specimen from the WA sample site (Figure 1) to determine whether point sampling of foliage provides a reasonable estimate of the bulk-leaf $\delta^{15}N$ value. We found moderate variation (~2‰) in $\delta^{15}N$ across the entire leaf, which followed coherent trends when plotted alongside $\delta^{13}C$, C/N, and total nitrogen (TN) as a function of distance from attachment to the stem (Figure 2). Since the $\delta^{15}N$ variability closely follows a change in C/N ratio, we hypothesize that the isotopic offset is due to different stoichiometry in foliage distal to versus adjacent to stems. Namely, a higher protein content in leaves (which is expected due to the high concentration of photosynthetic enzymes; Sterner & Elser, 2002) than in stems (which are relatively more enriched in structural compounds, namely cellulose and lignin) would explain lower C/N ratios and higher TN in the distal portions of the leaves, as we observed (Figure 2). Furthermore, protein is typically slightly enriched in ^{15}N relative to bulk biomass (Macko, Fogel, Hare, & Hoering, 1987), meaning that this increase in protein abundance could also explain the $\delta^{15}N$ trend.

Regardless of the precise mechanisms underlying the isotopic variability, the relevant question for our proxy calibration is whether sampling of foliage generates a reliable estimate of whole-plant $\delta^{15}N$. We addressed this question by calculating a weighted-mean $\delta^{15}N$, $\delta^{13}C$, and C/N value for the subsampled leaf and evaluating whether each point sample fell within the 1σ uncertainty interval of the weighted-mean value. We found that while sampling directly at the stem, or potentially at the very distal terminus of the leaf, could yield $\delta^{15}N$ values that are not representative of the bulk leaf

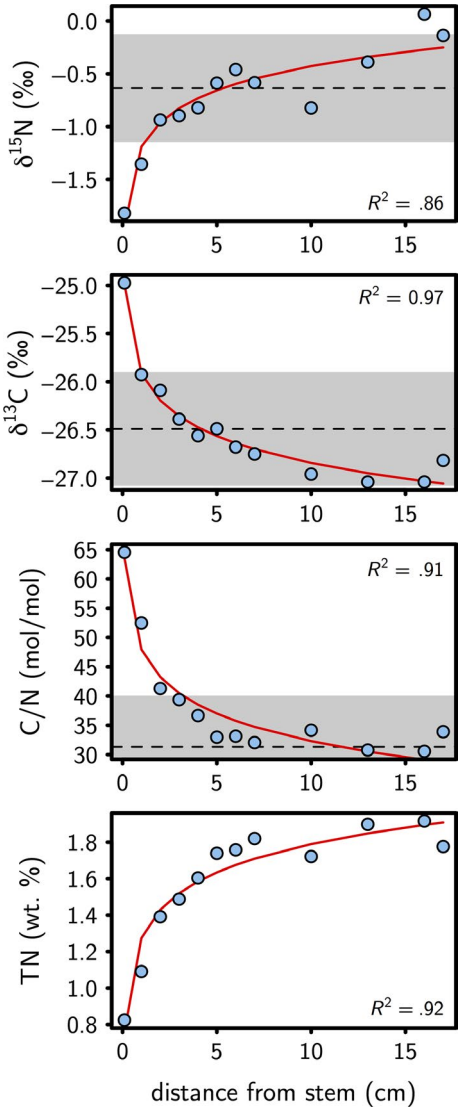

FIGURE 2 Trends in (a) $\delta^{15}N$, (b) $\delta^{13}C$, (c) C/N, and (d) TN along a transect of a single leaflet. Leaflet sampling transect is pictured on the bottom right of the leaf in Figure 1. Fitted curves denote logarithmic regression. Dotted lines denote the leaf-averaged value for the parameter being plotted; gray shaded region denotes 1σ confidence interval. The higher nitrogen content and isotopic depletion away from stems may reflect an increasing proportion of protein relative to structural material

