Symbiosis
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Diversity of symbiotic cyanobacteria in cycad coralloid roots using a short-read *rbc*L-X amplicon

Adriel M. Sierra[1,2] · **Sandrine Toupin**[1] · **Marta Alonso-García**[1,2] · **Juan Carlos Villarreal A.**[1,2,3,4]

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Abstract
Cycads are the only gymnosperms forming a symbiosis with nitrogen-fixing cyanobacteria in a specialized organ: the coralloid root. This paper investigates the endophytic bacterial community inhabiting the coralloid roots of two cycads from Panama. We sampled coralloid roots from *Zamia nana* (terrestrial) and *Zamia pseudoparasitica* (epiphytic). Then, we used the 16S rRNA amplicon marker to describe the entire bacterial community. We also designed a new marker to amplify the *rbc*L-*rbc*X spacer and around 100 bp of the *rbc*X gene, targeting cyanobacteria. We found that using 16S, endophytic bacteria diversity is represented mainly by the phyla Actinobacteria, Cyanobacteria, and Proteobacteria. In addition, 16S analyses showed that *Zamia* species do not share a core cyanobacterial community (using stringent 75% and 90% thresholds), while the two species shared 4 ASVs at a 50% threshold. The newly developed *rbc*L-*rbc*X marker revealed that both species share a core cyanobacterial community represented by a single amplicon sequence variant (ASV1) (*Nostoc sp.*) at 90% threshold that is found in the same phylogenetic clade of that contain mostly Panamanian symbiotic cyanobacteria. Using a 75% threshold, only three ASVs (ASV1, ASV2, ASV3) were present across samples, and five ASVs at 50% threshold. This new marker can effectively identify cyanobacteria ASVs and provide a better resolution for microbial analyses in autotroph cyanobacterial symbioses.

Keywords *Zamia* · 16S rRNA marker · New marker · Core cyanobacterial community · Panama

1 Introduction

Nearly all multicellular organisms have symbiotic interactions with microbes (fungi, bacteria, archaea, viruses), often rendering mutual benefits for both partners (i.e., mutualism) (Hassani et al. 2018). Since the beginning of the twenty-first century, endophytic plant bacterial symbiosis has become a central topic in evolutionary biology (de Vries and de Vries 2022; Griffin and Carson 2018; Trivedi et al. 2020). Endophytic bacteria (inside leaves, root, root nodules) are found across seed plant lineages with profound impact on host fitness and survival (de Vries and de Vries 2022; Griffin and Carson 2018; Moyes et al. 2016; Trivedi et al. 2020). Two lingering questions in plant-microbiome studies are whether host organs (below- and above-ground) actively recruit a distinct core microbiota, and whether those microbial taxa have a influence on the host metabolic functions (Neu et al. 2021; Ravanbakhsh et al. 2019; Trivedi et al. 2020). The core microbiota refers to a statistically determined consortium of microbial taxa present in a host species above a determined prevalence and frequency thresholds, typically between 50 and 100% (Neu et al. 2021; Risely 2020; Rodrigues et al. 2018). Identifying members of the core microbiota allows to link specific microbes to multiple key functions of the host plant, such as root elongation (Finkel et al. 2020) and nutrient supply (e.g., phosphorus and nitrogen) (Li et al. 2018; Oliverio et al. 2020).

Adriel M. Sierra and Sandrine Toupin contributed equally.

✉ Juan Carlos Villarreal A.
juan-carlos.villarreal-aguilar@bio.ulaval.ca

Adriel M. Sierra
adriel-michel.sierra-pinilla.1@ulaval.ca

Sandrine Toupin
sandrine.toupin@sbf.ulaval.ca

Marta Alonso-García
marta.alonso-garcia@sbf.ulaval.ca

[1] Institut de biologie intégrative et des systèms, Université Laval, Québec G1V 0A6, Canada

[2] Department de biologie, Université Laval, Québec G1V 0A6, Canada

[3] Université Laval, Québec G1V 0A6, Canada

[4] Smithsonian Tropical Research Institute, Balboa, Ancón, Panama

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Nitrogen-fixing bacteria are prevalent taxa in plant–microbe mutualistic interaction because of their fundamental role in providing biologically available nitrogen (ammonium) to the host (Li et al. 2018). The main phyla of nitrogen-fixing bacteria are Proteobacteria (e.g., order Hyphomicrobiales) and Cyanobacteria (e.g., order Nostocaceae and Stigonemataceae) (Bouchard et al. 2020; Lavoie et al. 2020; Nelson et al. 2021; Trivedi et al. 2020). Cyanobacteria are mostly free-living organisms but can also become endophytes and form an obligate or facultative symbiosis with all major plant lineages (Adams 2000; de Vries and de Vries 2022). They are present in all hornworts, in liverworts from the order Blasiales, in cavities of dorsal leaves of the fern *Azolla*, in the stem glands of the angiosperm *Gunnera,* and inside the coralloid roots of all cycads (Adams 2000; de Vries and de Vries 2022; Rikkinen and Virtanen 2008; Villarreal and Renzaglia 2006).

In cycads, cyanobacteria form a blue-green band between the inner and outer cortex of apogeotropic coralloid roots (Fig. 1). Inside the coralloid roots, cyanobacteria multiply and produce several structures called heterocysts that contain the enzymatic complex nitrogenase. This enzyme fixes atmospheric nitrogen (N_2) and makes it available to the host as ammonium (Chang et al. 2019; Lindblad 2009). The cyanobacterial-cycad symbiosis is ecologically important to the production and maintenance of their nutrient-rich persistent leaves (Deloso et al. 2020; Lindblad et al. 1985; Marler and Lindström 2021). Using Sanger sequencing, most of the bacteria inside the roots had been identified as members of the order Nostocales, with mainly a single strain per coralloid root (Costa et al. 1999, 2004; Lindblad 2009). In the last decade, the use of DNA-based amplicon sequencing (16S rRNA) on next generation sequencing tools has revealed a large consortium of bacteria from different phyla within the coralloid roots of several species of the family Zamiaceae (Bell-Doyon et al. 2020; Gutiérrez-García et al. 2019; Suarez-Moo et al. 2019).

The Zamiaceae is the predominant cycad family in the Neotropics (Fig. 1) (Calonje et al. 2019). The coralloid roots of species of Zamiaceae (genera *Dioon* and *Zamia*) have Nostocaceae as one of the most common bacterial families, including the genera *Nostoc* and *Calothrix* (Gutiérrez-García et al. 2019; Suarez-Moo et al. 2019). The only three root

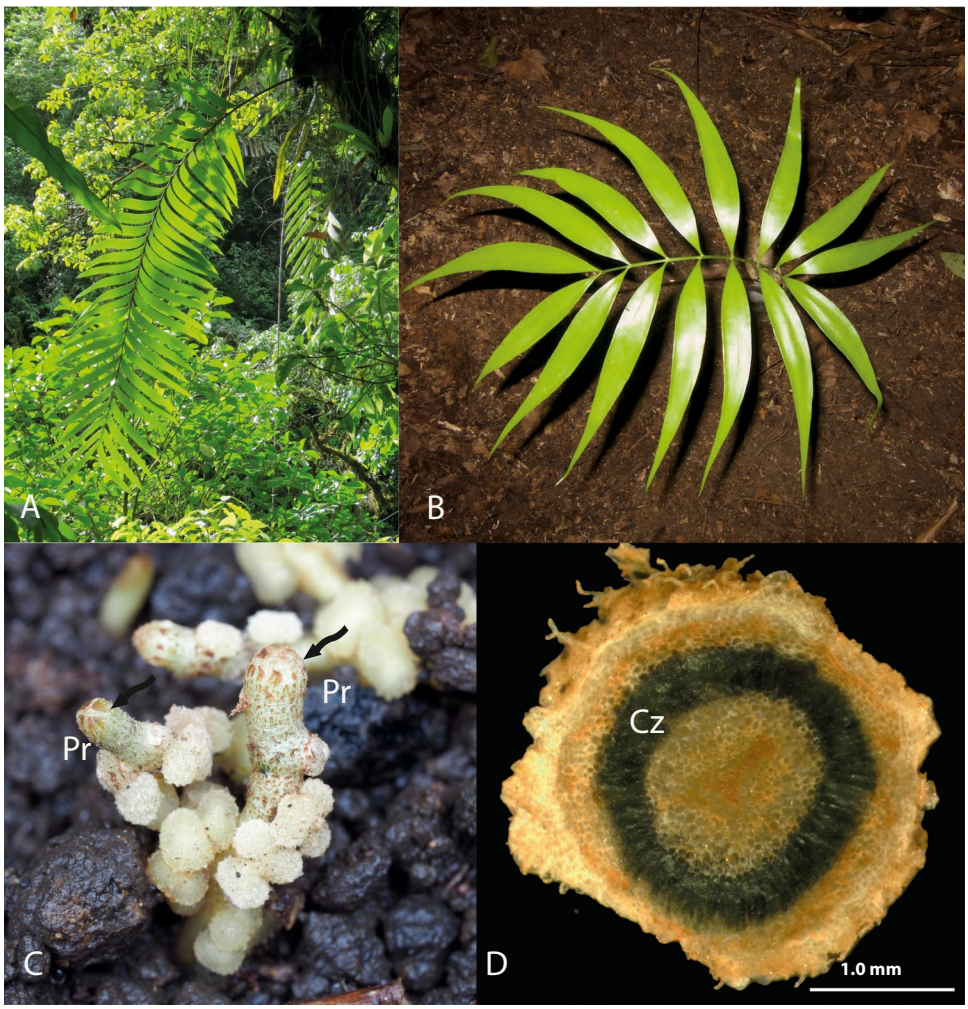

Fig. 1 **A**. Epiphytic *Zamia pseudoparasitica.* Note the large (nearly 2 m) and highly pinnate leaves. **B**. A young leaf of the terrestrial *Zamia nana*, the stem of the species is subterraneous. **C**. Young coralloid and pre-coralloid roots (PR) with abundant hairy papillae of *Zamia nana*. A young developing coralloid root shows lenticel (black arrows), potentially related to the cyanobacterial invasion. **D**. Transverse section of a young coralloid root of *Zamia pseudoparasitica*, showing the cyanobacterial zone (CZ). Scale = 1.5 mm. Photos by Maycol Madrid

