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Highly diverse endophytes in roots of *Cycas bifida* (Cycadaceae), an ancient but endangered gymnosperm[§]

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As an ancient seed plant, cycads are one of the few gymnosperms that develop a root symbiosis with cyanobacteria, which has allowed cycads to cope with harsh geologic and climatic conditions during the evolutionary process. However, the endophytic microbes in cycad roots remain poorly identified. In this study, using next-generation sequencing techniques, we investigated the microbial diversity and composition of both the coralloid and regular roots of *Cycas bifida* (Dyer) K.D. Hill. Highly diverse endophytic communities were observed in both the coralloid and regular roots. Of the associated bacteria, the top five families were the Nostocaceae, Sinobacteraceae, Bradyrhizobiaceae, Bacillaceae, and Hyphomicrobiaceae. The Nectriaceae, Trichocomaceae, and *Incertae sedis* were the predominant fungal families in all root samples. A significant difference in the endophytic bacterial community was detected between coralloid roots and regular roots, but no difference was observed between the fungal communities in the two root types. Cyanobacteria were more dominant in coralloid roots than in regular roots. The divergence of cycad root structures and the modified physiological processes may have contributed to the abundance of cyanobionts in coralloid roots. Consequently, the colonization of cyanobacteria inhibits the assemblage of other endophytes. Our results contribute to an understanding of the species diversity and composition of the cycad-endophyte microbiome and provide an abbreviated list of potential ecological roles of the core microbes present.

***Keywords*:** coralloid root, cyanobacteria, *Cycas*, diversity, endophyte, next-generation sequencing technique

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Introduction

As the oldest and most primitive taxon of living seed plants, the cycads retain characteristics of their ancestors such as the adaptation to barren soils and dry climates. This evolutionary conservatism is proposed to be associated with the ability to form a symbiotic relationship with endophytes, predominantly cyanobacteria, in specialized apogeotropic roots (coralloid roots) (Lobakova *et al.*, 2003). Three types of roots have been described within cycads: taproots, lateral roots and coralloid roots (Rai *et al.*, 2002). Recently, Lu (2006) argued that cycads have developed a fibrous root system, including regular roots, fleshy roots and coralloid roots. The regular roots are characterized by a thick periderm and structured vascular tissue, which protect the plants against attack by pathogens and promote the uptake and transfer of nutrients, respectively (Wu, 2006). The fleshy roots are characterized by parenchyma, which primarily contributes to the storage of nutrients (Wei, 2005). Instead of a developed vascular tissue, coralloid roots possess a zone of slime in the intercellular space of the cortical parenchyma that is colonized by symbiotic cyanobacteria (Huang, 2005). The primary physiological role of the coralloid roots is to provide nitrogen to the aboveground plant parts (Halliday and Pate, 1976).

The morphological divergence between coralloid roots and the other two root types in cycads (collectively termed regular roots in this paper) results largely from the adaptation to nutrient-poor soil. Cycads are scattered among environments that are characterized by hot dry summers and wet cool winters. Their niches include abrupt carbonate hillsides, precipices and rocks, i.e., zones with thin soil layers (Lobakova *et al.*, 2003). For example, *Cycas sexseminifera* F.N. Wei grows in the crevices of rugged karst limestone, often with little available soil (Hill, 2008). The survival of cycads in such habitats is strongly associated with their ability to form symbioses with nitrogen-fixing cyanobacteria (Gehringer *et al.*, 2010), which relieve severe environmental stress by supplying nutrients.

With the exception of *Azolla* and possibly lichen symbioses, it has been proposed that each new offspring of the cyanobacterial host is infected *de novo* (Rai *et al.*, 2002), implying that the cyanobacteria in the coralloid roots of cycads are primarily derived from the surrounding environment. Most of the cyanobionts of cycads belong to the genera *Nostoc*, a group of filamentous cyanobacteria in subsection IV (Nostocales: Costa *et al.*, 2004; Tomitani *et al.*, 2006; Yamada *et al.*, 2012). Compared with free-living cyanobacteria, the cyanobionts of cycad plants are characterized by enhanced nitrogen fixation, and a decreased or complete suppression of photosynthetic activity (Baulina and Lobakova, 2003). The fixed

nitrogen is typically transferred to the host plant rather than excreted to the external environment through the coralloid roots (Halliday and Pate, 1976). Therefore, the cyanobionts are of great importance to the nitrogen equilibrium of cycads.

However, nearly all of the published research has focused on the cyanobacterial community in the coralloid roots of cycads, including studies on the origin and development of cyanobionts (Nathanielsz and Staff, 1975; Ahern and Staff, 1994) and the species diversity and structure of cyanobacterial communities in coralloid roots (Lobakova *et al.*, 2003; Costa *et al.*, 2004; Yamada *et al.*, 2012); no investigation has been conducted comparing the species diversity and composition of endophyte assemblages between regular roots and coralloid roots. Plant endophytes, an assemblage of bacteria and fungi colonizing the tissues of plants without apparent negative impact, are ubiquitous and have been found in all plant species (Schulz *et al.*, 2006). In plants, these endophytic partners are able to improve the growth of the hosts, impart stress tolerance, induce systemic resistance, or supply the host with nutrients, resulting in mutualistic interactions (Schulz *et al.*, 2006).