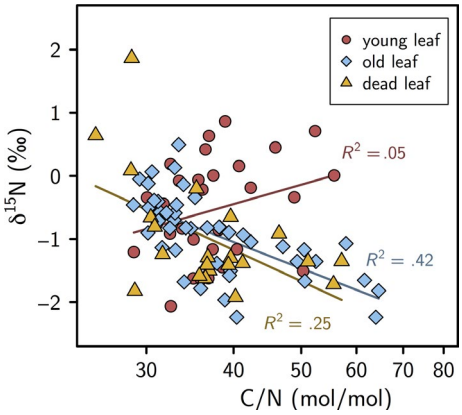

FIGURE 3 Relationship between $\delta^{15}N$ and C/N ratios in modern cycad leaves. Note log scale on x-axis. Includes samples from New South Wales and Western Australia from this study. Young leaves show no significant correlation between log(C/N) and $\delta^{15}N$ values ($p > .1$). Old and dead leaves show weak, negative correlations ($p < 10^{-5}$; $p < .01$). The gray band marks the typical range of $\delta^{15}N$ values generated via N_2 fixation in free-living cyanobacteria (Zhang et al., 2014)

(Figure 2), it is easily achievable to collect foliage samples that have representative $\delta^{15}N$ values. This can be done by either targeting the middle of the leaves, or more thoroughly, by homogenizing 5–10 cm of leaf material (as was done for the rest of our dataset). We favor the latter option and conclude that homogenization of leaf material provides an accurate assessment of the $\delta^{15}N$ value of cycads. With regard to nitrogen isotopic measurements on fossilized cycad foliage, sampling of multiple discrete leaves should provide a fairly accurate assessment of the bulk-leaf $\delta^{15}N$ value.

The last component of intra-plant isotopic fractionation is the offset due to transport from symbiont to host. We did not directly constrain this value in our study, since we conducted a separate microbial incubation and intra-plant sampling regime. However, given that the isotopic fractionation during N_2 fixation in vitro by isolated cyanobacteria (−0.8 ± 1.5‰) entirely overlaps with the observed data for whole-leaf $\delta^{15}N$ values, we estimate that there is minimal isotopic fractionation during transport of nitrogen from symbiont to host.

A possible reason for this lack of fractionation is that the cyanobionts in coralloid roots have glutamine synthetase (GS) activity similar to free-living cyanobacteria (Lindblad & Bergman, 1986). This marks the cycads as unique among plants with microbial N_2-fixing symbioses (Vessey et al., 2005), as others suppress the GS activity of their symbionts, causing nitrogen to be transported from symbiont to host as NH_3. Loss of NH_3 from root nodules could potentially explain the observed isotopic enrichment of legume roots relative to shoots (e.g., Wanek & Arndt, 2002). In contrast, since cycads receive nitrogen in the form of glutamine or citrulline (Pate, Lindblad, & Atkins, 1988), the transmission of nitrogen from symbiont to host may be more efficient or less prone to isotopic fractionation. Additionally, the persistence of cyanobionts in the interstitial tissue of the coralloid root structures could enable efficient nitrogen transport to neighboring cycad tissue, perhaps also leading to a smaller isotopic fractionation than that observed between root nodules and shoots in legumes.

We also note that recent work (e.g., Gutiérrez-García et al., 2018; Zheng, Chiang, Huang, & Gong, 2018; Suarez-Moo, Vovides, Griffith, Barona-Gomez, & Cibrian-Jaramillo, 2019) has revealed additional taxonomic diversity in coralloid roots beyond the dominant cyanobacterial strains that are typically described (i.e., *Nostoc* and *Calothrix*), including other diazotrophic bacteria such as rhizobia. While it is not known whether these other taxa contribute significant amounts of nitrogen to the host plant, our coupled pure culture and foliar $\delta^{15}N$ dataset suggests that any additional nitrogen supply to cycad tissues has an isotopic composition similar to that of the

