GeoChip 5.0 microarray: a descriptive overview of genes present in coralloid root microbiomes of *Cycas debaoensis* and *Cycas fairylakea*

[1,2,3]Aimee Caye G. Chang, [1,2,3]Melissa H. Pecundo, [1]Jun Duan, [1]Hai Ren, [2]Tao Chen, [2]Nan Li

[1] South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China;
[2] Fairy Lake Botanical Garden, Chinese Academy of Sciences, Shenzhen, China;
[3] University of Chinese Academy of Sciences, Beijing, China. Corresponding authors: A. C. G. Chang, aimee.caye.chang@gmail.com; N. Li, andreali1997@126.com

Abstract. This study deals with specialized roots of cycads called coralloid roots accommodating a small population of heterotrophic bacteria and significantly abundant endosymbiotic cyanobacteria functioning as nitrogen fixers. This paper aimed to identify what other genes are present in the coralloid roots aside from genes for nitrogen metabolism to determine the functional repertoire of symbiotic coralloid roots. Genomic functional gene profiles were examined using DNA-based microarray GeoChip 5.0. GeoChip analysis suggests that the functional profiles between the two species are highly identical and almost all identified genes present in the coralloid roots were similar. Functional gene inventory identified elevated gene signals for metal homeostasis, carbon metabolism, stress responses, antibiotic resistance, organic contaminant degradation and nitrogen metabolism. This supports the role of symbionts residing in coralloid roots as nitrogen fixers and in increasing host tolerance to various environmental stressors as most genes covering major pathways for nitrogen cycling, metal resistance and toxin degradation were detected in our study. Moreover, indications of active photosynthesis and carbon fixing potential of symbionts in coralloid roots were also evident due to the detection of various carbon cycling genes. This could probably be associated to its apogeotropic growth via phototropism suggesting that symbionts require access to sunlight causing the negative geotropic growth of coralloid roots.
Key Words: GeoChip, coralloid roots, symbiosis, cyanobacteria, *Cycas*, genes.

Introduction. Cycads are the only members of the gymnosperms that forms an obvious and stable symbiotic relationship with a photosynthetic prokaryote, the cyanobacterium. In fact, apart from its normal taproots and lateral roots, the host has developed a specialized, dichotomously-branched root system called the coralloid roots to house these endosymbiotic cyanobacteria, or cyanobionts (Chang et al 2019; Gutierrez-Garcia et al 2019). Inside the coralloid roots, cyanobionts distinctly form a green circular layer positioned in the middle of cortex tissues forming the cyanobacterial zone (Ahern & Staff 1994). Most cyanobionts are capable of cell differentiation and are generally filamentous types wherein a single filament consists of vegetative cells interrupted by regularly-spaced intervals of heterocyst cells that fix nitrogen for its host and is believed to assist cycads in withstanding contaminated and infertile soils (Halliday & Pate 1976; Meeks et al 2001). When unfavorable conditions arise, it can also form resistant spores called akinetes along the cyanobacterial filament and prior to host invasion, it can also transform into motile hormogonia for locomotion (Meeks et al 2001; Golden & Yoon 1998). This cycad-cyanobacterial association differs from usual plant-microbe symbioses as the cyanobionts do not migrate to other parts of the hosts' body but remains confined in the coralloid root tissues (Chang et al 2019). Moreover, it was also believed that cyanobacteria in coralloid roots do not actively photosynthesize (Costa et al 1999; Yamada et al 2012). This leads to the question as to why cyanobionts retain their full

photosynthetic machineries, associated pigments and enzymes (Lindblad et al 1985; Adams et al 2013) as well as why cycads maintain their ability to form coralloid roots when it seems unnecessary to accommodate facultative cyanobionts for them to survive (Fisher et al 2017).

Symbiotic cyanobacteria usually belong to the orders Nostocales and Stigonematales (Castenholz et al 2001). In cycads, individuals under genus *Nostoc* are the predominant symbionts but members belonging to the genera *Calothrix*, *Scytonema* and *Richelia* were also identified (Grobbelaar et al 1987; Costa & Lindblad 2002; Gehringer et al 2010). Several strains of cyanobionts were discovered in a single cycad host (Zheng et al 2002; Thajuddin et al 2010), but in a study by Gehringer et al (2010), only a single strain of *Nostoc* was isolated from the cycad host *Macrozamia* (Yamada et al 2012). Interestingly, other heterotrophic bacteria were also reported to co-exist with cyanobionts in coralloid roots (Chang et al 1988), but bacterial population are somehow scarce. In a recent study comparing the microbial communities between coralloid and regular roots, it was determined that bacterial communities differ significantly as cyanobionts overpopulate the cortical tissues of coralloid roots but when cyanobacterial population was disregarded in the data set, no discernible difference was found between the two root types (Zheng et al 2018). Cycads produce a wide range of compounds, which could be secreted by cyanobionts as well, which might limit population of bacteria without affecting cyanobacterial growth and this requires molecular-level communication be established between the symbiont and the host (Adams et al 2013; Obukowicz et al 1981; Grilli Caiola 1980). Even with continuous effort of scientists to decipher the origin and function of coralloid roots, its purpose still remains poorly understood due to the limitations of bacterial and tissue culture methods and more importantly, in capturing the exact behavior of cyanobionts while in symbiosis with cycad coralloid roots (Barona-Gomez et al 2019; Yamada et al 2012; Lindblad 2009; Grilli Caiola 2001; Costa et al 1999). Therefore, we aim to examine the functional gene profiles in coralloid roots using GeoChip 5.0 that will disregard the DNA of host tissues concentrating only on the gene abundance of microbes.

Functional gene microarrays are useful for establishing gene profiling and assessing changes in microbial gene expression from different sites in the context of ecology (He et al 2007; Mason et al 2010; Kang et al 2013; Bayer et al 2014). DNA-based GeoChip has oligonucleotide probes that detect functional genes of microorganisms and is a type of functional gene array that was introduced in 2002 and has been constantly upgraded by increasing number of probes for the detection of more genes. GeoChip 5.0 is the latest version with 180,000 probes that could detect 17 major functions related to carbon, nitrogen, sulfur and phosphorus cycling, heavy metal resistance, antibiotic resistance, pathogenicity, secondary metabolism, organic pollutant degradation, electron transport, stress responses, virulence and viruses (Van Nostrand et al 2016). Aside from updating previous functional gene families, over 1,000 new functional gene families were added in this new version (Shi et al 2019).

In this study, GeoChip 5.0 technology was utilized to determine the functional gene diversity of root-associated microbiomes in two different species of cycads which are *Cycas debaoensis* Zhong & Chen and *Cycas fairylakea* D. Y. Wang. Belonging to the 25 recognized Chinese species out of the 300 species in the world under the genus *Cycas* L., these two cycad species are endemic in China that thrives in evergreen, broad-leaved and coniferous forests in tropical and subtropical regions. *C. debaoensis* originated from Guangxi province (Zhong & Chen 1997) whereas the natural population of *C. fairylakea* was found in Guangdong province (Wang 1996). Thus, the objectives of this research were to answer (1) what are the abundant genes, (2) what are the functions from the microbial community in the coralloid roots diverse, and (3) do microbes, particularly cyanobionts, perform other functions aside from nitrogen fixation? Assessing the functions of endophytic bacteria may provide insights for future studies on symbiotic mechanism in the coralloid roots as well as better conservation strategies for cycads.

