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Cyanobiont diversity within coralloid roots of selected cycad species

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Abstract

The diversity and host specificity of the cyanobionts of several cycad species (*Cycas circinalis* L., *C. rumphii* Miq., *Encephalartos lebomboensis* I. Verd., *E. villosus* Lem., and *Zamia pumila* L.) collected in a botanical garden were examined using the $tRNA^{Leu}$(UAA) intron sequence as a genetic marker. Nested PCR was used to specifically amplify the $tRNA^{Leu}$(UAA) intron directly from the freshly isolated symbiotic cyanobionts. By direct amplification of the biological material the laborious isolation of the cyanobionts can be avoided. A single DNA fragment, ranging in size from 287 bp to 329 bp, was consistently amplified from the different biological samples. The intron sequences obtained from the cycad cyanobionts show high similarities to the corresponding sequences in the free-living strains *Nostoc* sp. strain PCC 73102 and *N. muscorum* as well as in several lichen cyanobionts. Although different *Nostoc* strains were found in the present study, no sequence variation was observed when analyzing a single coralloid root. However, different coralloid roots from a single cycad individual may harbor different cyanobacteria. Moreover, cyanobionts in coralloid roots of both *Encephalartos lebomboensis* and *E. villosus* were found to possess the same intron sequence.

Keywords: Cyanobacterium; Cycad; Diversity; *Nostoc*; Symbiosis; $tRNA^{Leu}$ intron

1. Introduction

Cyanobacteria are able to form symbiotic associations with a variety of organisms. Among plants, heterocystous cyanobacterial strains form symbiotic associations with bryophytes, pteridophytes (*Azolla*), gymnosperms (cycads) and angiosperms (*Gunnera*) [1–3]. The symbiosis between cyanobacteria and cycads is the only known example of a symbiosis between a gymnosperm and an organism capable of nitrogen fixation [4]. Cycads are an ancient group of plants, naturally occurring in the tropic and subtropic regions. The approximately 156 species of cycads are divided into three families: Cycadaceae (one genus; *Cycas*), Stangeriaceae (one genus; *Stangeria*), and Zamiaceae (eight genera; *Bowenia*, *Ceratozamia*, *Dioon*, *Encephalartos*, *Lepidozamia*, *Macrozamia*, *Microcycas*, and *Zamia*) [5]. Anatomically the cycads produce three types of roots: (i) a tap root which is equivalent to the primary root system found in most types of plants, (ii) lateral roots, and (iii) 'coralloid roots' which are a highly specialized type of lateral root containing the symbiotic cyanobionts.

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Coralloid roots have been recorded in all cycad species examined and they are formed by the plant even before being invaded by the cyanobiont [4]. In seedlings of *Macrozamia communis*, the coralloid roots are first produced endogenously within the hypocotyl [6]. Since Reinke [7] first described that the symbiont entered through injured parts of the root, several hypotheses for how the infection process occurs have been suggested: (i) through lenticels, (ii) through the papillose sheath, or (iii) through breaks in the dermal layer (see [4]). However, the process of infection is not completely understood. Invasion may occur at any stage of development and the number of times a coralloid root is infected is not yet known. Inside the root, the cyanobiont occupies a specific layer. This cyanobacterial zone is filled with mucilage, and made up of a large number of elongated cycad cells transversing the zone interconnecting two adjacent cortical layers. It has been suggested [8] that these elongated cells are specialized cells responsible for the transfer of metabolites between the symbionts.

The filamentous heterocystous cyanobacteria are located intercellularly between the elongated cycad cells [4]. However, there are some reports of an intracellular location [9,10]. Based on morphological characters, cyanobionts have mostly been classified as *Nostoc* [4,11]. Little work has been done to elucidate the host specificity and diversity of the cycad cyanobionts, and only few studies used molecular techniques for discriminating between different cyanobionts. In a study by Zimmerman and Rosen [12], different morphological *Nostoc*s were isolated from two different cycad species, and the morphological differences were confirmed using zymograms. However, only cultivated cyanobionts were included in this study. In a different study, Southern hybridizations using probes against conserved genes and genomic cyanobacterial DNA from natural populations of different cycad species indicated the presence of several cyanobionts in a single cycad species [13]. However, in order to get sufficient amounts of genomic DNA, samples from many coralloid roots from different cycad individuals were pooled for each sample being analyzed, so no conclusion can be drawn concerning whether a single plant, or coralloid root, hosts multiple cyanobionts.

