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Review

The cycad coralloid root: is there evidence for plant-microbe coevolution?

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Cycads are survivors, ancient plants originating in the Carboniferous. We hypothesize that cycad resilience and recent diversification could be partially explained by their specialized coral-like (coralloid) roots and their microbiome and that these symbiotic partners are co-evolving. The coralloid root is unique in gymnosperms and rare in vascular plants. Coralloid roots and their associated microbes have been studied since the late 19th century, but a deeper understanding of their taxonomy and function has taken place only recently. And yet, we are at the 'tip of the root' as there are many open questions regarding this specialized organ and its evolutionary history. This review provides an overview of cycad coralloid roots and their microbiome, the technical limitations of their study to date, and the exciting questions that remain to be answered.

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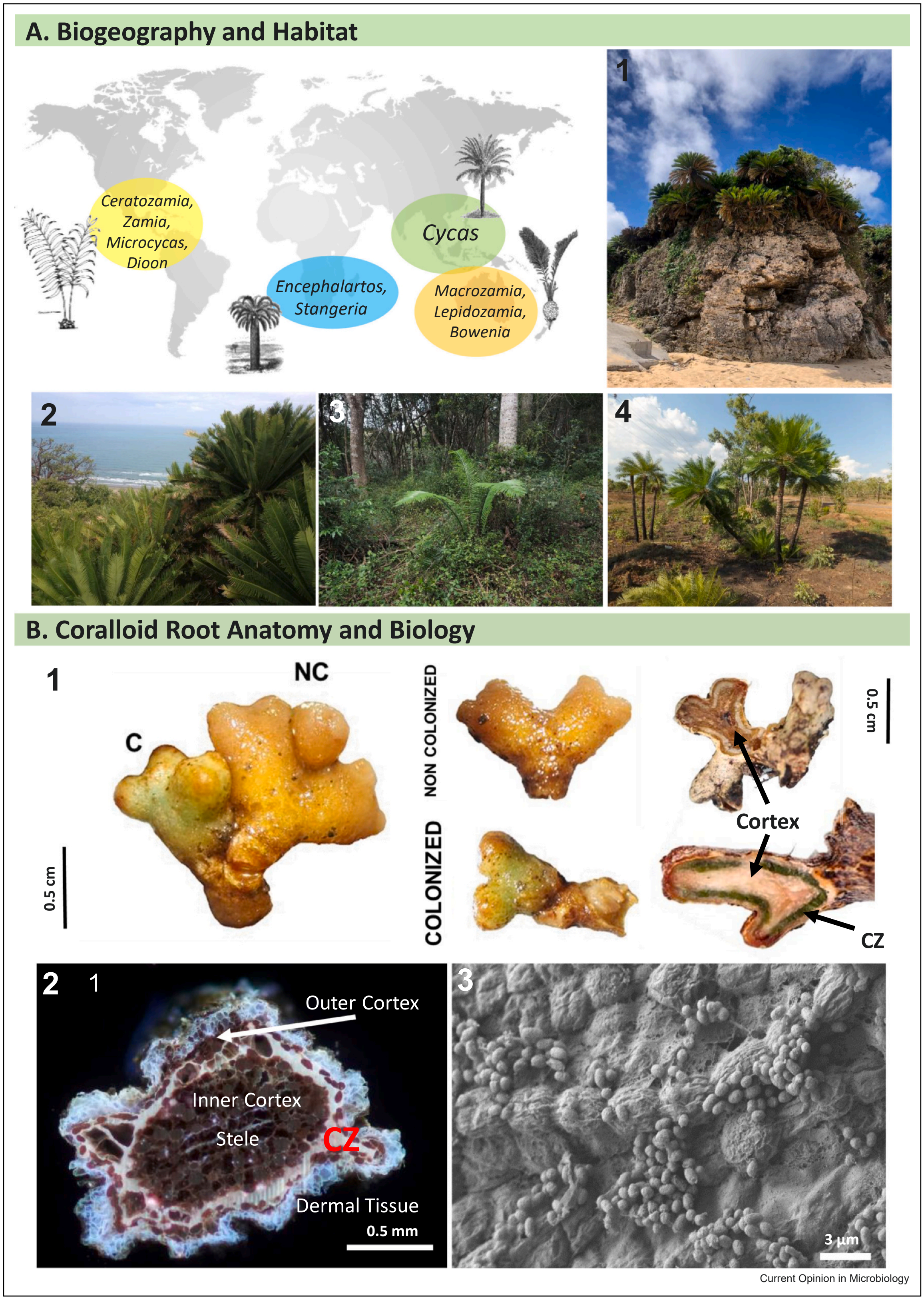
Introduction