microbiome studies available for species of Zamiaceae suggest a geographical pattern, in which, related cyanobacterial strains are found within the coralloid roots of geographically neighboring species (Bell-Doyon et al. 2020; Gutiérrez-García et al. 2019; Suarez-Moo et al. 2019). Cycads root endophytes are recruited from the plant, the substrate (e.g., soil), and the rhizosphere by horizontal transmission (Cuddy et al. 2012; Santoyo et al. 2016; Suarez-Moo et al. 2019). As in other plant systems, microbial endophyte communities are influenced by interactions with both the host and the environment (Brigham et al. 2023; Zheng and Gong 2019). Likewise, the colonization of coralloid roots by cyanobacteria may modulate the abundance of endophytic bacteria (Pecundo et al. 2021; Zheng et al. 2018).

For this study, we characterized the coralloid root bacterial community of two Panamanian endemic *Zamia* species that inhabit contrasting environments on the eastern Caribbean slope of the Central America mountain range (Fig. 1). Both species have a recent origin and belong to the same clade of Central America Isthmian species (Calonje et al. 2019). *Zamia pseudoparasitica* is found in mature forests growing on trees 10–30 m above the ground (Bell-Doyon and Villarreal 2020). Contrastively, *Zamia nana* is a narrowly endemic terrestrial species located about 62 km from the nearest known *Z. pseudoparasitica* site, in El Valle de Antón (Lindström et al. 2013). We used two amplicon-sequencing markers, the V3-V4 region of the 16S rRNA gene and *rbc*L-*rbc*X marker to characterize the bacterial community associated with the coralloid roots of *Zamia*, with specific emphasis on the cyanobacteria community.

Our main objectives are i) to address whether the endophyte bacterial community composition of coralloid roots depends on host species identity, and ii) to evaluate the efficiency of the two markers to detect and identify with a phylogenetic approach the cyanobacterial strain in *Zamia*'s coralloid roots. We hypothesize that *Zamia* species occurring in contrasting environments (terrestrial/epiphytic) will assemble different bacterial/cyanobacterial communities in their coralloid roots and present distinct members in their core microbiota. We also predict that i) *Z. pseudoparasitica* will present a more diverse core cyanobacterial community than *Z. nana*, due to the lower nutrient availability in the forest canopy (Bell-Doyon et al. 2020), and ii) the specific marker for cyanobacteria, *rbc*L-*rbc*X, will be more effective than 16S rRNA marker for differenciating cyanobacterial strains.

2 Material and methods

2.1 Study sites

We study two *Zamia* species in the humid premontane tropical rainforest in Central Panama, where daily temperatures range from 18–29 °C and annual rainfall ranges from 2,500–4,000 mm (Bell-Doyon and Villarreal 2020). Rainfall is concentrated through a long period from April to early January with mean values per month ranging between < 60 to 353 mm. Coralloid roots were retrieved from *Z. pseudoparasitica* from two populations: i) in the Donoso district (Colón province) in the territory of Minera Panama, and ii) in the National Park General de División Omar Torrijos Herrera (El Copé, PNGDOTH) (Coclé province). The latter corresponds to individuals sampled in the high canopy accessed using a single-rope and prusik-knot rappel techniques by Bell-Doyon et al. (2020). Coralloid roots of *Z. nana* were sampled at ca. 0.5 m deep in the soil, from a single population in Cerro Gaital, El Valle de Antón (Coclé province).

2.2 Sampling and handling of coralloid roots

A total of seventeen coralloid roots of *Z. pseudoparasitica,* and ten of *Z. nana* were newly collected from May to June 2018 (Table 1). The coralloid roots were stored on ice inside a hermetic container (4 °C) for less than 2 h until they were processed in the laboratory. A representative herbarium specimen for each *Zamia* species was deposited in the Panama University Herbarium (PMA).

We disinfected each coralloid root surface using the following procedure: a rigorous wash was done with double distilled water (ddH_2O) until all dirt was removed. Then we dipped the coralloid root in Triton 1% X-100 for 1 min 1% commercial bleach. The coralloid root was rinsed in ddH_2O for 5 min to make sure all the Triton X-100 and bleach were removed. Finally, a series of ethanol washes of 95% × 30 s, 70% × 60 s, 40% × 120 s, and subsequently rinsed 3 times with ddH_2O. Surface sterilized coralloid root was placed in a sterile 1.5 ml microcentrifuge tube for subsequent DNA extractions.

2.3 DNA extractions and amplicon sequencing library preparation

Coralloid root samples were frozen in liquid nitrogen and pulverized using a tissue homogenizer with metallic beads. Genomic DNA was extracted using the QIAGEN DNeasy Plant Mini Kit (QIAGEN Canada, Inc.). The amplicon sequencing library was prepared with a two-step dual-indexed PCR approach specifically designed for Illumina instruments as indicated in Bell-Doyon et al. (2020). Following this protocol, we amplified two loci to target the root endophytic bacterial community: 16S rRNA gene V3-V4 region (target all bacteria) and *rbc*L-X (exclusive for cyanobacteria). The primers used for the 16S region were: 16SF: 5'CCTACG GGNGGCWGCAG 3', 16SR: 5'GACTACHVGGGTAT CTAATCC 3'. To amplify the *rbc*L-X region, we developed a primer pair that targeted the spacer and nearly 100 bases

Table 1 List of samples collected to study the endophytic bacterial community in the coralloid roots of *Zamia pseudoparasitica* and *Z. nana*. The 16S ribosomal rRNA gene V3-V4 region and *rbc*L-X region were amplified. For each sample, we provided collector names and voucher information

Species	Sample ID	Biosample accession	Collection date	Habitat	Geographical location	Geographical coordinates	16S accession	*rbc*L-X accession
Zamia nana	JC1S	SAMN38713292	Jun-18	Terrestial	Panama: Coclé, El Valle, Cerro Gaital	8.6183877 N, 80.1250187 W	SRR27150574	SRR27145844
	JC25S	SAMN38713298	Jun-18	Terrestial	Panama: Coclé, El Valle, Cerro Gaital	8.6183877 N, 80.1250187 W	SRR27150568	SRR27145838
	JC26S	SAMN38713299	Jun-18	Terrestial	Panama: Coclé, El Valle, Cerro Gaital	8.6183877 N, 80.1250187 W	SRR27150567	SRR27145837
	JC27S	SAMN38713300	Jun-18	Terrestial	Panama: Coclé, El Valle, Cerro Gaital	8.6183877 N, 80.1250187 W	SRR27150566	SRR27145836
	JC28S	SAMN38713301	Jun-18	Terrestial	Panama: Coclé, El Valle, Cerro Gaital	8.6183877 N, 80.1250187 W	SRR27150565	SRR27145835
	JC3S	SAMN38713302	Jun-18	Terrestial	Panama: Coclé, El Valle, Cerro Gaital	8.6183877 N, 80.1250187 W	SRR27150563	SRR27145833
	JC4S	SAMN38713303	Jun-18	Terrestial	Panama: Coclé, El Valle, Cerro Gaital	8.6183877 N, 80.1250187 W	SRR27150562	SRR27145832
	JC5S	SAMN38713304	Jun-18	Terrestial	Panama: Coclé, El Valle, Cerro Gaital	8.6183877 N, 80.1250187 W	SRR27150561	SRR27145831
	JC6S	SAMN38713305	Jun-18	Terrestial	Panama: Coclé, El Valle, Cerro Gaital	8.6183877 N, 80.1250187 W	SRR27150560	SRR27145830
	JC7S	SAMN38713306	Jun-18	Terrestial	Panama: Coclé, El Valle, Cerro Gaital	8.6183877 N, 80.1250187 W	SRR27150559	SRR27145829
Zamia pseudoparasitica	JC10S	SAMN38713282	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150576	SRR27145846
	JC11S	SAMN38713283	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150575	SRR27145845
	JC12S	SAMN38713284	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150564	SRR27145834
	JC13S	SAMN38713285	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150556	SRR27145826
	JC14S	SAMN38713286	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150555	SRR27145825
	JC15S	SAMN38713287	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150554	SRR27145824
	JC16S	SAMN38713288	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150553	SRR27145823
	JC17S	SAMN38713289	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150552	SRR27145822
	JC18S	SAMN38713290	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150551	SRR27145821
	JC19S	SAMN38713291	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150550	SRR27145820
	JC20S	SAMN38713293	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150573	SRR27145843
	JC21S	SAMN38713294	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150572	SRR27145842
	JC22S	SAMN38713295	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150571	SRR27145841
	JC23S	SAMN38713296	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150570	SRR27145840
	JC24S	SAMN38713297	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150569	SRR27145839
	JC8S	SAMN38713307	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150558	SRR27145828
	JC9S	SAMN38713308	May-18	Epiphyte	Panama: Colón, Donoso	8.83312 N, 80.64010 W	SRR27150557	SRR27145827

from the 5' region of *rbc*X. We use the following primers: *rbc*LX-F: 5' GAGTTTGARGCAATGGATACC 3', *rbc*LX-R: 5' TTHGTTTCGCCTAGCTGTGC 3'. Details of the protocol for the PCR reactions are provided as supplementary methods. The libraries from the first PCR reactions were purified with magnetic microbeads and successive washes in 80% ethanol on a magnetic rack, then recovered in a TE elution buffer (10 mM Tris–HCl (pH 8.0) + 0.1 mM EDTA). Final products were quantified spectrophotometrically using a Spark 10 M plate reader (Tecan US Inc., Raleigh, USA) and pooled in equimolar concentration ratio for sequencing on an Illumina Miseq 300-bp paired-end run (Illumina Inc., San Diego, USA) at Plateforme d'analyses génomiques (IBIS, Université Laval, Québec, Canada).

