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Nitrogen isotopes reveal independent origins of N_2-fixing symbiosis in extant cycad lineages

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Cycads are ancient seed plants (gymnosperms) that emerged by the early Permian. Although they were common understory flora and food for dinosaurs in the Mesozoic, their abundance declined markedly in the Cenozoic. Extant cycads persist in restricted populations in tropical and subtropical habitats and, with their conserved morphology, are often called 'living fossils.' All surviving taxa receive nitrogen from symbiotic N_2-fixing cyanobacteria living in modified roots, suggesting an ancestral origin of this symbiosis. However, such an ancient acquisition is discordant with the abundance of cycads in Mesozoic fossil assemblages, as modern N_2-fixing symbioses typically occur only in nutrient-poor habitats where advantageous for survival. Here, we use foliar nitrogen isotope ratios—a proxy for N_2 fixation in modern plants—to probe the antiquity of the cycad–cyanobacterial symbiosis. We find that fossilized cycad leaves from two Cenozoic representatives of extant genera have nitrogen isotopic compositions consistent with microbial N_2 fixation. In contrast, all extinct cycad genera have nitrogen isotope ratios that are indistinguishable from co-existing non-cycad plants and generally inconsistent with microbial N_2 fixation, pointing to nitrogen assimilation from soils and not through symbiosis. This pattern indicates that, rather than being ancestral within cycads, N_2-fixing symbiosis arose independently in the lineages leading to living cycads during or after the Jurassic. The preferential survival of these lineages may therefore reflect the effects of competition with angiosperms and Cenozoic climatic change.

Nitrogen is an essential nutrient and plays an important role in regulating the productivity and trophic structure of ecosystems[1]. However, despite its abundance at Earth's surface as atmospheric N_2, bioavailable nitrogen is scarce in many habitats. This is because only some prokaryotic microbes (~15% of phyla[2])—and no eukaryotes—possess the metabolic capacity for splitting the N_2 molecule and forming bioavailable nitrogen (N_2 fixation). Thus, almost the entire supply of nitrogen to the biosphere flows through these N_2-fixing prokaryotes.

Most plants obtain their nitrogen from nitrate (NO_3^-), ammonium (NH_4^+) or organic-bound nitrogen that is available for uptake from soil

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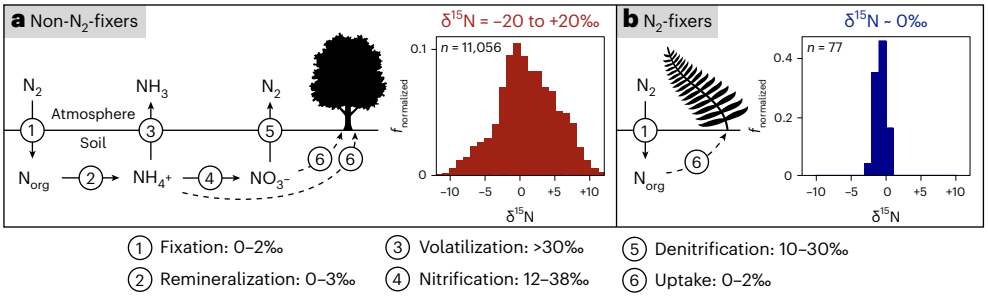

Fig. 1 | Nitrogen isotope fractionation in the terrestrial nitrogen cycle. a,**b**, Isotopic fractionations compiled from ref. 7, non-N_2-fixer foliar $\delta^{15}N$ data from TRY database (Methods) (**a**), N_2-fixer data from modern cycads[23] (**b**). Fluxes and plants are not drawn to scale.

either directly via roots or through associations with mycorrhizal fungi. These nitrogen compounds all require previous activity of prokaryotes that fix atmospheric N_2 into bioavailable form. In contrast, a few plant groups have forged symbiotic associations with bacteria that are capable of N_2 fixation. These include the nodulating legumes, actinorhizal taxa (for example, in the Betulaceae and Rosaceae), some liverworts and hornworts and cycads, among others[3,4]. The ability of these plants to obtain nitrogen directly from atmospheric N_2 via their symbionts allows them to persist in low-nutrient soils[5] or in the midst of other species that might otherwise outcompete them for fixed nitrogen[6].

Given the ability of nitrogen to regulate biological productivity and influence ecosystem structure, it follows that access to bioavailable nitrogen may have played an important role in major evolutionary or ecological events in Earth's history. While these dynamics have been explained in the context of the marine biosphere[7], at present we lack a record of the role of nitrogen in terrestrial biospheric evolution. An important example of such an event may be the ecological decline and subsequent rapid speciation of cycads in the Cenozoic.

Several traits suggest that symbiotic N_2 fixation played an important role in the evolutionary ecology of the cycad lineage (Cycadales). First, modern cycads harbour cyanobacteria (for example, *Nostoc* and *Calothrix*[8]) as their symbionts, as opposed to the rhizobia hosted by legumes or *Frankia* hosted by actinorhizal plants[4]. Also, these symbiotic cyanobacteria reside directly within unique, subaerial to shallow subterranean coralloid roots[9,10] which are swollen, dichotomously branching and upwards-growing, distinct from the deep root nodules characteristic of other plant–microbe symbioses[4]. Further, nitrogen is transported from these endophytic cyanobacterial symbionts to cycad hosts in the form of amino acids (glutamine and citrulline[11]), in contrast to ammonia (NH_3), which is the nitrogen transport substrate in other symbioses[4]. Last, symbiosis with N_2-fixing cyanobacteria is observed in all extant cycad species[9,12,13], unlike the patchy distributions in other plant clades, such as the legumes[4].

Today's occurrence of cycads in nutrient-poor soils, where their capacity for N_2 fixation enables their persistence[5], stands in stark contrast to their wide geographic, climatic and habitat distribution during the Mesozoic[14]. This begs the question: is the cycad–cyanobacterial N_2-fixing symbiosis ancestral, established in the lineage since at least the Triassic, when major lineages of extant cycads diverged[15–17]? The universal occurrence of N_2-fixing symbioses in extant cycads implies such a scenario[13]; however, the ubiquity of cycads in Mesozoic floras contradicts the observation that, in modern ecosystems, N_2 fixation is a costly process only undertaken when necessary for survival in nitrogen-poor soils[3,4]. On the other hand, if cycads did not forge symbioses with cyanobacteria (or other N_2-fixing prokaryotes) during their early evolution, did their unique mode of N_2-fixing symbiosis arise independently in extant lineages? Phylogenetic analyses of fossil and modern cycads suggest that, although modern species diversity was not generated until the Miocene[18,19], most extant genera diverged evolutionarily in the Mesozoic[15–17]. Thus, a late Mesozoic or Cenozoic appearance of N_2-fixing symbiosis in cycads would imply large-scale convergent evolution across the clade. Finally, what environmental or ecological changes could have promoted this strategy to be adopted by cycads, whether through a one-time acquisition in the ancestor of crown-group cycads or through later, convergent evolution across the clade?

A nitrogen isotope record of symbiotic N_2 fixation

We investigated the antiquity of N_2 fixation in fossil cycads by using a proxy that is well-studied in modern plants: foliar nitrogen isotope ratios ($^{15}N/^{14}N$, expressed in delta notation as $\delta^{15}N$). This proxy is derived from the observation that the foliage of plants obtaining nitrogen from N_2-fixing symbionts tends to be distinct from that of plants assimilating nitrogen from soil[20]. Specifically, plants with N_2-fixing symbionts mostly have foliar $\delta^{15}N$ values near 0‰ (Fig. 1), which is the value of atmospheric N_2 because the process of microbial N_2 fixation only slightly fractionates nitrogen isotopes (typically <2‰ relative to the N_2 source[7,21]). In contrast, bioavailable nitrogen in soils tends to be isotopically variable due to redox transformations occurring in the soil environment and is often fractionated relative to atmospheric N_2 by more than a few permil[20,21] (that is, $\delta^{15}N \neq 0‰$; Fig. 1). Thus, in a given habitat, N_2-fixing plants can be distinguished from non-N_2-fixing plants if the former have foliar $\delta^{15}N$ values near 0‰, while the latter have $\delta^{15}N$ values ≠ 0‰ (ref. 20).

While built on sound logic, this proxy has limitations. First, isotopic fractionation during transport of nitrogen from symbiont to host can lead to elevated foliar $\delta^{15}N$ values despite active N_2 fixation[22]. However, such a process is not observed in cycads[23], perhaps due to their transport of nitrogen as amino acids rather than NH_3, which is volatile and thus prone to leakage from the plant–symbiont system with an accompanying isotopic fractionation[7].