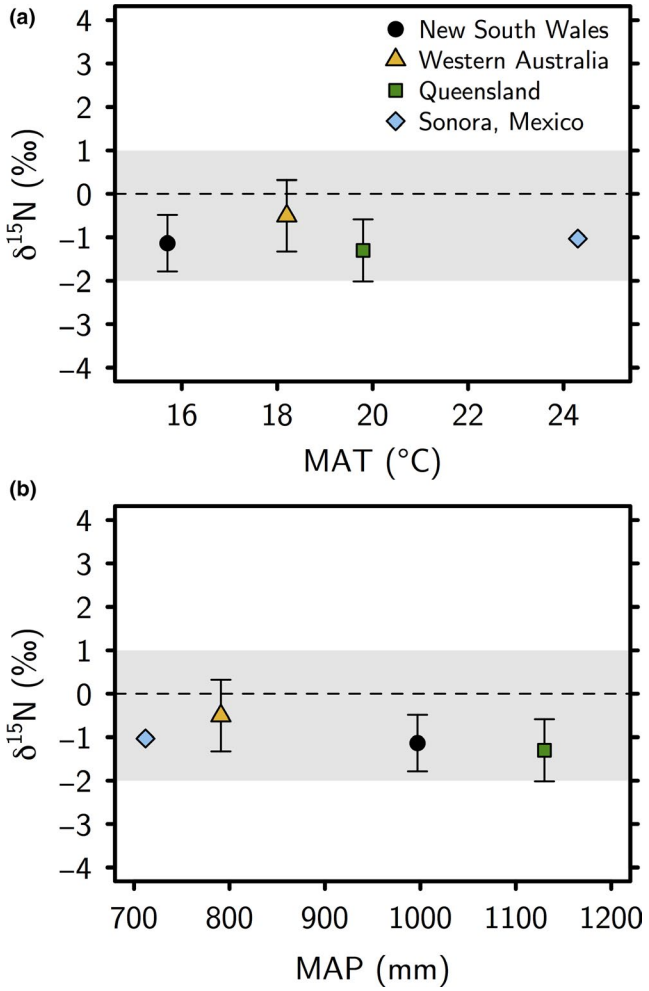

FIGURE 4 Relationship between $\delta^{15}N$ values of cycad foliage and (a) mean annual temperature, and (b) mean annual precipitation. Neither MAT nor MAP is significantly correlated with cycad $\delta^{15}N$ values. All cycad foliage has $\delta^{15}N$ values within the range expected for N_2 fixation using Mo nitrogenase (denoted as gray shaded region)

cyanobacterial isolates. This means that overall the $\delta^{15}N$ proxy in cycads is still a valid way to track symbiotic N_2 fixation, though strictly speaking it is a metabolic proxy and alone cannot be used to infer the taxonomic affinity of the symbionts.

In sum, we take the cyanobacterial culture data and intra-plant data as evidence that nitrogen isotopic fractionation within the cycad–cyanobacteria symbiosis is fairly small. If accounted for by proper sampling of foliage, intra-plant isotopic variability should introduce less uncertainty into reconstructions of atmospheric $\delta^{15}N$ than the small isotopic fractionation imparted during microbial N_2 fixation.

4.3 | Effects of life stage, sex, and environment on foliar $\delta^{15}N$

We considered leaves of different ages (young vs. old vs. dead) to determine whether ontogeny or early degradational processes could influence $\delta^{15}N$ in cycad foliage. We found that $\delta^{15}N$ values were slightly higher in young leaves than in old and dead leaves. This would be consistent with the stoichiometric control on intra-plant $\delta^{15}N$ variability if young leaves have proportionally more protein than structural material, with a greater investment in vascular tissue coming with leaf maturation. This inference is further supported by the relationship between $\delta^{15}N$ and C/N, where young leaves show no correlation but old and dead leaves show negative correlations between foliar $\delta^{15}N$ values and C/N ratios (Figure 3). Regardless of the precise mechanism, the isotopic offsets across these groups are small (<1‰) and all fall within the range of $\delta^{15}N$ values observed during N_2 fixation by the cyanobionts in vitro, suggesting that these effects should not interfere with cycads' ability to record the $\delta^{15}N$ of atmospheric N_2 in their foliage, even after early decomposition of leaf material.