2.4 Sequence assembly and filtering

The data were processed and analyzed using the R Core Team (2023). Sequence adapters were removed with *Trimmomatic* (Bolger et al. 2014). Raw reads of the 16S marker were processed following the package *DADA2* (Callahan et al. 2016, 2019) based on the quality profile (Phred quality score) and trimmed as follows: forward reads at 250 bp and reverse reads at 225 bp with default expected errors (maxEE = 2), ambiguous nucleotide (maxN = 0), and probability of erroneous assignment (truncQ = 2). Sequences were dereplicated with the function 'dada' and merged by aligning forward and reverse reads with an overlap of a minimum of 20 base pairs. Amplicon Sequence Variants (ASVs) were then inferred for each contig, and chimera sequences were removed (Callahan et al. 2016) with the *DADA2* taxonomic implementation of the naive Bayesian RDP classifier. Twenty-seven published amplicon sequencing libraries of *Z. pseudoparasitica* from PNGDOTH (Bell-Doyon et al. 2020; Bioproject: PRJNA591613) were added to our dataset of 17 newly sequenced increasing to 44 samples for this species. On the other hand, *rbc*L-*rbc*X raw reads (seventeen samples belonging to *Z. pseudoparasitica* and ten to *Z. nana*) were processed and trimmed with forward reads at 160 bp and reverse reads at 400 bp, because of the unequal length of the *rbc*L-*rbc*X spacer. Additional parameters were set with default expected errors (maxEE = 2), ambiguous nucleotide (maxN = 0), and probability of erroneous assignment (truncQ = 2). The taxonomy was assigned based on a custom Genbank database of over 2,793 *rbc*L-*rbc*X (henceforth *rbc*L-X) sequences spanning all cyanobacterial clades (Supplementary Table 1).

In both cases (16S and *rbc*L-X), removed any ASV sequences that was identified as chloroplast, mitochondria, or eukaryotes using the package *phyloseq* (McMurdie and Holmes 2013). For 16S, a total of 292 sequences contigs corresponding to chloroplast and mitochondria were removed from the dataset. Fifteen ASVs assigned to the phyla Armatimonadota, Deinococcota, Dependentiae, Bdellovibrionota, Spirochaetota, FCPU426, and Fusobacteriota, were removed because they were represented by less than six ASVs.

2.5 Analyses of bacterial diversity using 16S and *rbc*L-X markers

We created three datasets comprising the: 1) The total bacterial community from 44 samples of *Z. pseudoparasitica* and 10 samples of *Z. nana* identified using the 16S marker; 2) the phylum Cyanobacteria from 44 samples of *Z. pseudoparasitica* and 10 samples of *Z. nana* identified with 16S marker; and 3) the phylum Cyanobacteria from 17 samples of *Z. pseudoparasitica* and 10 of *Z. nana* identified with the *rbc*L-X marker. For the three datasets, alpha diversity was assessed using four indexes: i) the Chao index which shows the observed richness, ii) the exponential of Shannon which weights the ASVs by their frequency, iii) the Simpson's diversity index which considers the presence and relative abundances of ASVs present in a sample, and iv) the Pielou index which measures diversity along with species richness.

The alpha diversity indexes for the three datasets were visualized using boxplots with the package *ggplot2* (Wickham 2016). The assumptions of normality and homogeneity of variance were tested with the Shapiro–Wilk (Shapiro and Wilk 1965) and Levene tests, respectively. The four alpha diversity indexes were not normally distributed according to Shapiro–Wilk tests ($p\text{-}value = < 0.01$). Levene's test was not significant for the four indexes of alpha diversity ($p\text{-}value = > 0.05$) indicating homogeneity of the variance. Therefore, the significant differences between *Zamia* species were assessed using the Kruskal–Wallis nonparametric test. *Post hoc* pairwise comparisons were performed with the exact sum of Wilcoxon ranks. Additionally, for the cyanobacterial datasets (datasets 2 and 3), we tested for statistically significant differences in microbial diversity within the coralloid roots of the two species in relation to the amplicon marker used with the Kruskal–Wallis nonparametric test.

2.6 Comparison of cyanobacterial ASVs using 16S and *rbc*L-X markers

We compared the variability in cyanobacteria ASVs detectability of both markers (16S – dataset 2, r*bc*L-X – dataset 3) in relation to sequence read number and number of coralloid roots sampled. First, we evaluated if sequencing depth bias toward over or underestimation of cyanobacterial community diversity by correlating the observed species richness and the read count by sample (coverage) with the Kendall method.

For the two *Zamia* species separately, species accumulation curves (100 permutations) were generated from sample-based rarefactions using the Coleman method (Coleman et al. 1982) with the function 'specaccum' from the package *vegan* (Oksanen et al. 2016). Estimated species richness was also extrapolated for each species with three non-parametric methods. The Chao Index with standard error (Chao 1987) was estimated using the small sampling corrections to reduce estimation bias by multiplying (N-1)/N to the basic Chao equation (Gotelli and Colwell 2001) as implemented by the function 'specpool' from the package *vegan*. The First-order Jackknife estimator and the Bootstrap estimator with standard error were also used, as it is considered a more accurate measure of species richness from datasets with a high number of singletons and doubletons (Smith and van Belle 1984). Pearson's $\chi 2$ test was used to test for significant differences between the observed species and extrapolated richness with the three non-parametric methods. Rank abundance curves were generated to evaluate whether coralloid root cyanobacterial communities of the two *Zamia* species vary in the frequency of common and rare species. The package *Biodiversity* (Kindt and Coe 2005) was used to build dominance-diversity curves.

2.7 Assessing *rbc*L-X primers efficacy: a mock community approach

To assess the efficacy of the new primers (*rbc*L-X region) in detecting the real community diversity, we created a mock community, i.e., a defined mixture of cyanobacterial strains from cultures. Cyanobacterial cultures with three known strains already sequenced were selected as positive control. Cyanobacterial DNA was extracted from putatively pure laboratory cultures using a CTAB protocol (Bell-Doyon et al. 2020). DNA extraction from laboratory cultures were combined in equimolar concentration following these three combinations randomly selected: a community of three cyanobacterial strains (GenBank Accession: SRR27132443), two cyanobacterial strains (GenBank Accession: SRR27132442), and a single cyanobacterial strain (GenBank Accession: SRR27132440) (Supplementary Fig. 1). The pooled DNA was used as a control template for PCR amplification. We amplified the *rbc*L-X region of the three combinations of the mock communities using the two primers described above (*rbc*LX-F and *rbc*LX-R). Library preparation and sequencing procedures follow those previously described above. We applied the bioinformatic pipeline used to assemble and filter amplicon sequence reads for the *rbc*L-X marker.

2.8 Comparison of the core cyanobacterial community using 16S and *rbc*L-X markers

We identified the core cyanobacterial community of the coralloid root of *Z. pseudoparasitica* and *Z. nana* and compared the results between the two markers (16S – dataset 2 and *rbc*L-X – dataset 3). We used two methods to identify the specific association with the host species, the threshold-based frequency method (Salonen et al. 2012), and the indicator species analysis (Dufrene and Legendre 1997). The indicator species analysis uses relative abundance and relative frequency of ASVs to identify the specific association with the host species.

For the threshold-based frequency method, we used the package *microbiome* (Lahti and Shetty 2017). Due to the lack of consensus about a fixed threshold (Risely 2020), we tested for the presence of a core cyanobacterial community as defined by any taxon with a prevalence of 50%, 75%, or 90% (Jorge et al. 2020; Neu et al. 2021). We pruned out the specific samples to avoid bias due to the low coverage < 50 reads. We estimated the total abundance of specific members of the core cyanobacterial community in each sample.

For the indicator species analysis, the package *labdsv* (Roberts 2016) was used to identify which ASVs were relatively more abundant and predominantly found in one *Zamia* species compared to the other. We calculated an Indicator Value (IndVal) index between each ASV and the two species: i) group of samples belonging to *Z. pseudoparasitica*, and ii) group of samples belonging to *Z. nana*. The statistical significance of the association between ASVs and each group of samples was tested using 1000 permutations. ASVs were considered indicators of the host core cyanobacterial community based on a maximum probability of $p\text{-}value = 0.01$.

To identify the core taxa at the species level from both methods (threshold-based frequency and Indicator species analysis), ASV sequences were blasted against the NCBI database and retained those with BLAST hits with 100% identity.

2.9 Phylogenetic placement of cyanobacterial ASVs using *rbc*L-X

We inferred the phylogenetic placement of the most abundant cyanobacterial ASVs found in both species of *Zamia*. We selected ASVs, identified with *rbc*L-X, with a relative abundance threshold of 0.03% as suggested by Nelson et al. (2021) in a similar plant-cyanobacterial system, excluding rare ASVs with low read number. For the *rbc*L-X marker, a total of six sequences contigs corresponding to chloroplast and ribosomal DNA were removed from the dataset. Eight ASVs were present in both species. Two ASVs (ASV3 and ASV21) were presented exclusively in *Z. pseudoparasitica* and ASV5 and ASV9 exclusively in *Z. nana.* This resulted in a total of 12 ASVs.