Cycads are among the most ancient plant lineages alive today, with an evolutionary history since the Carboniferous [1]. They have survived despite major changes in biota, climatic conditions, the fall of the dinosaurs (their seed dispersers [2]), and the competitive rise of the angiosperms. Cycads are usually single-stem plants with circinate leaves and, as gymnosperms, have cones (dioecious) instead of flowers (Figure 1a). Cycad phylogenies based on morphological and genomic markers [3] generally agree on the existence of two sister clades, Cycadaceae and Zamiaceae. Although these gymnosperms are often referred to as 'living fossils' along with their sister group ginkgo, cycads have diversified into c.a. 380 species and counting [4]. They may have undergone the most recent diversification 12 Mya in which they had to adapt to angiosperms and changing climates [4], leading to the striking phenotypic diversity observable today, mostly in their leaves [3]. Cycads' beauty and rarity have been their demise, as they are widely traded illegally for ornamentals, which combined with habitat loss, makes them one of the plant groups with the most endangered species today [2,5].

Cycads' long-term survival is likely due in part to their coevolution with specialist butterflies and beetles and their intricate chemical ecology [6]. Several cycad anatomical and life traits have also aided, such as retractile pachycaul stems that can bury underground and thus tolerate fire [7]; their slow growth, and their capacity to live hundreds and maybe thousands of years. But among the most intriguing, and the focus of this review, is their coralloid root. The coralloid root has been the subject of studies for 153 years (Table 1). It is an above-ground transitory specialized root, which is colonized by Cyanobacteria, other bacterial groups, and fungi [8,9] (Figure 1b). The coralloid root and its symbiotic microbiota have been suggested to have a history of coevolution as critically reviewed here. Given cycads' longevity, slow growth and time to reproductive stage of a decade or more (e.g. *Cycas* species), the plasticity of the coralloid root via its rapidly adapting microbiome could be a key trait in cycad biology and long-term survival. There are examples of coevolution between other plants and their microbes that have allowed them to thrive [10], but few have formed specialized organs to contain diverse microbiomes, such as the cycad coralloid roots.

Just as in other symbiotic systems in plants with differentiated roots [11], it is reasonable to believe that the coralloid root and its symbiotic microbiota have a history of coevolution. Yet, this remains to be tested more explicitly. It is not known if the coralloid microbes and the coralloid root cells evolved independently than other

Figure 1



(caption on next page)

(a) Biogeography and habitat of cycads shown in a world map and four examples of cycads in different environments. 1 - *Cycas revoluta*, Japan; 2 - *Dioon edule,* Mexico; 3 - *Encephalartos villosus,* South Africa; 4 - *Cycas armstrongii*, Australia (Photos from World List of Cycads [65]). **(b)** Coralloid root anatomy and biology. 1 - Uncut colonized and uncolonized coralloid root (left) and cross-section of the colonized and uncolonized coralloid root, showing the Cyanobacterial Zone in green (right) (Photos with permission by Naishla M. Gutierrez-Arroyo). 2 - LAT picture of colonized coralloid root with annotated tissues showing the CZ in white (Photo by K.L. Zonneveld). 3 - SEM picture of co-culture extracted from a coralloid root from *Dioon edule*. Beaded strings are cyanobacteria, covered in additional microbes and extracellular material, showing the high diversity of taxa inside of the coralloid root (Photo by K.L. Zonneveld and F. Barona-Gómez).

cells in the plant, such as the bacteria found in the leaves [12]. Cycad leaf bacteria are part of the plant–microbial holobiont, but cycad leaves lack the anatomical differentiation to contain microbes such as the coralloid root cyanobacterial zone (CZ). One way of testing if microbes and plant cells of the coralloid roots have their own evolutionary trajectory would be to search for genomic signatures in both. Antagonistic coevolution, for example, would be expected to produce recurrent selective sweeps or signatures of balancing selection in the genomes of both hosts and symbionts, as in legumes and their *Rhizobium* spp, which seemed to have evolved together under stabilizing selection [13].

Here, we suggest that rather than relics from the past, cycads are resilient holobionts with their root microbes as key evolutionary partners. To begin addressing this possibility, we update the knowledge of the anatomy and biology of the root and provide the first overview of the taxonomy and systematics of their microbial diversity , including genomic studies. And for the first time, we discuss the technical limitations in the study of this root and identify knowledge gaps for future research.

