# Title page

**The cycad coralloid root contains a diverse endophytic bacterial community with novel biosynthetic gene clusters unique to its microbiome**

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## Keywords

Cycad, *Dioon*, coralloid root, microbiome, sub-community co-culture, cyanobacteria, *Nostoc*, specialized metabolites.

## Abstract

Cycads are the only gymnosperms and ancient seed plants that have evolved a specialized coralloid root to host endophytic bacteria. There are no studies exploring the taxonomic, phylogenetic and functional diversity of the bacterial endophyte microbiome of this 300 million-year old symbiosis. We provide a genomic characterization of the cycad coralloid root microbiome of the Mexican cycad *Dioon merolae* collected from their natural environment. We employed a co-culture-based metagenomics experimental strategy jointly with phylogenomic analyses to reveal both predominant and rare bacteria, to capture biological diversity, and also the presence of biosynthetic gene clusters associated with specialized metabolites. Most taxa were identified as diazotroph plant endophytes that include undescribed taxa and at least 27 genera belonging to 17 bacterial families in addition to Cyanobacteria. Three cyanobacteria genomes obtained from our samples formed a monophyletic group, suggesting a level of specialization characteristic of co-evolved symbiotic relationships. This contrasted with our finding of their large genome sizes and their broad biosynthetic potential, distinctive of facultative endosymbionts of complex alternative lifestyles. Nine out of 23 novel biosynthetic gene clusters identified after detailed genome mining are specific to these coralloid root endophytes, including a NRPS system predicted to direct the synthesis of nostoginins, protease inhibitors whose biosynthetic pathway remains to be discovered. Combined, our results show that the highly diverse taxonomic composition of the coralloid root and

its biosynthetic repertoire, correlate more with a degree of specificity to the cycad plant host than to other closely related plant endosymbionts or to the environment. We support the growing notion that plant-bacteria relations occur under heavy influence of chemical and genomic interactions, and we add to the understanding of the evolution of cycad-bacteria microbiome, with a bearing on bioprospecting of natural products for drug discovery and other applications.

## Background

Cycads (Cycadales) are the only early seed plants and the only gymnosperms that develop coralloid roots, a specialized root dichotomous and coral-like in appearance typically growing above ground, which acquires and maintains bacteria [1] (**Fig. 1**). The coralloid root is present in all cycad lineages, likely due to its adaptive value as a significant source of fixed nitrogen for the plant [2]. In natural habitats coralloid roots appear in the most vulnerable early life stages [3], or as adults in habitats with poor or inaccessible nutrients [4] such as sand dunes, sclerophyll forests, steep rock outcrops with high exposure to salt, and lowland forests with recurrent fires. The cycad coralloid root is probably a key trait that enabled cycads to thrive and adapt to novel environments for millions of years.

Coralloid root endophytes have been studied since the 19$^{th}$ century ([5] and references therein). However, most studies have focused on resolving the biology or taxonomy of the Cyanobacteria, and most samples have been collected from botanic garden collections or grown in greenhouses, typically outside of the cycad host natural range [6-12]. Anatomical studies have shown the presence of mucilaginous or protein-rich material that hosts other unidentified bacterial groups [5, 13, 14], with only a few

72 specific bacterial taxa suggested [15-19]. Studies testing for the specificity of
73 cyanobacteria and the cycad host have been conducted in plants collected outside of
74 their native distribution, with contrasting results regarding the specialization of coralloid
75 root symbionts [5, 15, 20]. Moreover, the handful of field-based studies from wild
76 cycad populations, focused only on cyanobacteria identified with molecular markers
77 [11, 21], and show that diversity ranges from a single cyanobacteria strain inside an
78 individual root, to diverse species complexes among roots, and within and among
79 various cycad genera. Studies on the origin and transmission of bacterial endophytes are
80 also inconclusive [12], thus the degree of cycad-bacteria co-evolution in this symbiotic
81 system remains a mystery.

82 In addition to nitrogen fixation there have been suggestions of additional
83 -unknown- roles for the coralloid root, but there is no clear evidence of its broader
84 function to date [5]. Likewise, various chemical, physical and physiological
85 mechanisms appear to regulate the cycad-bacteria interaction [22, 23], but no genes
86 involved in novel specialized metabolite production in the light of the symbiosis have
87 been identified. In all, the taxonomic composition and the function of the cycad
88 coralloid root microbiome, defined as the bacteria living inside this specialized organ
89 plus their genes and products, remains undescribed almost entirely. What is more, the
90 evolutionary history of the microbiome within a *ca.*300 million-year-old symbiotic
91 plant-bacteria relationship is still incipiently explored.

92 Our goal in this study is to investigate the microbiome of the coralloid roots of
93 *Dioon merolae* [24]. *Dioon merolae* is a long-lived, entomophilous, dioecious, and
94 arborescent cycad native to Mexico [25]. We collected coralloid root samples from wild
95 populations in two different habitats from its natural range, currently distributed in

96 moderate population sizes of a few hundreds of individuals throughout Chiapas and
97 Oaxaca in the south of Mexico [25]. The availability of whole-genome and
98 metagenomic sequencing enabled us to provide insights on the diversity and
99 phylogenetic distribution of its endophytes and their cycad-related specialized
100 functions.

101 The presence of uniquely specialized metabolites in the cycad coralloid root
102 microbiome was of particular interest to us because they may be a result of co-evolution
103 between the cycad host and the endophyte bacterial community. Bacteria have dynamic
104 genomic diversity and the capacity to synthesize specialized metabolites with
105 overwhelming chemical diversity that are produced to cope with biotic and abiotic
106 pressures [26]. Bacteria codify specialized metabolites in rapidly evolving genetic units
107 called biosynthetic gene clusters (BGCs) of about 25-40 Kbp. The ability to capture and
108 retain bacteria in the coralloid root could provide a mechanism for cycads to adapt
109 quickly to local conditions by increasing their specialized metabolite repertoire, in a
110 known host and environment. From a more anthropocentric view, conserved BGCs of
111 the coralloid root bacterial endophytes may also be of interest as a source of novel
112 natural products for drug discovery.

113 To overcome technical difficulties in characterizing the breadth of microbial
114 diversity in environmental samples, we used an enrichment co-culture strategy of sub-
115 communities obtained from the original sample [27]. We employed complementing
116 microbiological, genomic and metagenomic sequencing, and phylogenomic approaches
117 to characterize the coralloid microbiome's taxonomic diversity and gain insights into its
118 function. Our study is the first to characterize the taxonomy and function of the

119 coralloid root beyond cyanobacteria, providing a glimpse into the evolutionary history
120 of the cycad-bacteria coralloid root system.
121

122 ## Methods

123 ***Overall strategy.*** We used a combined co-culture, metagenomics and phylogenomic
124 strategy to detect and measure taxonomic diversity, phylogenetic relationships and
125 biosynthetic potential in the endophytes of the cycad coralloid root, as previously
126 described under the term of EcoMining [27] (**Fig. 1**). In this approach, we grew and
127 isolated bacteria from environmental samples using a diverse set of media that aim to
128 capture all possible cultivable bacterial diversity (*t0*). Simultaneously, we enriched the
129 same samples in co-cultures grown under specific conditions for cyanobacteria using
130 BG11 media. In addition to this autotrophic bacterial group, this approach captures
131 other bacterial groups that have interactions with cyanobacteria, present in the original
132 sample at low titers. We allowed the co-culture to grow over time and sampled it after
133 one month (*t1*) and at the end of a year (*t2*) to capture organisms that depend on other
134 bacteria of the community to grow. We isolated axenic bacteria (*t0* and *t1*) and sub-
135 communities in co-cultures (*t1* and *t2*), and reconstructed phylogenetic relationships and
136 assessed taxonomic diversity, using 16S rRNA and metagenomic OTUs (mOTUs) data,
137 respectively. Furthermore, genomes of isolated endophytes were obtained and
138 thoroughly mined together with metagenomes for BGCs potentially directing the
139 synthesis of specialized metabolites.
140

141 ***Field collections***. We sampled coralloid roots from two wild cycad populations
142 previously reported [25]. In March of 2014 we sampled from two sites in deciduous