Although cyanobacterial diversity has been investigated in coralloid roots, the identification of other endophytic microbes, for example, the four most frequently identified bacterial phyla, Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria (Hardoim *et al.*, 2015; Niu *et al.*, 2017), remains poorly studied. Moreover, most of the previous research has used culturing techniques or has been based on methods with insufficient resolution such as microscopic inspection or traditional Sanger sequencing, which inevitably underestimates the diversity of microbes (Huang *et al.*, 2014). Therefore, the knowledge of cycad-endophyte symbioses is still relatively limited.

The invention and rapid development of next-generation sequencing (NGS) techniques has provided access to the genetic information available in environmental samples regardless of the ability to culture microorganisms (Cowan *et al.*, 2005). In this study, we used an Illumina MiSeq sequencing approach to investigate the diversity and composition of endophytic communities in the roots of *Cycas bifida* (Dyer) K.D. Hill. This is a dioecious, entomophilous, arborescent cycad growing in karst limestone or loamy soils and frequently has developed coralloid roots (Hill, 2008). However, no study investigating the root endophytic microbes of *C. bifida* has been conducted. The aim of this study is to unravel the endophyte microflora of *C. bifida* by answering the following questions:

What is the diversity and composition of microbes in *C. bifida* roots?

What is the difference between the endophyte communities of coralloid roots and regular roots?

What are the potential ecological roles of the core endophytes of *C. bifida*?

Materials and Methods

Sample preparation

In this study, three coralloid root samples (B6, B10, and B12) and three regular root samples (B6X, B10X, and B12X) of *C. bifida* were collected from the field in Cao Bang, Vietnam and separately stored at 4°C prior to DNA extraction. The coralloid roots and regular roots were paired samples; B6 and B6X were collected from the same sample plant, B10 and B10X were collected from another sample plant, and B12 and B12X were collected from a third cycad plant. Prior to DNA extraction, the soil and other plant debris on the root samples were washed away under running water. Microorganisms in the rhizosphere or attached to the root epidermis were eliminated by immersing the roots in a 2% sodium hypochlorite solution for 5 min and subsequently washing them with sterile water for additional 5 min. The sterilization steps were repeated twice followed by five subsequent rinses in sterile water. The sterilized samples were then ground into a powder after treatment with liquid nitrogen.

Genomic DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle, 1991). For bacteria, the V4-V5 hypervariable region of the 16S rRNA gene was amplified using the primer pair 515F and 907R (Xiong *et al.*, 2012). This variable region is universal for nearly all bacterial taxa and accurately represents the taxonomic classification of sequences (Ramirez *et al.*, 2014). For fungi, the primer pair 1737F and 2043R targeting the internal transcribed spacer (ITS) region was used for amplification (White *et al.*, 1990). The PCR reaction was conducted in a 20 μl sample with 10 ng of template DNA, 0.4 μl TransStart Fastpfu DNA Polymerase, 4 μl 5X Fastpfu Buffer, 2 μl dNTP (2.5 mM), and 0.8 μl of each primer (5 μM). For the bacterial primers (515F, 907R), the PCR amplification consisted of 27 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec with a final extension of 72°C for 10 min. For the fungal primers (1737F, 2043R), the PCR amplification consisted of 30 cycles at 95°C for 30 sec, 59°C for 30 sec, and 72°C for 45 sec with a final extension of 72°C for 10 min. DNA sequencing was conducted using an Illumina MiSeq PE300 platform at Shanghai Majorbio Bio-pharm Technology Co., Ltd., following the manufacturer's protocols. Prior to sequencing, the libraries were validated by qPCR (Liu *et al.*, 2015).

Sequence processing

The raw data yielded by the Illumina MiSeq sequencing was quality filtered using a Perl-scripted pipeline (Huang *et al.*, 2014; Liu *et al.*, 2015) as follows. First, all the raw reads obtained were assigned to each sample based on primer sequences. Second, reads with an average quality value of < 20 and without both universal primer sequences were discarded. Third, all qualified paired reads were merged, and chimeras, as well as single-copy reads, were discarded to minimize possible sequencing errors. Qualified sequences were then clustered into operational taxonomic units (OTUs) at 97% sequence similarity based on mapping to the Greengenes database for bacteria (August 2013 release; http://greengenes.lbl.gov) and UNITE v7.0 for fungi (Kõljalg *et al.*, 2013) using the function 'pick_closed_reference_otus.py' implemented in the QIIME software package v.1.9.0 (Caporaso *et al.*, 2010).