Second, the facultative nature of microbial N_2 fixation means that plants may only periodically receive nitrogen from their symbionts, whereas at other times they assimilate nitrogen from soil[3]. Such fluctuations would cause foliar $\delta^{15}N$ values to integrate the isotopic composition of the two pools (soil and atmospheric N_2), perhaps leading to non-zero $\delta^{15}N$ values despite active N_2 fixation. All modern cycads studied until now have $\delta^{15}N$ values that fall within the range generated by microbial N_2 fixation[23–26], consistent with cycads receiving most of their nitrogen from their symbionts. However, if aiming to identify N_2 fixation in deep time, it must be recognized that the $\delta^{15}N$ proxy is specifically tracking the physiological and ecological expression of nitrogen uptake, not simply the capacity for N_2 fixation (that is, phenotype rather than genotype).

Last, if a habitat has soil $\delta^{15}N$ values close to the atmospheric value (~0‰), then plants assimilating nitrogen from those soils will have similar $\delta^{15}N$ values to plants that are receiving nitrogen from N_2-fixing symbionts[27]. Such data do not preclude the possibility of active N_2 fixation but rather make the $\delta^{15}N$ proxy inconclusive in those cases, as cycads and non-N_2-fixing plants would have similar $\delta^{15}N$ values[23].

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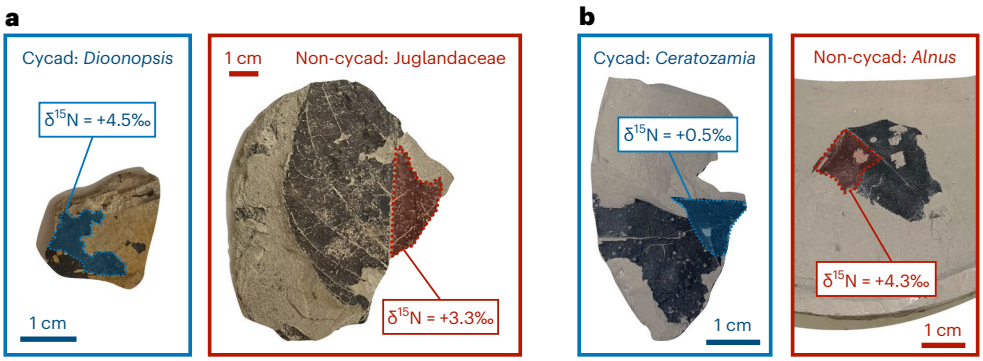

Fig. 2 | Nitrogen isotopic analyses of select fossil samples. a,**b**, Cycad and non-cycad foliage from the Denver Formation (Castle Rock flora, 64 Ma) (**a**) and Most Formation (20 Ma) (**b**) are depicted. Dashed lines and shading denote portions of fossils that were sampled for isotopic analysis.

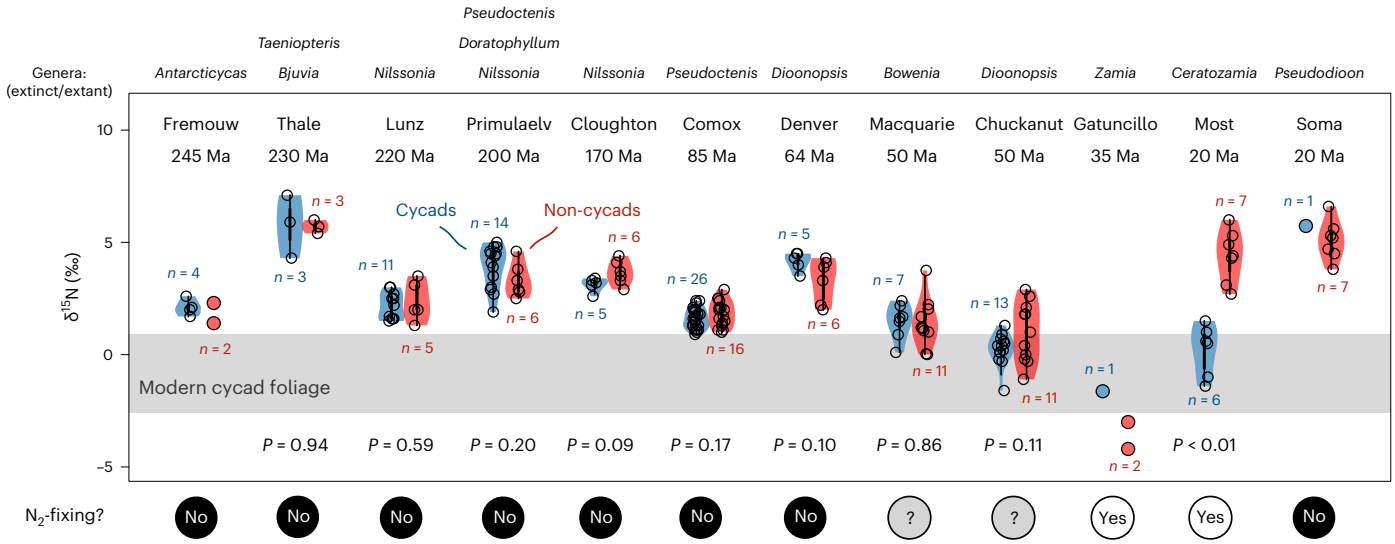

Fig. 3 | Foliar nitrogen isotope data from cycad (blue) and non-cycad (red) fossils across the last ~250 Myr. *P* values were calculated using two-sided *t*-tests or Wilcoxon tests where $n > 2$; the only significant difference is observed in the Most Formation ($P = 4 \times 10^{-5}$). Fossil-bearing units listed with approximate ages. Extinct cycad genera have foliar $\delta^{15}N$ values that are indistinguishable from other fossil plants in the same units and typically elevated relative to atmospheric nitrogen. These observations are most parsimoniously interpreted as reflecting nitrogen uptake from the same soil nitrogen pools by cycads and co-existing non-cycads. In contrast, Cenozoic fossil representatives of two extant genera (*Zamia* and *Ceratozamia*) show foliar $\delta^{15}N$ patterns that are consistent with symbiotic N_2 fixation (near-zero in cycads, fractionated in other plants). This suggests that cycads did not rely on symbiotic N_2 fixation until late in their evolutionary history and that this metabolic capacity may have played an important role in their survival and speciation through the Cenozoic.

With these limitations in mind, we applied this proxy to fossilized cycads to search for the presence of N_2 fixation through their evolutionary history. While stable carbon isotopes of carbonaceous compression fossils have been investigated as a palaeoecological proxy[28], nitrogen isotope ratios in fossilized foliage remain unexplored. This is largely because of the low nitrogen content of carbonaceous compression fossils and recalcitrant nature of fossil organic matter, which together make isotopic analysis difficult. We adapted methods designed for nitrogen recovery from nitrogen-poor Precambrian rocks[7] to enable us to measure nitrogen stable isotope ratios in fossilized plant foliage (Fig. 2; Methods).

We analysed a large sample set ($n = 178$) of carbonaceous compression fossils from 12 localities, including both cycads and other plants from the same strata. The study sites span much of the evolutionary history of cycads: *Antarcticycas* from the Early Triassic Fremouw Formation of Antarctica[15], *Taeniopteris* and *Bjuvia* from the Middle Triassic flora of Thale, Germany[29], *Nilssonia* from the Late Triassic flora of Lunz, Austria[30], *Pseudoctenis, Doratophyllum and Nilssonia* from the end-Triassic to Early Jurassic Primulaelv Formation of East Greenland[31], *Nilssonia* from the Middle Jurassic Cloughton Formation (Yorkshire flora) of England[32], *Pseudoctenis* from the Late Cretaceous Comox Formation of Vancouver Island, British Columbia, Canada[33], *Dioonopsis* from the earliest Palaeocene Denver Formation of Colorado, United States[34], *Bowenia* from the Eocene Macquarie Harbor Formation of Tasmania[35], *Dioonopsis* from the Eocene Chuckanut Formation of Washington, United States[36], *Zamia* from the Late Eocene Gatuncillo Formation of Panama[37], *Ceratozamia* from the Miocene Most Formation of Bohemia[38] and *Pseudodioon* from the Miocene Soma of Turkey[39]. We targeted sites that had been the focus of previous studies, allowing us to use robustly identified cycad foliage. Additionally, we analysed non-cycad foliage from the same lithological units—representing other vascular plants living in the same habitat as cycads—to discern whether cycads were assimilating nitrogen from soil or whether they relied on symbiotic microbial N_2 fixation.

We found (Fig. 3) that cycad fossils from two extant genera have foliar $\delta^{15}N$ values that are similar to those of modern cycads[23–26] and consistent with symbiotic N_2 fixation. These include *Ceratozamia* from the Most Formation (~20 Ma) and *Zamia* from the Gatuncillo Formation (~35 Ma). Furthermore, at these sites a dichotomy between

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cycad and non-cycad $\delta^{15}N$ values suggests that non-cycads were assimilating nitrogen from an isotopically fractionated soil reservoir. At a third site with fossil representatives of an extant genus (*Bowenia* from the Macquarie Harbor Formation, ~50 Ma), a similarity in cycad and non-cycad $\delta^{15}N$, as well as values at the upper threshold of what is observed in modern cycads, may reflect a lack of symbiosis. At the sites with isotopic differences between cycads and non-cycads, the C/N ratios of analysed cycad and non-cycad material is similar and not elevated relative to that of modern plants, suggesting that these trends are unlikely to be influenced by poor preservation (Extended Data Fig. 1). We therefore conclude that while the *Bowenia* data are somewhat ambiguous, symbiotic N_2 fixation is the most parsimonious explanation for the *Zamia* and *Ceratozamia* data.