143 tropical forests, at Jiquipilas, Mexico (JP or dry; Lat 16° 37' 26.87''N, Long 93° 34'
144 34.64" O) at 560m above mean sea level, with an average annual precipitation of 320
145 mm and average annual temperature of 18 °C; and Raymundo Flores Mexico (RF or
146 humid; Lat 16° 3' 26.75''N, Long 93° 35' 55.26" O) at 900m above mean sea level,
147 with 2500 mm and 25°C annual average precipitation and temperature, respectively. In
148 some cycad plants, coralloid roots were easily visible aboveground, while in others we
149 dug to about 30 cm around the main root until coralloid roots were found. In a
150 population of approximately 40 individuals, we generally found 10-12 coralloid roots,
151 in almost exclusively juvenile plants. A total of 10 coralloid apogeotropic roots were cut
152 from 10 plants, cleaned with sterile distilled water to remove soil excess, placed in 15
153 ml sterile Falcon tubes (Beckton Dickinson), and transported immediately to the
154 laboratory at room temperature.
155
156 ***Coralloid root processing.*** We focused our effort on three samples of three individuals
157 with the largest coralloid roots, in each of the two sites, Jiquipilas (JP or dry) and
158 Raymundo Flores (RF or humid) for a total of six coralloid root samples (JP1, JP2, JP6
159 and RF1, RF3 and RF9), and stored the remaining samples at -80 °C for subsequent
160 studies. When DNA samples from these individuals were pooled for sequencing
161 purposes they are referred to as JPPOOL or RFPOOL, respectively. We treated the
162 coralloid root in a laminar flow hood (Nuaire Model Nu-126-400) with a series of
163 washes to remove exogenous bacteria from the rhizosphere or other contamination
164 sources. Each root was introduced in 50 ml sterile Falcon tubes containing 10 ml of
165 each of the following solutions, and gently stirred for: three minutes in hydrogen
166 peroxide ($H_2O_2$), seven minutes in 70% ethanol, 30 seconds in sterile dd-MilliQ water,

167 four minutes in 6% sodium hypochlorite (NaClO), and three one-minute washes in
168 sterile dd-MilliQ water. After this procedure, we plated out water from the last wash in
169 Petri dishes containing the five media described below. Lack of growth in the last wash
170 was considered a negative control, and only samples complying with this criterion were
171 used for endophyte isolation. We undertook two approaches to bacterial isolation (Fig.
172 1): sampling without enrichment directly from field samples (*t0*), and sampling from the
173 enriched co-cultures (*t1*), as described in the following sections.
174
175 ***Bacterial isolation.*** To isolate bacteria from field samples before (*t0*) and after (*t1*)
176 enrichment, macerated roots or co-culture broth were used as inoculant, respectively.
177 Loss of some bacterial groups that were present in the sample collected from the
178 environment (*t0*) is expected. However, after enrichment (*t1*) we recover bacteria that
179 were initially present in low abundances and required time to grow, and that did so as a
180 response to the community nutritional interactions (e.g. amino acids derived from the
181 process of fixing nitrogen) [27]. Roots were macerated in 10 ml of sterile water using a
182 pestle and mortar until plant material was completely disintegrated. We used 100 µl
183 from the root macerate to directly isolate bacteria in Petri dishes containing six different
184 media, chosen to selectively (oligotrophic, four media) or non-selectively (eutrophic,
185 two media) recover bacterial diversity as much as possible. The four selective media
186 used were chosen to target bacterial groups that are known to be either plant endophytes
187 or rhizosphere bacteria, and included: 1) *Caulobacter* medium (glucose: 1 g/L; peptone:
188 1g/L; yeast extract: 1.5 g/L; trace metals solution: 1 mL/L; and 10 g/L of agar for solid
189 medium) [28]; 2) *Rhizobium* medium (mannitol: 10 g/L; dipotassium phosphate: 0.5
190 g/L; magnesium sulfate: 0.2 g/L; yeast extract: 1 g/L; sodium chloride: 0.1 g/L; final pH

191 6.8; and 20 g/L for solid medium [29, 30]; 3) ISP4, for isolation of actinomycetes
192 (starch: 10.0 g/L; dipotassium phosphate: 1 g/L; magnesium sulfate: 1 g/L; sodium
193 chloride: 1 g/L; ammonium sulfate: 2 g/L; calcium carbonate: 2 g/L; ferrous sulfate: 1
194 mg/L; magnesium chloride: 1 mg/L; zinc sulfate: 1 mg/L; final pH 7.0; and 20 g/L for
195 solid media) [31]; 4) BG-11, a cyanobacteria medium (sodium nitrate: 1.5 g/L;
196 dipotassium phosphate: 0.04 g/L; magnesium sulfate: 0.075 g/L; calcium chloride:
197 0.036 g/L; citric acid: 0.006 g/L; ferric ammonium citrate: 0.006 g/L; EDTA (disodium
198 salt): 0.001 g/L; sodium carbonate: 0.02 g/L; final pH 7.1 and agar solid media 10.0
199 gr/L [32]. The non-selective, rich media, included: 5) Nutrient Broth (BD Bioxon,
200 Mexico); and 6) As in *Caulobacter* medium, but supplemented with mannitol
201 (*Caulobacter* + mannitol medium): 1g/L, with aim of providing a carbon source closer
202 to that hypothetically encountered inside the cycad root.
203
204 ***Bacterial sub-communities cultivation.*** We took 100 µl of the macerated roots that
205 passed the negative growth controls after the final washing step (i.e. samples JP1, JP2,
206 JP6 and RF1, RF3 and RF9, which also lead to JPPOOL and RFPOOL samples as
207 described next), and inoculated 100 ml of media in 250 ml flasks. The remaining
208 macerated roots not used for fresh cultures were kept as frozen stocks for future studies
209 (-80 °C in 10% glycerol), although community viability after freezing is expected to
210 diminish over time. We used one non-selective eutrophic medium, i.e. enriched
211 *Caulobacter* + mannitol medium (medium No. 6), which we expected to favor growth
212 of the majority of the generalist taxa in the root bacterial community; and one selective
213 oligotrophic medium, i.e. BG11 (medium No. 4). This medium lacks a carbon source
214 but contains a limited amount of inorganic nitrogen. BG11 cyanobacteria-centric co-

215 cultures were grown for up to one year with constant stirring, with cycles of 16/8 hours
216 of light/darkness per day. Eutrophic cultures were sampled after 72 hours, and their
217 DNA extracts pooled (JPPOOL and RFPOOL), whereas sampling of the oligotrophic
218 co-cultures was done after 1 month (*t1*) and 1 year (*t2*), and treated independently.
219 Moreover, bacterial isolates were only obtained for the former, whereas for both time
220 points shotgun metagenomics were obtained, allowing for genome mining of
221 specialized metabolites.
222
223 ***Genomics and shotgun metagenomics.*** To sequence metagenomes from enriched sub-
224 community co-cultures, we collected their biomass by centrifugation (6000 RPM during
225 15 minutes) and used for DNA extraction using a CTAB-phenol chloroform standard
226 protocol. Isolate 106C, obtained from sample JP6, and isolate T09, obtained from
227 coralloid roots of *Dioon caputoi* from an unrelated environment (Xeric shrubland,
228 Tehuacan valley, Mexico), were both grown on BG11 plates. Genomic DNA from these
229 cultures was obtained with exactly the same CTAB-phenol chloroform protocol.
230 Genomic and metagenomic DNA samples were processed with truseq nano kit Q28 and
231 were sequenced at Langebio, Cinvestav (Irapuato, Mexico) using the MiSeq Illumina
232 platform in the 2X250 Paired end reads format (T09) and the NextSeq mid output
233 2X150 paired end read format (106C y RF3-1yr). The reads for each library were
234 filtered with fastQ and trimmed using Trimommatic version 0.32 [33], and assembled
235 using Velvet 1.2.10 [34] with different *k*-mers: the assemblies with the largest length
236 and the smaller number of contigs were selected and annotated using RAST [35]. The
237 assembly of "*Nostoc* sp. 1031Ymg" was obtained from metagenomic reads of co-
238 culture RF3- t2. These reads were filtered by mapping them against the assembly of