Next, we considered whether sex-specific differences in nutrient utilization could influence foliar $\delta^{15}N$ values. This was motivated by the findings of Krieg, Watkins, Chambers, and Husby (2017), who studied cycads grown in a botanical garden to investigate differences in nutrient acquisition between male and female plants. Across their entire dataset, they found that males on average had slightly higher $\delta^{15}N$ values (+0.9‰) than females (+0.4‰) (Krieg et al., 2017). This difference was attributed to a greater reliance on cyanobacterial N_2 fixation in female cycads than in males. However, the trend was only found to be significant within the species *Cycas micronesica*; no other species (from the genera *Cycas* and *Zamia*) differed in foliar $\delta^{15}N$ values between sexes. The *M. riedlei* samples in this study did not differ in foliar $\delta^{15}N$ values between sexes, similar to most of the species previously explored for sex-specific differences. This suggests that sex-specific differences in nutrient acquisition are not prevalent in wild populations of *Macrozamia*, and the similarity to many botanical garden specimens might indicate that this is a broader trend among cycads. Furthermore, even in the botanical garden specimens, the observed $\delta^{15}N$ values of both males and females fall within the range expected for N_2 fixation by cyanobionts. We therefore conclude that sex does not interfere with the ability of cycads to record the $\delta^{15}N$ of atmospheric N_2 in their foliage.

Beyond biological factors, we considered whether environmental conditions could influence cycad $\delta^{15}N$ values. For instance, environmental parameters such as MAT and MAP correlate with foliar $\delta^{15}N$ values in recent global compilations of broad plant populations (Amundson et al., 2003; Craine et al., 2009), with foliar $\delta^{15}N$ values increasing at higher MAT and lower MAP (Craine et al., 2009). These trends are thought to be related to changes in the isotopic composition of nitrogen in soils and therefore should not be observed in plants that always rely on a supply of nitrogen from symbiotic N_2-fixing bacteria. Thus, to a first order it is notable that cycads display no correlation between foliar $\delta^{15}N$ values and MAT (p = .2) or MAP (p = .6) (Figure 4). However, with only four sample sites this inference is necessarily based on limited climatic coverage. More importantly, while slight isotopic differences are observed among sites (e.g., Western Australia is slightly heavier than New South Wales and Queensland), foliar $\delta^{15}N$ values of cycads at all sites are tightly

clustered within the range seen during N_2 fixation by the isolated cyanobionts (Figure 4). Interestingly, the global foliar $\delta^{15}N$ compilation by Craine et al. (2009) showed that N_2-fixing plants have a larger range in $\delta^{15}N$ values at higher MAT. This was not observed in our dataset, although our sites only spanned a limited range of MAT (15–25°C). Put most simply, our initial survey suggests that climatic gradients (as manifest in the observed differences in MAT and MAP) do not seem to influence the foliar $\delta^{15}N$ values of these wild cycad populations.

4.4 | Foliar $\delta^{15}N$ values of cycads and non-N_2-fixing plants

Taking into account the findings described above, we then consider the foliar $\delta^{15}N$ values of cycads and non-N_2-fixing reference plants from our different sites. The early work of Yoneyama et al. (1993) and Pate and Unkovich (1999) demonstrated that foliar $\delta^{15}N$ values can effectively identify N_2 fixation in wild cycads at field sites in Thailand and Western Australia, respectively, through comparison of cycad foliage with non-N_2-fixing reference plants. They found that cycads consistently had near-zero foliar $\delta^{15}N$ values, while other plants had more elevated $\delta^{15}N$ values. More recently, Álvarez-Yépiz et al. (2014) showed that cycads (*Dioon sonorense*) in the Sonora region of Mexico also have foliar $\delta^{15}N$ values that are consistent with N_2 fixation; however, they did not measure $\delta^{15}N$ values in non-N_2-fixing reference plants. While these studies provide a starting point for investigations of foliar $\delta^{15}N$ values in cycads, they are limited in their taxonomic and geographic coverage: *D. sonorense* is a rare species with a limited geographic range, and only two measurements were made on *Cycas* spp. in Thailand in the study of Yoneyama et al. (1993). We therefore sought to extend these findings to other sites and with larger datasets. First considering our cycad (*M. riedlei*) data from the UWA Jandakot Reserve in Western Australia, the $\delta^{15}N$ values obtained in this study (−0.5 ± 0.8‰) agree well with previous work in the region (Pate & Unkovich, 1999) and are thus consistent with a substantial reliance of these cycads on N_2 fixation.