In contrast to the representatives of extant genera, all extinct cycad genera (*Antarcticycas*, *Taeniopteris*, *Bjuvia, Nilssonia*, *Pseudoctenis*, *Doratophyllum*, *Dioonopsis* and *Pseudodioon*) show foliar $\delta^{15}N$ values that are indistinguishable from other plants growing alongside and most (eight of nine sites) are fractionated relative to atmospheric N_2 (Fig. 3). The Chuckanut Formation (~50 million years ago (Ma)) data are an exception, with cycads (*Dioonopsis*) resembling modern $\delta^{15}N$ values; however, the overlap with non-cycad data makes this signal ambiguous as to N_2 fixation status. The other eight units range from Early Triassic (~245 Ma) to Miocene (~20 Ma) in age. These isotopic trends are also unlikely to be artifacts of poor preservation, as the cycad fossils from all sites have foliar C/N ratios that fall within the range of modern plants and are similar to those of the co-occurring non-cycad foliage analysed (Extended Data Fig. 1). The most parsimonious interpretation of these data is that cycads at these sites (which include forested riverbank and floodplain understory environments[15,30,31,33,34]) predominantly assimilated nitrogen from the same soil pools as other plants growing in their midst. Hence, cycads at these sites seem not to have relied on a microbial N_2-fixing symbiosis.

We leveraged these isotopic constraints to conduct an ancestral state reconstruction (ASR) of N_2 fixation in cycads (Methods). The ASR strongly suggests at least two independent origins of N_2 fixation within the Cycadales. These are reconstructed as occurring within the genus *Cycas* and at the base of the crown-group Zamiaceae (Fig. 4a). Dating of the tree suggests that these acquisitions occurred by the Jurassic and Late Cretaceous in the Zamiaceae and Cycadaceae, respectively. Both the ASRs and dates are highly sensitive to the phylogenetic distribution of fossils with isotopic data. Within Zamiaceae, the distribution of extant and recent fossil taxa, which are all N_2-fixing (or ambiguous), suggests that N_2 fixation was acquired at the base of this crown group. However, there is a ~150 Myr gap between this node and the oldest unambiguous isotopic evidence for N_2 fixation in the group (*Zamia nelliae*, ~35 Ma). This gap includes many fossil taxa for which we were unable to acquire isotopic data and thus it is possible that more data from these taxa would reveal a more complicated history of the acquisition of N_2 fixation in the Zamiaceae. Indeed, there is evidence to suggest that *Antarcticycas*, which our isotopic data constrain as lacking N_2 fixation, had affinities to taxa nested within the Zamiaceae[40]. While *Antarcticycas* was not included in the recently published phylogeny[17] used in our reconstructions, its inclusion would probably yield an inference of both more numerous and more recent independent origins of N_2-fixing symbiosis. Further isotopic and phylogenetic work is therefore needed to resolve these details.

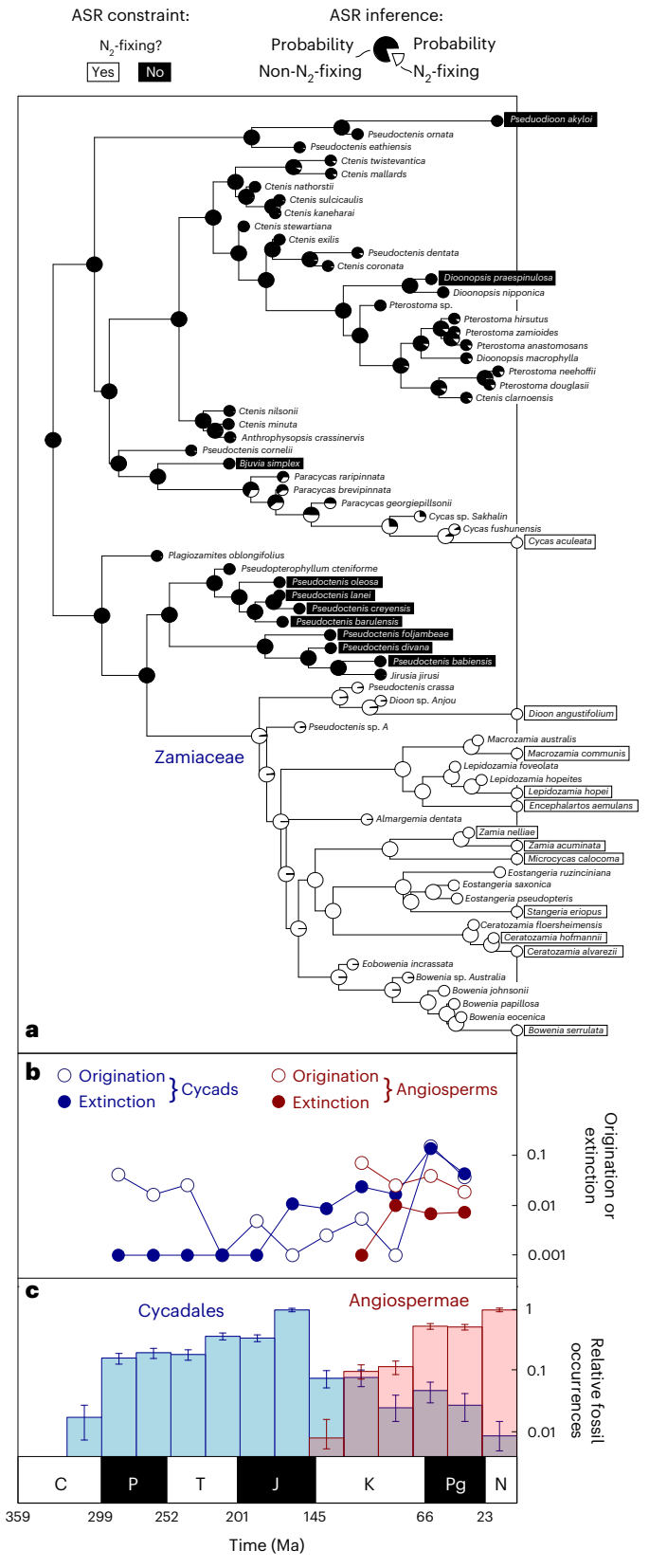

Fig. 4 | Evolutionary ecology of cycads. a, ASR of N_2-fixing capacity. **b**, Origination and extinction rates of Cycadales and Angiosperms. **c**, Relative abundance of cycad and angiosperm fossils through time. See Methods for details of all calculations. In **a**, white shading in pie charts denotes probability of N_2 fixation (100% in all modern taxa; inferred from ASR in fossil taxa); black shading in pie charts marks lack of N_2 fixation. Taxa in black boxes are constrained as non-N_2-fixing via isotopic data; taxa in white boxes show isotopic evidence of N_2 fixation. Fossil flora trends in **b** and **c** are from the Paleobiology Database (https://paleobiodb.org/#/). Relative fossil occurrence rates in **c** are plotted as bootstrap resampled (n = 1,000) means with 5th–95th percentile error bars. The ASR implies a lack of N_2 fixation in fossil taxa before ~200 Ma, indicating that reliance on N_2 fixation is not ancestral to cycads and requiring that N_2 fixation emerged more than once in the clade. The emergence of this trait coincides roughly with an increase in extinction rate in the Cycadales, as well as a decline in cycad abundance relative to angiosperms.

While the $\delta^{15}N$ values and ASR indicate a shift in nutrient acquisition strategy of cycads between the Mesozoic and Cenozoic, some inherent limitations of the $\delta^{15}N$ proxy cloud our understanding of these transitions. For instance, it is uncertain if the symbionts providing nitrogen to fossil *Zamia* and *Ceratozamia* were cyanobacteria or other prokaryotic taxa. Modern coralloid root microbial communities can contain several non-cyanobacterial taxa[41] and it is possible that similar variation was present in fossil taxa. Nevertheless, our data from the Eocene Gatuncillo Formation mark the oldest geochemical evidence of a terrestrial plant–prokaryote symbiosis in the geologic record.

Additionally, it is unclear whether any of these cycads facultatively used symbiotic N_2 fixation to supplement uptake from soil nitrogen pools. Quantifying this balance using foliar $\delta^{15}N$ values is uncertain even in modern settings[20,27] and nearly impossible in palaeoenvironments. It is possible that *Bowenia* in the Macquarie Harbor Formation and *Dioonopsis* in the Chuckanut Formation received some nitrogen from symbionts. But while ambiguous at those sites, our data provide a robust indication of nitrogen supply from symbiotic microbial N_2 fixation in extant cycad lineages by 35 Ma, with no clear evidence for such an association in extinct genera.