239 *Nostoc* sp. 106C with BWA [36]. The resulting reads were assembled with Velvet using
240 different *k*-mers: the assemblies with the largest length and the smaller number of
241 contigs were selected and annotated using RAST [35]. JPPOOL and RFPOOL
242 metagenomes from eutrophic conditions were obtained after pooling DNA samples
243 from JP and RF, respectively, and treated as individual samples.
244
245 ***Taxonomic diversity***. We first estimated taxonomic diversity using the 16S rRNA gene
246 as a marker for our entire bacterial endophyte collection. PCR products of 1.4 Kbp in
247 length, obtained using the F27 and R1492 primers [37], were obtained and sequenced
248 using the Sanger method (ABI 3730xl). The taxonomic identification was made using
249 Blastn with an initial cut-off e-value of 1e-5 against the SILVA database [38]. We used
250 the phylogenetic position of the top 10 hits from each search without duplicated
251 matches, to determine both taxonomic diversity and phylogenetic relationships.
252 To measure the taxonomic composition of the sub-community co-cultures from
253 metagenomes, we contrasted different methods of OTU identification and abundance
254 that we presumed would be able to capture the breadth of taxa in our samples. We were
255 particularly concerned with capturing cyanobacteria diversity. First, we used mOTUS, a
256 method based on single-copy marker genes obtained from metagenomes and reference
257 genomes [39]. We trimmed and filtered the Illumina reads and kept those with a
258 minimum cutoff identity of 93%, and all other parameters as default. Taxa abundance
259 from mOTUs, defined as the percentage of the genera present in each sample, was
260 calculated with the Vegan v2.3-5 package in R [40]. We estimated the efficiency of our
261 sequencing effort with respect to the total possible taxa per metagenome using the
262 rarefaction method based on [41]. To do this we calculated the proportional number of

263 sequences for each metagenome, in which the richness of mOTUs is sub-sampled
264 randomly from the entire community.
265 Second, we used Kraken, a taxonomic analyzer to assign taxonomic labels to
266 metagenomic DNA sequences based on exact alignment of *k*-mers [42]. Kraken is a
267 taxonomic analyzer based on assigned taxonomy to short DNA reads, using a reference
268 data base to identify alignments and the lowest common ancestor [42]. We implemented
269 Kraken using the pipeline available at http://ccb.jhu.edu/software/kraken/ in our cluster
270 Mazorka with five nodes each with 2 Intel Xeon E5-2650 @ 2.30GHz CPUs
271 ("Haswell", 10 cores/socket, 20 cores/node) and 768 GB of RAM memory. We used
272 Kraken-build to make a standard Kraken database using NCBI taxonomic information
273 for all bacteria, as well as the bacterial, archaeal and viral complete genomes in RefSeq
274 (October 2016). This database contains a mapping of every *k*-mer in Kraken's genomic
275 library to the lowest common ancestor in a taxonomic tree of all genomes that contain
276 that *k*-mer. We summarized the results in genera-level tables for each metagenome and
277 filtered taxonomy hits that had one or more reads assigned directly to a taxon.
278 Our third method to estimate metagenomic taxonomic diversity was MG-RAST
279 [43], which we used to annotate each metagenome at the level of genera using the
280 default parameters, and selected only taxa that had at least 10,000 number of reliable
281 hits. Each taxonomic annotation indicates the percentage of reads with predicted
282 proteins and ribosomal RNA genes annotated to the indicated taxonomic level.
283 To visualize shared taxa among metagenomes, and their abundance, we used
284 Cytoscape v3.4.0 [44], where each node and its size represent the abundance of an
285 OTU, and lines represent shared taxa between metagenomes. The network was made by
286 an interaction matrix, where each of the OTUs that had more than 14 readings assigned

287 directly by Kraken identification, was linked to the metagenome from which it came.
288 Identified nodes were manually ordered to prevent visual overlap. We also calculated
289 the Shannon-Weaver *H'* and Simpson *L* indices for OTUs from all three methods using
290 the Vegan v2.3-5 package in R [40].
291
292 ***Reconstruction of phylogenetic relationships.*** We aligned annotated 16S rRNA
293 sequences trimmed to 1.1 Kbp, using MUSCLE v3.8.31 with default parameters [45].
294 This matrix was used for phylogenetic reconstruction using MrBayes v3.2 [46] with a
295 gamma distribution type range with 1,000,000 generations. ModelTest [47] showed that
296 Kimura 2 parameters was the best substitution model. To explore major clades in more
297 detail, we estimated individual phylogenies for each of the genera in our main tree and
298 represented them graphically. To do this we first recovered a tree by generating a
299 consensus sequence from all genera within each clade in MUSCLE v3.8.31 with default
300 parameters [45]. Then a Bayesian phylogeny with a gamma distribution and a million
301 generations (additional generations did not change our results) was reconstructed using
302 MrBayes v3.2 for each individual genus dataset. The resulting trees were edited and
303 sorted with Environment for Tree Exploration Toolkit v3.0.0b35 [48] in Python v2.7.6.
304 To construct a complete phylogeny of cyanobacteria strains we used the amino
305 acid sequences of GyrB and RpoB as markers [49]. However, their corresponding
306 phylogenies lacked support and resolution even after concatenation, thus we included
307 into the matrix orthologs of the Carbamoyl-phosphate synthase large subunit (CPS),
308 Phenylalanine-tRNA ligase beta subunit (PheT) and the Trigger factor (Tig). Sequences
309 of RpoB, GyrB, CPS, PheT and Tig were extracted from an in-house database of
310 cyanobacterial genomes obtained from GenBank, and annotated using RAST [35]. The

311 sequences were obtained using Blast with a cut-off e-value of 1e-50 and a bitscore of
312 200. Each set of sequences were aligned using MUSCLE v3.8.31 with default
313 parameters [45], and trimmed manually. Independent phylogenies were performed for
314 each marker to filter out redundant and divergent sequences. The sequences that passed
315 this filter were included in the final array, which included the organisms for which all
316 five markers could be retrieved. The final matrix included 289 taxa, with 3617
317 aminoacids, and it was used to reconstruct a tree with MrBayes, using a mixed
318 substitution model based on posterior probabilities (aamodel[Wag]1.000) for proteins
319 for a 10 million generations. Convergence of runs was reached after 1 million
320 generations.

321 Finally, a high resolution cyanobacteria phylogenetic tree was constructed
322 using a set of 198 conserved proteins (Additional file 1: **Table S1**), which represent the
323 core of a set of 77 cyanobacterial genomes (Additional file 2: **Table S2**) including our
324 two isolates (T09 and 106C) and the RF31YmG assembly; and *Fischerella* sp. NIES
325 3754 and *Hassallia byssoidea* VB512170 as outgroups. We extracted and assembled the
326 cyanobacterial genomes from the metagenome RF3-T2. To obtain the RF31YmG
327 genome, contigs from the 106C assembly were used as reference to match and extract
328 reads from the RF3-t2 metagenome using BWA [36]. The obtained reads were
329 assembled using Velvet with the extension columbus with different *k*-mers. The best
330 assembly, considered as the largest assembly with the lower number of contigs, was
331 selected and annotated with RAST as previously. The core genome was obtained using
332 an in-house script available at https://github.com/nselem/EvoDivMet/wiki, which will
333 be reported elsewhere in due course. Then, a set of 198 core proteins was selected from
334 only 33 Nostocales genomes in our database to construct the final concatenated matrix,

335 which included 45477 amino acids. We used this matrix to reconstruct a phylogeny
336 using MrBayes v3.2 with a mixed model (not partitioned), for a million generations.
337
338 ***Genome mining for BGCs.*** To identify BGCs potentially directing the synthesis of
339 specialized metabolites among selected cyanobacteria, we annotated the genome of the
340 isolate 106C with antiSMASH [50]. The predicted BGCs were used as a reference for
341 further searches among the selected genomes. For this purpose we used our in-house
342 pipeline, called CORASON (available at https://github.com/nselem/EvoDivMet/wiki),
343 which will be reported elsewhere in due course. CORASON allows for the
344 identification of conserved and unique BGCs among the selected genomes. Prediction
345 of the chemical structures of the putative specialized metabolites associated with these
346 BGCs was done after domain identification and specificity prediction, mainly of
347 adenylation and acyl transfer domains, with NRPS-PKS server [51], PRISM [52] and
348 antiSMASH [50].
349

350 ## Results

351 Our experimental strategy (**Fig. 1**) to characterize the taxonomic diversity of the
352 coralloid root endophytic microbiome led to hundreds of bacterial isolates obtained
353 directly from the original sample (*t0)*; and from enriched sub-communities in
354 oligotrophic (BG11) medium (*t1*), aimed at promoting interactions between members of
355 the coralloid root community. Individual markers and genomic sequences obtained from
356 these isolates captured the taxonomic diversity of endophytes living in the root,
357 including bacteria present in low titers in the original sample (*t2*). It also provided a
358 mean to obtain insights into the biosynthetic potential specific to the cyanobacteria