Material and Method

Sample collection and processing. From three individual *Cycas debaoensis* (CD) host plants, a bulk of coralloid root samples were collected from each plant. All three biological replicates were collected in Fairy Lake Botanical Garden (FLBG), Shenzhen, China. For *Cycas fairylakea* (CF), quantity of coralloid root samples grown and cultivated in FLBG were not sufficient during the sampling period and thus, one of the biological replicate was collected from the wild, the native habitat in Meilin Reservoir (Shenzhen, Guangdong, China) and the other one is a plant that originated from the wild in North Guangdong, China eventually cultivated in FLBG. Sample collection was conducted from April to June 2018. During collection, coralloid root samples were visually examined for the presence of a cyanobacterial zone, or green ring, indicating a mature symbiotic root. Coralloid roots were placed in ice and immediately transferred to the laboratory for further processing. Soil debris were gently washed off with iced milliQ water repeatedly until the washed-off water became clear. Due to the delicate structure and condition of coralloid roots, tissue sterilization using sodium hypochlorite and ethanol was not conducted to keep the roots intact and the cyanobacterial zone inside the roots protected. Therefore, the roots were further rinsed with cold sterile distilled water until clear water was finally observed. Clean coralloid roots were then cut into small pieces, around 2 cm in length, and immediately frozen in liquid nitrogen and stored at -80°C until further processing.

DNA isolation. Frozen coralloid and normal root samples were pulverized to fine powder using a sterile mortar and pestle. Total genomic DNA was isolated using a modified CTAB method. CTAB extraction buffer (2X CTAB, PVP and 0.2% mercaptoethanol) was mixed with 3 g pulverized coralloid roots followed by 30 minutes incubation at 65°C. DNA was then extracted using phenol-chloroform-isoamylol (25:24:1) mixture and precipitated using isopropyl alcohol and 5M NaCl. A series of washing steps using 76% and 100% ethanol was then conducted. DNA quality and concentrations were evaluated using NanoDrop 2000 at A260/280. Only the DNA extracts with ratios of > 1.70 were used for further analyses.

GeoChip 5.0 loading and data processing. DNA concentration of 800 ng per sample was used for labelling following a 16.5-hour hybridization step. The subsequent steps: fluorescence labelling, array hybridization and scanning were conducted as described in Lu et al (2012). The optical signal of the probe was converted into a digital signal using ImaGene 6.0 software. Data normalization, removal of low quality spots and signal intensities based on signal-to-noise ratio, filtering of outliers and obtaining mean normalization signal intensities for each spot was provided by Magigene Biotechnology Co., Ltd. (Guangzhou, China) as client service. The normalized signal intensities were calculated as the sum of all probes per gene and divided by the signal intensity of all probes per category. Afterwards, the mean across all replicates per sample was obtained. GeoChip data were further analyzed in the R environment v3.5.1 (The R Core Team) for principal components analysis.

Results and Discussion

GeoChip analysis of microbial functional genes. Using GeoChip 5.0, a total of 199,891 probes were detected in both species that represent 889 functional genes classified in 17 gene categories. These probes with positive signals belong to metal homeostasis (53,058), stress (31,844), carbon cycling (29,939), antibiotic resistance (27,465), organic contaminant degradation (17,524) and nitrogen cycling (7,557). Other functional categories having low signals with only < 7,500 number of probes each are microbial defense, virulence, sulfur, phosphorus, pigments, *gyr*B, viruses, metabolic pathways, electron transfer, plant growth promotion and protists.

Principal components analysis of samples revealed that the gene abundance of two of the six samples (CF2 and CD3) deviated from the rest, the latter deviating more significantly, but the other four samples vary less from each other (Figure 1). CD1, CD2,

CF1 and CF3 were found to be more clustered together that presented a clear demarcation between species. As the age and vigor status of coralloid roots were difficult to determine during sampling, these might account for the differences in signal intensities read by GeoChip for samples CF2 and CF3. Nonetheless, hierarchical clustering of pooled normalized signal intensities of all gene categories between CD and CF revealed that functional genes were not different in both species in all categories identified by GeoChip (Figure 2A) and that similarities were more discernible rather than differences. This indicates that the functional genes occurring in coralloid roots might be similar between species of *Cycas* spp. regardless of where they thrive. Analysis of the microbial genes present between species also supports this hypothesis as 23,155 (96%) overlapping genes from the six samples were identified suggesting high similarity in the functional profiles of coralloid roots (Figure 2B). A plausible explanation to this high gene convergence is the dominance of cyanobacterial symbionts in the microbiome of coralloid roots in which majority of the positive signals collectively detected by GeoChip from all samples were in fact contributed by cyanobionts.

To our knowledge, this is the first time that the microbial functional genes in coralloid roots were examined and reported. Only minimal differences were observed between the two species of cycads in which the small number of identified differentially abundant genes belong to the categories of viruses and protists. Hence, only highly-abundant genes per functional category common between CD and CF were selected and further analyzed. Since metagenomics profiling from other studies revealed that majority of the microbial population identified in coralloid roots are cyanobacteria (Zheng et al 2018; Bell-Doyon et al 2020), cyanobionts will be considered as prime contributors of majority of the positive gene signals collected from GeoChip. Genes potentially belonging to other microbes will still be included in the discussion, if necessary and relevant to host-microbe symbiosis. But unless sufficient evidence suggests that certain genes actually belong to a particular group of microorganisms, the discussion on the succeeding sections will generally pertain to the abundance of cyanobacterial genes. Therefore, the top gene categories relevant to cyanobacterial-coralloid root symbiosis were individually presented below to assess the functional range and capacities of the symbiotic coralloid root-associated microbiomes.

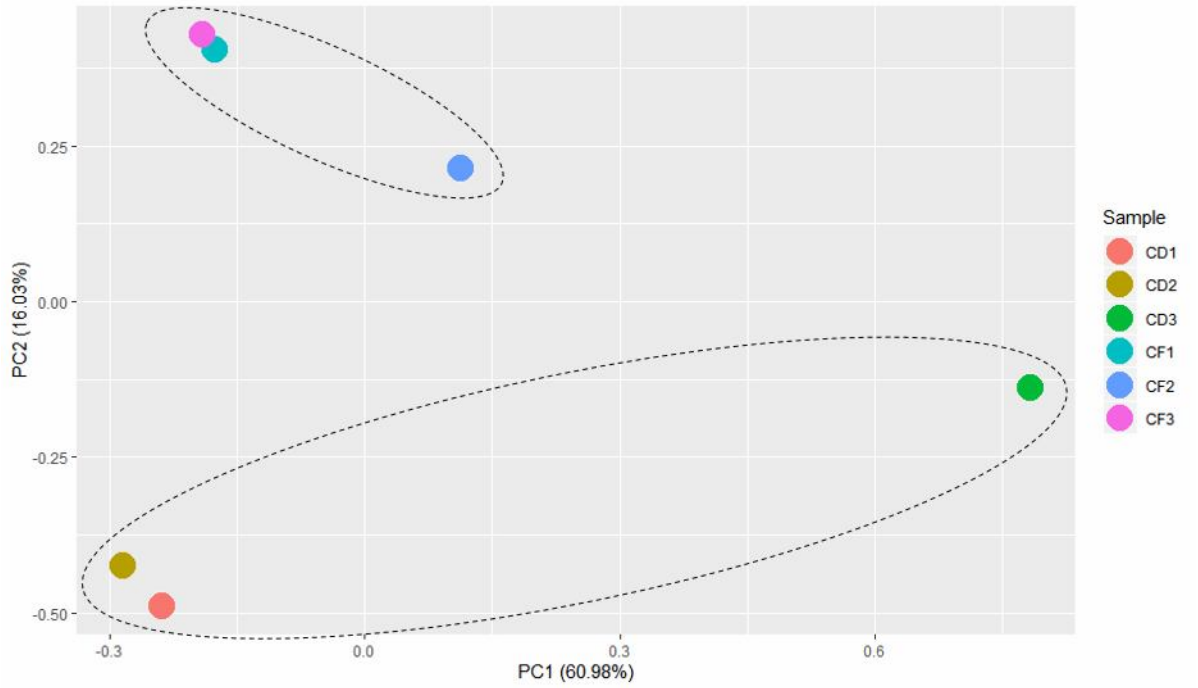

Figure 1. PCA plot showing percent variation of functional genes in all biological samples of *C. debaoensis* (CD1, CD2, CD3) and *C. fairylakea* (CF1, CF2, CF3).