The anatomy and biology of the coralloid root

The coralloid root is a specialized organ, developed in response to ecological cues, most that remain to be identified, that contains microorganisms (Figure 1b). While functionally similar to the root nodules found in legumes with regard to nitrogen fixation, it evolved independently within the gymnosperms, where it is unique. All modern cycad species can make coralloid roots. Its pervasiveness across genera and species, its appearance in young life stages [14] or when the plant is under environmental stress [15] suggests its role in the plant's survival. The chemical basis of attraction and colonization of microbes in cycad coralloid roots first described in 1872 [8] is still not well understood. It may be facilitated by diacylglycerols that are hormogonium-inducing factors [16], and by the production of mucilage, but metabolome, chemical, and microscopy studies are still required to describe the mechanisms that trigger and sustain the symbiosis.

While coralloids exhibit the basic anatomical architecture of lateral gymnosperm roots, there are differences in their development and tissue distribution [14] (Figure1b). Observations made in different coralloid roots show anatomical and developmental variations across cycad species and individuals. In their basic *bauplan*, coralloid roots first develop opposite of protoxylem points in the stele of the primary and secondary roots. During its early differentiation process, which occurs after the pre-coralloid root shoots out from the pericycle of either primary or secondary roots, the precoralloid roots branch dichotomously into short and thick 'coral-like' structures [17] that typically grow apogeotropically (above ground) [14]. They can shift to a geotropic pattern after the precoralloid bundles are colonized by microorganisms. This colonization triggers the organ's maturation, irrevocably changing its anatomy. During maturation, the papillose sheath that envelops precoralloid roots differentiates into dermal tissue containing lenticels, while cortex cells that surround the stele dedifferentiate into unique meristematic cells that form the region that houses most of the symbiotic microbiome [18]. In a cross-section of the coralloid, cyanobacteria appear visible to the naked eye, forming the 'CZ' or coralloid zone, consisting of a green ring, or the extracellular space between the inner and outer coralloid root cortex [14,19] (Figure1b). It is possible microorganisms inhabit or migrate through the cortex, but the handling of the root in most anatomical research mechanically ruptures the CZ, making it difficult to know if loose cyanobacteria are simply a result of such rupture.

It is unknown how long the coralloid root lives for, if a few months, decades, or hundreds of years like the rest of their host plants [20]. But in any of these scenarios, generation time would translate from hundreds to millions of microbial generations evolving in a mostly contained ecosystem within the differentiated root tissues, in contrast to a few generations of the plant. Through horizontal gene transfer to the plant, genome innovations, reconstruction of regulation networks of the microbiome, or other mechanisms known in legumes and other symbiotic systems with specialized structures, the microbiome of the coralloid is likely playing a role in the survival of the plant by adapting to changing local, ecological conditions.

Evidence from angiosperms shows this could be the case [21,22], but this attractive hypothesis requires further testing in cycads on multiple levels. For instance, we lack experimental evidence such as long-term cross-inoculation experiments to test mutual adaptation of root and symbionts; lack-of-function root mutants to test the role of bacteria and fungi in root development itself. Plant fitness experiments, in particular, under nitrogen limitation (temporarily or not) are lacking. Perhaps most

Table 1

Brief history of cycad coralloid root research.

Reference	Breakthrough
1753	First description of cycads [55]
Reinke, 1871 [56]	Early known description of the coralloid roots, here mentioned as dichotomously branching lateral roots.
Reinke, 1872 [8]	First mention of the bacteria in the coralloid roots, then still thought to be parasitic.
Hariot, 1892 [57]	First report suspecting of nostocales as symbiotic partner.
Schneider, 1894 [58]	First mention of symbiosis in coralloid roots, rather than parasitism.
Life, 1901 [52]	First in-depth description of the coralloid roots, where the term coralloid roots is used. Also suggests that there is a nitrogen fixation function in the roots.
Bottomley, 1909 [59]	Identification of *Pseudomonas radicicola* and *Azotobacter* sp. in coralloid roots and suggestion that they are powerful nitrogen fixers.
Zach, 1910 [60]	One of the earliest mentions of microbiome in coralloid roots. Mentions the importance of fungi and sees this symbiosis as parasitism.
Horejsi, 1910 [61]	This paper states that the Cyanobacteria are the main part of the symbiosis, and that the other microbes (which have been noticed) are not important.
Spratt, 1911 [53]	Fully describes the Cyanobacteria
Fowden, 1958 [62]	Isotope ($15N_2$) experiments prove that *Anabaena* and/or *Nostoc* species in *Ceratozamia* and *Encephalartos* coralloid roots fix atmospheric nitrogen.
Caiola & Vecchi, 1980 [63]	First usage of SEM to visualize the roots.
Hill et al., 2003 [24]	First comprehensive genetic research into cycads and the relationships between genera.
Lobakova et al., 2003 [64]	Cell-degrading microorganisms (most notably Myxobacterales) were found on the surface of coralloid roots and may facilitate the colonization of symbiotic cyanobacteria filaments by generating channels through the root periderm.
Costa et al., 2004 [41]	Pioneer fingerprinting work of *Nostoc* strains in coralloid root using *trnL* as marker.
Gehringer et al., 2010 [36]	Using an approach combining cultures,16S *rRNA* amplification and microscopy, 21 genotypes of *Nostoc* spp. and one of *Calothrix* sp. were identified in coralloid roots of 31 species of *Macrozamia* spp. in natural habitat. Nonetheless, no specialization was detected in the symbiosis, and the same cyanobiont occurred in several different species and individuals.
Zheng et al., 2018 [9] and Suarez-Moo et al., 2019 [25]	First looks into the microbiome beyond the Cyanobacteria.
Gutierrez-Garcia et al., 2019 [29]	First example of microbiome interaction mediated by the metabolic product of a BGC.
Bustos-Díaz et al., 2024 [27]	Phylometagenomics analysis reporting the occurrence of sympatric and paraphyletic symbiotic cyanobacteria.