359 inhabiting the coralloid root, which could be driving community interactions. In the
360 following sections we describe the results obtained from this effort in three sub-
361 sections, overall taxonomic diversity, cyanobacteria phylogenetic relationships and
362 specificity of BGCs present in the *Dioon* coralloid roots.
363
364 ***Dioon* coralloid roots show ample endophyte diversity of taxa beyond and within**
365 **cyanobacteria.**
366
367 *Taxa assessment based in 16S rRNA.* Cultivable bacteria constitute only a biased subset
368 of the total endophyte biodiversity, yet from our 16S rRNA sequences alone we found
369 470 isolates grouped into 242 OTUs, distributed in 17 families and 11 bacterial orders,
370 with 27 genera in total, representing most of the known bacterial groups (**Table 1**. See
371 also Additional file 3: **Table S3**). As seen in our 16S rRNA phylogenetic reconstruction
372 (**Fig. 2**), all of our sequences grouped within monophyletic clades, and most trees
373 within each clade show that there are new species that remain to be described, in almost
374 all of the genera found within the cycad coralloid root (see also Additional file 4: **Fig.**
375 **S1**). An 87% of the taxa identified can be taxonomically classified as diazotrophic plant
376 endophytes, validating our endophyte isolation procedures (see Materials & Methods).
377 Indeed, most OTUs grouped within the genera *Streptomyces, Bacillus, Rhizobium,*
378 *Stenotrophomonas, Pseudomonas, Mitsuaria, Achromobacter* and *Burkholderia*, which
379 are known for their extraordinary taxonomic diversity, their ability to establish
380 symbiont relationships across the tree of life, or are commonly found in the soil or the
381 plant rhizosphere.

382 We confirmed previous reports of other bacteria associated to the cycad
383 coralloid root, namely, *Bacillus*, which was previously reported as associated to the
384 outside of the coralloid root; *Streptomyces*, previously isolated as an epiphyte [23],
385 which grew on our selective media (ISP4); and *Pseudomonas* [19] growing indistinctly
386 in our four non-selective media. As expected, we confirmed endophytes that belong to
387 *Nostoc* [5], but also found *Tolypothrix*, a previously unreported genus of Nostocales
388 living in the coralloid root. We isolated six strains belonging to this genus according to
389 16S rRNA characterization.
390 Our results also show that OTUs are shared among samples and species, with no
391 specific distribution among the various isolation culture media (**Fig. 2**). There are
392 environment-specific trends such as higher diversity in the dry environment. We
393 observed a tendency in the 16S rRNA data showing that some genera occur only in dry
394 (JP; e.g. *Rhizobium*), or only in humid (RF; e.g. *Xanthomonas*) forest environments,
395 with a few genera occurring in both (e.g. *Burkholderia*). In terms of species diversity
396 and abundance, the Shannon-Weaver and Simpson biodiversity indices based on genera
397 abundance from 16S rRNA sequences have higher diversity in the dry environment than
398 in the humid environment (Additional file 5: **Table S4**). We consider these results
399 preliminary and limited by the use of cultivable approaches, but valid as they compare
400 samples treated under the same conditions and thus informative to define further
401 ecological studies.
402
403 *Taxa assessment based in co-cultures metagenomics.* We extracted and sequenced
404 whole-community metagenomic DNA from *t1* and *t2* subcommunity co-cultures with
405 the aim of enriching for specific interactions in response to growth conditions. We were

406 able to sequence metagenomes from six different individuals grown on eutrophic
407 conditions after 72 hours, whose DNAs were pooled as limited diversity was expected
408 (JPPOOL and RFPOOL); from four different individuals after 30 days of culture in
409 oligotrophic conditions, two from each of the two environments (JP2, JP6 and RF1,
410 RF3); and after 365 days, same conditions, one from each environment (JP6 and RF3)
411 (**Table 2**. see also Additional file 6: **Table S5**).

412 In terms of taxonomic diversity, each OTU-assignment strategy recovered
413 different taxa and in different proportion (Table 2). Notably, despite visual confirmation
414 of the occurrence of heterocyst-forming cyanobacteria in green cultures (Additional file
415 7: **Fig. S2**), mOTUS revealed only a minor proportion of cyanobacteria, only 6%. In
416 contrast, MG-RAST likely overestimated diversity at 39%. Kraken provided and
417 intermediate result with 12%. Kraken is also a sequence classification technique that
418 can exclude sequence contaminants from the draft assembly, allowing us to generate a
419 symbiotic cyanobacteria marker database as reference for future classification. Thus,
420 Kraken-identified OTUs were used for all subsequent analyses.

421 In Kraken-based OTUs, specifically associated to one of the metagenomes (JP),
422 we also found *Calothrix*, previously reported in *Encephalartos* [16, 17] and in *Cycas*
423 *revoluta* [18]; and *Caulobacter*, which can be found associated to cyanobacteria [19].
424 Of the Nostocales we were unable to recover *Tolypothrix* in the metagenomes. Notably,
425 taxa identified in the four metagenomes mostly overlap (**Fig. 3**. See also Additional file
426 8: **Figure S3**). The few exceptions that were unique to a sample include species such as
427 *Shewanella* specific to JP2 from the dry environment, and *Cronobacter* specific to RF3
428 in the humid environment. Likewise, the original taxonomic diversity from the
429 environmental isolates (*t0*), as revealed by their 16S rRNAs sequences, and that found

430 in the co-culture sub-communities (*t1*), measured as OTUs by Kraken, overlap only
431 partially. Specifically, we recovered 12 OTUs with 16S rRNAs that were not recovered
432 with Kraken, and 79 OTUs discovered only with Kraken, showing the complementarity
433 of our approaches.

434 Biodiversity indices showed the same tendency as in the 16S rRNA results, in
435 which the dry environment is more diverse than the humid (Additional file 5: **Table**
436 **S4**). In all cases results from BG11 co-cultures show higher diversity than those
437 obtained from the *Caulobacter* + mannitol medium. Similar to the process of
438 eutrophication in biofilms, in which nutrient availability affects biofilm diversity and
439 composition [53], rapid growers and presumably primary producers colonized and took
440 over in the eutrophic medium, resulting in overall low diversity. In contrast, the results
441 of the oligotrophic conditions suggest a cyanobacteria-centric community enables
442 diversity. Indeed, rarefaction curves based on Kraken estimates suggest we captured 40-
443 60% of the microbial community in the BG11 media (15 genera in JP6), with the least
444 being the results obtained from the co-cultures grown on the *Caulobacter* + mannitol
445 medium (Additional file 9: **Figure S4**).

446 Differences in genera identified with 16S rRNA and metagenomes could be
447 explained because our metagenomes may not be deep enough to recover cyanobacteria-
448 associated OTUs; because taxa presence may fluctuate in the cultures; and/or because
449 cycanobacteria sequences are too divergent to be captured. It is likely that all three
450 factors influenced our results. Despite these issues and differences in the media, we
451 confirmed the occurrence of many of the bacterial endophyte taxonomic groups in the
452 metagenomes, which were previously isolated and characterized with 16S rRNA. In
453 sum, it is clear from these results that we have captured a significant fraction of the

454 taxonomic diversity of the endophytes in the cycad coralloid root, and that the
455 combination of isolation and shotgun metagenomics results in a realistic representation
456 of the cycad coralloid bacterial community.
457
458 ***Dioon* cyanobacteria belong to the family *Nostocaceae* and are a monophyletic**
459 **group**
460 In order to explore the specificity of our cyanobacterial isolates, we reconstructed a
461 phylogeny from five markers (**Fig. 4a**. See also Additional file 10: **Figure S5**).
462 Although cyanobacteria phylogenetic history is likely reticulated [54], our tree is
463 congruent with previous phylogenies that grouped cyanobacteria into mostly
464 monophyletic clades, and we recover and support various known taxa relationships. For
465 instance, we support the lack of monophyly of *Chlorogloeopsis* and *Fischerella* with
466 *Chlorogloeopsis* strains grouped with the nostocalean *Scytonema* [55]. We also support
467 the monophyly of heterocyst and akinete-bearing cyanobacteria of the sections IV and
468 V [56, 57]. A deeper discussion of the phylogeny is out of the scope of this article, but it
469 will serve as additional evidence in the complex relationships of the cyanobacteria.
470 Hereafter we focus on the Nostocaceae as they are the closest to our samples, and
471 species from the IV and V group are able to establish various types of symbiotic
472 associations [58].
473 Previous molecular studies and our own data show that choice of genome-wide
474 markers, and the type of OTU assignment methods, significantly affect the ability to
475 recover Nostocaceae phylogenetic history. Our results were contingent on using 198
476 genome-wide orthologs from a broad and curated database (Additional file 1: **Table**
477 **S1**; Additional file 2: **Table S2**), combined with Kraken to assign OTUs, which was

478 best at detecting cyanobacteria. Overall, our phylogeny (**Fig. 4b**) shows that *Calothrix*
479 PCC 7507 fails to group within the *Rivulariaceae* and is instead nested within the
480 Nostocaceae. We confirmed the presence of *Anabaena* (metagenomes) first mentioned
481 as algae in the cycad literature [13]; and of *Nostoc* (isolates) [18], and show that they
482 each separate clearly in our phylogeny. Also, *Nostoc* is sister to *Anabaena,*
483 *Aphanizomenon* and *Trichormus* [59, and references therein]. A previously recognized
484 clade using 16S rRNA, constituted by *Anabaena* species associated to *Aphanizomenon*
485 species, with *A. cylindrica* as sister to the rest [60], is also distinct in our phylogeny
486 (Clade I). This group includes the fern endophyte *Nostoc azollae* 0708, supporting
487 original descriptions of *Anabaena* fern symbionts [61] and similar findings with 16S
488 rRNA [59]. The *Nostoc* free-living PCC 7120 grouped distantly to strains of symbiotic
489 origin.