We used Geneious 11.1.4 (https://www.geneious.com) to align the 12 ASVs with 610 sequences from Nostocales downloaded from GenBank. Alignments were performed automatically using the default settings (65% similarity,

gap open penalty = 12, gap extension penalty = 3, refinement iterations = 2), and were checked manually. We excluded the *rbc*L-*rbc*X spacer for alignability issues.

A second alignment, including the *rbc*L-*rbc*X spacer, was performed using 30 of the 610 sequences. These 30 sequences were selected because they were the closest blast match to our 12 ASVs identified with *rbc*L-X. By aligning a reduced number of sequences, we validated the accurate placement of our 12 ASVs in the larger phylogenetic tree (610 sequences). The Maximum likelihood phylogenetic tree was inferred using *RaxML-HPC2* v8.2.12 (Stamatakis 2014) on CIPRES (Miller et al. 2015), with 500 replicates of rapid bootstrapping to assess branch support. The putative placement of the ASVs was mapped on the tree and was visualized in *FigTree* v1.4.3 (Rambaut 2010).

3 Results

3.1 Diversity patterns of bacterial community

3.1.1 16S ribosomal RNA marker

The total endophytic bacterial community retrieved with the 16S marker in coralloid roots of *Z. pseudoparasitica* and *Z. nana* comprised 1551 ASVs (1295 ASVs in *Z. pseudoparasitica* and 294 ASVs in *Z. nana*) (dataset 1). The mean sample coverage was 9326.73, with either low (< 400 reads) or very high coverage (29,539 reads). Rarefaction curves did not reach an asymptote with the number of individuals sampled for both species (Supplementary Fig. 2A), meaning that further sampling would increase bacterial ASVs. Supplementary Table 2 shows the number of ASVs of the most abundant phyla and their respective orders and families. Dominant phyla with over 100 ASVs corresponded to Proteobacteria (593 ASVs), Actinobacteriota (337 ASVs), Acidobacteriota (143 ASVs), Planctomycetota (106 ASVs), and Cyanobacteria (101 ASVs). The five most dominant orders were Hyphomicrobiales (119 ASVs), Burkholderiales (86 ASVs), Frankiales (75 ASVs), and Pseudonocardiales (74 ASVs).

Richness based on the Chao1 index indicated that there was not a significant difference in alpha-diversity between host plant individuals, with similar observed average ASVs (*Z. pseudoparasitica* = 40.84; *Z. nana* = 43.89) (Supplementary Fig. 3). The Simpson index was high (almost 1) in both plant species highlighting that some bacterial ASVs are overabundant in relation to the rest of the community (Supplementary Fig. 3), particularly in samples from the PNGDOTH (Supplementary Fig. 3). The Pielou index also suggested high community evenness. The Shannon index was high for both species, consequently, the communities were highly diverse, with some dominant ASVs. All the richness indexes indicated there were no significant differences in diversity between both *Zamia* species (Shannon: $\chi 2 = 2.1572$; *p-value* = 0.1419; Simpson: $\chi 2 = 2.0881$; *p-value* = 0.1485; Pielou: $\chi 2 = 2.434$; *p-value* = 0.1187). Regarding the relative abundance analyses, one to three phyla (Actinobacteria, Cyanobacteria, and Proteobacteria) seem to be overrepresented in most samples in both species (Supplementary Fig. 4).

The endophytic cyanobacterial community retrieved with the 16S marker in coralloid roots of *Zamia* species comprised 101 ASVs (81 ASVs in *Z. pseudoparasitica,* and 21 ASVs in *Z. nana*) (dataset 2). The mean sample coverage for cyanobacteria was 3780, with either low (72 reads) or very high coverage (20,631 reads). Sample rarefaction curves did not reach an asymptote with the number of individuals sampled for the two *Zamia* species (Supplementary Fig. 2B). When only the cyanobacterial ASVs were considered, the richness indexes indicated that the two species host significantly different cyanobacterial communities, where *Z. nana* presented higher evenness (Chao: $\chi 2 = 2.015$; *p-value* = 0.15; Shannon: $\chi 2 = 6.09$; *p-value* = 0.01; Simpson: $\chi 2 = 3.82$; *p-value* = 0.05; Pielou: $\chi 2 = 8.66$; *p-value* = 0.003).

3.1.2 *rbc*L-X marker

The total cyanobacterial community retrieved with *rbc*L-X maker in the coralloid roots of the *Zamia* species comprised 176 ASVs (141 ASVs in *Z. pseudoparasitica,* and 71 ASVs in *Z. nana*) (dataset 3). Mean sample coverage was 85,006.6, with samples varying in coverage between 38,287 reads and 109,232 reads. With the current sample size, the rarefaction curves for both species did not reach an asymptote (Supplementary Fig. 2C). Observed species based on the Chao1 index was slightly significantly different in alpha-diversity between host species ($\chi 2 = 3.58$; *p-value* = 0.05), with *Z. pseudoparasitica* showing a higher number of species (25.2 ASVs), when compared to *Z. nana* (13.2 ASVs). All the other richness indexes showed a similar pattern to the one observed with the marker 16S, yet between host species differences were not significant (Shannon: $\chi 2 = 1.11$; *p-value* = 0.29; Simpson: $\chi 2 = 0.372$; *p-value* = 0.54; Pielou: $\chi 2 = 0.196$; *p-value* = 0.65).

3.2 Differences in cyanobacterial ASV detectability between the two markers

When comparing the ability of both markers (16S and *rbc*L-X) to detect cyanobacterial ASVs, we first observed that the marker 16S detected a lower number of ASVs (101 ASVs for 16S and 176 for *rbc*L-X). The 16S marker was highly dependent on library size (Fig. 2A). For example, lower richness was biased toward samples with low coverage

($R^2 = 0.28$; *p-value* = 0.004). Whereas the *rbc*L-X did not significantly change with sample read coverage ($R^2 = 0.069$; *p-value* = 0.64).

Rarefaction curves showed that *rbc*L-X tends to retrieve more species of cyanobacterial ASVs by sample than 16S. Species richness extrapolation of the cyanobacterial

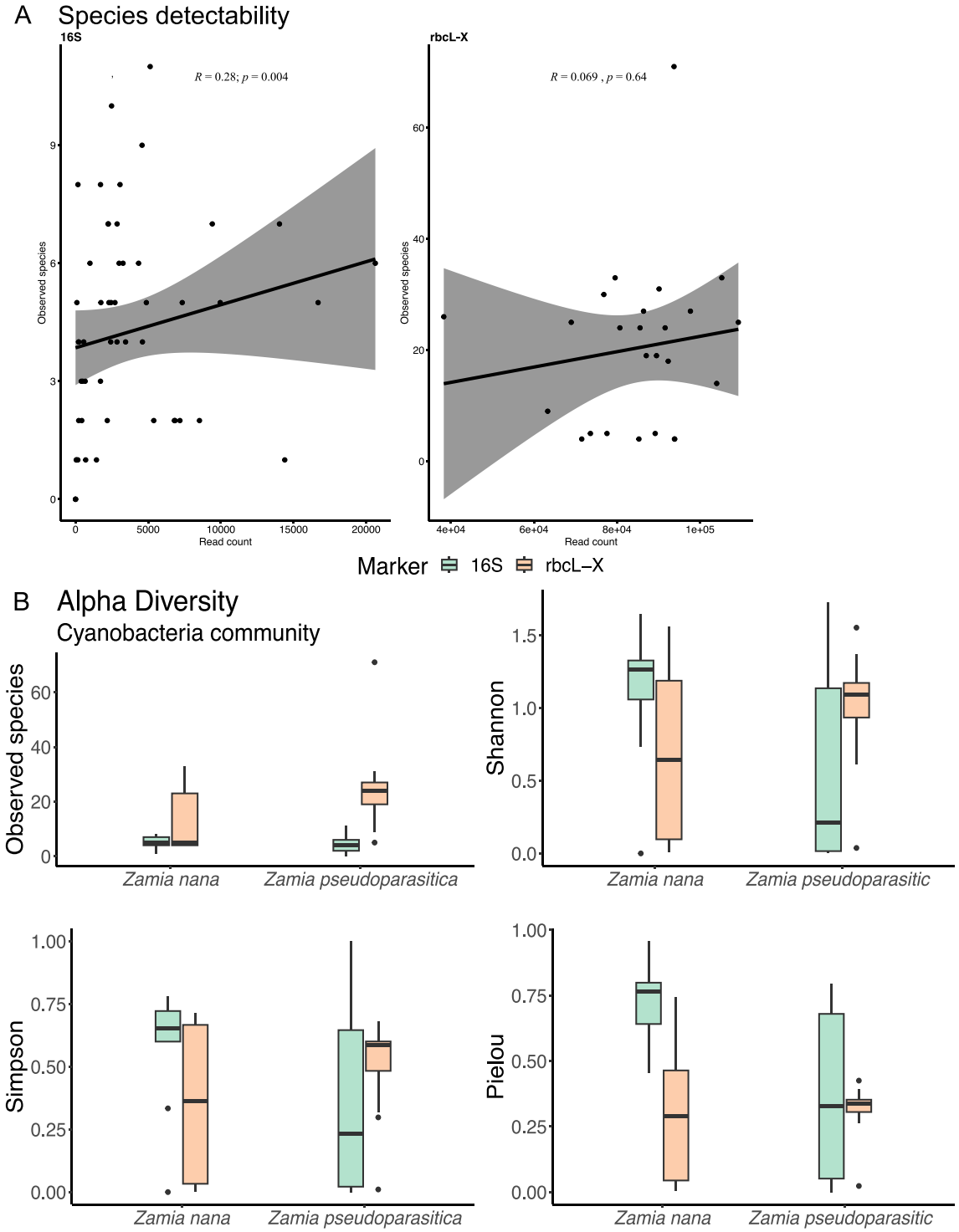

Fig. 2 Alpha diversity of the cyanobacterial community in coralloid roots of the two *Zamia* species (*Z. pseudoparasitica* and *Z. nana*). **A**. Species detectability of 16S and *rbc*L-X markers: correlation of observed species in relation to read count (coverage). **B**. Alpha diversity estimates of the cyanobacterial community observed in *Z. pseudoparasitica* and *Z. nana.* The cyanobacterial community was identified with two molecular markers (16S in green and *rbc*L-X in orange)