intriguing, work on the microbiome has focused on the bacteria, which has left fungi and viruses generally absent or underrepresented, even though both are known to have impact in plant–microbe symbioses [23]. Research in any of these areas is bound to find surprises on the coralloid root.

Biogeography of the coralloid root and its microbiome

Several studies have focused on explaining the current distribution of cycads, either for both of the main lineages or for separate genera [3,5]. Coiro et al. [3] highlight the geographical evolutionary history of all extant cycad species, proposing that the origin of cycads was in the Western and Eastern Palearctic during the Paleozoic. When continents separated in the early Jurassic period, cycads did too, resulting in a disjunct distribution of 10 genera *Ceratozamia*, *Zamia*, *Microcycas*, and *Dioon* in America; *Encephalartos* and *Stangeria* in Mid-South Africa; *Cycas* in East and South Asia; and *Macrozamia*, *Lepidozamia*, and *Bowenia* in Australia [24] (Figure 1a).

The biogeographic factors influencing speciation and distribution of the coralloid roots' microbiome are less clear. The microbiome is recruited from the rhizosphere [25], where local and regional (a)biotic factors will influence the microbial community. The species-level taxonomic bacterial composition of the root microbiome varies between studies, cycad species, and even individual specimens and individual roots [9,25–37]. It remains to be seen if these earlier patterns are biased due to differences in sampling and sequencing methodologies (see technical section). In one of the few studies including fungi, Zheng and Gong [26] assessed how the microbiome of cycads differed between cone, leaves, stem, and seeds, and among individuals within a species from different botanical gardens. They found that coralloid roots had a unique bacterial composition, with similar taxa among con-specifics but different among locations. For fungi, there was no significant difference among plant organs but differed among individuals [26].

We have yet to test for local adaptation at the population level. We also lack regional phylogeographic studies.

Microbiomes may respond to local environments irrespective of the plant species, or respond to the plant species' distribution, or could be conserved at a global or regional scale. The cosmopolitan distribution of Nostocales (e.g. *Nostoc punctiforme* PCC 73102), a key group of bacteria in the coralloid microbiome, would suggest that at least the most abundant species will be cosmopolitan. It has indeed been suggested there is a group of bacterial orders shared ('core') in the coralloid roots across all specimens and species regardless of their geographical origin or plant species [27,37]. So far, there is no global or regional sampling of the cycad microbiome to test what factors shape the distribution of the coralloid microbiome at various spatial scales. Biogeography remains an open area of cycad coralloid root research.

Systematics: a cyanobacteria-centered yet taxonomically diverse 'core' microbiome

The capacity to make coralloid roots by all known cycad species is remarkable. The most parsimonious explanation to this shared ability is that it is an ancient trait that originated in the cycad ancestor, more than 250 Mya. If so, the coralloid root could predate legumes' specialized nodule symbiosis, which may have originated 100 Mya, *Gunnera*'s opportunistic stem symbiosis and plants with coralloid-like roots such as Thismiaceae [38,39], making cycads the oldest specialized symbiotic root in existence. Unfortunately, recovering fossil records is difficult given the fragile constitution of the coralloid root tissue, and a single cycad genome published [40], limits comparative phylogenomic studies that can date the appearance of the coralloid root.