490 Importantly, our *Dioon* isolates from T09, 106C and RF31YmG form a
491 monophyletic clade. This contradicts previous studies in which different species of
492 cycads host multiple cyanobacteria and do not form cycad or host-specific clades [6, 62,
493 63]. The isolate T09 was obtained from coralloid roots of *Dioon caputoi,* collected
494 previously by our group in dry shrubland from the Tehuacan Valley in Puebla, and
495 added as a control. This result suggests specificity of Nostocaeae symbionts within
496 *Dioon* species. It also shows diverging evolutionary trajectories of *Nostoc* species
497 associated with cycads, from those of the free-living Nostocaceae (**Fig. 4b**). Congruent
498 with these findings, a 16S rRNA phylogeny of Nostocacean cyanobacteria shows that
499 hormogonia-producing species symbiotic to *Gunnera* ferns, *Anthoceros*, and cycads,
500 tend to cluster together [59].

501 The name of the new *Dioon* cyanobacteria symbionts remains to be determined.
502 *Tolypothrix* sp PCC 7601 is sister taxon to our *Dioon* isolates, and they are sister to two
503 other plant symbionts: *Nostoc* sp KVJ20 (PRJNA310825), which lives in special
504 cavities located on the ventral surface of the gametophyte of the Norway liverwort
505 *Blasia pusilla* [64]; and *Nostoc punctiforme* PCC73102 (ATCC 29133), associated with
506 the Australian cycad *Macrozamia* [65]. *Calothrix* sp. PCC 7507 and *Fortiea contorta*
507 PCC7126 are sister taxa to our isolates clade (Clade II). Thus, it is concluded that *Dioon*
508 cyanobacteria endophytes belong to the family Nostocaceae, and that they show a
509 monophyletic origin. This suggests that our isolates may be specialized bacteria, with
510 unique metabolic and other phenotypic features that warrant further characterization and
511 polyphasic taxonomic determination.
512
513 **Identification of BGCs in sub-community metagenomes suggests metabolic**
514 **specialization of *Dioon* cyanobacteria**
515 Mapping the size of each bacterial genome onto the phylogeny showed that our *Dioon*
516 coralloid endophytes have larger genomes sizes than all other close relatives, while
517 maintaining their (G+C)-content (**Fig. 4b**). Large genomes correlate with the ability of
518 bacteria to produce specialized metabolites. Thus, we aimed at exploring the coralloid
519 root microbiome functions in detail by identifying examples of BGCs putatively
520 directing the synthesis of specialized metabolites (**Fig. 5**). Genome mining of isolate
521 106C revealed 18 BGCs (Additional file 11: **Table S6**). The analysis of the distribution
522 of these BGCs among the selected Nostocaceae genomes (Additional file 12: **Table S7**)
523 revealed that the heterocyst glycolipid (BGC 16), the only BGC with a defined product
524 [66], and BGC 2, a terpene of unknown structure, were present in all analyzed genomes.

525 Mining of other known molecules associated with cycad cyanobionts, such as nodularin
526 [67], or other known BGCs found in members of the genus *Nostoc*, yielded negative
527 results.

528 In contrast, half of the BGCs were uniquely found within *Dioon* symbionts
529 including isolate 106C. Remarkably, these nine BGCs are absent in the well-annotated
530 genome of *Nostoc punctiforme* PCC73102, a strain isolated from an Australian *Zamia*.
531 These observations support the metabolic specialization of *Dioon* cyanobionts. Among
532 the *Dioon*-specific cyanobacterial BGCs we found four coding for lantipeptides,
533 namely, BGC 1, 9, 10, 17 (**Fig. 5**, see also Additional file 13: **Text S1**). BGC 20
534 includes genes coding for one adenylation domain, one thiolation domain and one
535 thioesterase domain, which may be involved in the synthesis of modified amino acids,
536 or in the formation of a yet-to-be discovered metabolite. The remaining four BGCs code
537 for NRPSs, including one NRPS-PKS hybrid, BGC 21, which codes for a PKS-NRPS
538 hybrid system potentially directing the synthesis of a hybrid peptide with three residues
539 (Phe-Thr-Phe) and a hydroxyl-iso-butyrate group as the C-terminal substituent.

540 BGC12, which caught our attention, codes for an assembly line predicted to
541 direct the synthesis of an N-terminal acylated hexapeptide with several modifications,
542 such as the epimerization of four of its residues, the N-acylation of its second amidic
543 bond, and the reduction of its C-terminal end to yield an aldehyde group. The N and C
544 terminal modifications on this peptide are typical of small peptide aldehyde protease
545 inhibitors, which have been previously reported on cyanobacteria [68]. Alternatively,
546 the product of this biosynthetic system may be a siderophore, as iron-related genes were
547 found next to the NRPS coding-genes and previous reports have shown that reductase
548 domain-containing NRPS systems such as in myxochelin [69], are linked to iron

549 chelators. The BGC 22 encodes a small NRPS system for a dipeptide (Gly-Val), which
550 in 106C and RF3Mg seems to be associated to genes coding for chemotaxis proteins,
551 also present in the corresponding region in T09.
552 BGC 23, the most interesting of all, codes for a NRPS system putatively
553 directing the synthesis of a tripeptide consisting of leucine, valine and tyrosine residues,
554 as well as an N-terminal acylation, an N-methylation at an amide bond of the isoleucine
555 residue, plus a domain of unknown function likely modifying the tyrosine residue.
556 Remarkably, the order of the domains in the BGC suggests lack of co-linearity, which
557 may imply domain skipping or recycling. A search for peptides containing such
558 modifications, performed with the server PRISM that includes a feature for de-
559 replication of known chemical structures [52], directed our attention to nostoginins, a
560 specialized metabolite whose biosynthetic pathway remains unknown. Nostoginin A is
561 an acylated tripeptide (Leucine-Valine-Tyrosine) with N-acylations at the isoleucine
562 and tyrosine residues, originally isolated from a member of the genus *Nostoc* [70], and
563 shown to be a protease inhibitor with specificity towards aminopeptidases. Similar
564 bioactivity has been found for its congeners nostiginin B, microginins FR1 and SD755,
565 and oscillaginins A and B [71]. Interestingly, a nostoginin congener (Nostoginin B),
566 which includes an extra tyrosine group at the C- terminal end, was also isolated from
567 the same *Nostoc* strain as nostoginin A. The amino acid specificity of BGC 23
568 adenylation domains, the location of the modification on the leucine and tyrosine
569 residues, the lack of collinearity, the presence of N-terminal acylation domains, the
570 occurence of peptidase coding genes in the BGC, and the taxonomic origin of
571 nostoginins, strongly suggest that BGC 23 is linked to these metabolites (**Fig. 5**).