Table 2 Pearson's Chi-square test between observed species and extrapolated richness (using three non-parametric methods) of the cyanobacterial community in coralloid roots of *Zamia pseudoparasitica* and *Zamia nana* identified with the markers 16S and *rbc*L-X

16S				*rbcL-X*			
Zamia pseudoparasitica				*Zamia pseudoparasitica*			
	X^2	*Df*	*p-value*		X^2	*Df*	*p-value*
Chao	3,545,237	40,918	< 0.001	Chao	1,063,395	11,976	< 0.001
Jackknife 1	31,199	40,918	1	Jackknife 1	100,462	11,976	< 0.001
Bootstrap	21,277	40,918	1	Bootstrap	66,470	11,976	< 0.001
Zamia nana				*Zamia nana*			
	X^2	*Df*	*p-value*		X^2	*Df*	*p-value*
Chao	66,182	5988	< 0.001	Chao	164,038	6986	< 0.001
Jackknife 1	4926.7	5988	1	Jackknife 1	15,079	6986	< 0.001
Bootstrap	3359.4	5988	1	Bootstrap	12,171	6986	< 0.001

community in coralloid roots of the two *Zamia* species indicated that a large proportion of cyanobacterial ASV remains undetected with both markers (Supplementary Fig. 5–8). A less conservative method, Chao Index, extrapolates to over a thousand cyanobacterial ASVs for *Z. pseudoparasitica* for the two markers (summarized in Supplementary Table 3). Overall, in *Z. pseudoparasitica* and *Z. nana*, the observed cyanobacterial ASVs were significantly lower than the estimated cyanobacterial ASVs with both markers (Table 2). However, *rbc*L-X showed to some extent, more accuracy based on the smaller standard error in extrapolation simulations. The more conservative richness estimator Jackknife1 estimated 146.48 cyanobacterial ASVs for *Z. pseudoparasitica* and 41.89 for *Z. nana* when using the marker 16S. In contrast, *rbc*L-X estimated additionally 236.2 cyanobacterial ASVs for *Z. pseudoparasitica* and 110.6 for *Z. nana*. Bootstrap estimation ranges between 105.65 cyanobacterial ASVs more for *Z. pseudoparasitica* and 31.79 for *Z. nana* when using the marker 16S, while the estimation ranges between cyanobacterial 178.22 ASVs for *Z. pseudoparasitica* and 87.71 for *Z. nana* when using the marker *rbc*L-X. Overall, Jackknife and Bootstrap methods with *rbc*L-X tended to to estimate significantly more cyanobacterial ASVs when compared to the observed cyanobacterial ASVs (*p-value* = 0.001), while estimated from data with the 16S marker were not significantly higher to the observed cyanobacterial ASVs richness.

We also compared if a marker biases cyanobacterial diversity in coralloid roots of the two *Zamia* species using four diversity indexes (Fig. 2B). Of the four alpha-diversity indexes the Chao1 index showed significantly higher richness with the *rbc*L-X marker (Chao: *p-value* = < 0.001), while others showed non-significant statistical differences between the two markers used (*p-value* = > 0.05). Results from statistical results are summarized in Table 3.

Finally, based on the rank abundance curves for datasets 2 and 3, we observed that, with both markers, the cyanobacterial community in coralloid roots of *Zamia* species was dominated by few ASVs with relative abundances over 50% (Fig. 3). Specifically, *Z. pseudoparasitica* showed dominances of three ASVs, and five in *Z. nana*. With the marker *rbc*L-X, rare ASVs were represented at higher relative abundance compared to 16S.

3.3 Efficacy of *rbc*L-X marker: Mock community

In the mock community I (cultures 52, 54, and 103) we recovered exactly five ASVs, which had three sequences identical to those from the three input strains (Fig. 4), two strains had lower abundance and were not part of the mock community, meaning that the cultures were not entirely pure. Visually inspecting the cultures, all belonged to Nostocales (Supplementary Fig. 1). As predicted, the mock community

Table 3 Kruskal–Wallis rank sum test summary statistics. Differences in alpha diversity with four richness indexes for cyanobacterial community in the coralloid roots of *Zamia pseudoparasitica* and *Zamia nana*. Statistical comparison between host cyanobacterial community with 16S (101 ASVs), and with *rbc*L-X (176 ASVs). Comparison of the cyanobacterial community between the two amplicon sequencing markers used (16S vs *rbc*L-X). Significant results are depicted in bold

	Richness index	*Df*	*χ2*	*p-value*
Cyanobacteria (16S) between host	Observed	1	2.0154	0.156
	Shannon	1	6.0991	**0.013**
	Simpson	1	3.8255	**0.05**
	Pielou	1	8.6687	**0.003**
Cyanobacteria (*rbc*L-X) between host	Observed	1	3.5873	0.058
	Shannon	1	1.1108	0.292
	Simpson	1	0.37231	0.542
	Pielou	1	0.19692	0.657
Cyanobacteria between markers (16S vs *rbc*L-X)	Observed	1	27.148	**< 0.001**
	Shannon	1	3.377	0.066
	Simpson	1	0.6538	0.419
	Pielou	1	2.2036	0.138

II (cultures 52 and 103) recovered two ASVs (ASV10, ASV11), in nearly equal abundance, and the mock community III (culture 103) recovered a single ASV (Fig. 4).

3.4 Core cyanobacterial community

Using the 16S marker (dataset 2), no ASV was identified as part of the core cyanobacterial community when the threshold was set at 90% and 75%. Three shared ASVs (ASV2, ASV3, and ASV4) were found when the threshold was set at 50%. The ASV2 is an identical match (using blastn) to an existing *Nostoc* sp. (sample identity: HA4356-MV1), ASV3 matched *Nostoc linckia,* and ASV4 matched *Nostoc* sp. from the lichen *Peltigera*. The cyanobacterial community identified did not find a significant associated bacterial community with the indicator species analysis.

Using the *rbc*L-X marker (dataset 3), the ASV1 was the only member of the core cyanobacterial community present in nearly all samples with a 90% prevalence. The relative abundance of ASV1 ranges from 0.002–99.8% of the reads (Fig. 5). Using a 75% threshold, only three ASVs (ASV1, ASV2, ASV3) were present across samples and five ASVs at a 50% threshold. When there were more than three ASVs per root, other ASVs were found at lower abundance below 100 reads to even eleven reads. Out of the 27 samples of both *Zamia*, only two *Z. nana* individuals did not contain the ASV1 (Fig. 5). With the indicator species analysis, only the ASV2 was determined to be significantly associated with *Z. pseudoparasitica* (*p-value* = 0.004).

3.5 Phylogenetic placement of the cyanobacterial strains based on *rbc*L-X

We inferred the phylogenetic placement of the most abundant cyanobacterial ASVs found in both species of *Zamia*. The relative abundance threshold of 0.03% (Nelson et al. 2021) identified eight ASVs in both species. In addition, two ASVs (ASV3 and ASV21) were presented exclusively in *Z. pseudoparasitica*, only found in two samples, and ASV5 and ASV9 exclusively in *Z. nana* only found in two and one sample respectively. This resulted in a total of 12 ASVs. The average ASV number was 5.4 (min 3, max 7) per coralloid