However, a recent study suggests that the coralloid originated recently, as late as 12 Mya [20]. Kipp et al. [20] use foliar nitrogen isotope ratios as an indirect measure of nitrogen fixation to test for the antiquity of the cycad–cyanobacterial symbiosis. They find signatures of microbial (vs soil) nitrogen fixation in two Cenozoic representatives of extant genera. Extinct cycad genera have nitrogen isotope ratios similar to co-existing non-cycad plants and are inconsistent with microbial nitrogen fixation. They conclude that nitrogen-fixing symbiosis arose independently in the lineages leading to living cycads during or after the Jurassic. They also conclude that the survival of cycad lineages that were able to concentrate and host nitrogen-fixing symbionts had an adaptive advantage in the competition with angiosperms and in adapting to Cenozoic climatic change.

It is also possible that the coralloid root could have existed in the extinct lineages fulfilling biological roles different to nitrogen fixation, for example, for other metabolic functions encoded by enriched Biosynthetic Gene Clusters (BGCs) [29]. We propose that nitrogen fixation is only one of its many functions (next section), which could have evolved relatively recently on par with similar systems in angiosperms. In either scenario however, the findings of Kipp and co-authors [20] support our hypothesis that the coralloid root is a result of coevolution and has a key role in cycad survival.

Aside from the date of its appearance, an increasingly exciting area of research is coralloid root microbiome taxonomy and systematics. In the past 20 years, the amount of phylogenetically informative data obtained from the main symbiont order, the *Nostocales* (Cyanobacteria), has steadily increased. Multiple genetic markers, such as the leu-tRNA intron [41], cyanobacterial highly iterated palindromic sequences [42], and the 16S *rRNA* and/or *rbcL-rbcX* gene [33,34], have revealed that cyanobionts are phylogenetically diverse. This finding has been corroborated by whole-genome phylo(meta)genomic studies [27,29]. The exact number of Nostocales lineages capable of becoming cyanobionts remains unknown.

Other bacterial and fungal taxa have been found in the coralloid roots (Table 2), but not much is known about them aside from their taxonomy. In total, a striking 29 orders, 31 families, and 19 genera of bacteria have been reported. A total of 13 orders, six families, and 12 genera were detected in more than one study [9,25–37] (Table 2). Although fungi are part of the microbiome coralloid roots, only three studies report their occurrence [9,35,36]. Twelve fungal orders, 26 families, and 45 genera have been reported, seemingly not shared across studies. Despite this diversity, several bacteria lineages have been found consistently in several studies in different biomes, supporting the idea of a 'core' (i.e. shared bacterial orders) microbial community, likely preferred by cycads [27], including the orders Nostocales, Hyphomicrobiales, Caulobacterales, Sphingomonadales, Burkholderiales, Xanthomonadales, and Sphingobacterales.

Are there phylogenetic signatures of microbiome and plant coevolution in these taxa? Some microbial strains labeled as *Aulosira*, *Calothrix*, or *Nostoc* genera seem to have co-evolved with cycads, as they are found across host species sampled to date [27]. The unambiguous taxonomic identification of these microorganisms therefore deserves further investigation as to define with clarity the identity and natural history of these cyanobionts. Thankfully, we have made some progress in understanding the coralloid microbiome's functions, which could be the basis for co-phylogenies and other comparative evolution studies to get such answers.

Functional traits: nitrogen fixation and beyond

Cycads often inhabit karsty outcrop soils with limited nitrogen availability. Studies have shown that cycad roots interact with their subtending soils and increase

Table 2

Overview of publications and microbial groups reported, separated by order, family and genus. Groups in bold appear in more than one study. Bacterial groups are in black, fungal groups in gray.