572 In addition to our genome-driven analysis, we also assembled, annotated and
573 mined, *de novo*, the metagenomes of *t1* and *t2* oligotrophic co-cultures in an iterative
574 fashion. First, by identifying sequence signatures of biosynthetic enzymes using
575 antiSMASH, and second, by extending the contigs with hits by iterative mapping and
576 assembly. This approach only revealed in all metagenomes together of *t1* five short
577 signal sequences (less than 3.5 Kbp) that are suggestive of enzyme genes that could be
578 part of BGCs. It seems that although representative of the rich biological diversity of the
579 root, the lower coverage of these metagenomes hampered our ability to obtain loci long
580 enough to allow proper annotation of presumed BGCs. In contrast, for *t2*, where
581 bacterial diversity has been enriched we found two complete BGCs in the RF3 sub-
582 community metagenome, both clearly coming from cyanobacteria, the most abundant
583 taxa in the co-culture (**Table 2**). Indeed, these BGCs coincided with those found in the
584 RF31YmG genome extracted from RF3 metagenome, showing that a computational
585 pangenomic analysis of metagenomes is a promising approach to capture the
586 biosynthetic potential of co-cultures.
587

588 **Discussion**

589 Our combined strategy of co-cultures at different timescales and genomic and
590 metagenomic sequencing analyzed with a phylogenomic framework enabled us to study
591 bacterial endosymbionts that coexist in the same cycad host, and identify the BGCs
592 associated to their coralloid root-specific niche. We focus our discussion on the taxa
593 found in the bacterial isolates, and OTUs present in the metagenomes, and we refer to
594 species and OTUs interchangeably.

595

596 ***The microbiome of the cycad coralloid root reveals a biodiverse community, with***
597 ***monophyletic grouping of cyanobacteria***

598 Our evidence undoubtedly shows that within the cycad coralloid root there is a highly
599 diverse bacterial community within the cycad coralloid root of at least 27 genera
600 identified with 16S rRNA of which 12 were not recovered with Kraken, and 79
601 additional genera identified in the metagenomes, which includes all of the previously
602 reported Nostocales and newly reported genera. We validated previous reports of taxa
603 for which their endophytic origin and presence was unclear or doubtful. Cyanobacteria
604 are present, but also many other taxa that interact in a community.

605 We also support previous morphological observations that showed that an
606 individual cycad plant could harbor diverse communities that differ in their taxonomic
607 composition and life-strategy [23], from soil dwellers to well-known plant symbionts.
608 Morphological studies observing mucilaginous material inside the coralloid root [14,
609 20] are also congruent with the microbiome consortium we describe. However, most of
610 the abundant genera were shared among samples, which suggests weak taxonomic
611 specificity in different environments. Similarly, the majority of the taxa identified in the

612 phylogeny can be taxonomically classified as diazotrophic plant endophytes, which
613 points toward functional congruence associated with nitrogen fixation, rather than
614 phylogenetic filtering, and suggests a taxonomic and functional core.

615 Although many other groups are worth exploring, we focused on cyanobacteria
616 as the main group of interest given previous records of this group in cycads, their ability
617 to establish symbiosis with most lineages of eukaryotes in many different types of
618 tissues, and in plants with known co-evolutionary histories [72]. This bacterial group is
619 also renowned for its potential to synthesize specialized metabolites of applied and
620 evolutionary interest.

621 Among our most interesting findings is the monophyletic placement of our
622 cyanobacterial samples, which confirm a single morphological observation of possible
623 specificity among cyanobacteria coralloid root endophytes (then termed phycobionts),
624 and their hosts, including *Dioon* [5], and contrasts with several previous notions
625 regarding relationships between *Nostocaceae* and their hosts. Cyanobionts in other
626 systems, such as cyanobacteria from a single lichen species, are often more closely
627 related to free-living microorganisms, strains belonging to other species, or to plant
628 symbionts, than to each other. Likewise, other studies of symbiotically competent
629 *Nostoc* isolates suggest that they are not specialized and strains isolated from one plant
630 species are capable of infecting phylogenetically distant hosts [59, 73, 74]. These
631 contrasting previous observations could be biased by partial taxon identification in what
632 we know now is a diverse cycad coralloid root microbiome, including several different
633 cyanobacteria genera. Additionally, those phylogenies were based on samples collected
634 growing outside of their place of the cycad's native distribution [75]. As data is
635 gathered from more genomes of bacterial cycad symbionts, it will be possible to test for

636 other co-evolutionary relationships, including horizontal gene transfer between bacteria
637 and the eukaryote host, and other patterns that suggest close evolutionary histories.
638
639 ***Cultivated bacterial sub-communities are useful to assess functional interactions of***
640 ***the root microbiome***
641 We found congruent results in diversity patterns among 16S rRNA and metagenomes,
642 yet there are clear limitations of 16S rRNA and even genome-wide markers to carry out
643 in-depth microbiome analyses, depending on how OTUs are assigned. There are even
644 more limitations to understanding their functional interactions. We increased our ability
645 to identify a diverse array of organisms using cultivated bacterial sub-communities (*t1,*
646 *t2*) and exploring their metagenomes with phylogenomic tools. Most of the genera with
647 only a few species were recovered in *t1*, and genera with many species were recovered
648 in both *t0* and *t1*. The differences in composition with genera identified without
649 enrichment (*t0*) was expected, because environmental sampling and enriched inoculant
650 complement each other, and aim to recover distinct aspects of the microbiome's
651 composition [27]. These patterns can also be explained by various scenarios: i) rare
652 groups present in low abundance can only be recovered in sub-community co-cultures
653 on which they increase in biomass; ii) some organisms are fast growers irrespective of
654 media, and will dominate in OTUs, simply by chance, iii) some groups are more media-
655 specific; and/or iv) groups in BG11 (*t1*) are recovered as a result of functional
656 interactions to pre-adapted cyanobacteria-associated groups.
657 The long-term one-year co-culture (*t2*) allowed us to explore at least some of the
658 aforementioned possibilities. Although dynamic, the initial amount of inorganic
659 nitrogen available in these co-cultures became a limiting factor over time. Hence, the

660 establishment of stable communities after a year with emerging and surviving taxa
661 suggests that Nitrogen fixation is at least one of the main driving forces in the assembly
662 of the coralloid root community. Plant-associated and slow-growing actinobacterial
663 taxa, renowned for being prolific producers of specialized metabolites, are abundant in
664 these communities. Further exploration of the metabolic-driven hypotheses emerging
665 from these observations in different conditions, with an emphasis on Nitrogen fixation
666 and physiological studies of the community, is required to understand the complexity of
667 such community. For now, we can conclude that co-cultures are a strategy that allows
668 assessing deeper sub-community functional interactions within the microbiome of a
669 specialized organ, as it is the cycad coralloid root.
670
671 ***Large genome size as a signature of facultative lifestyles in cycad cyanobacteria***
672 ***symbionts***
673 Most bacterial endosymbionts of plants or animals show a reduction in genome size
674 compared to free-living relatives [76], yet our endosymbiont samples have larger
675 genome sizes than all other closely related taxa in their phylogeny. Large genome sizes
676 in endosymbionts are usually attributed to a facultative relationship that requires
677 retaining free-living stages. For instance, rhizobial nitrogen-fixing bacteria in root-
678 nodules of legumes that exhibit multiple lineages with genome expansions compared to
679 closely related taxa ([77] and references therein), are also more similar in genome
680 content and size to other plant symbionts than to closely related species [78]. Other
681 facultative symbionts which form Nitrogen-fixing root nodules in angiosperms have
682 large genome sizes adapted to shifting from the soil to the plant environment [79], while
683 others such as *Brucella, Wolbachia* or *Agrobacterium* have favored expansions of

genome size to cope with complex and varying life-styles [80]. Thus, a feasible hypothesis is that the *Nostocaceae* taxa we found associated to the cycad coralloid root, have experienced a large genome expansion driven by selection to initially survive the structural, ecological and biological complexity of the soil from which they are recruited.

Additionally, a large repertoire of genes would be required to maintain the developmental phenotypic plasticity of the cyanobiont cells to adapt to the inside of the cycad host. Extremely plastic symbionts, such as *Nostoc* species, have notorious complex life cycles that require cell differentiation of the organism to be able to enter the host plant and disperse [81]. The only other cyanobacteria cycad symbiont sequenced, *Nostoc punctiforme* from an African cycad *Macrozamia* [65], is phenotypically plastic and ranges from photoautotrophic to diazotrophic, to facultatively heterotrophic. Its vegetative cells can develop into nitrogen-fixing heterocysts and have transient differentiation into hormogonia. Its genome shows 29% unique protein-encoding sequences of known function, with roles in its cell differentiation and symbiotic interaction properties [65]. It also has numerous insertion sequences and multilocus repeats, as well as genes encoding transposases and DNA modification enzymes, which would be congruent with genomic plasticity required to sense and respond to the environment outside and inside the plant [65].