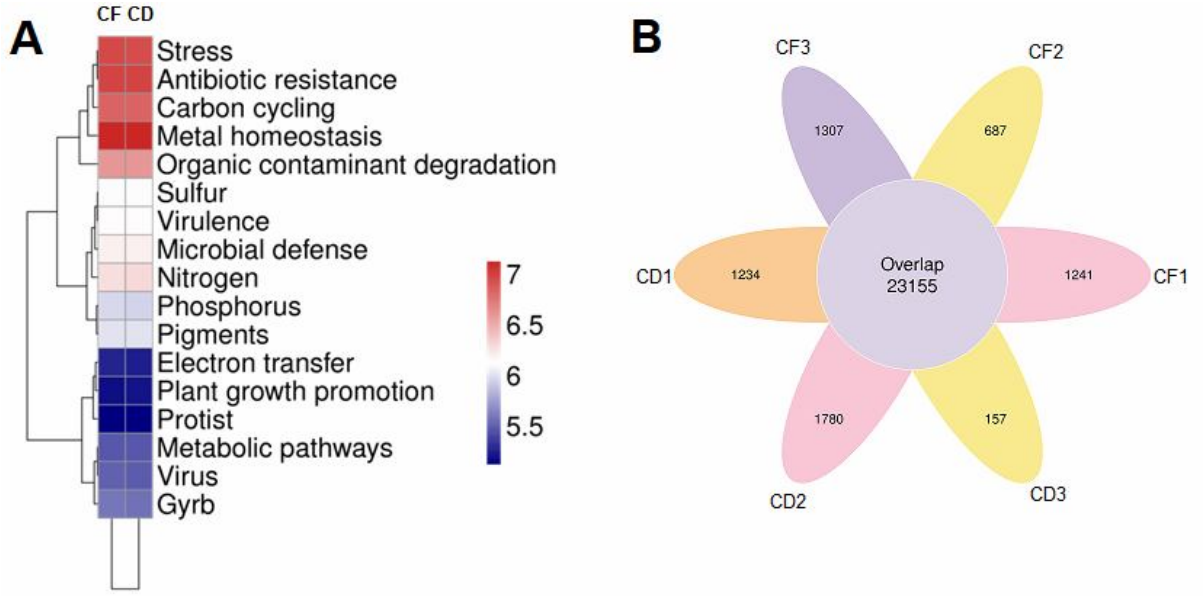

Figure 2. (A) Hierarchical clustering of pooled normalized signal intensities of all gene categories between *C. debaoensis* (CD) and *C. fairylakea* (CF) and (B) Venn diagram of overlapping genes between all samples of *C. debaoensis* (CD1, CD2, CD3) and *C. fairylakea* (CF1, CF2, CF3).

Metal homeostasis. Heavy metals are usually present in the environment especially in soil. Copper, nickel, iron, magnesium, cobalt, manganese and zinc are trace metals used by cyanobacteria for key cellular metabolism (Huertas et al 2014). However, levels of these metals should be regulated as either deficient or excess amounts could affect viability and performance of the cell. Other metals such as chromium, lead, cadmium and arsenic are not essentially needed for survival but can only be tolerated by the cells in low quantities (Shilpi et al 2015). Beyond a certain threshold, viability could be compromised that could affect vital cell processes eventually leading to cell death (Hudek & Ackland 2017). Since cyanobacteria that invades coralloid roots originate from the surrounding soil, cyanobionts are typically exposed to varying levels of metals and over time, must have developed a mechanism to increase metal tolerance. Likewise, cyanobacteria might also play an important role in increasing accessibility and resistance to metals for their respective hosts as symbionts (Dupont et al 2010). It has already been reported that a symbiotic cyanobacterium isolated from coralloid roots of cycads, *Nostoc punctiforme*, served as a facilitator in increasing host tolerance to elevated levels of heavy metals (Hudek & Ackland 2017). The cycad hosts which are an ancient plant group originating from Jurassic period are also known to withstand dry and rocky habitats with infertile soil (Brenner et al 2003). The coralloid roots seem to be an adaptive feature where it evolved to host microbes including cyanobacteria to aid the survival of cycads from harsh environments by utilizing high levels of metals (Halliday & Pate 1976).

This is also evident in our study as the highest average raw signal intensities (13,066,735±388,202) and most number of probes (8,843±1,161) detected belong to various metal homeostasis genes (Figure 3). The most abundant gene in all samples is *nik*A receiving an average of 707.16±68.20 normalized signal intensity. *nik*A gene is the periplasmic nickel-binding protein responsible for nickel transport. According to Woodward et al (2000), if nickel metal transporter genes are present in phototrophic microorganisms, it usually is involved in photosynthetic pathways as part of the large subunit of hydrogenase complex coupled with iron creating a nickel-iron (Ni-Fe) active binding site for production of mature hydrogenases. Hydrogenase enzymes release hydrogen gas in oxygen-limited environments thereby increasing bioavailability of hydrogen as an alternative energy source (Waldron & Robinson 2009). Each metal has a specific channel for influx and efflux of metals across cell membranes and therefore, different transporters are also required (Waldron & Robinson 2009; Ma et al 2009). Aside from nickel and iron transporters, elevated normalized probe signals (> 300) were also

detected for *chr*A, *cor*A, *cop*A, *mgt*A and *znu*C genes for transport of chromium, cobalt/magnesium, copper, magnesium and zinc, respectively. As previously mentioned, these metals are essential for cyanobacterial metabolism except chromium. The increased gene intensity of *chr*A genes (459.06±39.01) in coralloid roots shows evidence that symbionts in coralloid roots have potentially gained a strategy in regulating chromium to non-deleterious levels for itself and its host as well. Similar strategy might be employed for arsenic and cadmium as genes *ars*B, *ars*C and *cad*A obtained > 100-200 normalized signal intensities. The wide array of metal homeostasis genes that was identified in both CD and CF suggests that coralloid roots can indeed regulate metal uptake and increase metal tolerance of its host.

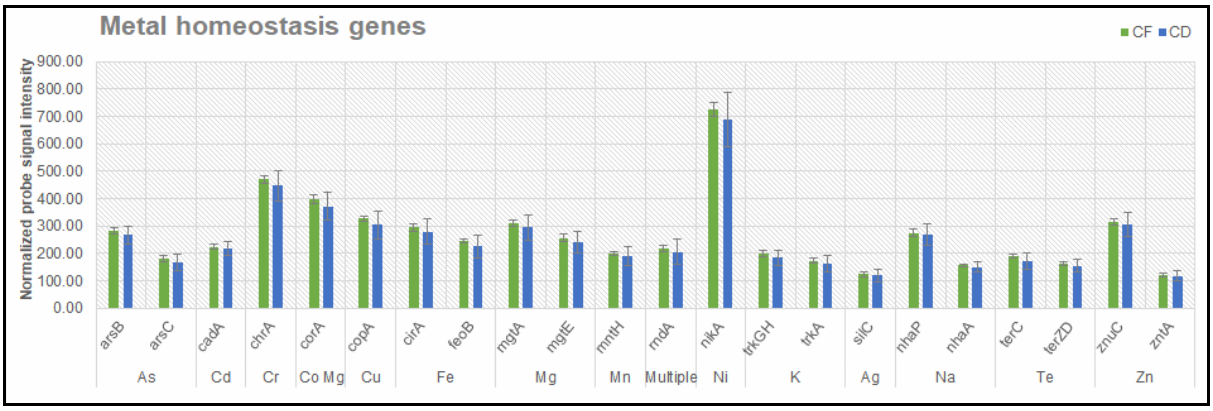

Figure 3. Genes obtaining high normalized probe signal intensities for metal homeostasis. Error bars represent standard deviation of the means. [*C. debaoensis* (CD) and *C. fairylakea* (CF)].