Reference	Order	Family	Genus
Zheng et al., 2018 [9]	**Hypocreales**	Nectriaceae	
Suarez-moo et al., 2019 [25]	**Sphingomonadales, Rhizobiales, Hyphomicrobiales,** Mycobacteriales, **Streptomycetales**	Streptomycetaceae	*Sphingomonas, Bradyrhizobium, Rhizobium, Agrobacterium, Devosia, Mycobacterium, Amycolatopsis, Pseudonocardia, Streptomyces*
Bustos-Diaz et al., 2024 [27]	**Sphingomonadales, Burkholderiales, Caulobacterales, Hyphomicrobiales, Pseudomonadales, Xanthomonadales, Actinomycetales**		*Pseudomonas*
Gutierrez-Garcia et al., 2019 [29]	**Burkholderiales, Sphingomonadales, Rhizobiales, Caulobacterales, Enterobacterales, Xanthomonadales, Streptomycetales,** Pseudonocardiales, Micrococcales, Bacillales	Streptomycetaceae	*Bulkholderia, Ralstonia, Sphingomonas, Sphingobium,* ***Bradyrhizobium,*** *Mesorhizobium,* ***Rhizobium****, Caulobacter, Pseudomonas, Serratia, Xanthomonas, Amycolatopsis, Microbacterium,* ***Bacillus***
Ndlovu et al., 2023 [31]	**Rhizobiales, Bacillales**	Beijerinckiaceae	*Beijerinckia, Lysinibacillus, Paenibacillus,* ***Bacillus***
Zheng et al., 2021 [32]	**Rhizobiales, Burkholderiales, Pseudomonadales, Enterobacterales**	**Burkholderiaceae,** Bradyrhizobiaceae, Rhizobiaceae, Sinobacteraceae, Pseudomonadaceae, Enterobacteriaceae	
Bell-Doyon et al., 2020 [33]	Acetobacterales, **Rhizobiales, Caulobacterales, Frankiales,** Acidobacteriales, Corynebacteriales, Propionibacteriales, Solibacterales, Isosphaerales		
Sierra et al., 2024 [34]	**Burkholderiales, Hyphomicrobiales, Frankiales, Pseudonocardiales, Pseudomonadales, Xanthomonadales**		
Pecundo et al., 2021 [35]	Burkholderiales, **Rhizobiales, Enterobacterales, Enterobacteriaceae, Xanthomonadales, Pseudonocardiales,** Micromonosporales, **Hypocreales**, Capnodiales, Eurotiales, Chaetothyriales, Verrucariales, Glomerales, Agaricales	**Burkholderiaceae,** Xanthomonadaceae, Micromonosporaceae, **Nectriaceae,** Ophiocordycipitaceae, Bionectriaceae, Hypocreaceae, Clavicipitaeceae, Aspergillaceae, Herpotrichiellaceae, Verrucariaceae, Amisporaceae	***Rhizobium,*** *Mesorhizobium, Kosakonia, Acidibacter, Xanthomonas, Amycolatopsis, Actinoplanes, Fusarium, Clonostachys, Gliocladiopsis, Simplicilium, Scytalidium, Aspergillus, Exophiala, Penicilium, Ambispora, Mycena*
Liu et al., 2023 [37]	Rhodospirillales, **Actinomycetales**, Cytophagales	Nocardiaceae, Cytophagaceae	*Niastella, Rhodococcus, Edaphobacter*

recalcitrant carbon and nitrogen, which increases spatial heterogeneity of soil chemistry [43]. So if it is not because of the cyanobionts fixing nitrogen (in the form of diverse amino acids, such as glutamine, glutamate, and citrulline [19]), the coralloid root would be extremely poor in nitrogen, needed not only by the plant but also by the cyanobiont and the remaining members of the coralloid microbiome to thrive. It is therefore reasonable to expect that its microbiome must be capable of carrying out biological nitrogen fixation as one of its biological functions.

Nitrogen fixation requires the nitrogenase enzymes found in multiple bacterial species, including species from the Nostocales and some Hyphomicrobiales orders [44], which are part of the ‘core’ coralloid root microbiome [27]. Nostocales are presumed to be the main nitrogen fixers due to their abundance and the measurable physiological and metabolic changes that they undergo to facilitate and boost nitrogen fixation inside the root [45–47]. Other bacterial species, such as those belonging to the Hyphomicrobiales order, have been isolated from the coralloid roots and might be capable of nitrogen fixation too [27,29,32], but their roles remain to be studied.

With such a diverse microbiome, it is tempting to speculate that the nitrogen fixation taking place within the coralloid is supporting the entire microbiome in addition to (or instead of) the plant. For instance, strains from the Gram-negative alphaproteobacteria Hyphomicrobiales undertake partial nitrogen denitrification in the *Azolla* fern, a known host of Nostocales symbiotic microbiomes [48]. Furthermore, specific

Hyphomicrobiales isolated from mosses associated with cyanobacteria-dominated microbiomes have been shown to provide the cycad cyanobiont *Nostoc punctiforme* PCC 73102 with bicarbonate to compensate for its inefficient carbon concentration mechanisms [49]. The presence of these specific Hyphomicrobiales microbiome members (i.e. *Agrobacterium* sp.) was also found to trigger downregulation of the expression of ferredoxins and proteins involved in nitrogen fixation and heterocyst formation. They also trigger the upregulation of flavodoxins, chemotaxis-related proteins, stress markers, and two natural products, a siderophore and a nostopeptolide involved in the regulation of hormogonia differentiation [50].