Overall, this dataset is consistent with the absence of fossilized coralloid roots in well-preserved Permian–Cretaceous cycad fossils (for example, *Antarcticycas*[42]). The implication of this analysis is that either the unique coralloid roots evolved convergently in extant cycad lineages or, perhaps more likely, that predecessors of coralloid roots, the genetic machinery to effect N_2 fixation via cyanobacterial symbiosis, or both, were present in most or all cycads but served a different purpose or were at least not widely used for N_2 fixation[10]. To test these hypotheses, the poorly known genetic mechanisms involved in the cycad–cyanobacterial symbiosis[10] should be further explored.

Drivers of acquisition of N_2 fixation

What drove this stark shift in cycad ecology? By far the most conspicuous change in terrestrial flora during the Mesozoic and into the Cenozoic was the rise of angiosperms to ecological dominance[14]. From their origins in or before the earliest Cretaceous[43], angiosperms came to dominate most biomes in terms of diversity and biomass by the Late Cretaceous[44]. Alongside this rise of angiosperms was a pronounced decline in the abundance of gymnosperms and of cycads in particular[44], in fossil assemblages from the Cretaceous to present (Fig. 4b,c). While multiple factors are likely to have influenced this restructuring of terrestrial ecosystems[45]—including extrinsic drivers such as terrestrial biomass deposition and associated changes in weathering rates[46], the K-Pg bolide impact[47] or the Cenozoic trend of declining CO_2 levels and global cooling[48]—direct competition between angiosperms and gymnosperms is often invoked as playing an important mechanistic role[45,48].

In modern habitats, angiosperms commonly outcompete gymnosperms for access to essential nutrients, including nitrogen[6,49]. It has traditionally been assumed that this competition is the reason why living cycads use the costly strategy of forging a symbiosis with N_2-fixing prokaryotes[3,6,24]. In contrast, early Mesozoic cycads were widespread, ecologically diverse and abundant in a broad range of habitats[50,51], indicating that they were not always limited by such competition for nutrients. Our data support the idea of a switch in ecological strategy, by implying that two or more disparate cycad lineages independently became reliant on symbioses with N_2-fixing prokaryotes between the late Mesozoic and early Cenozoic. This may have been a response to the expansion of angiosperms into habitats where cycads previously thrived. While many characteristic Mesozoic plant lineages went extinct during the Cenozoic[14], the cycads survived, albeit in limited numbers. With the isotopic signature of N_2 fixation only observed in fossil representatives of extant genera (Fig. 3), our data might therefore suggest that the capacity of cycads to harbour N_2-fixing symbionts played a critical role in their survival through Cenozoic time.

Conclusions

Cycad evolutionary history is marked by the loss of large swaths of diversity since the Jurassic[17,19]. Our results offer a mechanistic explanation for the preferential survival of certain lineages, namely those in which symbiotic N_2 fixation had been acquired. In contrast, lineages lacking this ecological strategy perished, perhaps as a result of competition with angiosperms and Cenozoic climate change. We therefore propose that the universal occurrence of N_2 fixation across extant cycads reflects a selective pressure for survival in an angiosperm-dominated world, thus reconciling the early history of cycads as abundant understory flora with their modern niche as rare, N_2-fixing plants.

Methods

Recovery of organic leaf residues

Organic residues were removed from the background rock matrix using one of two methods. In most cases, the carbonaceous film was separated from the matrix using a stainless steel razor blade. Blades were wiped with methanol between all samples and air-dried to avoid cross-contamination. In some samples that were not amenable to handling with a razor blade, an ultrafine drill was used to abrade a thin layer of organic matter from the fossil.

Under both protocols, the same method was applied to a portion of the rock matrix adjacent to the fossil in which no carbonaceous fossil remains were evident. This allowed a quantification of the background carbon and nitrogen content that could potentially be contaminating the signal obtained from the recovered fossil material. The concentration of nitrogen (TN, total nitrogen) in the isolated fossil material was consistently higher than in the background matrix, on average by a factor of 9.1. Similarly, carbon concentrations (TC, total carbon) were higher in isolated fossil material than the matrix by a factor of 20.3 on average. Furthermore, the isotopic composition of nitrogen does not covary with the difference in TN (Extended Data Fig. 2). These observations lead us to conclude that the methods used for isolating fossilized organic matter were indeed capturing the signature of the discrete fossilized specimens and not disseminated organic matter in the matrix or nitrogen bound in clay minerals. If anything, it is more likely that the nitrogen in the adjacent sediment matrix was contributed in part by degradation of the leaf specimens, as has been observed in studies of animal degradation in soil environments[52].

Isotopic analysis

The concentration and isotopic composition of carbon and nitrogen in powders isolated from fossil and matrix material were measured on a Costech ECS 4010 Elemental Analyzer coupled to a Thermo Finnigan MAT253 continuous flow isotope ratio mass spectrometer in IsoLab at the University of Washington following published protocols[23]. The instrument was operated using the commercial (Thermo Electron Gmbh) Isodat (v.2.0) software. Combustion was carried out at 1,000 °C with a 20 ml pulse of O_2. The resulting gases were then passed through a reduced copper column held at 700 °C to reduce NO_x species to N_2 and scrub excess O_2 from the gas stream. A magnesium perchlorate trap was then used to remove water from the gas stream, after which the gases were separated using gas chromatography and fed into the mass spectrometer using a Thermo Finnigan Conflo III.

Raw isotopic data were corrected using a two-point calibration[53] with three inhouse standards: two glutamic acids (GA1, TC = 40.8%, TN = 9.5%, $\delta^{13}C = -28.3‰$, $\delta^{15}N = -4.6‰$; GA2, TC = 40.8%, TN = 9.5, $\delta^{13}C = -13.7‰$, $\delta^{15}N = -5.7‰$) and dried salmon (SA, TC = 45.7%, TN = 11.8%, $\delta^{13}C = -21.3‰$, $\delta^{15}N = +11.3‰$), which are calibrated against international reference materials USGS-40 and USGS-41. Each inhouse standard was analysed four times per analytical sequence. Analytical blanks resulting from combustion were measured and subtracted from nitrogen data; analytical blanks were below detection limits for carbon.

All nitrogen isotopic data are reported in delta notation relative to air. The external reproducibility (1σ) of isotopic measurements,

as determined by replicate analyses of inhouse standard UW-McRae ($\delta^{15}N$ = +5.6‰), was ±0.2‰. Average precision (relative error) of concentration measurements was ±1.7% for TN and ±1.4% for TC.

Statistical analyses of isotopic data

For all sites with multiple cycad and non-cycad specimens, we statistically compared their isotopic compositions. We first used the Shapiro–Wilk normality test to determine whether data adhered to a normal distribution. In cases when the null hypothesis was rejected, we proceeded with a non-parametric (Wilcoxon) test; in the rest of cases, *t*-tests were used to compare the means of populations. The resulting *P* values are presented in Fig. 3.

Ancestral state reconstruction

To evaluate number of acquisitions of N_2 fixation within the Cycadales, as well as their timing, we ran ASRs using the recently published, time-calibrated phylogeny of ref. 17. All taxa included in the phylogeny were coded for the presence or absence of N_2 fixation. Where no data were available, taxa were assumed to have equal probability of possessing either trait and their status as N_2-fixers was inferred by the model. Ancestral states were estimated using stochastic character mapping with 1,000 simulations under an 'all rates different' model of character evolution, which allows for the gain and loss of N_2 fixation to occur at different rates. Akaike information criterion supported this over a simpler 'equal rates' model. Analysis was completed using the phytools[54] package (v.1.0-1) in the R software environment[55] (v.4.1.2).

Members of seven fossil genera for which we obtained isotope data were included in the phylogeny of ref. 17. Three of our sampled fossils (*Ceratozamia hofmannii*, *Pseudodioon akyoli* and *Zamia nelliae*) were identified to the species level, while the remainder could only be identified to the genus level. Isotope data for the latter were coded as follows. *Bjuvia simplex* was coded as non-N_2-fixing, although fossils were only identified to genus, this is the only included member of *Bjuvia* in the phylogeny. The N_2 fixation states of all fossil *Bowenia* were treated as uncertain due to the equivocal isotope data for this taxon. Isotopic data from *Dioonopsis* fossils show clear absence of N_2 fixation in specimens of the Denver Formation, while more recent Chuckanut fossils are ambiguous. As such, we coded *Dioonopsis praespinulosa*, the closest geographic and temporal match for the Denver Formation fossils, as lacking N_2 fixation and left the remaining species ambiguous. One study[17] demonstrated that the genus *Pseudoctenis* is highly polyphyletic. For our analyses, we treated all species of *Pseudoctenis* that fell within the clade that included most Laurasian species as non-N_2-fixing. This excluded the Gondwanan fossils assigned to this genus, the early Late Triassic *P. cornelii* and two Late Jurassic taxa (*Pseudoctenis eathiensis* and *Pseudoctenis* sp. A). While not monophyletic, the remaining taxa are closely related (Fig. 4a) and their geographic and temporal distribution suggests this is probably the clade sampled in our isotope dataset (section on Geologic context). Attributing our isotope data to specific species within this clade is not feasible, so we make the assumption that all '*Pseudoctenis*' within this clade lacked N_2 fixation. The strong signal rejecting N_2 fixation in the two sampled members separated by >100 Ma suggests that this assumption may be justified.