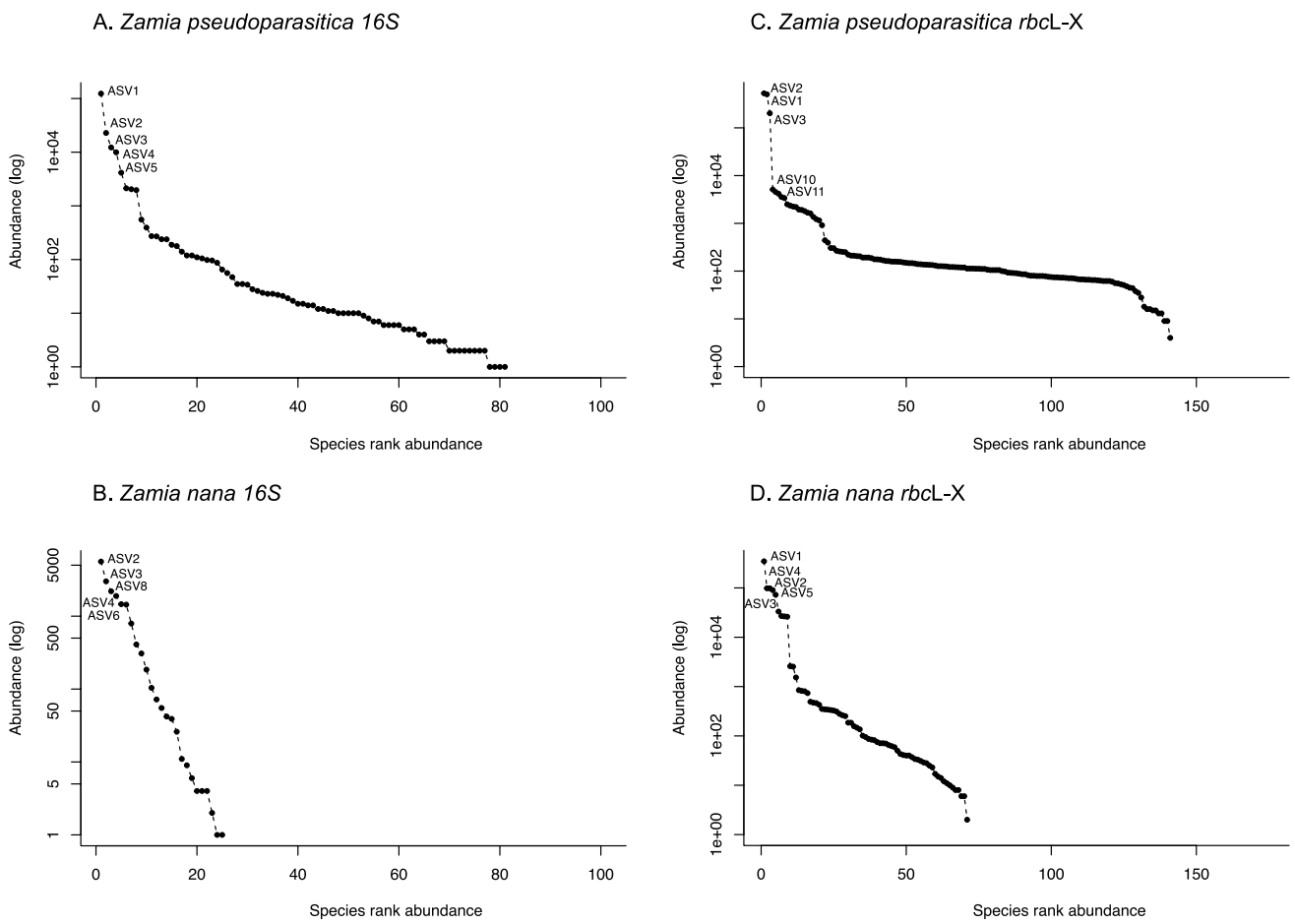

Fig. 3 Rank-abundance plot of cyanobacterial ASVs in coralloid roots identified with the markers 16S and *rbc*L-X for the two *Zamia* species (*Z. pseudoparasitica* and *Z. nana*)

root of *Z. pseudoparasitica* and 3.9 ASVs (min 1, max 8) of *Z. nana* (Fig. 6). Generally, there were one to three frequent ASVs, ASV1 being the most abundant and present in nearly all samples. In three *Z. nana* samples, the ASV1 accounted for nearly 100% of the cyanobacteria present in the root. The ASV1 was an identical match (using blastn) to an existing *rbc*L-X sequence from *Z. pseudoparasitica* from the site in El Copé (Supplementary Table 4).

Maximum likelihood (ML) analyses of the 12 ASVs revealed three main clades with abundant ASVs with over > 75–90% of prevalence nested in clades with ASVs occurring in less than 50% and in low abundance or ASVs occurring in less than 10% of the samples but with high abundance (Fig. 7; Supplementary Table 4). When mapped into the *rbc*L-X phylogeny, the *Zamia* cyanobacterial strains fell into five clades. One group (including ASV1, ASV3, ASV7, ASV8, ASV9, ASV13, ASV16, and ASV21) formed one clade including *Z. pseudoparasitica* El Copé, to a close cyanobacterial strain from the Panamanian hornwort *Leiosporoceros dussii,* and few sequences from *Zamia nana* sequenced here. The other clade containing ASV2 was related to cyanobacterial strains found in southern temperate lichens such as *Collema* spp. The other highly abundant cyanobacterial strain (ASV4) from samples of *Z. nana* was quite isolated from the other cyanobacterial strains in the phylogenetic tree. The ASV6 was closely related to the cyanobacterial strains found in *Leiosporoceros dussii*, a plant found 10 km from the nearest *Z. nana* population.

4 Discussion

4.1 Bacterial endophyte diversity inside the coralloid roots

A total of 1551 ASVs of bacteria within coralloid roots is estimated in both species, mainly represented by three main phyla: Actinobacteria, Cyanobacteria, and Proteobacteria, in agreement with previous studies on Zamiaceae (Bell-Doyon et al. 2020; Gutiérrez-García et al. 2019; Suarez-Moo et al. 2019). For example, the coralloid roots of Mexican *Dioon* species show a greater diversity of bacteria (~5740 OTUs) based on OTUs 97% similarity, with bacteria in the genera *Caulobacter* (Alphaproteobacteria), *Burkholderia* (Proteobacteria), *Amycolaptosis* (Proteobacteria), *Variovorax*

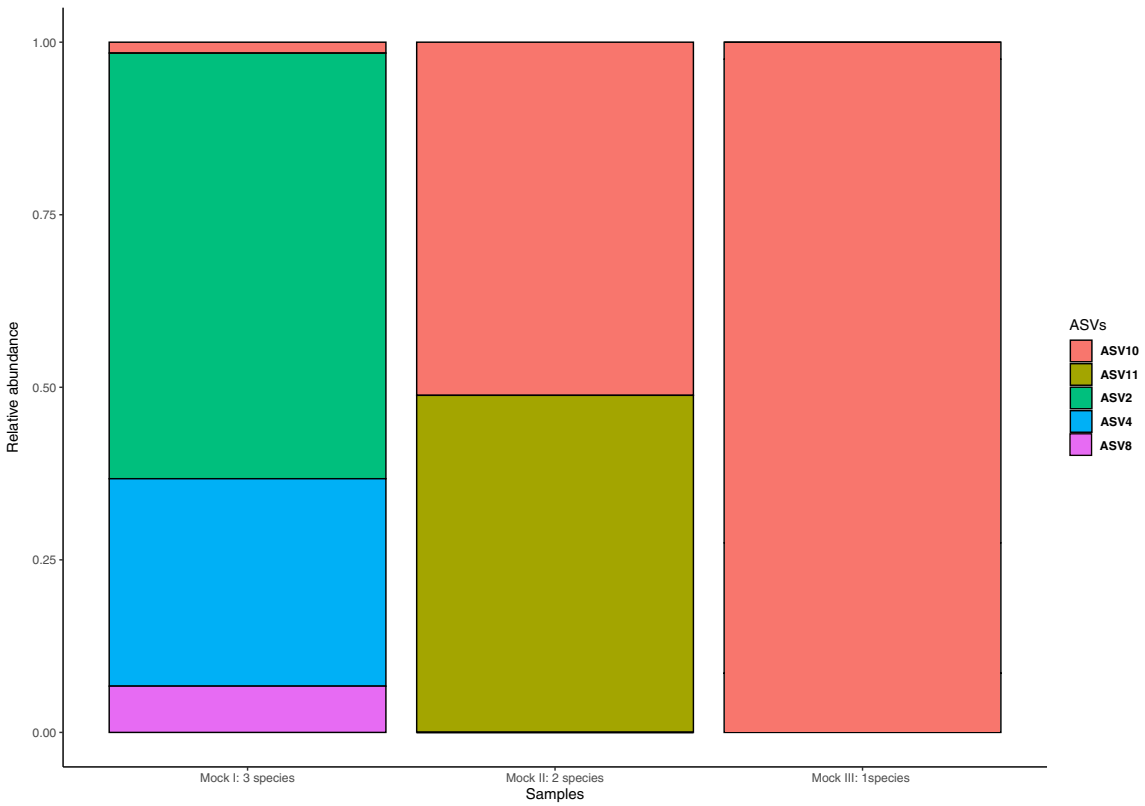

Fig. 4 Relative abundance of the mock communities of cultured cyanobacteria. ASVs were identified with the *rbc*L-X marker. Each vertical bar represents one mock community. The mock community with one cyanobacterial strain recovered one ASV (ASV10), the mock community with two cyanobacterial strains recovered two ASVs (ASV10, ASV11), in nearly equal abundance, and the mock community with three cyanobacterial strains recovered four ASVs

(Proteobacteria) and *Nostoc* (Cyanobacteria) (Suarez-Moo et al. 2019). Such difference estimating the number of microbial taxa may be related to the higher sensitivity of the ASV-based method used here in contrast to the OTU-based clustering method that tends to overestimate true diversity (Callahan et al. 2016). Most of the bacterial strains found in previous studies on coralloid roots of cycads and the ones observed here are found associated with roots of seed plants (Beckers et al. 2017; Trivedi et al. 2020). In addition, Hyphomicrobiales (formerly Rhizobiales, Proteobacteria) seem to be present in coralloid roots of all cycads sequenced so far. Within Hyphomicrobiales, many genera are widely known for performing biological nitrogen fixation (Chen et al. 2021) . The association of the two *Zamia* species with diverse nitrogen-fixing taxa suggests coralloid roots can assemble phylogenetically disparate bacterial taxa with functional redundancy (Chang et al. 2019; Pecundo et al. 2021). In cycads, coralloid roots hosting cyanobacteria are characteristic of each one of the 375 species of the phylum. Cyanobacteria occupy most of the tridimensional space of the coralloid root (Fig. 1). Thus, a cyanobacterial-specific marker is quite useful to further uncover the pattern of cyanobacterial diversity across cycad species and test clade (genera/species) specificity. The newly developed marker (*rbc*L-X) targeting cyanobacteria can be used for other cyanobacterial symbiotic systems such as hornworts, the angiosperm *Gunnera*, cyanolichens, and Blasiales (liverworts) (Adams 2000; de Vries and de Vries 2022).