Table 1. Summary of sequencing reads from all barcodes and samples

	Assignation	B6	B6X	B10	B10X	B12	B12X	Total
16S	Total	23 388	13 408	15 708	14 025	21 031	11 310	98 870
	Processed to haplotypes	13 928	6807	9107	10 500	11 277	6703	58 322
	Map to Greengenes OTU	13 082	6588	8608	9946	10 760	6258	55 242
	Bacteria	11 601	3469	7530	5345	9575	6147	43 667
	Cyanobacteria	9809	1	4841	50	4621	2	19 324
	Archaea	3	2	0	4	3	20	32
	Chloroplast	1302	2980	969	3989	1025	74	10 339
	Mitochondria	176	137	109	608	157	17	1204
ITS	Total	17 469	13 070	10 418	10 482	14 408	17 031	82 878
	Processed to haplotypes	17 302	12 888	10 372	10 373	14 342	16 711	81 988
	Map to GenBank OTU	3860	4099	4074	5680	4278	9452	31 443

Evaluation of diversity and composition

To estimate the alpha diversity of the microbial community, the Shannon diversity index (Shannon, 1948) and species richness were generated for each sample using the QIIME package. Before evaluating diversity, all samples were rarefied based on the number of sequences obtained from the library using the lowest sequencing depth (3469 for bacteria based on B6X and 3860 for fungi based on B6, Table 1). A principal component analysis (PCA) was carried out using the website METAGENAssist (Arndt *et al.*, 2012). To illustrate differences in the composition of microbial communities, Venn diagrams were drawn using BIOINFOGP (Oliveros, 2007). An analysis of similarity (ANOSIM) was conducted to assess the significance of differences in species diversity between coralloid roots and regular roots using the procedure 'anosim' in the R 'vegan' package v.3.4.1 (Clarke, 1993; Clarke *et al.*, 2016).

Results

Sequencing results and diversity indices

A total of 181,748 reads were obtained from six samples through the Illumina MiSeq sequencing analysis, and 3256 OTUs were identified, of which 3001 OTUs (98,870 reads) were bacteria and 255 OTUs (82,878 reads) were fungi (Tables 1 and 2). Specifically, 16 OTUs (19,324 reads) in the bacteria dataset belonged to the Cyanobacteria (Table 2). All rarefaction curves tended to approach a saturation plateau, indicating that the volume of sequenced reads was adequate; a large number of these reads accounted for only a small proportion of the total number of OTUs (Supplementary data Fig. S1). In addition, the rarefaction curves revealed significant variation in the total number of OTUs among different samples.

The Shannon diversity index, which shows the species richness and the evenness of species (Zhou *et al.*, 2015), indicated that in the six root samples, bacteria were more abundant than fungi (Table 3). Regular roots contained more bacterial species than the coralloid roots (ANOSIM, R = 0.6667, P = 0.001). However, the species richness of the fungal communities in the coralloid roots and the regular roots were not significantly different (ANOSIM, R = 0.2593, P = 0.003). Among the six samples, B12X exhibited the highest level of species diversity for bacteria, whereas B6 showed the lowest diversity. High fungal species diversity was detected in samples B6 and B6X, but low fungal diversity was observed in sample B12 (Table 3).

Principal component analysis (PCA) based on the relative abundance of OTUs at the phylum level differentiated the bacterial community of the coralloid roots from regular roots of *C. bifida* (Fig. 1A). However, after removing the Cyano-

Table 2. Summary of haplotypes and operational taxonomical unit (OTU) numbers detected using two markers

	Assignation	B6	B6X	B10	B10X	B12	B12X	Total
16S	Haplotypes	8336	6820	5973	4530	10 193	5601	41 453
	Total OTU	364	467	326	513	681	650	3001
	Bacteria	349	455	316	497	667	639	2923
	Cyanobacteria	4	1	5	3	2	1	16
	Archaea	3	2	0	4	1	6	16
	Chloroplast	10	8	9	10	10	2	49
	Mitochondria	2	2	1	2	3	3	13
ITS	Haplotypes	1318	1082	818	1028	1141	1724	7111
	Total OTU	56	56	28	30	29	56	255

Table 3. Shannon diversity index estimated with two markers

Shannon diversity index	B6	B6X	B10	B10X	B12	B12X
Bacteria	1.48	4.60	2.17	4.31	3.23	4.86
Fungi	2.05	1.96	0.62	1.18	0.57	1.20