Quantitative analyses of the fossil record

Temporal trends in the abundance and diversity of cycads and angiosperms were assessed using data compiled in the Paleobiology Database. Data were downloaded through the paleobioDB package[56] (v.0.7.0) in the R software environment on 30 March 2023 by separately querying all fossil occurrences of Cycadales and Angiospermae. As the fossil record of cycad data is complicated by misdiagnoses (typically *Bennettitales*), the downloaded data were filtered to only include taxa of unambiguous cycad affinity. This reduced the number of fossil genera from 51 to 34 (Source Data). We further filtered the occurrence data to only include fossil taxa that represent foliar features (that is, excluding taxa ascribed to other organs such as stems or cones). This further reduced the number of fossil genera from 34 to 24 (Source Data), although we note this filtering step did not substantively change the observed trends. The final, filtered dataset contained 2,285 unique cycad fossil occurrences. The angiosperm dataset contained 36,206 fossil occurrences.

Three types of information were gleaned from these fossil occurrence compilations: (1) abundance, (2) diversity and (3) origination/extinction rate. For each of these calculations, data were divided into 25 Myr bins to allow comparison of intervals of equal length. Fossil occurrence ages were taken as the midpoint of the maximum and minimum ages in the Paleobiology Database. While some ages have large (>10 Myr) uncertainty, using only maximum or minimum ages does not affect the observed trends.

Fossil abundance trends were assessed in two ways. First, the raw abundance was calculated as the number of fossil occurrences (Cycadales or Angiospermae) per time interval. Second, the cycad and angiosperm datasets were bootstrap resampled with replacement (1,000 iterations, each time sampling up to 1,000 occurrences) and the mean number of occurrences per interval was reported with a 95% confidence interval (2σ). All occurrence data are plotted as relative abundance (that is, scaled to the highest interval) to simplify comparison across clades of different size (Cycadales versus Angiospermae).

Fossil diversity trends were also assessed via multiple methods. First, raw genus richness was calculated as the number of genera (in Cycadales or Angiospermae) observed per time interval. In this and subsequent genus richness calculations, range through genus richness was calculated by adding to each interval genera that are observed in time bins both before and after. Second, the cycad and angiosperm datasets were bootstrap resampled as above and the mean genus richness per interval was reported with a 95% confidence interval (2σ). Third, the bootstrap resampled datasets were used to estimate 'true' genus richness using two extrapolation methods: TRiPS[57] and Chao1 (ref. 58). These approaches aim to provide more robust estimates of 'true' richness by leveraging the abundance of rare taxa and making statistical assumptions about the distribution of occurrence data. Both methods have merits and shortcomings[59]. Here, we aimed to compare these approaches and raw genus richness estimates to determine whether coherent trends in cycad and angiosperm diversity can be identified. All inferred genus richness estimates are plotted as relative genus richness (that is, scaled to the highest interval) to simplify comparison across clades of different size (Cycadales versus Angiospermae). We find that all approaches broadly agree that cycad genus richness shows no clear secular trend, whereas angiosperm genus richness increases in tandem with abundance from the Cretaceous to present (Extended Data Fig. 3).

Last, the cycad and angiosperm occurrence data were used to estimate origination and extinction rates following the approach of ref. 60. Rates were calculated for each time bin besides the first and last, where available data are insufficient.

TRY database

Nitrogen isotope data from foliage of non-N_2-fixing plants was downloaded from the TRY database[61], resulting in 11,056 isotopic data that are plotted in Fig. 1a.

Geologic context

Soma Formation. The Soma Formation in western Turkey is a sedimentary unit that overlies clastic sediments and carbonates of the Izmir-Ankara zone and conformably underlies the Denis Formation[62]. The Soma Formation is comprised of basal conglomerates overlain by sandstones, mudstones, marls and coal beds. Non-marine deposition has been interpreted for the lignite-bearing horizons and intervening marls that contain leaf fossils[63]. The flora is dominated by Fagaceae, with a notable presence of Lauraceae and the gymnosperms *Pinus* and *Glytpostrobus*, among other taxa[39,64]. The unit has been assigned

an early to middle Miocene age on the basis of palynological observations[64–66] and radiometric ages of associated volcanic rocks[67–69].

Carbonaceous cycad and non-cycad fossils from the Soma Formation were obtained from the Hungarian Natural History Museum in Budapest. The cycad material was identified as *Pseudodioon akyoli*[39] on the basis of cuticular and macroscopic morphological characteristics. Non-cycad specimens analysed include representatives of Fagaceae, Cupressaceae, Rosaceae and *Daphnogene*.

Most Formation. The Most Formation is a sedimentary unit in northern Czech Republic that overlies Proterozoic gnessic basement and conformably underlies the Strezov Formation. Collectively the Most and Strezov Formations comprise the sedimentary fill of the Most Basin, which contains ~0.5 km of siliciclastic material thought to have been deposited in fluvial, deltaic and lacustrine environments[70]. Coal-bearing horizons in the Most Formation have been extensively explored and contain rich fossil plant assemblages[71,72]. The timing of deposition is constrained to be Burdigalian (early Miocene, 16–21 Ma) on the basis of mammalian biostratigraphy[73] and palaeofloristic correlation[71,72], as well as palaeomagnetostratigraphy and cyclostratigraphy[70].

Carbonaceous cycad and non-cycad fossils from the Most Formation were obtained from the National Museum in Prague, Czech Republic. Cycad material was previously identified as *Ceratozamia hofmannii* on the basis of stomatal and macroscopic morphology[38]. The non-cycad material analysed here includes several angiosperms identified to the genus or species level. All analysed specimens were recovered from the Lom Member (~16.5 Ma; ref. 74) of the Most Formation in drillcore material from Osek, Czech Republic.

Gatuncillo Formation. The Gatuncillo Formation comprises several hundred metres of terrestrial and marine sedimentary deposits that outcrop across Panama[75,76]. The unit rests atop Cretaceous basement and deposition is estimated as late Eocene to early Oligocene on the basis of U-Pb detrital zircon geochronology[77], Sr isotope chemostratigraphy[78] and foraminiferal biostratigraphy[79–81]. The depositional environment is thought to have been a series of volcanic islands surrounded by mangrove swamps and limestone reefs[82] and represents an interval of localized terrestrial sedimentation before the complete closing of the Isthmus of Panama in the Pliocene[83].

A cycad specimen identified as *Zamia nelliae* was collected from a carbonaceous sandstone bed in the Gatuncillo Formation near Buena Vista[37]. The bed containing the cycad specimen has since been destroyed by quarrying activity but contained various other plant taxa and overlay a marine mudstone succession estimated as Bartonian–Priabonian and Priabonian–Rupelian on the basis of nannoplankton and foraminiferal biostratigraphy, respectively[37]. Conformable deposition of the cycad-bearing unit atop the marine mudstone horizon suggests that the age of the cycad specimen is Priabonian (37.7–33.9 Ma) or Rupelian (33.9–27.8 Ma). Fragments of *Zamia nelliae* and of adjacent non-cycad carbonaceous material were provided by the Florida Museum of Natural History.

Chuckanut Formation. The Chuckanut Formation is a several kilometre thick sedimentary package[84] in northwestern Washington State, United States. The unit was first named by ref. 85, with type sections later characterized by ref. 86 and ref. 87, who identified deposition of the unit in a coastal plain setting. The present view is that deposition occurred in several small fault-bounded basins before the uplift of the Cascade Range[88–90], although some contend that deposition occurred as large, sheet-like deposits spanning the Formation[91] that were later dissected by strike-slip faulting[92]. In either case, deposition occurred in a coastal plain before mid-Tertiary Cascade uplift.

One study[84] divided the Chuckanut stratigraphy into two periods of deposition. The earlier period is represented by the lower units, the Bellingham Bay (~3.3 km thick) and Slide (~1.9 km thick) Members, which include alternating beds and arkose sandstone and siltstone with minor conglomerate and coal[84]. The upper units identified by ref. 84 were the Padden Member (~3.0 km thick) and the minor Governor's Point, Maple Falls, Warnick and Bald Mountain Members, which are comprised of massive arkose sandstone and conglomerate with alternating mudstone and siltstone. Palaeontological data suggest a climatic change between deposition of the lower and upper Chuckanut Formation, from subtropical flora in the lower units to warm temperate in the upper units[92,93].