As cyanobacteria retain full photosynthetic machineries and accessory pigments even as symbionts, the high abundance of *nik*A gene, as well as *cir*A and *feo*B genes for iron transport, supports the possibility that cyanobionts are capable of fixing carbon even in environments deprived of light and necessary photosynthetic reactants by use of an alternative mechanism yet to be determined. In line with this, it is apparent that metal demands for cyanobacteria are much higher compared to other non-photosynthetic prokaryotes as various metals are required for photosystems I and II to properly function (Zak et al 2001; Shcolnick & Keren 2006). Photosynthetic processes require metals and consequently, explains the high demands for metals in cells. Moreover, the main enzyme for nitrogen fixation, nitrogenase, forms a dinitrogenase heterotetramer which requires an iron protein (Dos Santos et al 2012; Jasniewski et al 2018). Hence, apart from gaining insight that coralloid roots are indicative of assisting cycads to withstand poor-conditioned soils, it appears that the significantly-elevated metal homeostasis genes detected in GeoChip is correlated to nitrogen and carbon-fixing potential of cyanobacteria in coralloid roots. Thus, separate discussions on carbon and nitrogen metabolism will be expanded in the subsequent sections.

Carbon metabolism. Several carbon cycling genes were detected by GeoChip in coralloid roots having an average raw signal intensity of 6,699,473±275,295 and a total of 4,990±667 positive probes. Coralloid roots are oftentimes found embedded in soil or covered by large foliage of its cycad host limiting its access for sufficient air and sunlight. Hence, it was previously thought that cyanobacteria in coralloid roots do not photosynthesize despite retaining complete apparatuses for carbon fixation (Lindblad et al 1985; Lindblad et al 1987; Adams et al 2013). However, our study is telling otherwise because a number of abundant genes for Calvin cycle were identified indicating active photosynthetic activities are occurring in coralloid roots (Figure 4).

Figure 4. Genes obtaining high normalized probe signal intensities for carbon fixation and degradation. Error bars represent standard deviation of the means. [*C. debaoensis* (CD) and *C. fairylakea* (CF)].

Highest normalized signals were obtained from *tkt*A gene (160.02±18.57), which codes for transketolases. This gene is responsible for production of NADPH (nicotinamide adenine dinucleotide phosphate) as well as several other sugar phosphates intermediates (Iida et al 1993). NADPH is a critical step in carbohydrate production and regenerating ribulose biphosphates (RuBP) in Calvin cycle. The Calvin cycle consists of 3 main steps – carboxylation, reduction and regeneration (Abebe et al 2009) – in which all genes garnering high intensities under Calvin cycle category from GeoChip were involved in each step of the carbon fixation cycle. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) genes were detected obtaining an average normalized signal intensity of 91.61±7.98 which is the key enzyme in the carboxylation step of carbon fixation pathway. Presence of genes for reduction (pgk, GAPDH, FBPase, TIM) and regeneration (RPI, prk) steps were also found to be present. In almost comparable mean normalized intensities with *tkt*A gene is the multiple systems *pcc* gene (157.96±16.95) for citrate cycle or tricarboxylic acid (TCA) cycle that codes for propionyl-CoA-carboxylase commonly observed in archaea for autotrophic carbon fixation pathway (Berg et al 2007). In relation to this is the presence of *cod*H gene, which is commonly characterized in anaerobic bacteria. This gene plays a part in carbon cycle by reversibly transforming carbon dioxide

to carbon monoxide and capable of forming a cluster with acetyl-CoA-synthase (Tan et al 2006). Significant levels of FTHFS (formyltetrahydrofolate synthetase) genes typically found in sulfate-reducing bacteria were also identified. In addition, bacterial microcompartments (*ccm* and *cso*) genes were also present. In cyanobacteria, these genes codes for carboxysomes which are structures for carboxylation and has a significant role in global carbon cycling by storing RuBisCo enzymes (Kerfeld et al 2018). Active carbon metabolism within coralloid roots are evident in both species of *Cycas* used in this study. It even showed higher intensities for carbon compared to nitrogen metabolism, wherein the latter was more popularly known as the primary function of symbiotic coralloid roots. One interesting feature of coralloid roots is its negative geotropic growth. Instead of growing downwards like a normal root, it prefers to grow upwards until it reaches the soil surface - also referred to as apogeotropic growth (McLuckie 1922; Chang et al 2019) which might have evolved throughout the course of geologic history to facilitate survival and reproduction. Relating this to the functional profiles obtained from GeoChip, it appears likely that endosymbiosis in coralloid roots with cyanobacteria might be causing the roots to grow apogeotropically, for exposure and more contact to air and sunlight, as the identified carbon fixation genes validate its dynamic involvement in the global carbon cycling. These findings corroborate a study done by Perraju et al (1986) where high photosynthetic rates were discovered in the coralloid roots of *Cycas circinalis* that develop at close proximity to the soil surface where cyanobacteria may gain access to more light.

Surpassing signal intensities of carbon fixation genes are genes for carbon degradation. Significant signals came from *amy*A gene that is responsible for starch degradation. In contrast to carbon fixation genes which could easily be attributed to cyanobacteria because of their characteristic as pigmented, photosynthesizing microbes, therefore setting it apart from fungi and other microbes, the same cannot be applied for carbon degradation genes. However, it must be mentioned that some strains from the phylum Proteobacteria, which were also detected in coralloid roots (Zheng et al 2018; Bell-Doyon et al 2020), are known to produce bacteriochlorophyll a (Bchl a) and carotenoid pigments making them able to photosynthesize as well (Molouba et al 1999). Hence, in the matter of carbon degradation, gene signals from other microbes might also be contributing to these functions and cannot be solely attributed to cyanobionts. Nonetheless, the identified microbiomes of coralloid roots in our samples seem to be capable of breaking down various aromatic compounds and carbon-based molecules thereby playing a role in recycling and stabilizing carbon levels in the environment.

Nitrogen metabolism. Cyanobacteria in coralloid roots are known as nitrogen fixers increasing bioavailability of usable forms of nitrogen for its host. As some soil microbes were also capable of fixing nitrogen, it is good to note that other microbes might also be contributing to the signal intensities detected by GeoChip. The average raw signal intensities and number of probes detected for nitrogen metabolism are 1,931,158±114,209 and 1,260±181, respectively. Major pathways for nitrogen cycling (ammonification, nitrification, denitrification, assimilatory and dissimilatory nitrogen reduction and nitrogen fixation) were identified in our study (Figure 5).

Nitrification genes, *amo*A and *hao*, were identified that indicates presence of ammonia oxidizers in the microbial community of coralloid roots. Elevated normalized gene intensities were from *nar*G, *nir*K, *nir*S and *nos*Z genes involved in denitrification processes. These genes aid in converting nitrate (NO_3^-) or nitrites (NO_2^-) to dinitrogen (N_2) atoms. Significant signals for nitrogen fixation *nif*H gene were also detected that sustains the nitrogen cycle by converting dinitrogen atoms to useful nitrogen forms such as ammonia (NH_3) or ammonium (NH_4^+). And finally, ammonification genes, *ure*C and *gdh*, were present that transforms organic nitrogen to NH_3 (Hoffman et al 2014). GS-GOGAT (glutamine synthetase-glutamate synthase) is the main pathway for nitrogen assimilation (Muro-Pastor et al 2005). Nitrogen assimilation genes, as well as *nir*B, *nas*A, *nrf*A and *nap*A genes for assimilatory and dissimilatory nitrogen reduction, were also identified but detected at lower gene intensities. Likewise, the main enzymes for GS-GOGAT pathway, glutamine synthetase and glutamate synthase, were consistently present as expected

from a nitrogen-fixing organism. Free-living and symbiotic bacteria both have *nif* genes which can also be found in other nitrogen-fixing organisms (Corbin et al 1982; Fay 1992). The main function of these genes is to form nitrogenase complexes for the conversion of unusable atmospheric dinitrogen to useful forms like ammonia and in this process, other *nif* genes (*nif*D and *nif*K) encode a dinitrogenase heterotetramer creating an active site for reducing dinitrogen atoms (Dos Santos et al 2012). The n*if*DK genes combine with an iron protein encoded by *nif*H gene to complete the structure and function of the formed nitrogenase complex (Jasniewski et al 2018). Hence, *nif* genes are essential for microbial nitrogen fixers as lack of these genes may alter their ability to efficiently fix nitrogen for their host (Spaink et al 1998) making them inadequate as beneficial symbionts.