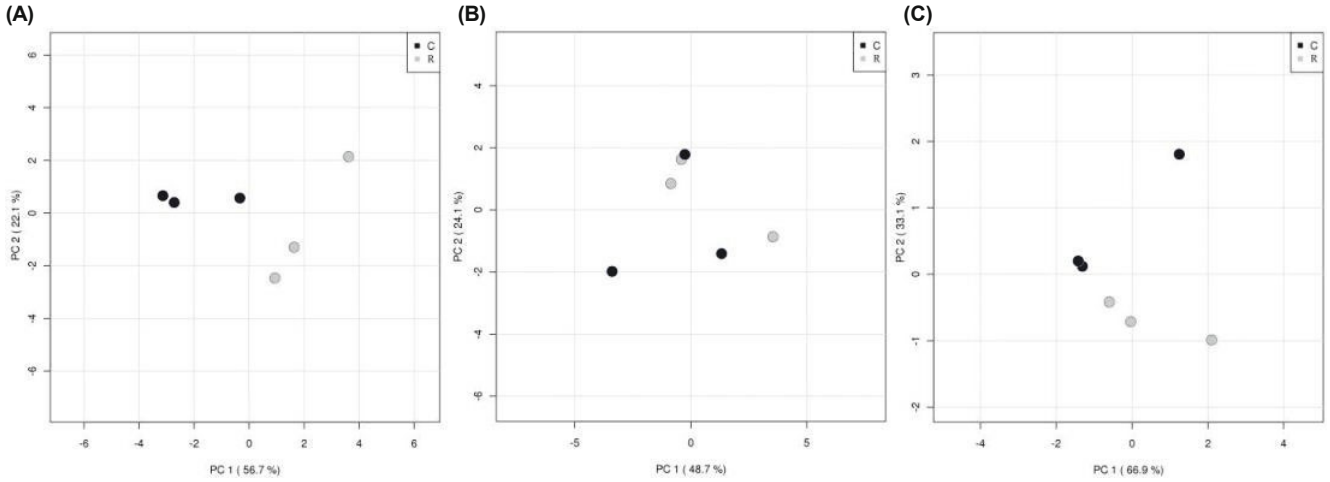

Fig. 1. Principal component analyses of endophytic microorganisms in *Cycas bifida* roots at the phylum level. Comparison analysis between coralloid roots and regular roots based on 16S rRNA (A), 16S rRNA without cyanobacteria OTUs (B), and ITS (C) sequences. Black circles represent the coralloid roots (C) and gray circles represent the regular roots (R) as indicated by the top-right corner legends.

bacteria OTUs, there was no significant difference detected between coralloid roots and regular roots (Fig. 1B). There was also no significant difference in fungal community composition between these two root types (Fig. 1C). Although the number of reads retrieved from coralloid roots was higher than from regular roots, the number of OTUs varied little (Tables 1 and 2). Interestingly, among the Cyanobacteria species, only *Nostoc* and *Dolichospermum* (Nostocaceae) were identified from the 19,324 reads.

Bacterial and fungal taxonomic characteristics

In this study, all the bacterial sequences could be classified at the phylum level (Fig. 2A). Phyla with fewer than ten reads were combined and marked as 'other' for further analysis. Apart from the 'other' phyla, nine phyla predominated across the samples: Cyanobacteria (0.03–84.55%), Proteobacteria (8.03–63.18%), Actinobacteria (1.95–37.64%), Acidobacteria (1.06–13.40%), Bacteroidetes (0.54–13.33%), Firmicutes (0.07–7.15%), Planctomycetes (0.31–4.27%), Nitrospirae (0.10–1.71%), and Chloroflexi (0.01–0.99%). At the class level, approximately 99.98% of sequences could be identified (Fig. 2A); the most abundant classes included the Nostocophycideae, Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, Thermoleophilia, and Acidobacteria-6. Nostocophycideae belongs to the Cyanobacteria, Alphaproteobacteria and Gammaproteobacteria are members of the Proteobacteria, and Thermoleophilia belongs to the Actinobacteria. At the order level, 41–50 orders were identified across the samples. At the family level, approximately 88.39% of reads were assigned to 59–71 families across the samples (Figs. 2A and 3A). At the lower taxonomic ranks, only 51.67% and 3.52% of sequences could be identified to the genus and species level, respectively (Fig. 2A).

In the fungal community, Ascomycota was the dominant phylum in all six root samples. In contrast, three other phyla were not shared across the samples; Glomeromycota and Zygomycota occurred only in B6, B6X, and B12X. With the exception of B12, all five of the other samples contained fungal species from the Basidiomycota. Eleven taxa were identified at the class level with Sordariomycetes (91.06–99.14%) as the dominant class followed by Dothideomycetes (0.07–3.50%) and Eurotiomycetes (0.05–1.92%). The dominant fungal order was the Hypocreales (88.29–99.07%) and the dominant family was the Nectriaceae (73.66–96.24%) (Fig. 3B). At the genus and species level, only 53% and 23.88% sequences could be identified, respectively (Fig. 2B).