In sum, taxonomic diversity of the coralloid root, combined with monophyly of the large *Nostocaceae* genomes found in the cycad coralloid root, could be a result of imposed constrains of the facultative symbiotic lifestyle, and the broad symbiotic competence with the plant host. The facultative nature of cyanobionts of *Dioon* would suggest they are secondary endophytes acquired from environmental sampling with

host-specificity to *Dioon*.

It remains to be examined how the genomes of our *Dioon* cyanobionts expanded. Upcoming work on the comparative genomics of the cycad coralloid root microbiome should test for trends in genome size, AT content, changes in the content and distribution of repeats and mobile elements, distribution of accumulated mutations and type of genes gained or lost and pseudogenization. All these factors could inform the nature of the cycad-bacterial interactions in ecological and evolutionary time. Of particular interest to us, is how metabolic functions are retained or acquired in relation to loci present within the root microbiome. We begin exploring this by identifying and analyzing the distribution of BGCs in our bacterial genomes, which we discuss in the final section below.

***BGCs are conserved and unique to the cycad cyanobionts***

The bacterial repertoire of specialized metabolites can correlate to environmental selective pressures [82] and result in conserved metabolic and genetic repertoires among species facing similar challenges, including those from plant symbiotic relationships. In Nostocales, although free-living strains are often competent and will form symbiotic interactions under laboratory conditions with many hosts [83], most recruited cyanobacteria are capable of producing specific compounds to survive within the plant. A remarkable example of a specialized metabolite involved in symbiosis is nosperin, a polyketide produced by a lichen-associated *Nostoc* cyanobacteria [84]. This molecule belongs to the pederin family, which includes molecules produced by non-photosynthetic bacterial symbionts from beetles and sponges [84], suggesting a role on eukaryote-prokaryote interaction. Nosperin has also been found in the liverwort *Blasia*-

732 associated and in free-living *Nostoc* cyanobacteria [64] suggesting that in cycads,
733 nosperin producers are selected for symbiosis, although production is not necessarily
734 induced while inside the coralloid roots.

735 None of the BGCs for specialized metabolites previously reported for *Nostoc*
736 cyanobionts of lichens, bryophytes or other cycads, namely, nosperin, mycocystin or
737 nodularin, could be found in the *Dioon* cyanobionts. Our unique biosynthetic repertoire
738 of several BGCs provides an example of metabolic specialization that correlates more
739 with the plant host biology than with the environmental conditions or geography.

740 A chemical insight derived from our genome mining efforts, which may have a
741 strong bearing on the evolution and biology of the *Dioon*-bacteria symbiosis, relates to
742 the potential of *Dioon* cyanobionts to produce at least two small peptide protease
743 inhibitors: the nostoginin-like peptides predicted to be produced by BGC 23; and the
744 acylated penta-peptide aldehyde predicted to be produced by BGC 12. The specific
745 presence of these metabolites in the cyanobionts may imply that proteolysis is involved
746 in the cyanobacteria-cycad interaction. Protease activity in the coralloid roots may be
747 linked to the reconfiguration of the root architecture or the filtering of the microbiome.
748 This is an interesting possibility as the involvement of proteases in root nodule
749 symbiosis has been observed previously between arbuscular mycorrhiza and legumes
750 [85]. Within this context, our sub-community metagenomics approach provided a
751 platform for BGC discovery that can be applied to other microbial-host interactions.
752 Also, the BGC patterns found in the coralloid root add to the growing notion that
753 symbiotic relations occur under heavy influence of chemical interactions, providing a
754 rich source of novelty for drug discovery [84].
755

## Conclusions

Our work shows that the coralloid root microbiome is a highly diverse community, with most genera shared within *Dioon* species regardless of their original environment or plant host. Our methods of enriched sub-community metagenomics and phylogenomics were able to recover a good portion of the taxonomic and phylogenetic diversity and reveal genes underlying the production of previously unreported specialized metabolites that result from bacterial functional interactions. We also provide emerging evidence of co-evolution between cyanobacteria and their plant hosts, suggested by monophyly of the samples and the presence of unique BGCs to their clade.

The coralloid root microbiome is likely established by dual forces of host-driven selection and environmental recruitment of cyanobacteria and possibly other taxa that are capable of transitioning from free-living to endosymbiotic lifestyles, and the functional capacities of the bacterial consortium itself. Future phylogenomic work on the cycad coralloid root microbiome via an integrated analysis of genome organization and expression of specialized metabolite production, as well as of their relationship to the fitness of the host, will further facilitate our understanding of the evolutionary history of the cycad microbiome.

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994

995 **Table 1.** Taxonomic composition of endophytes isolated from *Dioon* coralloid roots

| Phylum | Class | Order | Family | Genus | OTUs [a] |
|---|---|---|---|---|---|
| **Bacteroidetes** | Sphingobacteriia | Sphingobacteriales | Sphingobacteriaceae | *Mucilaginibacter* | 3 |
| | | | | *Sphingobium* | 1 |
| | | | | *Sphingomonas* | 2 |
| | | | | *Variovorax* | **1** |
| | Cytophagales | Cytophagales | Cytophagaceae | *Dyadobacter* | 1 |
| **Cyanobacteria** | Cyanobacteria | Nostocales | Microchaetaceae | *Tolypothrix* | *6* |
| | | | Nostocaceae | *Nostoc* | ***2*** |
| **Firmicutes** | Bacilli | Bacillales | Bacillaceae | *Bacillus* | ***16*** |
| | | | Paenibacillaceae | *Paenibacillus* | ***2*** |
| | | | Staphylococcaceae | *Staphylococcus* | 1 |
| **Proteobacteria** | Alphaproteobacteria | Rhizobiales | Rhizobiaceae | *Rhizobium* | ***32*** |
| | | | | *Shinella* | 2 |
| | | | Brucellaceae | *Ochrobactrum* | 1 |
| | Betaproteobacteria | Burkholderiales | Alcaligenaceae | *Achromobacter* | ***33*** |
| | | | Burkholderiaceae | *Burkholderia* | ***39*** |
| | | | | *Ralstonia* | ***2*** |
| | | | | *Mitsuaria* | **8** |
| | | | Comamonadaceae | *Variovorax* | 1 |
| | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | *Enterobacter* | ***3*** |
| | | | | *Luteibacter* | 1 |
| | | | | *Pantoea* | ***1*** |
| | | Pseudomonadales | Pseudomonadaceae | *Pseudomonas* | ***21*** |
| | | Xanthomonadales | Xanthomonadaceae | *Luteibacter* | **2** |
| | | | | *Stenotrophomonas* | ***35*** |
| | | | | *Xanthomonas* | **2** |
| **Actinobacteria** | Actinobacteria | Micrococcales | Microbacteriaceae | *Microbacterium* | 5 |
| | | Streptomycetales | Streptomycetaceae | *Streptomyces* | ***19*** |

996 [a] Taxa identified in the literature as endophytes (italics) and/or diazotroph (bold) are shown.

997 **Table 2.** Taxonomic composition of sub-communities isolated from *Dioon* coralloid roots

| Sample [a] | Growth conditions | Genera identified with different methods: total number (bold), most abundant (%) | | |
|---|---|---|---|---|
| | | mOTUs | Kraken | MG-RAST |
| JPPOOL | Eutrophic, 72 hours, *Caulobacter* medium + mannitol | **6**, *Bacillus* (87%) | **22**, *Bacillus* (84%) | **512**, *Bacillus* (86%) |
| RFPOOL | | **8**, *Bacillus* (99%) | **25**, *Bacillus* (65%) | **524**, *Bacillus* (80%) |
| JP2 | Oligotrophic, 30 days, BG-11 | **42**, *Agrobacterium* (45%) | **57**, *Rhizobium* (7%) | **1273**, *Nostoc* (21%) |
| JP6 | | **38**, *Pseudoxanthomonas* (22%) | **69**, *Xanthomonas* (2%) | **1253**, *Xanthomonas* (8%) |
| RF1 | | **33**, *Stenotrophomonas* (83%) | **63**, *Nostoc* (3%) | **1157**, *Stenotrophomonas* (20%) |
| RF3 | | **25**, *Stenotrophomonas* (42%) | **61**, *Xanthomonas* (7%) | **1065**, *Xanthomonas* (22%) |
| JP6 | Oligotrophic, 1 year, BG-11 | **70**, *Deinococcus* (25%) | **69**, *Deinococcus* (4%) | **1957**, *Deinococcus* (26%) |
| RF3 | | **67**, *Stenotrophomonas* (33%) | **63**, *Nostoc* (3%) | **1592**, *Nostoc* (13%) |

998 [a]JPPOOL= JP1, JP2, JP6; RFPOOL = RF1, RF3, RF9.