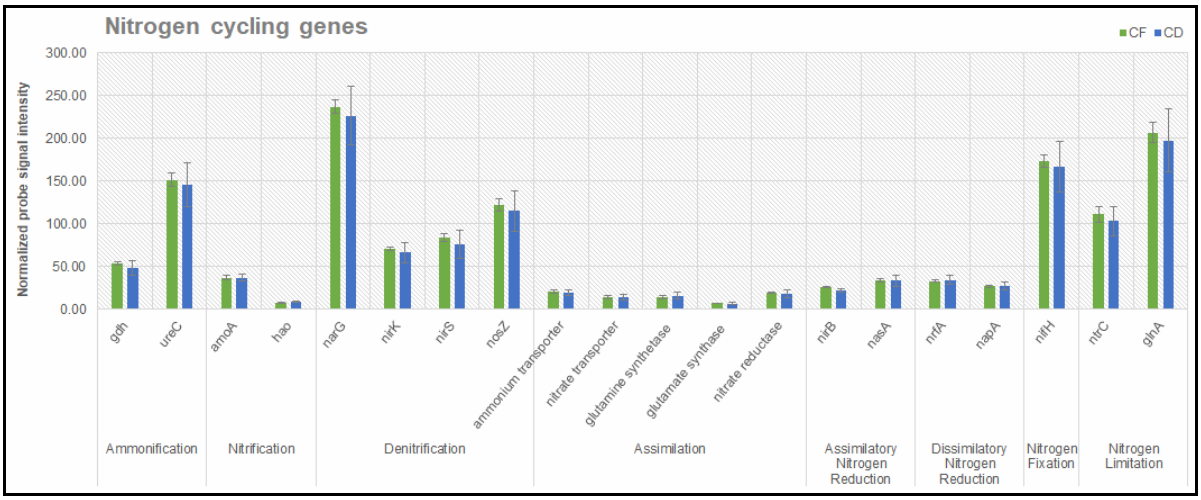

Figure 5. Genes obtaining high normalized probe signal intensities for nitrogen cycling. Error bars represent standard deviation of the means. [*C. debaoensis* (CD) and *C. fairylakea* (CF)].

GeoChip functional profiles showed evidences that nitrogen metabolism is active in coralloid roots with the presence of most functional genes involved in nitrogen cycling. Supporting this are the identified *ntr*C and *gln*A genes for nitrogen limitation where it is widely accepted that formation of heterocyst cells, where nitrogen fixation occurs, are triggered by nitrogen starvation (Bergman et al 1996; Zhang et al 2006). Hence, an increase in nitrogen metabolism, as a response to low nitrogen levels, was evident in the functional profiles of both species of cycads used in this study. Comparing the signal intensities of carbon and nitrogen metabolism, it is apparent that carbon cycling genes surpassed the latter. However, this does not indicate that carbon fixation is the main function of cyanobacteria in coralloid roots. It is best to recall that cyanobacterial chains are made up of vegetative cells where carbon fixation occurs, whereas in regularly-spaced intervals between these cells are heterocysts for nitrogen fixation (Kumar et al 2010). In a single cyanobacterial chain, the ratio of vegetative cells to heterocyst cells are indeed significantly higher and thus, might account for the elevated signals for carbon metabolism detected by GeoChip (Figure 6).

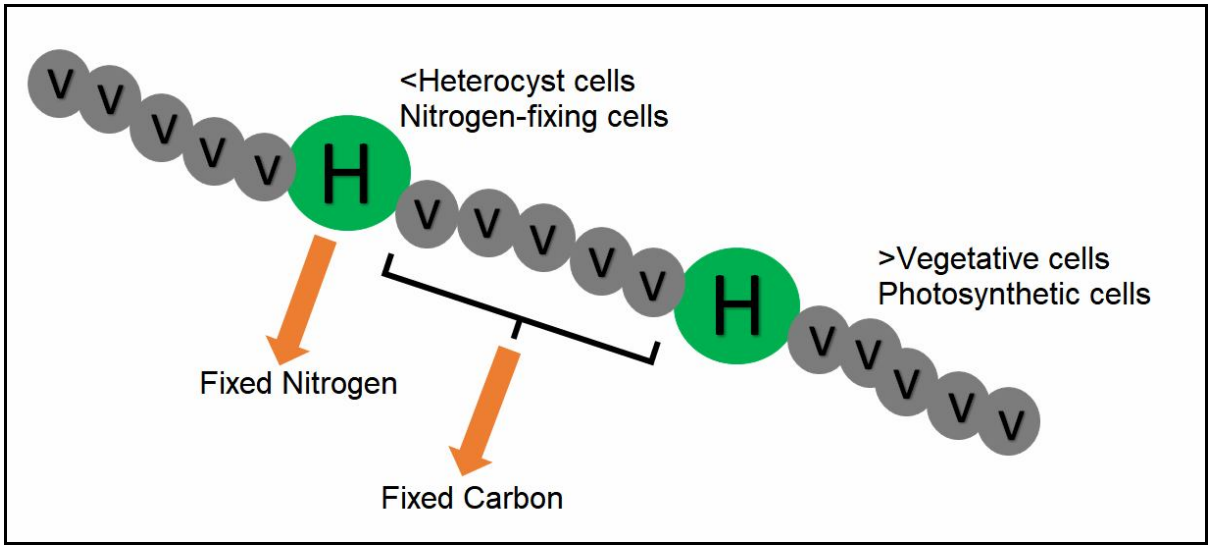

Figure 6. A depiction of ratio between vegetative (v) and heterocyst (H) cells in a cyanobacterial filament accounting for higher presence of carbon fixation genes compared to genes for nitrogen metabolism.

Stress, virulence and other genes. Since coralloid roots are typically exposed to harsh environments in the soil, presence of genes coding for stress-related genes are significant obtaining average raw signal intensities of 8,788,618±265,435 and a total of 5,307±721 probes (Figure 7). Sigma factors 24 and 70 are abundant alongside stress genes for oxygen limitation (*fnr*), osmotic stress (*omp*R) and heat shock proteins (*clp*P). Also considerably high normalized probe signals were detected for genes involved in MFS (2,663±225) and Mex (982±95) antibiotic resistance categories.

MFS, or major facilitator superfamily and Mex systems are bacterial multidrug efflux pumps, which are energy-dependent efflux pumps for the extrusion of various antibiotics making them incapable of effectively damaging their targets (Nikaido 2009). ABC family of antibiotic efflux pump is also present in coralloid roots which is similar to MFS which could help increase resistance to broad-spectrum antibiotics (Lubelski et al 2007). On the contrary, beta-lactamases genes target only specific group of antibiotics (Vila & Martinez 2008). Aside from antibiotics, it is also assumed that multidrug efflux pumps can eliminate toxic compounds such as antimicrobials produced by competing microbes, heavy metals and organic contaminants (Blanco et al 2016). This brings us to the evidences of organic contaminant degradation occurring in coralloid roots as genes for degrading chlorinated solvents (*exa*A and *deh*H), hydrocarbons (*alk*B) and herbicides-related compounds (*atz*A) were also found to be present. And lastly, virulence genes for iron uptake (*iro*), adherence (pilin), toxins (*hly*), antiphagocytosis (*cap*) and type III secretion systems were identified. Some of these genes might be involved in host invasion, with emphasis on entry of hormogonium, the motile stage of cyanobacterium, into the cortical layer of coralloid roots. Though detected at low signals (only < 20 normalized probe signals), virulence factors for cell adhesion, host cell disruption, invasion and intracellular survival was also present which is needed by potential symbionts to successfully enter host tissues.