The Chuckanut Formation originally was mistakenly correlated with the Upper Cretaceous Nanaimo Group[85,93,94] but more recent work has demonstrated an Eocene age for the former unit. Palynological data are consistent with late Palaeocene to early Oligocene deposition[95]. This is corroborated by geochronological work, including a 49.9 ± 1.2 Ma zircon fission track age from a tuff bed of the lower Chuckanut[84], a 44.5 ± 4.5 Ma zircon fission track age from a bentonite bed in the upper Chuckanut[90], K-Ar ages of 40.5 ± 5 Ma and 36.8 ± 9.2 Ma on volcanic rocks overlying the Chuckanut[96] and zircon fission track ages of 52.7 ± 2.5 Ma (ref. 97) and 52.5 ± 4.8 Ma (ref. 90) from a rhyolite flow and tuff, respectively, in the lower Chuckanut. We therefore follow these studies in inferring a ~50 Ma age for deposition of the lower Chuckanut Formation, noting that uncertainty on this age estimate does not influence the conclusions of this study.

Carbonaceous compression fossils from the Chuckanut Formation were obtained from the Burke Museum of Natural History and Culture. Cycad specimens used in this study were previously[36] referred to the extant genus *Dioon*. However, our closer analysis of the specimens here reveals a more likely assignment of *Dioonopsis*, on the basis of anastomosing or dichotomizing veins, insertion of the leaflets onto the rachis and the overall shape of the leaflets. Non-cycads were identified to varying taxonomic levels as allowed by specimen morphology. All specimens were collected from the lower Chuckanut Formation in the Bellingham Bay Member.

Macquarie Harbor Formation. The Macquarie Harbor Formation in southwest Tasmania comprises a thick sequence of fossiliferous mudstones filling a rift basin[98]. The flora is thought to represent estuarine vegetation growing in a warm and humid climate[99,100]. Mangroves[101], seed ferns[102], rainforest angiosperms[103,104] and conifers (including Araucariaceae, Cupressaceae and Podocarpaceae[105,106]) are present in the assemblage. The age of the sediments is constrained to 53–50 Ma on the basis of palynological and marine biostratigraphic records[107].

Carbonaceous compression fossils were obtained from the Lowana Road and Regatta Point sites within the Macquarie Harbor Formation through the University of Adelaide. The cycad material studied here was identified as a member of the extant genus *Bowenia* on the basis of stomatal and macroscopic morphology[35]. The non-cycad material analysed includes several angiosperms and one conifer (*Araucarioides*).

Castle Rock flora, Denver Formation. The Castle Rock flora is an exceptionally diverse fossilized forest floor deposit first discovered in 1994 (refs. 34,108,109). The fossil site occurs within the D1 sequence of the upper Denver Formation in the Denver Basin in Colorado, United States[110]. The depositional age of the site is constrained to 63.8 ± 0.1 Ma on the basis of five zircon U-Pb analyses from a stratigraphically proximal ash layer[111].

In contrast to other Paleogene fossiliferous deposits, the Castle Rock flora evidently records autochthonous or parautochthonous burial of a forest floor, inferred to have occurred during multiple flooding events[108]. In addition to this unusual depositional style, the flora is markedly diverse and exhibits leaf morphology and plant diversity indicative of modern rainforests[34,108], indicating that in at least some habitats, terrestrial biodiversity had rebounded substantially within ~2 Myr of the Cretaceous–Paleogene mass extinction[34].

The flora is dominated by angiosperms (94% dicots[108]) and an exceptionally well-preserved cycad individual and isolated leaves were also recovered[34,112].

Carbonaceous compression fossils from the Castle Rock flora were obtained from the Denver Museum of Nature & Science. Cycad specimens used in this study were previously characterized[112] to the genus level (*Dioonopsis*) and may derive from a single individual; non-cycads were identified to at least the family level (Lauraceae, Platanaceae and Juglandaceae). All specimens were collected from the Castle Rock site excavated by the Denver Museum of Nature & Science.

Comox Formation, Nanaimo Group. The Comox Formation is the lowermost unit of the Upper Cretaceous Nanaimo Group on Vancouver Island, British Columbia. The Nanaimo Group is comprised of several kilometres of non-marine, shallow marine and deep marine facies that are thought to have been deposited in a forearc or peripheral foreland basin[113–115] known as the Georgia Basin, which to the south and east contains Tertiary deposits including the Eocene Chuckanut Formation described above. The age of the Comox Formation is estimated as Santonian (~86-83 Ma) on the basis of marine biostratigraphy[113] and a U-Pb zircon age[116] of 82.5 ± 1 Ma (earliest Campanian) from a tuff in the Dunsmuir Member in the upper Comox Formation.

Cycads were identified in the Comox Formation near the town of Nanaimo in the late nineteenth century[117] and have been studied ever since[33,36]. A recent study[33] noted that while angiosperms dominate the Nanaimo Group flora overall, cycads in the Saanich Member of the Comox Formation were disproportionately found in gymnosperm-rich deposits that were floristically distinct from other angiosperm-dominated sites, the latter of which tend to be more species-rich. This overall pattern is consistent with the inference of widespread ecological dominance of angiosperms by the Santonian stage[44] and perhaps suggests that the cycad-rich sites represent refugia that mimic earlier Mesozoic ecological conditions.

Carbonaceous compression fossils from the Comox Formation were obtained from the Royal BC Museum. Cycad specimens used in this study were previously characterized to the genus level (*Pseudoctenis*); non-cycads were identified to varying taxonomic levels as allowed by specimen morphology. Specimens were collected from three sites within the Comox Formation: BR-1 and BR-2 on the Saanich Peninsula and the no. 8 Mine near Courtenay.

Yorkshire flora, Cloughton Formation. The Yorkshire flora of England is a long-studied palaeobotanical archive[32,118–120]. Mesozoic deposition in the Cleveland Basin created a thick stratigraphic package, of which the Cloughton Formation in the Ravenscar Group is the largest unit[121]. The Cloughton Formation comprises the Sycarham, Lebberston and Gristhorpe Members, with the Sycarham and Gristhorpe being non-marine and fossiliferous[119,121,122]. Biostratigraphic constraints place Cloughton Formation deposition in the Lower Bajocian stage[119]. Early work by ref. 32 noted the presence of cycads in the Cloughton Formation and up to now more than 300 taxa have been identified, including bennettitaleans, sphenophytes and an abundance of ferns[119,120,122].

Carbonaceous cycad and non-cycad specimens from the Yorkshire flora were obtained from the Yale Peabody Museum. All cycad (*Nilssonia*) and non-cycad (*Cladophlebis*, *Phlebopteris*, *Sagenopteris* and *Nilssoniopteris*) specimens were identified to the genus level.

Primulaelv Formation, Kap Stewart Group. The Kap Stewart Group in eastern Greenland is comprised of sedimentary rocks spanning the Triassic–Jurassic boundary. The Group is divided into the Innakajik, Primulaelv and Rhaetelv Formations, which transition from conglomerate and sandstone dominated alluvial plain deposition, to mixed sandstone–shale deposition in a delta plain, to mixed sandstone and shale deposition in a lacustrine setting, respectively[123–125]. The age of the Kap Stewart Group was initially constrained biostratigraphically by ref. 126, who noted a Rhaetian (209–201 Ma) flora characterized by *Lepidopteris* that gave way to a Hettangian (201–199 Ma) assemblage characterized by *Thaumopteris*. An >80% species turnover between the assemblages led ref. 126 to locate the Triassic–Jurassic boundary (~201 Ma), reflecting the mass extinction of terrestrial flora[125].

The flora of the Kap Stewart Group is gymnosperm-dominated, containing an abundance of cycads, ginkgos, conifers and ferns[31,127,128], similar to other Triassic assemblages in Europe (for example, the Lunz and Thale flora described below). For this study, carbonaceous compression fossils from the Primulaelv Formation at Astartekløft were obtained from the Field Museum. The studied cycad specimens were previously characterized[31] to the genus level (*Nilssonia*, *Doratophyllum* and *Pseudoctenis*); as were non-cycads (all Bennettitales: *Pterophyllum, Cycadolepis* and *Anomozamites*), on the basis of stomatal morphology.

Lunz Formation. The successions of the Lunz Formation of Austria are comprised of shallow marine marls and terrestrial sandstones, shales and coal[129,130]. Marine biostratigraphy from marine successions bracketing the continental plant-bearing beds[131,132] and palynology[133] indicate a Carnian (237–227 Ma) age for the Formation. Sedimentological, palaeogeographic and palaeoecological study has suggested that deposition of the Lunz Formation occurred in a lowland swamp to deltaic environment[130].

A rich fossil flora has been studied in the Lunz Formation for over a century[134,135]. The assemblage is dominated by ferns, cycads and bennettitaleans[129,130,136]. For this study, carbonaceous compression fossils from the Lunz Formation were obtained from the Swedish Museum of Natural History. The studied cycad specimens were previously characterized[30,137] to the genus level (*Nilssonia*), as were non-cycads[138,139] (all Bennettitales: *Pterophyllum* and *Nilssoniopteris*), on the basis of stomatal morphology.