4.2 *rbc*L-X reveals the phylogenetic placement and diversity of symbiotic *Nostoc*

The *rbc*L-X region has been routinely used for exploring phylogenetic relationships among symbiotic cyanobacteria associated with lichens (Lavoie et al. 2020; Magain et al. 2017; O'Brien et al. 2005, 2013; Rudi et al. 1998) and bryophytes, specifically hornworts (Bouchard et al. 2020; Nelson et al. 2021). In *Nostoc,* the *rbc*L-X spacer has been typically

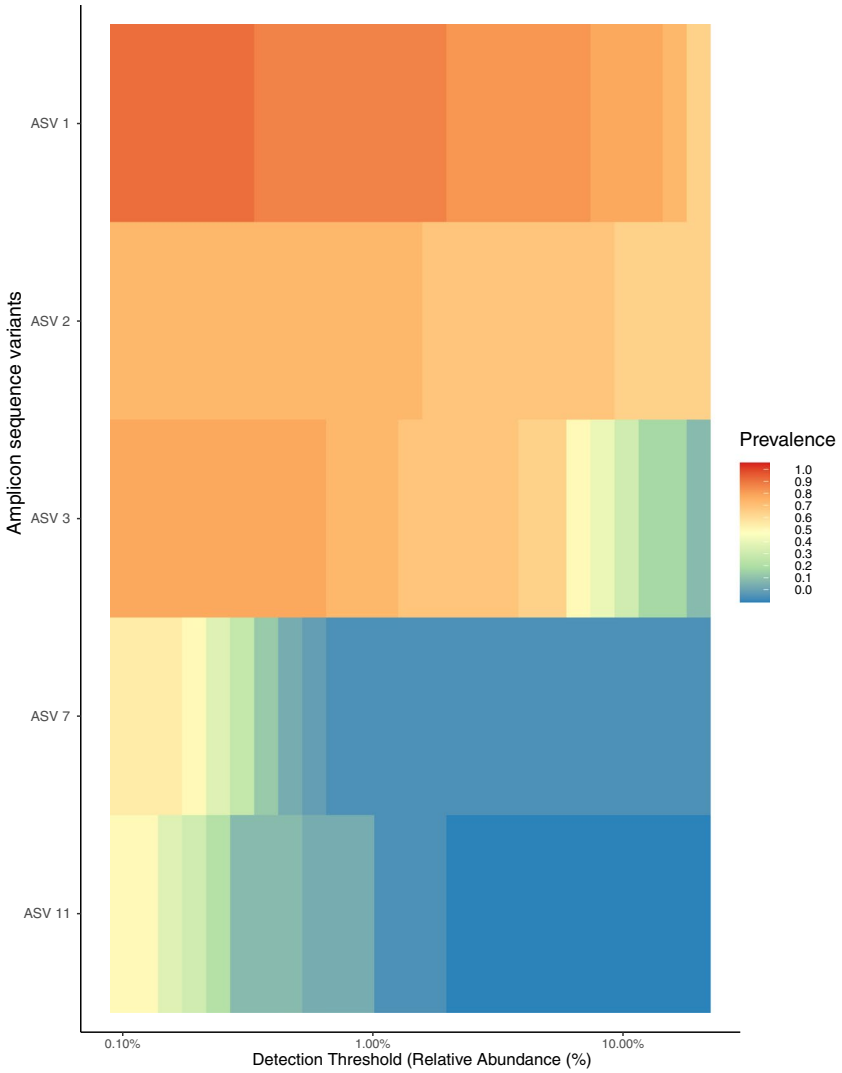

Fig. 5 Core cyanobacterial community in the coralloid roots of the two species of *Zamia* (*Z. pseudoparasitica* and *Z. nana*). The core community was identified with the *rbc*L-X marker. Prevalence and relative abundance are shown for the five most common ASVs at a 50% threshold. At a 75% threshold, only 3 ASV are recovered (ASV1, ASV2, ASV3), at the 90% threshold only the ASV1 is recovered

excluded from phylogenetic analyses because of its difficulty when aligning the region across strains. However, the spacer seems to be highly informative to identify a single strain due to its variability. The database compiled here and conserved regions allow the creation of specific primers for amplicon sequencing. The newly designed amplicon targets 100 bp of the gene *rbc*X (out of the 400 bp of the locus), which is quite useful for aligning the sequences to existing phylogenies and comparing to available sequences in databases, as presented here. The low cost per sample using Illumina technology, including low DNA input material, makes this amplicon suitable for routine metabarcoding studies, which will improve the detectability of low abundant cyanobacterial strains that are underrepresented with previously used markers like 16S and *trn*L (Costa et al. 2004).

Using the *rbc*L-X sequence, we found that the most prevalent ASVs (ASV1, ASV3) are included in a clade with known cyanobionts associated with *Z. pseudoparasitica*, the Panamanian hornwort *Leiosporoceros dussii* and few lichen species from Southern America (Bell-Doyon et al. 2020; Bouchard et al. 2020). Most of the other ASVs (in lower prevalence) are found scattered throughout the Nostocales phylogeny. Our results are in line with those from Suarez-Moo et al. (2019) in which the endophytic *Nostoc* from Mexican cycads are clustered in a single clade. In addition, our results show that there is more than a single *Nostoc* strain per coralloid root, suggesting an existing competition among cyanobacterial strains, selectivity by the host, or low diversity in the underlying substrate.

Using Sanger-sequencing and targeting two loci (*trn*L, 16S) several researchers have proposed that there is a single or a few *Nostoc* per coralloid root (Costa et al. 2004; Yamada et al. 2012). The Sanger sequencing is well-known to amplify the most common symbiont instead of capturing the total diversity of the symbiotic spectrum (Bouchard et al. 2020). The advent of metagenomic sequencing has broadened the spectrum of local diversity and clearly shows a higher local diversity within a single coralloid root (Bell-Doyon et al. 2020; Gutiérrez-García et al. 2019; Pecundo et al. 2021; Suarez-Moo et al. 2019; Zheng et al. 2018). An amplicon protocol targeting the entire region *rbc*L-*rbc*X-*rbc*S region was developed using PacBio long reads uncovering a large cyanobacterial diversity in the targeted hornwort species (Nelson et al. 2021). This study recovered similar number of ASVs for the hornwort species studied. This method, however, remains costly for studies

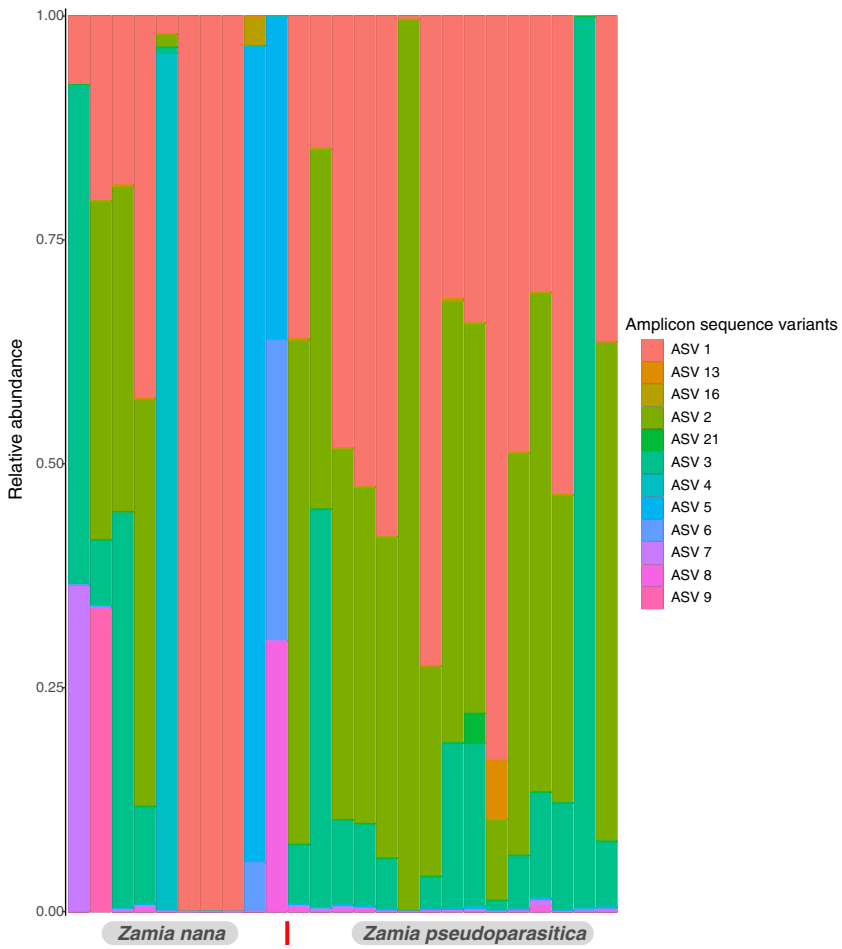

Fig. 6 Relative abundance of cyanobacterial ASVs in the coralloid roots of the two species of *Zamia* (*Z. pseudoparasitica* and *Z. nana*). ASVs were identified with the *rbc*L-X marker. Each vertical bar represents one sample of *Zamia*. ASV1 is found in nearly all samples with high relative abundance (up to 99.99%) in some samples

addressing plant-cyanobacteria diversity, especially in species with small, microscopic colonies with low DNA yield per extraction. A cost-efficient short-read amplicon sequencing of cyanobacteria, such as the *rbc*L-X, will be accessible in developing countries where most symbiotic cyanobacteria remain unexplored (Helmy et al. 2016).