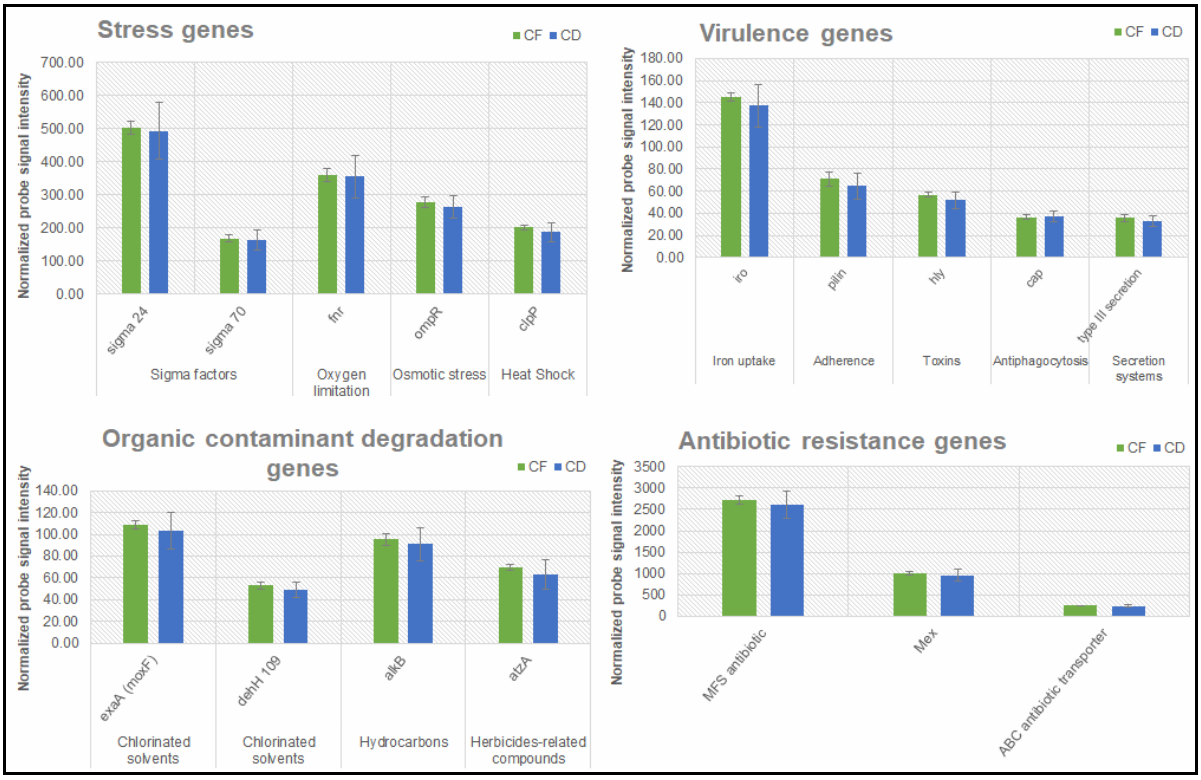

Figure 7. Genes obtaining high normalized probe signal intensities for stress, virulence, organic contaminant degradation and antibiotic resistance. Error bars represent standard deviation of the means. [*C. debaoensis* (CD) and *C. fairylakea* (CF)].

Conclusions. Functional gene array analysis revealed that community gene profiles of coralloid root-associated microbiomes in two species of cycads are significantly common and that the genes detected from microbes are the same regardless of their host species. GeoChip analysis revealed that microbes in coralloid roots primarily work on maintaining metal homeostasis which can be associated to the ability of cycads to grow in poor-conditioned soil and habitats. Cyanobionts, with the help of other microbes in the coralloid roots, appears to be increasing the tolerance and utilization of their hosts to high levels of metals. With respect to nitrogen metabolism, all major genes for nitrogen cycling were successfully identified by GeoChip. Our study therefore supports the role of coralloid roots as nitrogen fixers upon forming a stable symbiotic association with cyanobacteria. The microbiome of coralloid roots also showed active carbon metabolism which might also be attributed to cyanobionts as they are microorganisms capable of photosynthesis. It was formerly believed that cyanobacteria as symbionts of coralloid roots only retain photosynthetic machineries and accessory pigments but do not fix carbon as some enzymes might be lacking. Here, evidences that cyanobacteria are capable of performing Calvin cycle were presented and that it appears to be actively involved in various carbon cycling activities. This also provided insights as to why coralloid roots grow apogeotropically as our data suggest that symbionts probably require access to sunlight causing the negative geotropic growth of coralloid roots via phototropism. Even though GeoChip could only identify existing functional genes, it was still able to provide valuable insights and validate existing theories that gave us a better understanding of how coralloid root functions with its symbionts.

Gene profiling and expression studies for in-depth analysis of the coralloid root symbionts is still in its initial stages and a lot of areas are still waiting to be explored. Upon successful identification of genes proven beneficial for the host, this research will validate the potential use of cyanobacterium and other endophytic microbes as effective bio-inoculants for a more sustainable approach in improving soil fertility and also opens an opportunity to create more effective biofertilizers or root symbionts to a wide range of plants by engineering symbiotic cyanobacterium and other bacteria. Furthermore, it will

uncover the unexplored areas and dynamics involved in cycads-cyanobacteria endosymbiosis. For a more comprehensive approach, we recommend using de novo RNA-Seq technology for the identification of novel transcripts and comparing expression patterns between symbiotic coralloid roots and non-symbiotic normal roots.

Ethics approval and consent to participate. We are declaring that our whole research process did not violate any ethical guidelines.

Consent for publication. All authors read and approved the final manuscript.

Competing interests. All authors declare that there is no conflict of interests.

Acknowledgements. We would like to acknowledge CAS-TWAS President's Fellowship for their academic and material contributions to the main authors. We also would like to thank Mr. Christian Supsup of De La Salle University, Manila, Philippines for his assistance in data analysis using R.

Funding statement. This research was supported by the special financial project from the National Forestry and Grassland Administration for Rescues and Breeding of Wild Rare and Endangered Species (Li N. 2017, 2018, 2019), the Shenzhen Urban Management Bureau (Li N. 201411) and the Shenzhen Key Laboratory of Tropical and Subtropical Plant Diversity, Fairy Lake Botanical Garden, Chinese Academy of Sciences (Chen T. 2017-2019), and South China Botanical Garden (Duan J. 2017–2019), Chinese Academy of Sciences. There was no additional external funding received for this study.

Author contributions. AC, TC and NL designed research. AC and MP performed research and analyzed data. AC, MP, TC, HR, JD and NL wrote the paper.

References

Abebe T., Wise R. P., Skadsen R. W., 2009 Comparative transcriptional profiling established the awn as the major photosynthetic organ of the barley spike while the lemma and the palea primarily protect the seed. The Plant Genome 2(3):247-259.

Adams D. G., Bergman B., Nierzwicki-Bauer S. A., Duggan P. S., Rai A. N., Schüßler A., 2013 Cyanobacterial-plant symbioses. In: The prokaryotes – prokaryotic biology and symbiotic associations. Rosenberg E., DeLong E. F., Lory S., Stackebrandt E., Thompson F. (eds), Berlin: Springer-Verlag pp. 359-400.

Ahern C. P., Staff I. A., 1994 Symbiosis in cycads: the origin and development of coralloid roots in *Macrozamia communis* (Cycadeceae). American Journal of Botany 81(12):1559-1570.

Barona-Gomez F., Corona-Gomez A., Gutierrez-Garcia K., Cruz-Morales P., Suarez-Moo P., Selem-Mojica N., Perez-Farrera M. A., Cibrian-Jaramillo A., 2019 The cycad coralloid root contains a diverse endophytic bacterial community including cyanobacteria encoding specific biosynthetic gene clusters. Genome Biology and Evolution doi: https://doi.org/10.1101/121160.