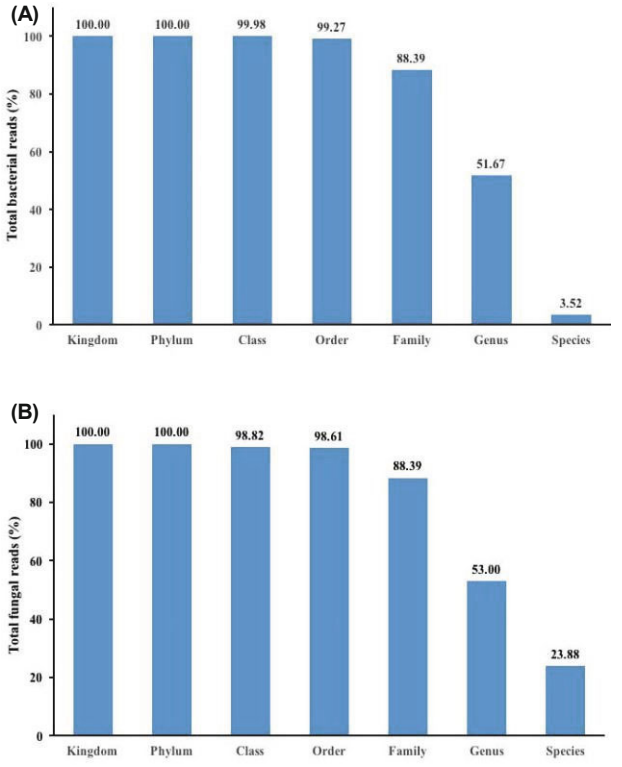

Fig. 2. Proportion of total bacterial (A) and fungal (B) reads assigned to taxonomic ranks.

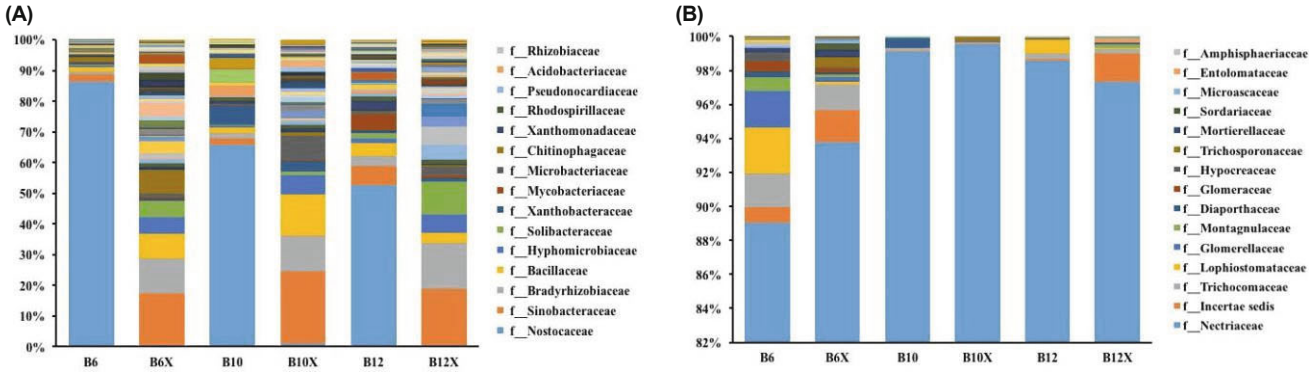

Fig. 3. Taxonomic composition of endophytes in *Cycas bifida* roots. Top 15 bacterial (A) and fungal (B) families are listed in the legends to the right.

Variation in bacterial composition between coralloid and regular roots

The composition of the microbial community based on the 16S rRNA gene amplicons differed significantly between coralloid and regular roots. As indicated by the Shannon diversity index, samples from the regular roots contained more bacterial taxa (66–70 families) than the coralloid roots (54–59 families); the exception was sample B12, which displayed the highest number of bacterial families (71). Combining the bacterial families within the same root type, 74 families were identified in the coralloid roots, and 75 families were identified in the regular roots, with an overlap of 98.7% (Supplementary data Fig. S2A). Interestingly, all of the bacteria families identified from the coralloid roots were found in the regular roots.

Within a plant, most of the bacteria were shared between the coralloid and regular roots (mean = 81.6%). Regular roots contained relatively more bacterial families than the coralloid roots, with the exception of B12 and B12X in which the coralloid roots had one more family than the regular roots. For the coralloid roots, 45 families (60.8%) were shared among the three samples. In addition, B6 shared another two families with B10 and 11 families with B12; B10 shared seven unique families with B12. Eight families exclusively occurred in B12. In the regular roots, 56 families (74.7%) were shared among the samples. Four additional families were shared between B6X and B10X, six were shared between B10X and B12X, and six were shared between B6X and B12X. One family was found exclusively in B6X and two families were found exclusively in B12X.

Variation in fungal communities between coralloid and regular roots

The results of PCA at the OTU level based on the ITS barcode indicated that there was no significant difference in the composition of endophytic fungal between coralloid roots and regular roots (Fig. 1C). After removing the unidentified families and combining fungal families within the same root type, a total of 29 fungal families were detected in the cycad roots, of which 13 (44.8%) were shared between coralloid roots and regular roots (Supplementary data Fig. S2B). Coralloid roots contained seven exclusive families. In comparison, regular roots had two more families than the coralloid roots.

The differences in fungal composition between the coralloid roots and regular roots within individuals were relatively high compared with the bacterial composition (mean = 60.77%). Both B6 and B6X contained five extra fungal families. B10 contained four exclusive families, which was one more family than B10X. B12 had one exclusive family, whereas B12X had ten exclusive families. For the coralloid roots, three families were shared: Nectriaceae, Trichocomaceae, and *Incertae sedis*. B6 shared one and two additional families with B10 and B12, respectively, whereas B10 and B12 shared one. In addition to the families held in common, all three samples of coralloid roots possessed unique families: ten in B6, two in B10 and one in B12. For the regular roots, species from the families Nectriaceae, Trichocomaceae, Glomerellaceae, and *Incertae sedis* were shared. B6X shared two and six additional families with B10X and B12X, respectively, and also contained four exclusive families. Nevertheless, there was no other family shared between B10X and B12X. B10X did not contain any unique families, whereas B12X contained six exclusive families.