Thale flora, Lower Keuper. The Thale flora is a rich fossil assemblage in the vicinity of the village of Thale in central Germany. The existing specimens were collected by a school teacher in the early nineteenth century and posthumously deposited in German and Swedish museums[140,141]. The plant-bearing successions were later dated to early Keuper[140,141], giving them a Ladinian (242–273 Ma) age.

A detailed and comprehensive study of both macroflora and palynoflora revealed that a lush vegetation grew at the site. The flora is interpreted as autochthonous, probably representing growth on a floodplain. The macroflora is dominated by ferns, horsetails and cycads while the palynological assemblages are dominated by bisaccate pollen grains typical of conifers[142]. For this study, carbonaceous compression fossils from the Thale flora were obtained from the Swedish Museum of Natural History. The studied cycad specimens were previously characterized[29] to the genus level (*Bjuvia* and *Taeniopteris*), as were non-cycads (here all representing the seed fern *Scytophyllum*).

Fremouw Formation. The Triassic Fremouw Formation is located in the central Transantarctic Mountains and consists of ~750 m of sandstones, carbonaceous shales and volcaniclastic deposits[143]. The upper member of the Fremouw Formation contains a larger proportion of carbonaceous shale, including a silicified horizon representing permineralized peat[143]. Deposition is inferred to have occurred via braided streams in a floodplain setting[143,144]. Palynology and vertebrate fossils suggest an Anisian (247–242 Ma) age for the unit[145,146].

The floral assemblage of the Fremouw Formation reflects a high-latitude forest ecosystem characterized by the *Dicroidium* foliage morphotype[144]. Permineralized cycad fossils have also been recognized and studied in the Fremouw Formation since the 1980s (refs. 15,40,42,147,148). For this study, silicified cycad stems and *Dicroidium* leaf layers from the Fremouw Formation were obtained from the Natural History Museum at University of Kansas. The studied cycad specimens were previously characterized[15,42] to the genus level

(*Antarcticycas*) and all analysed non-cycad specimens were of *Dicroidium* affinity.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Fossil occurrence data were downloaded from the Paleobiology Database (https://paleobiodb.org/) by means of the paleobioDB package in R (v.0.7.0). Non-N_2-fixing plant nitrogen isotope data were downloaded from the TRY plant trait database (https://www.try-db.org/). Source data are provided with this paper.

Code availability

All code generated in this study is available in Supplementary Codes 1, 2, 3 and 4 as well as at https://github.com/m-kipp/cycad-evolution.

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Author contributions

M.A.K., E.E.S., C.A.E.S. and R.B. designed the study. C.A.E.S., V.M.A., B.E., R.S.H., K.R.J., J.K., J.C.M., I.M.M., M.S. and V.V. provided fossil specimens. M.A.K. and E.E.S. conducted the isotopic measurements. M.A.K., E.E.S., C.A.E.S. and R.B. analysed the data. W.H.B. conducted the ASR, with input from M.A.K., E.E.S., C.A.E.S. and R.B. M.A.K. wrote the manuscript, with input from all authors.

Competing interests

The authors declare no competing interests.

Additional information

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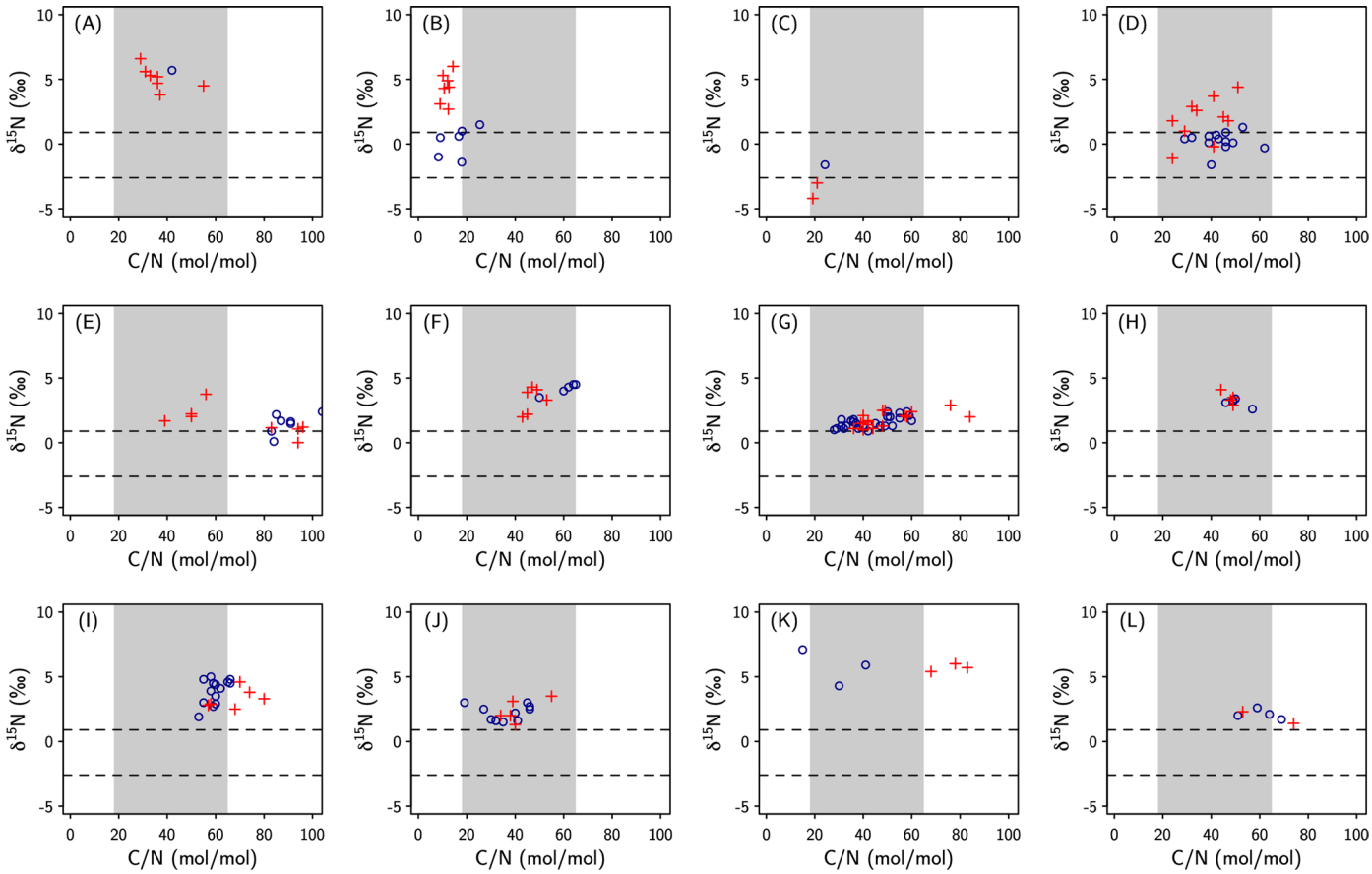

Extended Data Fig. 1 | δ15N vs. C/N for all studied units. (**A**) Soma flora, (**B**) Most Formation, (**C**) Gatuncillo Formation, (**D**) Chuckanut Formation, (**E**) Macquarie Harbor Formation, (**F**) Castle Rock flora, Denver Formation, (**G**) Comox Formation, Nanaimo Group, (**H**) Yorkshire flora, Cloughton Formation, (**I**) Primulaelv Formation, Kap Stewart Group, (**J**) Lunz flora, Lunz Formation, (**K**) Thale flora, Lower Keuper (**L**) Fremouw Formation. Cycad data shown as blue circles; non-cycad data as red crosses. Grey bands denote range of C/N ratios observed in modern cycads23; dashed lines denote range of δ15N values observed in modern cycads23. Fossil cycad foliage predominantly falls within the range of C/N ratios observed in modern plants. Fossil cycad foliage also overwhelmingly overlaps with the C/N ratios of other analysed plants, with one stark exception (Thale flora, panel K). In that case, the lack of δ15N vs. C/N correlation within either cycads or non-cycads suggests that diagenetic processes (which would impart a δ15N vs. C/N correlation) did not appreciably alter the isotopic composition of either group, or create a postdepositional isotopic offset between the two groups. Overall, the C/N data suggest that postdepositional alteration is unlikely to have imparted or obscured the isotopic trends observed across units.