We then consider the Queensland and New South Wales sites, which differ in that they have more nutrient-rich soils (~10% organic carbon by dry weight instead of 1%–2% typical of *Banksia* woodland and <1% typical of sandplain heath in Western Australia; Foulds, 1993; Bui & Henderson, 2013). At both of these localities, foliar $\delta^{15}N$ values in cycads (−1.3 ± 0.7‰ QLD; −1.1 ± 0.7‰ NSW) also fall near zero and within the range seen in pure cultures of the isolated cyanobionts, suggesting a consistent reliance on microbial N_2 fixation across these different habitats. However, a survey of a wide variety of non-N_2-fixing reference plants at each site showed similar mean foliar $\delta^{15}N$ values (−0.8 ± 1.8‰ QLD; −0.9 ± 2.6‰ NSW) albeit with a significantly larger variance in both cases (Figure 5). In contrast to field studies of legumes, which can target non-N_2-fixing reference plants of close taxonomic affinity (occasionally even non-nodulating legume species), the paucity of gymnosperms makes selection of reference plants for cycads a bit more difficult. Two coniferous gymnosperms (*Araucaria cunninghamii* and *Podocarpus elatus*) present at the Queensland site had foliar $\delta^{15}N$ values of +1.4‰ and +2.3‰, respectively, which are >2‰ heavier than co-occurring cycads. With only two specimens, though, a statistical comparison to the cycad population is not feasible. We therefore considered a wide variety of non-N_2-fixing plants, within which we did not observe any systematic bias in foliar $\delta^{15}N$ values. At both sites, monocots and dicots had statistically indistinguishable $\delta^{15}N$ values (p = .5 QLD; p = .9 NSW) and many of the sampled leaves had $\delta^{15}N$ values that overlapped with the range seen in cycads.