More than 80% of land plants are thought to have developed symbiotic relationships with arbuscular mycorrhizal fungi (AMF) from the Glomeromycota (Kawaguchi and Minamisawa, 2010). In this study, species from family Glomeraceae were only detected in the samples B6, B6X and B12X. The failure to detect other AMF was ascribed to the low resolution of the single ITS region (Bellemain *et al.*, 2010; Stockinger *et al.*, 2010) and/or the inadequate reference database (Huang *et al.*, 2014).

Discussion

The colonization of cyanobacteria inhibits the diversity and composition of other endophytes in cycad roots

Unlike the gut microbiota, which is partially inherited from the mother, the root endophytic microbiota is largely reestablished each time a seed germinates, and the microbes colonizing the plant roots are primarily derived from the soil environment (van der Heijden and Schlaeppi, 2015; Compant *et al.*, 2016). As observed in this study, 74 (98.7%) bacterial families were shared between the coralloid roots and the

regular roots of *C. bifida*. The Nostocaceae, Bradyrhizobiaceae, Bacillaceae, Hyphomicrobiaceae, and Sinobacteraceae were the top five taxa of the bacterial families. Although the formation of coralloid roots occurs slightly later than the regular roots (Ahern and Staff, 1994), the spectrum of core endophytic microbiota in the coralloid roots and regular roots did not differ greatly.

However, greater endophytic bacterial diversity was detected in the regular roots compared with the coralloid roots of *C. bifida*, and conversely, the cyanobacteria were dominant in coralloid roots. Four factors could explain such variation, although they remain to be tested. First, the priority effect, i.e., the influence of the arrival of a species on the subsequent community assemblage, has been shown to impact species composition in many organisms, including plants (Ladd and Facelli, 2008), amphibians (Alford and Wilbur, 1985), and arbuscular mycorrhizal fungi (Werner and Kiers, 2015). The cyanobacteria are among the most ancient taxa, with fossil akinetes identified 1650 to 1400 million years ago (Ma) (Tomitani *et al*., 2006). This taxon is characterized by enormous flexibility and environmental adaptation, low host specificity and rapid spreading of hormogonia, which have made them the perfect endophytic candidates/partners during plant evolution. It has been reported that cyanobacteria have developed symbiotic relationships with organisms from distinct lineages such as *Azolla*, bryophytes, gymnosperms (cycads) and the angiosperm genus *Gunnera* (Rai *et al.*, 2002; Usher *et al*., 2007). Therefore, cyanobacteria may have priority over other free-living microorganisms in the invasion of plant roots.

Second, the differentiated cycad root structure is easy for cyanobacteria to colonize. There are marked structural distinctions between the regular roots and the coralloid roots of cycads. The regular roots are characterized by a thick periderm and structured vascular tissue (Wu, 2006). In coralloid roots, the periderm and exodermis are thinner, and the endodermis is thicker (Huang, 2005). The invasion of cyanobacteria is initiated by a chemoattractant secreted by the coralloid roots, inducing the formation of motile hormogonia, which are the infective units in symbiotic cyanobacteria (Cuddy *et al.*, 2012). The thin periderm and exodermis of the coralloid roots facilitate colonization by cyanobacteria, and the thick endodermis serves as a firewall avoiding excessive interference by cyanobacteria.

Third, the ability of symbiotic cyanobacteria to fix nitrogen benefits the resident host by providing access to an abundance of nitrogen and other metabolites. Experiments comparing $^{15}N_2$ and CO_2 feeding by coralloid roots of *Cycas revoluta* revealed that glutamine was the dominant product of nitrogen fixation, with aspartate and alanine as the other major ^{14}C-labeled amino compounds (Pate *et al*., 1988). The host takes up as much nitrogen fixed by the symbiont cyanobacteria as possible, rather than excrete it outside the plant (Halliday and Pate, 1976). As a consequence, the substantial nutrient benefits acquired from the cyanobacteria weaken the host's immune reaction against them, and further promote the establishment of a stable and efficient symbiotic association between these two lineages. During the evolutionary process, the cyanobacteria-cycad symbionts have undergone several structural and physiological changes resulting in better adaptation to a mutualistic life. The most prominent physiological change in cyanobacteria is the enhanced rate of nitrogen fixation coupled with the increased frequency of heterocyst differentiation, which is the agent for nitrogen fixation (Meeks and Elhai, 2002). In addition, a decrease in or total suppression of photosynthetic activity has also been detected (Meeks and Elhai, 2002). Moreover, some parenchyma cells in the cycad root cortex are elongated, and the cell walls of heterocystous cyanobacteria are reduced, both of which could be responsible for the efficient transfer of metabolites between cyanobacteria and the host cycad.