Bayer K., Moitinho-Silva L., Brummer F., Cannistraci C. V., Ravasi T., Hentschel U., 2014 GeoChip-based insights into the microbial functional gene repertoire of marine sponges (high microbial abundance, low microbial abundance) and seawater. FEMS Microbiology Ecology 90(3):832-843.

Bell-Doyon P., Laroche J., Saltonstall K., Aguilar J. C. V., 2020 Specialized bacteriome uncovered in the coralloid roots of the epiphytic gymnosperm, *Zamia pseudoparasitica*. Environmental DNA 00:1-11.

Berg I. A., Kockelkorn D., Buckel W., Fuchs G., 2007 A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in Archaea. Science 318 (5857):1782-1786.

Bergman B., Matveyev A., Rasmussen U., 1996 Chemical signaling in cyanobacterial-plant symbioses. Trends in Plant Science 1(6):191-197.

Blanco P., Hernando-Amado S., Reales-Calderon J. A., Corona F., Lira F., Alcalde-Rico M., Bernardini A., Sanchez M. B., Martinez J. L., 2016 Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. Microorganisms 4(1):14.

Brenner E. D., Stevenson D. W., Twigg R. W., 2003 Cycads: evolutionary innovations and the role of plant-derived neurotoxins. Trends in Plant Science 8(9):446-452.

Castenholz R. W., Wilmotte A., Herdman M., et al, 2001 Phylum BX. cyanobacteria. In: Bergey's manual of systematic bacteriology. Vol 1, Boone D. R., Castenholz R. W., Garrity G. M. (eds), New York, NY: Springer, pp. 473-599.

Chang A. C. G., Chen T., Li N., Duan J., 2019 Perspectives on endosymbiosis in coralloid roots: association of cycads and cyanobacteria. Frontiers in Microbiology 10:1888.

Chang D. C. N., Grobelaar N., Coetzee J., 1988 SEM observations on cyanobacteria-infected cycad coralloid roots. South African Journal of Botany 54(5):491-495.

Costa J. L., Lindblad P., 2002 Cyanobacteria in symbiosis with cycads. In: Cyanobacteria in symbiosis. Rai A. N., Bergman B., Rasmussen U. (eds), Dordrecht: Kluwer Academic Publishers, pp. 195-205.

Costa J. L., Paulsrud P., Lindblad P., 1999 Cyanobiont diversity within coralloid roots of selected cycad species. FEMS Microbiology Ecology 28:85-91.

Corbin D., Ditta G., Helinski D. R., 1982 Clustering of nitrogen fixation (*nif*) genes in *Rhizobium meliloti*. Journal of Bacteriology 149(1):221-228.

Dos Santos P. C., Fang Z., Mason S. W., Setubal J. C., Dixon R., 2012 Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. BMC Genomics 13:162.

Dupont C. L., Butcher A., Valas R. E., Bourne P. E., Caetano-Anolles G., 2010 History of biological metal utilization inferred through phylogenomic analysis of protein structures. Proceedings of the National Academy of Sciences 107(23):10567-10572.

Fay P., 1992 Oxygen relations of nitrogen fixation in cyanobacteria. Microbiology Review 56(2):340-373.

Fisher R. M., Henry L. M., Cornwallis C. K., Kiers E. T., West S. A., 2017 The evolution of host-symbiont dependence. Nature Communications 8:15973.

Gehringer M. M., Pengelly J. J. L., Cuddy W. S., Fieker C., Forster P. I., Neilan B. A., 2010 Host selection of symbiotic cyanobacteria in 31 species of the Australian cycad genus: *Macrozamia* (Zamiaceae). Molecular Plant-Microbe Interactions 23(6):811-822.

Golden J. W., Yoon H. S., 1998 Heterocyst formation in *Anabaena*. Current Opinion in Microbiology 1(6):623-629.

Grilli Caiola M., 1980 On the phycobionts of the cycad coralloid roots. New Phytologist 85(4):537-544.

Grilli Caiola M., 2001 Cycad coralloid roots housing cyanobacteria. In: Symbiosis: cellular origin, life in extreme habitats and astrobiology. Seckbach J. (ed), Netherlands: Kluwer Academic Publishers, pp. 397-409.

Grobbelaar N., Scott W. E., Hattingh W., Marshall J., 1987 The identification of the coralloid endophytes of the southern African cycads and the ability of the isolates to fix dinitrogen. South African Journal of Botany 53:111-118.

Gutiérrez-García K., Bustos-Díaz E. D., Corona-Gómez J. A., et al, 2019 Cycad coralloid roots contain bacterial communities including cyanobacteria and *Caulobacter* spp. that encode niche-specific biosynthetic gene clusters. Genome Biology and Evolution 11(1):319-334.

Halliday J., Pate J., 1976 Symbiotic nitrogen fixation by coralloid roots of the Cycad *Macrozamia riedlei*: physiological characteristics and ecological significance. Australian Journal of Plant Physiology 3(3):349-358.

He Z., Gentry T. J., Schadt C. W., Wu L., Liebich J., Chong S. C., Huang Z. J., Wu W. M., Gu B. H., Jardine P., Criddle C., Zhou J., 2007 GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. The ISME Journal 1(1):67-77.

Hoffman B. M., Lukoyanov D., Yang Z. Y., Dean D. R., Seefeldt L. C., 2014 Mechanism of nitrogen fixation by nitrogenase: the next stage. Chemical Reviews 114(8):4041-4062.

Hudek L., Ackland M. L., 2017 Selective metal ion homeostasis in cyanobacteria. In: Prospects and challenges in algal biotechnology. Tripathi B. N., Kumar D. (eds), Springer, pp. 219-228.
Huertas M. J., Lopez-Maury L., Giner-Lamia J., Sanchez-Riego A. M., Florencio F. J., 2014 Metals in cyanobacteria: analysis of the copper, nickel, cobalt and arsenic homeostasis mechanisms. Life(Basel) 4(4):865-886.
Iida A., Teshiba S., Mizobuchi K., 1993 Identification and characterization of the *tkt*B gene encoding a second transketolase in *Escherichia coli* K-12. Journal of Bacteriology 175(17):5375-5383.
Jasniewski A. J., Sickerman N. S., Hu Y., Ribbe M. W., 2018 The Fe protein: an unsung hero of nitrogenase. Inorganics 6(1):25.
Kang S., Van Nostrand J. D., Gough H. L., He Z., Hazen T. C., Stahl D. A., Zhou J., 2013 Functional gene array-based analysis of microbial communities in heavy metals-contaminated lake sediments. FEMS Microbiology Ecology 86:200-214.
Kerfeld C. A., Aussignargues C., Zarzycki J., Cai F., Sutter M., 2018 Bacterial microcompartments. Nat Reviews Microbiology 16(5):277-290.
Kumar K., Mella-Herrera R. A., Golden J. W., 2010 Cyanobacterial heterocysts. Cold Spring Harbor Perspectives in Biology 2(4):a000315.
Lindblad P., 2009 Cyanobacteria in symbiosis with cycads. Microbiology Monographs 8:225-233.
Lindblad P., Hallblom L., Bergman B., 1985 The cyanobacterium-*Zamia* symbiosis: C2H2 reduction and heterocyst frequency. Symbiosis 1:19-28.
Lindblad P., Rai A. N., Bergman B., 1987 The *Cycas revoluta–Nostoc* symbiosis: enzyme activities of nitrogen and carbon metabolism in the cyanobiont. Journal of General Microbiology 133:1695-1699.
Lu Z., Deng Y., Van Nostrand J. D., et al, 2012 Microbial gene functions enriched in the deepwater horizon deep-sea oil plume. The ISME Journal 6:451-460.
Lubelski J., Konings W. N., Driessen A. J., 2007 Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. Microbiology and Molecular Biology Reviews 71(3):463-476.
Ma Z., Jacobsen F. E., Giedroc D. P., 2009 Metal transporters and metal sensors: how coordination chemistry controls bacterial metal homeostasis. Chemical Reviews 109(10):4644-4681.
Mason O. U., Nakagawa T., Rosner M., Van Nostrand J. D., Zhou J., Maruyama A., Fisk M. R., Giovannoni S. J., 2010 First investigation of the microbiology of the deepest layer of ocean crust. PLoS ONE 5:e15399.
McLuckie J., 1922 Studies in symbiosis II: the apogeotropic roots of *Macrozamia spiralis* and their physiological significance. Proceedings of the Linnean Society of New South Wales 47:319-328.
Meeks J. C., Elhai J., Thiel T., Potts M., Larimer F., Lamerdin J., Predki P., Atlas R., 2001 An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. Photosynthesis Research 70(1):85-106.
Molouba F., Lorquin J., Willems A., Hoste B., Giraud E., Dreyfus B., Gillis M., De Lajudie P., Masson-Boivin C., 1999 Photosynthetic Bradyrhizobia from *Aeschynomene* spp. are specific to stem-nodulated species and form a separate 16S ribosomal DNA restriction fragment length polymorphism group. Applied and Environmental Microbiology 65(7):3084-3094.
Muro-Pastor A. M., Reyes J. C., Florencio F. J., 2005 Ammonium assimilation in cyanobacteria. Photosynthesis Research 83:135-150.
Nikaido H., 2009 Multidrug resistance in bacteria. Annual Review of Biochemistry 78:119-146.
Obukowicz M., Schallerm M., Kennedy G. S., 1981 Ultrastructure and phenolic histochemistry of the *Cycas revoluta-Anabaena* symbiosis. New Phytology 87:751-759.
Perraju B. T. V. V., Rai A. N., Kumar A. R., Singh H. N., 1986 *Cycas circinalis-Anabaena cycadeae* symbiosis: photosynthesis and the enzymes of nitrogen and hydrogen metabolism in symbiotic and cultured *Anabaena cycadeae*. Symbiosis 1:239-251.