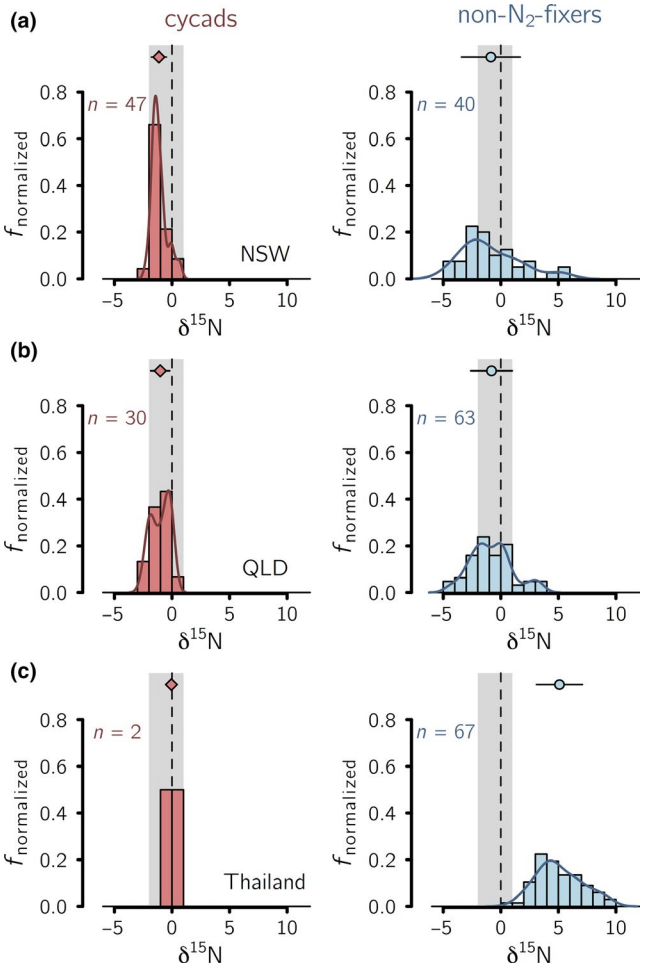

FIGURE 5 Density distribution of $\delta^{15}N$ values in cycads and non-cycads from (a) New South Wales and (b) Queensland sampling sites, and (c) from Thailand (Yoneyama et al., 1993). Gray shaded regions denote the typical range of $\delta^{15}N$ values generated via N_2 fixation in free-living cyanobacteria (Zhang et al., 2014). Dotted line denotes atmospheric $\delta^{15}N$ value. Circles mark mean $\delta^{15}N$ values ±1σ. Cycad foliage in all three settings has $\delta^{15}N$ values consistent with reliance on N_2 fixation. The similarity in mean $\delta^{15}N$ values between cycads and non-N_2-fixing reference plants in the Australian sites highlights a potential difficulty in unambiguously inferring N_2 fixation in ancient cycads; however, the larger variance in non-N_2-fixers is perhaps suggestive of assimilation of isotopically variable soil nitrogen

The similarity in mean $\delta^{15}N$ values between cycads and non-N_2-fixing reference plants precludes a quantitative assessment of N_2 fixation by cycads at these sites. However, this does not mean that cycads are not strongly reliant on symbiotic N_2 fixation in these settings. If anything, the greater variance in foliar $\delta^{15}N$ values of reference plants seems indicative of isotopically variable soil pools, which cycads are not accessing. Furthermore, the narrow range of foliar $\delta^{15}N$ values in cycads at all of our sites—as well as all other sites studied to date (e.g., Álvarez-Yépiz et al., 2014; Pate & Unkovich, 1999; Yoneyama et al., 1993)—is suggestive of a consistently high reliance on symbiotic N_2 fixation for their nitrogen demand. The occurrence of similar $\delta^{15}N$ values in non-N_2-fixing plants at these sites could even be related to N_2 fixation in the cycad–cyanobacteria symbiosis, since it has been determined that cycads can constitute a substantial input of nitrogen to their habitat (e.g., Halliday & Pate, 1976). Additionally, surveys of cyanobacterial diversity in the rhizosphere in cycad habitats have found that N_2-fixing cyanobacteria are often present and active (Cuddy, Neilan, & Gehringer, 2012), suggesting that plants without explicit N_2-fixing symbioses could receive some nitrogen with an atmospheric $\delta^{15}N$ value. The relative impact of these mechanisms could be assessed with further study of $\delta^{15}N$ values in the roots, symbionts, and leaves of cycads and non-N_2-fixing plants in a broader range of sites and species worldwide. In particular, future work should focus on cycad genera for which there are no published $\delta^{15}N$ data (e.g., *Encephalartos*, *Stangeria*, *Zamia*, *Ceratozamia*, *Microcycas*, and *Bowenia*) in order to corroborate our initial finding that foliar $\delta^{15}N$ values in cycads consistently record a signature of symbiotic N_2 fixation.

In any case, our results highlight a limitation of foliar $\delta^{15}N$ values as an N_2 fixation proxy. Distinct distributions of foliar $\delta^{15}N$ values in N_2-fixing versus non-N_2-fixing plants can provide strong evidence of microbial N_2 fixation, but the absence of a difference (i.e., near-zero $\delta^{15}N$ values in non-N_2-fixing plants) does not provide evidence for the absence of symbiosis. This limitation must be held in mind when applying the cycad $\delta^{15}N$ proxy to fossil assemblages in deep time.