Finally, secondary metabolites such as phenolic compounds (Lobakova *et al*., 2004), polysaccharides (Sasse *et al*., 2017) and cyanobacterial toxins (Cox *et al*., 2003; Gehringer *et al*., 2012; Liaimer *et al*., 2016) may also contribute to the predominance of cyanobacteria in coralloid roots. Phenolic compounds are primarily present in the cortical cells surrounding the gonidial zone. These compounds have been suggested to affect the formation of a symbiotic relationship as well as the metabolism between cyanobacteria and cycads (Lobakova *et al*., 2004), which may partially inhibit the colonization of the roots by other endophytic microorganisms (Caiola and Canini, 1993). Mucilage polysaccharides and proteins are abundant in the cyanobacterial zone, functioning as another barrier to restrict invasion by soil microorganisms (Sasse *et al*., 2017). The cyanobacterial toxins, e.g., microcystins and β-N-methylamino-L-alanine (BMAA), produced by symbiotic cyanobacteria could disrupt the physiological processes of other endophytes by inhibiting protein phosphatase activity (Liaimer *et al*., 2016). Hence, whether exuded by cyanobacteria or by the host plant, the majority of secondary metabolites appear to facilitate the predominance of cyanobacteria in coralloid roots.

Of the fungi, 13 (44.8%) families were shared, and the Nectriaceae, Trichocomaceae, and *Incertae sedis* were the top three families. No variation in the endophytic fungal community was detected between the coralloid roots and regular roots. As mentioned above, cyanobacteria have occupied the dominant position in coralloid roots. In return, the host receives an adequate source of nitrogen. If most nutrient requirements have been met by the mutualist, the host may down-regulate processes that facilitate further colonization (Werner and Kiers, 2015). In this case, if the cycad's nitrogen demands have largely been met by symbiotic cyanobacteria, then there is no need to form a symbiotic relationship with many fungal species given the energy required to house an endophyte (Usher *et al*., 2007). In support of this hypothesis is the absence of arbuscular mycorrhizal fungi, a well-documented phosphorus provider for host plants, in cycad coralloid roots (Fisher and Vovides, 2004). Intense competition for a limited root niche may also play a role in the structure of the endophyte assemblage (Werner and Kiers, 2015). Consequently, the aggressive colonization by cyanobacteria not only inhibits bacterial endophytes but also influences the diversity of fungal endophytes in cycads.

Based on the above-mentioned evidence and coupled with current results, we suggest that the specialized structure of coralloid roots may provide a suitable niche for cyanobacteria and that the colonization by cyanobacteria gradually affects the physiological processes in coralloid roots, par-

ticularly the enhanced rate of nitrogen fixation, which benefits the host and thus facilitates the development of cycad cyanobionts (Meeks and Elhai, 2002). Under such circumstances, cyanobionts may take over the root niches, inhibiting the growth of other endosymbiotic microbes, including bacteria and fungi, in part by excreting secondary metabolites. Additionally, because the host's nutrient demands have largely been met by the cyanobacteria and because the root niche is limited, the colonization of fungal endophytes may be greatly restricted. However, the exact mechanisms of the suppression effect caused by symbiotic cyanobacteria need to be tested.

Potential ecological roles played by core microbiomes in *Cycas* roots

Different core bacterial communities were found in coralloid roots and regular roots : In this study, the top five endophytic bacteria families (Nostocaceae, Bradyrhizobiaceae, Bacillaceae, Hyphomicrobiaceae, and Sinobacteraceae) were identified. The Nostocaceae belong to the Cyanobacteria, a taxon capable of both photosynthesis and nitrogen fixation. Interestingly, a significant difference between coralloid roots and regular roots was detected for this family, with a higher abundance of Nostocaceae present in coralloid roots (ANOSIM, R = 1, P = 0.001). Conversely, the Bradyrhizobiaceae (ANOSIM, R = 1, P = 0.001), Hyphomicrobiaceae (ANOSIM, R = 0.8148, P = 0.001), and Sinobacteraceae (ANOSIM, R = 0.9259, P = 0.001) were more abundant in regular roots than in coralloid roots. There was no significant difference between root types for the Bacillaceae (ANOSIM, R = 0.3330, P = 0.002).