Shcolnick S., Keren N., 2006 Metal homeostasis in cyanobacteria and chloroplasts. Balancing benefits and risks to the photosynthetic apparatus. Plant Physiology 141(3):805-810.
Shi Z., Yin H., Van Nostrand J. D., Voordeckers J. W., Tu Q., Deng Y., Yuan M., Zhou A., Zhang P., Xiao N., Ning D., He Z., Wu L., Zhou J., 2019 Functional gene array-based ultrasensitive and quantitative detection of microbial populations in complex communities. mSystems 4(4):e00296-19.
Shilpi G., Shilpi S., Sunita S., 2015 Tolerance against heavy metal toxicity in cyanobacteria: role of antioxidant defense system. International Journal of Pharmacy and Pharmaceutical Sciences 7(2):9-16.
Spaink H. P., Kondorosi A., Hooykaas P., 1998 The Rhizobiaceae: molecular biology of model plant-associated bacteria. Springer Netherlands, 566 pp.
Tan X., Volbeda A., Fontecilla-Camps J. C., Lindahl P. A., 2006 Function of the tunnel in acetylcoenzyme A synthase/carbon monoxide dehydrogenase. Journal of Biological Inorganic Chemistry 11:371-378.
Thajuddin N., Muralitharan G., Sundaramoorthy M., et al, 2010 Morphological and genetic diversity of symbiotic cyanobacteria from cycads. Journal of Basic Microbiology 50(3):254-265.
Van Nostrand J. D., Yin H., Wu L., Yuan T., Zhou J., 2016 Hybridization of environmental microbial community nucleic acids by GeoChip. Methods in Molecular Biology 1399:183-196.
Vila J., Martinez J. L., 2008 Clinical impact of the over-expression of efflux pump in nonfermentative Gram-negative bacilli, development of efflux pump inhibitors. Current Drug Targets 9(9):797-807.
Wang D. Y., 1996 Taxonomy of *Cycas* in China. In: Cycads in China. Wang F. X., Liang H. B., Chen T. Q., Wang D. Y. (eds), Guangdong Science and Technology Press, Guangzhou, pp. 13-19.
Waldron K. J., Robinson N. J., 2009 How do bacterial cells ensure that metalloproteins get the correct metal? Nature Reviews Microbimicrobiology 6:25-35.
Woodward J., Orr M., Cordray K., Greenbaum E., 2000 Enzymatic production of biohydrogen. Nature 405:1014-1015.
Yamada S., Ohkubo S., Miyashita H., Setoguchi H., 2012 Genetic diversity of symbiotic cyanobacteria in *Cycas revoluta* (Cycadaceae). FEMS Microbiology Ecology 81(3):696-706.
Zak E., Norling B., Maitra R., Huang F., Andersson B., Pakrasi H. B., 2001 The initial steps of biogenesis of cyanobacterial photosystems occur in plasma membranes. PNAS 98(23):13443-13448.
Zhang C. C., Laurent S., Sakr S., Peng L., Bedu S., 2006 Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. Molecular Microbiology 59(2):367-375.
Zheng Y., Chiang T. Y., Huang C. L., Gong X., 2018 Highly diverse endophytes in roots of *Cycas bifida* (Cycadaceae), an ancient but endangered gymnosperm. Journal of Microbiology 56(5):337-345.
Zheng W., Song T., Bao X., Bergman B., Rasmussen U., 2002 High cyanobacterial diversity in coralloid roots of cycads revealed by PCR fingerprinting. FEMS Microbiology Ecology 40:215-222.
Zhong Y. C., Chen C. J., 1997 *Cycas debaoensis* Y.C. Zhong et C.J. Chen - a new cycad from China. Acta Phytotaxon. 35(6):571.

Received: 23 June 2020. Accepted: 28 July 2020. Published online: 12 August 2020.
Authors:
Aimee Caye Garcia Chang, South China Botanical Garden, Chinese Academy of Sciences, No. 723 Xingke Road, Tianhe District, Guangzhou 510650, China; Fairy Lake Botanical Garden, Chinese Academy of Sciences, 160 Xianhu Road Liantang, Luohu District Shenzhen Guangdong 518004, China; University of Chinese Academy of Sciences, No. 80 Zhongguancun East Rd, Haidian District, Beijing, China, e-mail: aimee.caye.chang@gmail.com
Melissa H. Pecundo, South China Botanical Garden, Chinese Academy of Sciences, No. 723 Xingke Road, Tianhe District, Guangzhou 510650, China; Fairy Lake Botanical Garden, Chinese Academy of Sciences, 160 Xianhu Road Liantang, Luohu District Shenzhen Guangdong 518004, China; University of Chinese Academy of Sciences, No. 80 Zhongguancun East Rd, Haidian District, Beijing, China, e-mail: melissa.pecundo@gmail.com
Jun Duan, South China Botanical Garden, Chinese Academy of Sciences, No. 723 Xingke Road, Tianhe District, Guangzhou 510650, China, e-mail: duanj@scib.ac.cn
Hai Ren, South China Botanical Garden, Chinese Academy of Sciences, No. 723 Xingke Road, Tianhe District, Guangzhou 510650, China, e-mail: renhai@scib.ac.cn
Tao Chen, Fairy Lake Botanical Garden, Chinese Academy of Sciences, 160 Xianhu Road Liantang, Luohu District Shenzhen Guangdong 518004, China, e-mail: taochen.mobg@gmail.com
Nan Li, Fairy Lake Botanical Garden, Chinese Academy of Sciences, 160 Xianhu Road Liantang, Luohu District Shenzhen Guangdong 518004, China, e-mail: andreali1997@126.com

How to cite this article:
Chang A. C. G., Pecundo M. H., Duan J., Ren H., Chen T., Li N., 2020 GeoChip 5.0 microarray: a descriptive overview of genes present in coralloid root microbiomes of *Cycas debaoensis* and *Cycas fairylakea*. ELBA Bioflux 12(1):5-20.