999 **Legends to Main Figures**

1000

1001 **Figure 1. Pipeline to capture and characterize bacterial microbiome diversity.**
1002 Coralloid roots from cycads growing naturally in dry and humid deciduous tropical
1003 forests were sampled (photo of coralloid root of approx. 9cm in length shown, not to
1004 scale). Endophytes from the macerated root were isolated, following two strategies:
1005 directly from the sample (*t0*) and after enrichment using co-cultures of sub-
1006 communities, and sampled after 30 days (*t1*), although sampling can be done anytime
1007 (*t1…tn*). Cultivable bacteria were obtained using an array of six different media. Co-
1008 cultures were characterized using shotgun metagenomics, and the resulting data was
1009 used to select representative genomes from the endophyte culture collection that we
1010 mined for functional information using a phylogenomic and comparative genomic
1011 approaches.

1012

1013 **Figure 2. 16S rRNA Bayesian phylogeny of endophytes from coralloid roots of**
1014 ***Dioon merolae*.** The external ring refers to the two environments sampled: dry or JP (D
1015 - orange) and humid of RF (H - blue) deciduous tropical forests. The inner ring refers
1016 isolation strategy: directly from the sample (*t0* - white) or after enrichment using co-
1017 cultures of sub-communities (*t1* - gray). Major bacterial groups are highlighted in
1018 different colors across the tree.

1019

1020 **Figure 3. Network of taxa co-occurrence from different coralloid root samples.** The
1021 lines connecting the circles represent shared taxa identified with Kraken from the
1022 metagenomes. Orange lines correspond to samples from the dry (JP) forest and blue to

1023 samples from the humid (RF) forest. The most abundant genera in the four
1024 metagenomes are represented by circles. Circle diameters are scaled in accordance with
1025 the number of reads associated to each genus.

1026 **Figure 4. Phylogeny of *Cyanobacteria*. A. Multilocus phylogeny.** The tree was
1027 constructed with five molecular markers and genomes obtained from GenBank, plus our
1028 genomes from T09, 106C and Rf31Y. Branches names have been colored according to
1029 the genera originally assigned in GenBank (a larger version of the tree is available as
1030 additional file 10: Figure S5); **B. Genome-wide phylogeny of the family *Nostocaceae***.
1031 The tree was constructed with 45 conserved proteins, and includes *Dioon* cyanobionts
1032 106C, T09 and Rf31Ymg. The habitat type of each taxa is indicated with colored
1033 bullets. The bars show a relatively homogeneous (G+C)-content among *Nostocaceae*
1034 cyanobacteria, and a trend for larger genomes in *Dioon*-associated cyanobacteria.

1035 **Figure 5. *Dioon*-specific cyanobiont biosynthetic gene clusters for specialized**
1036 **metabolites predicted from their genomes**. Genes are shown as colored boxes, the
1037 tips of the boxes indicate the direction of their translation. Annotation color key is
1038 provided. Domain organization, biosynthetic logic and products are indicated below
1039 each BGC, except for lantipeptide encoded by BGCs 1, 9, 10 and 17, whose predicted
1040 products are shown as additional file 13: Text S1.
1041

# Declarations

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**

The genomes generated during the current study are available in the GenBank public repository as follows:

| SUBID | BioProject | BioSample | Accession | Organism |
|---|---|---|---|---|
| SUB2297132 | PRJNA360300 | SAMN06208854 | MTAV00000000 | *Nostoc* sp. T09 |
| SUB2299096 | PRJNA360305 | SAMN06208961 | MTAW00000000 | *Nostoc* sp. 106C |
| SUB2299173 | PRJNA360315 | SAMN06209042 | MTAX00000000 | *Nostoc* sp. RF31Y |

Metagenomes are available at sequence read archive (ID number pending), and directly from the corresponding author. Other data generated or analyzed during this study are included in this published article and its supplementary information or additional files, as enlisted:

**Additional file 1: Table S1.docx/ Proteins in the cyanobacterial core genome.** Annotated proteins used to reconstruct the cyanobacteria phylogenetic tree of 198 conserved proteins which represent the core of a set of 77 cyanobacterial genomes. We provide the name of the protein and the aminoacid sequence.

1065 **Additional file 2: Table S2.docs/Genomes used to obtain the core proteome.** List of
1066 species and their larger classification used to obtain the core genome.
1067
1068 **Additional file 3: Table S3.xlsx/List of 470 isolated bacteria with their 16S rRNA.**
1069 We enlist all of the identified taxa isolated from the *t0* samples and identified with 16S
1070 rRNA Sanger-sequencing.
1071
1072 **Additional file 4: Figure S1.pdf/Graphic representation of each group identified**
1073 **with 16S rRNA from isolates. A)** We generated individual phylogenies for each of the
1074 genera in our main tree and represented them graphically as shown here. **B)** We also
1075 show individual trees with support values. A full resolution of both figures as individual
1076 files is available at:
1077 https://www.dropbox.com/sh/ss5mmwujnynyc7m/AABqABxc5wS_wjd8NzkarHTca?dl
1078 =0.
1079
1080 **Additional file 5: Table S4.docx/ Biodiversity indices of 16S rRNA and OTUs.**
1081 Diversity indices estimated for samples from 16S rRNA data, and from the four
1082 metagenomes (MET) we sequenced. We calculated Shannon-Weaver *H'* (1962) and
1083 Simpson *L* (1964).
1084
1085 **Additional file 6: Table S5.docx/Statistics of metagenomes sequenced.**
1086 We provide detail on the sequencing depth, contigs, quality of contigs and other basic
1087 statistics on sequenced metagenomes.
1088

1089 **Additional file 7: Figure S2.jpg/ Pictures of cyanobacteria-centric co-cultures.** Co-
1090 cultures in 1L flasks. In the insets is a close up of the culture, where a mucilaginous
1091 biofilm mass can be observed, presumably polysaccharides generated by the
1092 cyanobacteria.
1093
1094 **Additional file 8: Figure S3. Kraken-based taxonomic diversity of metagenomes**.
1095 Taxa abundance from the metagenome mOTUs defined as the percentage of the genera
1096 present in each sample. Jiquipilas (JP) is the dry environment, while Raymundo Flores
1097 (RF) individuals are found in the humid environment. JP or RFPOOL refers the samples
1098 sequenced in pools from media No. 6.
1099
1100 **Additional file 9: Figure S4.pdf/ Rarefaction analysis of 16S rRNA and OTUs data.**
1101 Shown is the proportion of OTUs represented by sample, by type of culture and by
1102 environment for each of the metagenomes sequenced, and a total of possible samples
1103 (All samples) according to a rarefaction estimate.
1104
1105 **Additional file 10: Figure S5.pdf/Concatenated species-tree of cyanobacteria.**
1106 Complete phylogeny of the Nostocales using five molecular markers, RPOB, GyrB,
1107 CPS, PheT and Tig. See text for technical details.
1108
1109 **Additional file 11: Table S6.docx/Prediction of BGCs on the genome of isolate**
1110 **106C.** Biosynthetic Gene Clusters predicted by antiSMASH on the genome of isolate
1111 106C are enlisted, with their corresponding length in Kp.
1112

**Additional file 12: Table S7.docx/106C-specific BGCs throughout Nostocales.** We show the presence or absence of the 18 BGCs found throughout the Nostocales, to emphasize their presence of only some of them in our samples.

**Additional file 13: Text S1.docx/ Predicted lantipeptide from *Dioon* cyanobionts.** We show the sequence corresponding to the lantipeptides from the unique BGCs, whose prediction could not be fully shown in the main figures.

Any additional datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

Funding from this work is from CONACyT #169701 to ACJ, CONACyT #179290 and #177568 to FBG.

**Authors' contributions**

PC-M executed laboratory work, analyzed and interpreted data, and was a major contributor in writing the manuscript. AC-M executed laboratory work and analyzed data. NSM analyzed data. MAP-F identified and collected the plants. AC-J and FB-G equally co-designed and executed the study. AC-J was the main contributor in writing the manuscript. FB-G revised the manuscript critically for intellectual content. All authors read and approved the final manuscript.

## Acknowledgements

We acknowledge Rafael Rincón for his help with preparation of supplementary material. We also thank Juan Palacios, Pablo Suarez-Moo and Antonio Hernández for help during field collections, as well as Hilda E. Ramos-Abiotes and Flor Zamudio for technical support.

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