The overall improvement of ASV detectability with the marker *rbc*L-X will aid in disentangling microbial diversity change in host-cyanobacterial symbiotic interaction in natural and disturbed habitats. We compared the bias associated to sequence coverage and sampling size in estimating cyanobacterial diversity. We observed that while 16S

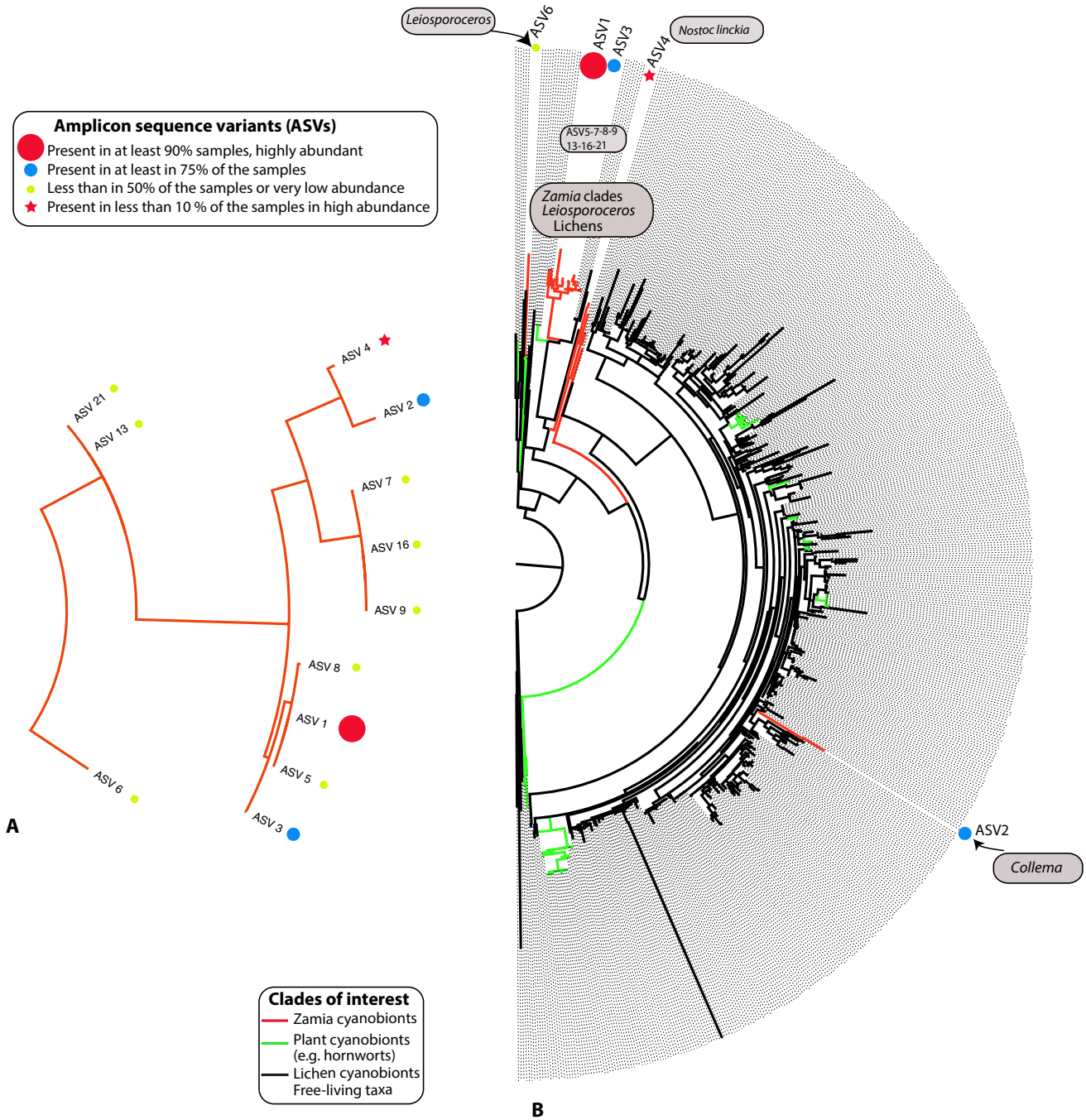

Fig. 7 **A**. Phylogenetic tree of cyanobacteria based on the *rbc*L-X region. The matrix is based on 610 samples from GenBank and 12 samples from this study. Genbank samples belong to Nostocales, they are mostly associated with cyanolichens, plants (*Gunnera*, cycads, hornworts, liverworts), or free-living strains. **B**. Phylogenetic tree of the *rbc*L-X region of the 12 cyanobacterial ASVs present in the coralloid roots of the two species of *Zamia* (*Z. pseudoparasitica* and *Z. nana*). The tree shows four main groups

capacity to detect cyanobacterial ASVs significantly depends on coverage, *rbc*L-X was able to detect the same number of ASVs even in samples with low coverage. On the other hand, sampling size was limiting for both markers. However, when we rarefied the number of ASVs, with a lower number of samples, the marker *rbc*L-X was able to detect a higher number of ASVs. This is highly beneficial when designing sampling and experimental research dealing with threatened species with low occurrence at regional scale, like *Zamia* species. Our approach using microbial mock communities shows that this marker can efficiently identify the respective taxa members in communities with a known diversity which can be applied to characterize unknown cyanobacterial symbiosis (Nelson et al. 2021). Here we demonstrate that with the *rbc*L-X amplicon marker we were able to find significant differences among host species, accurately detect both common and rare ASVs, and study specific members of the core microbiota Whereas there was a bias with the broad prokaryote spectrum 16S marker.

4.3 The *Zamia nana* and *Zamia pseudoparasitica* core cyanobacterial community

The core microbiome appears to have paramount biological and evolutionary importance; however, a threshold of prevalence has not been established (Neu et al. 2021; Risely 2020). In our study, we set three different thresholds (50%, 75% and 90%) for both 16S and *rbc*L-X. Using 16S, Gutiérrez-García et al. (2019) found that cycads of the genus *Dioon* shared a core bacterial community composed mainly of nitrogen-fixing microorganisms. With the current species of Zamiaceae studied so far, the root endophytic bacterial community is mainly composed of the orders Hyphomicrobiales and Nostocales (Bell-Doyon et al. 2020; Suarez-Moo et al. 2019). However, the two cycad genera differed in part in their five most abundant families. In the coralloid roots of the genus *Dioon* the most abundant families are Nostocaceae, Streptomycetaceae, Rhizobiaceae, Pseudonocardiaceae, and Chitinophagaceae (Suarez-Moo et al. 2019), while in *Zamia* roots are Pseudonocardiaceae, Nostococaceae, Acidothermaceae, Acetobacteraceae, and Isosphaeraceae. In contrast, in the genus *Cycas,* the more abundant families are Rhizobiaceae, Pseudonocardiaceae, Mycobacteriaceae, Bacillaceae, and Bradyrhizobiaceae (Zheng et al. 2018), where only Pseudonocardiaceae and Mycobacteriaceae are dominant in the Zamiaceae's coralloid root. These findings put into question the extent to which host identity and geography drive microbial community composition within coralloid roots.

The *rbc*L-X spacer provides a complementary story. We set three different thresholds (50%, 75% and 90%). Using a 90% threshold, a single ASV (ASV1), is highly abundant across most samples (Fig. 6). The ASV1 is followed by ASV3 (from the same clade) and ASV2 (in 75% of the samples). The presence of a clear pattern of dominance by a particular clade of *Nostoc* invites a wider sampling of widespread and local endemics throughout their range to test whether a pattern is widespread or localized. Sequencing soil from the substrate (rhizosphere or tree) of target plants would also shed some light on endophyte distribution, availability, and recruitment.

5 Conclusion

Plant–microbe and microbe-microbe interactions in the coralloid roots of cycads are unique systems to explore cyanobacteria assembly, ecological interactions, and evolution. In summary, we showed that coralloid roots are composed of a diverse microbial community, dominated by Nostocales. However, different alpha-diversity and microbial community composition are observed between *Zamia* species, and between cycad genera (Bell-Doyon et al. 2020; Suarez-Moo et al. 2019; Zheng et al. 2018). The amplicon marker *rbc*L-X described here to target cyanobacteria proves to be low-cost and effective at detecting cyanobacterial ASVs present in the coralloid roots and can be easily applicable to other cyanobacterial symbiotic systems such as cyano lichens, bryophytes, and vascular plants.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13199-024-00972-w.

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Author contributions AMS, ST performed metabarcoding analyses and wrote the first draft; JCV conceived the study and performed metabarcoding analyses; AMS, MAG, ST, and JCV wrote and edited the manuscript.

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Data availability Raw sequence data were deposited in the NCBI Sequence Read Archive (SRA) under the bioproject number PRJNA1049855 with their respective accession numbers.

The datasets, metadata, and scripts generated and/or analysed during the current study are openly available in the Github repository, https://github.com/jcarlosvillarreal/cycads_rbcLX_roots.

Declarations

Compliance with international conventions and regulations on biological diversity and endangered species *Zamia* species collections were done under the collecting permit SE/PB 1–16 from the Ministry of the Environment MiAmbiente, Panama, and from the administration of the National conservation parks visited for this study who granted permission to access the park facilities. Due to the CITES status of all cycad species, we have omitted the coordinates of the localities.
Samples from Donoso were accessed through the site facilities of the mining company (First Quantum Minerals). The company has not been consulted about the publication.

Benefit-sharing statement The present work is the result of an international scientific partnership that was developed with scientists from the country where samples were gathered, who are included as co-authors. The work was done under partnership between research institutions and the results will be shared with the provider communities and the broader scientific community.

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