4.5 | Outlook for foliar $\delta^{15}N$ values as a proxy for $\delta^{15}N$ of atmospheric pN_2 in deep time

Based on the data presented here, we consider that cycad foliage provides a reasonably robust archive of the nitrogen isotopic composition of atmospheric N_2 in modern environments. The isotopic fractionation associated with cyanobacterial N_2 fixation is small, and the isotopic effect of nitrogen transport within cycads seems to be even smaller. Sampling of homogenized leaf material provides adequate assessments of whole-leaf $\delta^{15}N$, and foliar $\delta^{15}N$ values do not appear to vary across biological or environmental gradients.

The major question that follows, then, is whether fossilized cycad foliage can (a) preserve primary $\delta^{15}N$ signals and (b) be robustly analyzed for nitrogen isotope ratios. To the first point, our investigation of isotopic fractionation during earliest diagenesis revealed no detectable fractionation of nitrogen isotopes. While we did not capture later diagenesis, other studies of nitrogen isotopes in ancient sedimentary rocks have shown that isotopic alteration during later diagenesis and thermal maturation in metamorphism tends to be minor (<1‰) in rocks that have remained at or below lower greenschist facies metamorphism (Bebout & Fogel, 1992; Boudou et al., 2008; Rivera, Puckette, & Quan, 2015; Stüeken, Zaloumis, Meixnerová, & Buick, 2017). Thus, given that many terrestrial deposits from the Cenozoic, Mesozoic, and latest Paleozoic have remained within this window, we surmise that thermal alteration of $\delta^{15}N$ values in fossil cycads will not preclude this proxy from being effective in a deep-time context.

Next, we consider whether the measurement of nitrogen isotope ratios in fossil plant material is analytically feasible. For modern cycad foliage, <1 mg of plant material is sufficient for a high-precision $\delta^{15}N$ measurement. For the carbonaceous cycad fossils that we are considering (here we use "carbonaceous" to mean any fossils that have an organic film preserved on the rock matrix, i.e., "carbonaceous compression fossils"), more material will likely be required due to loss of nitrogen during diagenesis of plant matter. However, studies of coals have shown that even the most thermally mature samples have C/N ratios <300 (Ader, Boudou, Javoy, Goffé, & Daniels, 1998; Ward, Li, & Gurba, 2005). In most samples, C/N ratios of immature coals were <100 (Ader et al., 1998; Ward et al., 2005), which is only a factor of 2–3 higher than modern cycad foliage. This increase in C/N ratios is likely due to the degradation of labile, nitrogen-rich components of plant biomass (e.g., proteins, DNA) relative to the more recalcitrant, nitrogen-poor components (e.g., cellulose). This compositional difference between modern and fossil plant biomass could lead to systematic isotopic offsets between the two if the different components of plant biomass have distinct $\delta^{15}N$ values (see Section 4.2); however, as we only observed minor $\delta^{15}N$ variability within leaves with C/N ratios ranging from 30 to 65, we suspect that such a preservational effect would not dramatically skew the fossil cycad record of atmospheric $\delta^{15}N$ values (except for very poorly preserved specimens). In order to screen for such effects in the fossil record, correlations between $\delta^{15}N$ and C/N ratios can be used to identify diagenetic or metamorphic alteration of primary $\delta^{15}N$ values. Most importantly, though, these low levels of nitrogen loss from fossilized plant organic matter imply that still only a few milligrams of material would be required for robust $\delta^{15}N$ measurements. Such material is readily available from fossil units reaching back to the late Paleozoic, and many such fossils have been studied for their organic carbon isotope ratios for decades (e.g., Bocherens, Friis, Mariotti, & Pedersen, 1993; Gröcke, 1998).

This all suggests that the nitrogen isotopic composition of carbonaceous cycad fossils—which are quite abundant through the Mesozoic and into the late Paleozoic (Taylor et al., 2009)—could provide a record of the $\delta^{15}N$ of atmospheric N_2 over the last 200–300 Myrs. Some have proposed that pN_2 has remained quite

constant throughout the last ~600 Myrs (Berner, 2006); under such a model, the $\delta^{15}N$ of atmospheric N_2 would be fairly stable. However, if the atmosphere has been steadily losing nitrogen through the Phanerozoic (Johnson & Goldblatt, 2018), a secular shift in the $\delta^{15}N$ of atmospheric N_2 might be expected.