Interestingly, BGCs predicted to direct the synthesis of natural products, including cyanotoxins, have been found to be enriched in cyanobiont species [27,50,51]. This suggests that these metabolites might be important for the symbiosis, including cyanotoxins such as the non-proteinogenic amino acid β-Methylamino-L-Alanine or BMAA [6], whose BGC remains to be discovered, even though the compound can be systematically detected. Specialized metabolites have been also found in the interaction between cycad cyanobionts and *Caulobacter* spp. In it, *Caulobacter* strains produce an indigoidin-like pigment in the presence of the *Nostoc* cyanobiont and in the absence of bioavailable nitrogen [29]. Finally, fungal endophytes isolated from the coralloid root have been shown to have antifungal activity against known pathogens [35], potentially implicating these microbes in biotic relationships still to be discovered.

Since the first descriptions of the coralloid root, other functions have been assigned to it, for example, aeration [52]. In the era of the microbiome, and based on their taxonomic identities, multiple functions have been attributed to different microbes, such as phosphorus solubilization, phosphorus and metal ion uptake, and even nitrogen fixation [29,31]. However, it remains to be seen if these traits are indeed functionally operational in the strains present in cycad microbiomes. Moreover, since the composition of this microbiome seems to be determined by the location and/or host [26–28], it is unclear if these auxiliary functional traits are local adaptations or common to different microbiomes. Altogether, this body of knowledge on cycad's coralloid root functional traits speaks to a more complex scenario beyond nitrogen fixation benefiting the plant and warrants further research with mechanistic resolution for the first time in the history of cycad's coralloid root research.

Technical challenges in cycad microbiome research

Methods in the field of cycad microbiomes research have made much progress. But different methods and criteria complicate the comparison of taxonomic and phylogenetic patterns to date. There is no comprehensive sampling (e.g. from all cycad species and/or at the global scale); experimental set-ups; or joint studies in physiology, chemistry, DNA barcoding, and/or genome sequencing of both cycads and the microbiome. Future coralloid studies require common protocols to collect and process the microbiome. We suggest a guideline that can help build comparative studies and be aware of possible biases of each method (Figure 2), which we overview below.

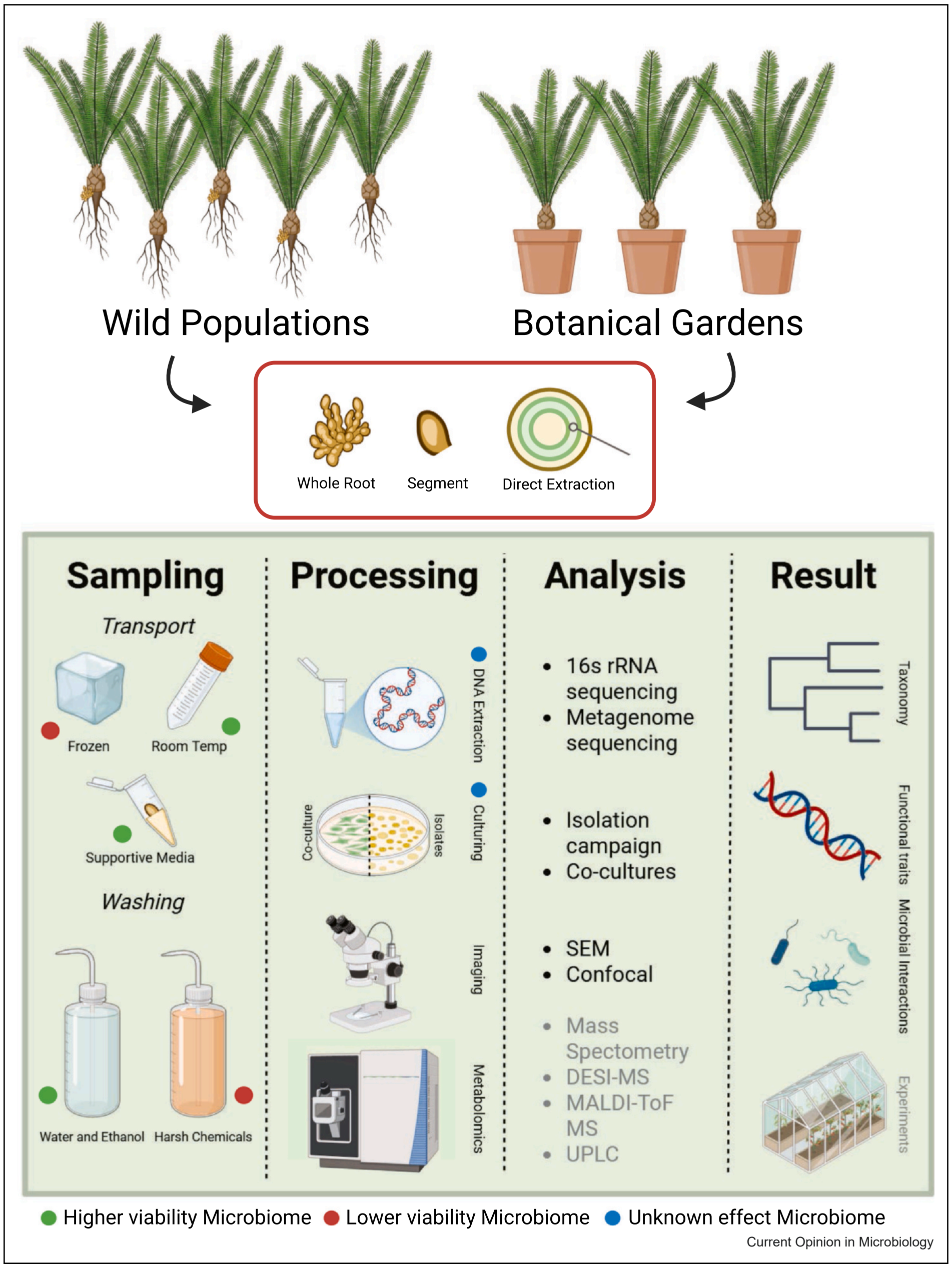
Sampling in the field is complicated due to the unpredictability of root presence and placement, coralloids are often above ground but sometimes underground in larger individuals. New technologies should be explored, such as field-based Computed Tomography scans. Once found, coralloid roots are cut off the host plant with sterile razors and often transported to a laboratory, ideally in sterile conditions; or frozen in liquid nitrogen immediately for RNA or similar studies. As early as 1911, it was suggested that "The root tubercles of *Cycas* are found to retain their vitality for weeks or even months, if they are wrapped in moist blotting-paper and kept in a tin box" [53]. Roots can be transported at room temperature to preserve most of the microbiome, or cooled, to limit metabolic activity. Ideally, transport in bacterial medium at room temperature or 4°C would allow for the more stable preservation of the microbiome while providing the root with nourishment and hydration.