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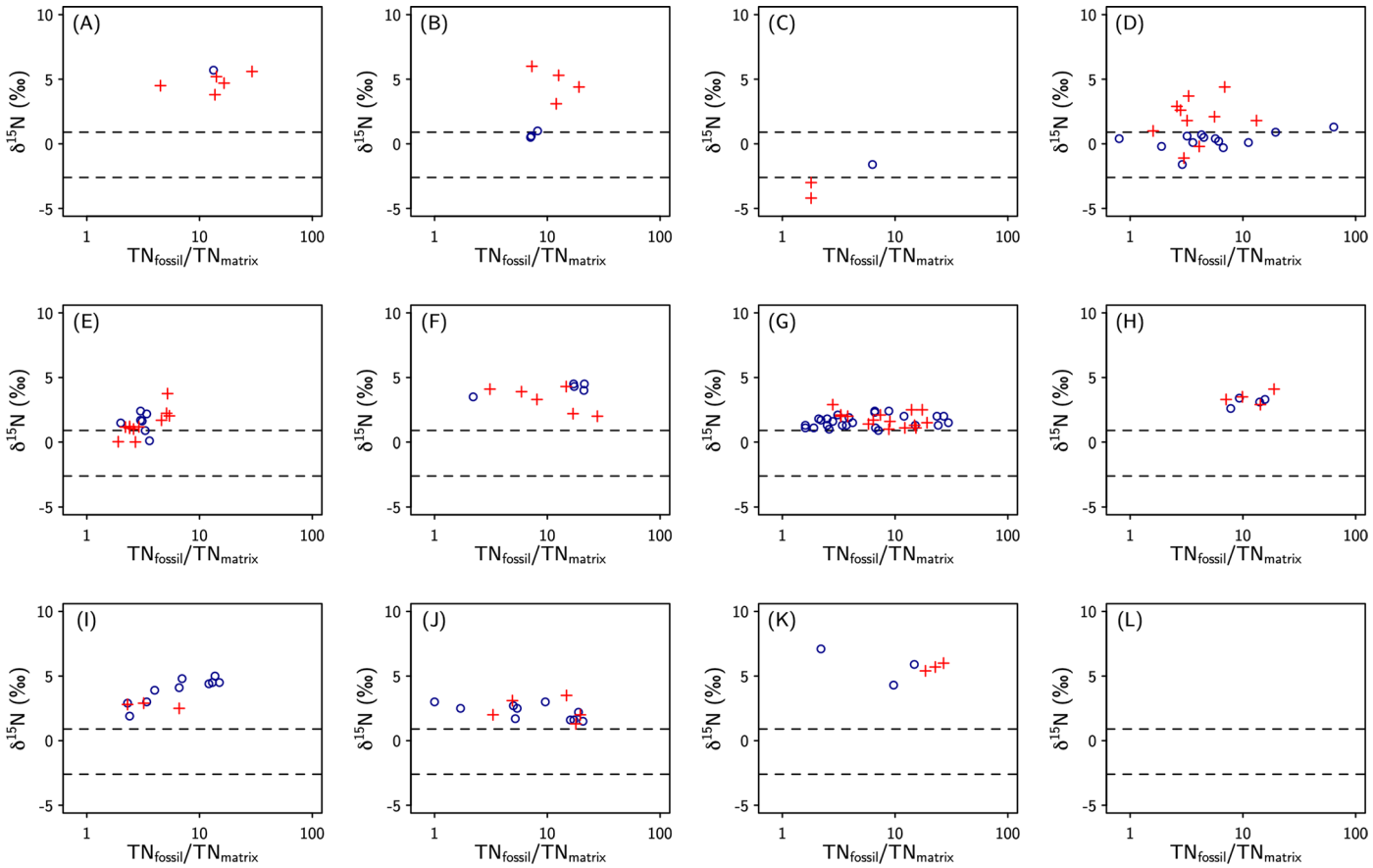

Extended Data Fig. 2 | TN content of fossil over matrix. (**A**) Soma flora, (**B**) Most Formation, (**C**) Gatuncillo Formation, (**D**) Chuckanut Formation, (**E**) Macquarie Habor Formation, (**F**) Castle Rock flora, Denver Formation, (**G**) Comox Formation, Nanaimo Group, (**H**) Yorkshire flora, Cloughton Formation, (**I**) Primulaelv Formation, Kap Stewart Group, (**J**) Lunz flora, Lunz Formation, (**K**) Thale flora, Lower Keuper, (**L**) Fremouw Formation. Fremouw Formation samples were permineralized and thus did not allow a separate characterization of carbonaceous compression fossil versus matrix. Cycad data shown as blue circles; non-cycad data as red crosses. Dashed lines denote range of δ15N values observed in modern cycads23. Isotopic trends within and between units are not correlated with the N concentration of recovered foliage. Recovered fossil material has on average an order of magnitude more nitrogen than the background matrix, indicating that the isotopic signatures derive from the foliage and not soil organic matter.

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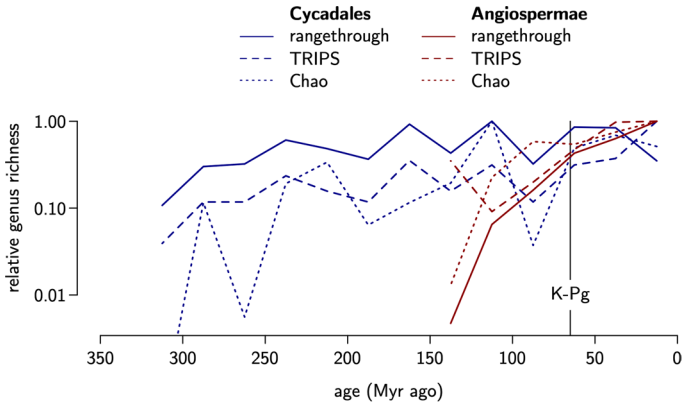

Extended Data Fig. 3 | Relative genus richness of Cycadales and Angiospermae. Lines separately denote genus richness estimated via bootstrap resampled range through genus richness, TRiPS estimated genus richness and Chao1 estimated genus richness. Calculations are described in Methods.

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
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☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Elemental and isotopic data were collected on a Thermo MAT 253 Isotope Ratio Mass Spectrometer (IRMS) that was operated using the commercial (Thermo Electron Gmbh, Bremen, Germany) Isodat (v. 2.0) software.
Data analysis	All statistical analyses were conducted in the statistical programming language R (v. 4.0.0).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
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All geochemical data collected in this study are available in the supplementary information. Fossil occurrence data were downloaded from the Paleobiology Database (https://paleobiodb.org/) via the paleobioDB package in R (v. 0.7.0). Non-N2-fixing plant nitrogen isotope data were downloaded from the TRY plant trait database (https://www.try-db.org/).

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences ☐ Behavioural & social sciences ☒ Ecological, evolutionary & environmental sciences

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Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	This is study sought to compare the nitrogen isotopic composition of fossilized cycad and non-cycad foliage to patterns observed in modern ecosystems.
Research sample	The research samples include carbonaceous compression fossils of cycads (and non-cycad plants from the very same depositional units). The samples span in age from Triassic to Miocene.
Sampling strategy	The sampling strategy was to analyze the maximum available number of suitable fossil cycad specimens from each collection. We then sought to analyze a similar number of non-cycad specimens for a robust statistical comparison.
Data collection	M. A. Kipp and E. E. Stüeken collected the isotopic data in IsoLab at University of Washington following published protocols.
Timing and spatial scale	Data were collected between summer 2014 and summer 2022. This time span reflects the time required to visit museum collections, obtain destructive sampling permits, and conduct analyses.
Data exclusions	Isotopic data were rejected if (i) the signal/noise ratio (sample intensity relative to analytical blank) was < 2, or if (ii) replicate analyses of the same homogenized powder were not reproducible to better than 1 part per thousand (standard deviation, SD). In practice this applied to very few analyses, as most samples had signal/noise > 50 and very good reproducibility (SD < 0.3 parts per thousand).
Reproducibility	When replicate analyses were conducted on the same homogenized material, data reproduced faithfully (as noted above). In cases where we conducted multiple separate sub-samples per fossil specimen, the isotopic trends also were reproduced.
Randomization	Randomization was not applicable, as we were studying two groups of specimens based on taxonomic affinity: cycad and non-cycad.
Blinding	Blinding was not applicable in this study, as the isotopic analysts were aware of the taxonomic affinity of the specimens due to the nature of the sampling.

Did the study involve field work? ☐ Yes ☒ No

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☐	☒ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Palaeontology and Archaeology

Specimen provenance	This study targeted specimens in existing palaeobotanical collections. A detailed description of the geological context for each sample set is provided in the Methods section. The institutions that provided access to these samples include: Burke Museum of Natural History and Culture, Denver Museum of Nature & Science, Royal BC Museum, Swedish Museum of Natural History, Field Museum, the Natural History Museum at University of Kansas, the Czech National Museum, the Florida Museum of Natural History, the Yale Peabody Museum, and the Hungarian Natural History Museum. Destructive sampling permits were obtained from the curatorial staff of each respective institution.

Specimen deposition	The specimens remain deposited in the original host collections as described above.
Dating methods	No new dates are provided.

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Ethics oversight	No formal ethical oversight was required by any of the collaborating institutions for the execution of this study.

Note that full information on the approval of the study protocol must also be provided in the manuscript.