All of the families dominating the regular roots belonged to the Proteobacteria. Specifically, both the Bradyrhizobiaceae and the Hyphomicrobiaceae are affiliated with the Alphaproteobacteria, Rhizobiales, whereas the Sinobacteraceae belong to the Gammaproteobacteria, Xanthomonadales. Species from the Bradyrhizobiaceae are common soil-dwelling microorganisms that form symbiotic relationships with leguminous plants, often providing nitrogen for the hosts (DeLong *et al.*, 2014). The Bradyrhizobiaceae are a major component of forest soil microbial communities, but strains isolated from these soils are not typically capable of nitrogen fixation or nodulation. Members of the family Hyphomicrobiaceae are ubiquitous, occurring in aquatic and terrestrial habitats and even in moderately hypersaline and hot environments (DeLong *et al.*, 2014). Some representatives within this family have been reported to be endosymbiotic with plants, including the genus *Devosia* with the marine ciliate *Euplotes magnicirratus* (Ciliophora, Hypotrichia) and *D. yakushimensis* with the root nodules of *Pueraria lobata* (Bautista *et al.*, 2010). *Pedomicrobium* spp. and *Seliberia* are known to deposit iron and manganese in their cells (Larsen *et al.*, 1999; Slepecky and Hemphill, 2006), which may relieve the damage from heavy metal pollution for the host plants. The Bacillaceae are affiliated with the Firmicutes, with some genera capable of producing antibiotics (Katz and Demain, 1977). The Sinobacteraceae are a group of bacterial phytopathogens affecting agriculturally important plants such as tomatoes, rice and coffee (Naushad *et al.*, 2015). However, no visible disease symptoms have been observed in cycads, which is primarily due to the balanced antagonism between pathogens and hosts (Schulz *et al.*, 2006).

Consequently, a differential preference for bacterial endophytes has been discovered between the coralloid roots and regular roots of *C. bifida*. Coralloid roots exhibit a specific preference for an association with the Nostocaceae, and regular roots show an extensive preference for species from the Sinobacteraceae, Bradyrhizobiaceae, and Hyphomicrobiaceae. The difference in preference for endophytic bacteria between the coralloid roots and regular roots of *C. bifida* likely results from differentiation in the root structures, as well as the various ecological roles that endophytic bacteria have played.

***Fusarium* species are likely the preferred fungi in *Cycas bifida* :** No difference in fungi was detected between the microbial communities of coralloid roots and regular roots (ANOSIM, R < 0.25). Among the 29 families detected, only three were found in all six root samples: Nectriaceae, Trichocomaceae, and *Incertae sedis*. The Nectriaceae are the most abundant family, belonging to the Ascomycota, Sordaromycetes, and Hypocreales. The Trichocomaceae and *Incertae sedis* were relatively low in abundance. The Trichocomaceae is a family of fungi in the order Eurotiales, whereas *Incertae sedis* refers to a taxonomic group in which the broader relationships are uncertain or undefined (Ludwig *et al.*, 2011).

Strains belonging to the family Nectriaceae are frequently isolated from gymnosperms and angiosperms (Kuldau and Yates, 2000), particularly within the genus *Fusarium*. *Fusarium* species are adapted to a wide geographical range with diverse ecological habitats and host plants. For example, *F. verticillioides* is capable of colonizing over 1,000 plant species. Moreover, several *Fusarium* species can detoxify benzoxazinoids (Glenn *et al.*, 2001, 2002). Benzoxazinoids are the native antimicrobial compounds of host plants, predominantly occurring in the roots of hosts, and they are toxic to fungi (Schulz *et al.*, 2006). The occurrence of *Fusarium* strains may reduce the infection of hosts by pathogenic fungi. Moreover, all *Fusarium* species produce the antibiotic fusaric acid, which shows activity against both Gram-negative and Gram-positive bacteria. The pathogenicity of *Fusarium* should not be ignored. The serious wilts such as Panama disease in bananas, which is induced by *F. oxysporum*, are among the most devastating plant diseases in the world (Schulz *et al.*, 2006).

Another important genus in the Nectriaceae is *Gibberella*, which produces gibberellin, a plant hormone that promotes cell elongation, flower formation, and seedling growth. *Penicillium* from the Trichocomaceae are common endophytes in plants (Vega *et al.*, 2006; Angelini *et al.*, 2012), and some species can improve phosphate solubility or produce gibberellic acid to stimulate plant growth (Wakelin *et al.*, 2007; Khan *et al.*, 2008).

In this study, species from *Fusarium*, *Gibberella*, and *Penicillium* were detected, with *Fusarium* predominating. Given the great abundance of fungi detected in the cycad roots and the adaptability of the Nectriaceae, we suggest that *C. bifida* has developed a fungal endophytic preference for the Nectriaceae, particularly for species from the genus *Fusarium*, which may protect the host from pathogens and toxic compounds.

Conclusion

This is the first study focusing on the species diversity and community composition of endophytic microflora in the roots of *C. bifida* using an NGS deep sequencing approach. Highly diverse endophytic species were detected within coralloid roots and regular roots. Bacteria displayed greater species diversity than fungi in the root samples. Specifically, a significant difference in the core bacterial community composition was detected between coralloid roots and regular roots, but no difference was observed in the fungal communities. Based on existing evidence coupled with the current analysis, we propose that the differentiation of coralloid root structures provides a suitable niche for cyanobacteria and that the nitrogen provided by this taxon reciprocally benefits the hosts, leading to aggressive colonization by cyanobacteria. The abundance of cyanobacteria may inhibit the colonization by other endophytes. In conclusion, the development of endosymbiotic relationships between cycad roots and cyanobacteria is mutually beneficial and may be conducive to the long-term survival of cycads in harsh environments. However, due to an inadequate sample size, the exact interplay between cycads/cyanobionts and their ecological roles remains to be discussed and tested.

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