In addition to providing insight into these differing models of pN_2 through time, the application of this proxy to the fossil record would ground future deep-time applications of the $\delta^{15}N$ proxy by constraining the $\delta^{15}N$ of atmospheric N_2. Fixation of atmospheric N_2 is the initial input to the biogeochemical nitrogen cycle and widely assumed in isotope mass balance models (e.g., Algeo, Meyers, Robinson, Rowe, & Jiang, 2014; Kipp, Stüeken, Yun, Bekker, & Buick, 2018) to have maintained a constant isotopic ratio through geologic time. Thus, identifying changes in the $\delta^{15}N$ of atmospheric N_2 would force a reinterpretation of ancient sedimentary $\delta^{15}N$ data.

Lastly, in addition to answering questions about the evolution of atmospheric N_2, the application of this proxy to the fossil record could provide insight into the longevity of the N_2-fixing symbiosis in cycads. Many N_2-fixing symbioses are thought to have fairly ancient origins (Sprent & Raven,1985), but empirical evidence for their establishment in antiquity is typically difficult to derive. While the occurrence of symbiotic cyanobacteria in all extant species of cycads is consistent with an ancestral origin, the current diversity of cycad species (within already existing genera) may have been largely generated in the last ~12 Myrs (Nagalingum et al., 2011). Thus, it is perhaps the case that N_2 fixation has become an obligatory feature of cycad physiology only recently and was not prevalent early in cycad evolution. This would be evident if fossilized cycad leaves showed highly variable $\delta^{15}N$ values, in contrast to the narrow range observed in all modern environments studied to date. These data would be further supported by the measurement of $\delta^{15}N$ values in non-cycad fossils from the same depositional setting (i.e., as in the comparison shown in Section 4.4). An observation of similar $\delta^{15}N$ values in cycads and non-cycads would support the inference of a lack of N_2-fixing symbiosis. In such a scenario, fossilized cycad foliage would therefore not provide a robust archive of the $\delta^{15}N$ of atmospheric N_2, but would rather be informative about the evolutionary ecology of the cycad lineage. Therefore, in many ways, the nitrogen isotopic study of carbonaceous cycad fossils holds promise as a means to learn about nitrogen cycling in ancient environments.

5 | CONCLUSIONS

We have conducted a survey of cycads in three environments across Australia to determine whether symbiotic N_2 fixation allows their foliage to accurately and consistently record the isotopic composition of atmospheric N_2. Cultured isolates of cyanobionts show fairly small and consistent isotopic offsets during N_2 fixation in vitro (−0.8 ± 1.5‰), and isotopic fractionations during transport within cycad tissues appear to be of smaller magnitude, with homogenized leaves providing accurate assessments of whole-leaf $\delta^{15}N$. Furthermore, no biological or environmental factors caused foliar $\delta^{15}N$ values to fall outside of the range expected for cyanobacterial N_2 fixation. Therefore, cycad foliage seems to provide a robust archive of atmospheric $\delta^{15}N$ values in modern settings. The measurement of $\delta^{15}N$ values in non-N_2-fixing reference plants can potentially corroborate the inference of N_2 fixation, but is unable to definitively refute it. Thus, assembly of large datasets from various units will likely prove critical in generating a robust record of atmospheric $\delta^{15}N$ values over the last 200–300 Myrs. Furthermore, diagenetic or metamorphic alteration of primary $\delta^{15}N$ signatures can be assessed in fossil cycads using C/N ratios as a proxy for degradation of leaf material. If viewed within the context provided by analysis of non-cycads, and rigorously screened for geochemical preservation of primary signatures, $\delta^{15}N$ data from fossil cycad tissues could potentially provide key insights to aspects of Earth's ancient nitrogen cycle.

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DATA AVAILABILITY STATEMENT

All data generated in this study are available in the Supporting Information section at the end of the article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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