Roots then need to be cleaned to remove rhizospheric bacteria. This can be done with water or 70% ethanol or with harsher chemicals such as hydrogen peroxide or bleach to limit the possibility of encountering epiphytes in further analyses. Ideally, the root is cut transversally, and samples are taken from the cyanobacterial ring using a sterile tool. Genetic analysis can be achieved through extraction, Polymerase Chain Reaction profiling, and sequencing of DNA libraries. Studies on functional traits and microbial interactions can be achieved through metagenome sequencing, microscopy, and culturing. To maintain the Cyanobacteria in a co-culture or culture, medium $BG11_0$ (no nitrogen source) is usually used, but different media might provide options for isolating parts of the microbiome, with proper controls throughout [29].

Extending the 'tip of the root'

Although we have highlighted many of the missing pieces of the holobiont puzzle, it is clear there are also many opportunities for discovery. Perhaps one of the most intriguing and exciting research areas in the coralloid root is the role of fungi and viruses, which were first described more than a hundred years ago. But also how their and other bacterial functions can help answer if this is indeed a co-evolved symbiotic system as we suggest. The chemistry of the microbiome and the functions they

Figure 2



Overview of methods that have been used (black font) or could be used (gray font) in cycad research, divided per subsection. Created in BioRender. Zonneveld, K. (2025) https://BioRender.com/6lrlx99.

sustain, the links between the coralloid roots and cycadivorous insects' microbiomes [54] and beyond into the soil microbiomes, are all waiting to be discovered, ideally with similar protocols and technical guidelines. And as we continue to discover, we propose that the coralloid microbiome itself should have an endangered conservation status, just like their host plants. After more than 150 years of its description, the coralloid root continues to be an exciting area of research worth protecting.

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Declaration of Competing Interest

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Declaration of Generative AI and AI-assisted technologies in the writing process

No use of AI in any part of this paper.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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Glossary

Coralloid roots: Specialized mostly aboveground cycad roots, coral-like in appearance that contain microorganisms within differentiated cells.

Precoralloid root: Young coralloid roots, often shorter than mature coralloids and without a differentiated cyanobacterial zone.

Holobiont: A host and its associated symbionts, expected to function as a contained evolutionary unit.

Functional genomic traits: Traits associated with gene clusters that in some cases produce metabolites.

Biological Nitrogen Fixation (BNF): The process by which microbes convert inorganic nitrogen into organic nitrogen sources that organisms can use.

Secondary or specialized metabolites: Natural chemical products with varying biological and ecological functions that result from the activity of metabolism.