The aim of this work was to further examine the genetic diversity of the cyanobionts in cycads. We analyzed the host specificity and diversity of the cyanobiont(s) in cycad symbioses using a genetic marker, the cyanobacterial $tRNA^{Leu}$(UAA) intron [14–16], and coralloid roots collected from some selected cycad species.

2. Materials and methods

2.1. Biological material

The coralloid roots used in the present study were collected from cycads growing in The Fairchild Tropical Garden, Miami, FL, USA. Cycad species used included *Cycas circinalis* L., *C. rumphii* Miq., *Encephalartos lebomboensis* I. Verd., *E. villosus* Lem., and *Zamia pumila* L. Different coralloid roots from the different cycad individuals were collected from different locations within the botanical garden (see Table 1).

2.2. Preparation of the biological material

The coralloid roots were sectioned with sterile scalpels and the cyanobiont(s) from each section was/were collected in 50 μl of sterile water. The cells were washed with 120 μl of 10% (w/v) sodium dodecyl sulfate (SDS) and centrifuged (10 000 × *g*, 5 min). The obtained pellet was washed twice with TE buffer (10 mM Tris (pH 8.0) containing 1 mM EDTA) and resuspended in 50 μl of TE. A series of dilutions were done from this suspension and 2 μl from each dilution was used directly in the PCRs. Similarly, cells of *Anabaena* sp. strain PCC 7120 and *Nostoc* sp. strain PCC 73102 were used directly for PCR.

2.3. Nested PCR

Two primer pairs (outer and inner, respectively), specific for the cyanobacterial $tRNA^{Leu}$(UAA) intron, were used in the PCRs (see [14]). These primers contain restriction sites to facilitate cloning of the obtained DNA fragments. The increased specificity and sensitivity obtained with nested PCR were necessary for good amplification from the freshly isolated cycad cyanobionts. Earlier work [14–16] dem-

onstrated that by sequencing the tRNA^{Leu}(UAA) intron it is possible to distinguish between different cyanobacterial strains of the same genus, as well as between structurally identical cyanobionts in symbiosis with different lichen species. The specific segment of the genome used in the present study was chosen for its high sequence variability in filamentous heterocystous cyanobacteria. Limitations include that there are only few sequences in the public data bases and that the value for general cyanobacterial systematics is unknown. However, it is a good marker for the specific biological questions examined in the present study. The primers were designed by Jeff Elhai (Department of Biology, University of Richmond, Richmond, VA, USA). PCRs were performed as described previously [14].

2.4. Cloning and sequencing

The obtained tRNA^{Leu}(UAA) intron fragments were digested with *Sma*I and *Eco*RI (Pharmacia) before being ligated into the vector pBluescript (Stratagene). The plasmids were transformed into *Escherichia coli* (Epicurian coli® XL-1 Blue, Stratagene) according to the protocol given by the manufacturer. The DNA sequence was determined, using T3 and T7 primers (Stratagene), from double-stranded plasmid DNA using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). The reactions were run and analyzed on a 373A automated DNA sequencer (Applied Biosystems). Computer-assisted sequence analyzes and comparisons were performed, using the GenBank database, at the National Center for Biotechnology Information (Washington, DC: http://www.ncbi.nlm.nih.gov/).

3. Results

3.1. Methodology

Direct amplification of the tRNA^{Leu}(UAA) intron was at first not reproducible in all samples. In order to improve the method, a dilution series and a cleaning step with a solution containing 10% SDS were

Table 1
Cycad coralloid roots collected from The Fairchild Tropical Garden (Miami, FL, USA) and used for amplification and sequencing of cyanobiont tRNA^{Leu}(UAA) introns

Sample	Cycad species	Biological material examined		GenBank accession number
C.c.1	*Cycas circinalis* L.	Specimen grown in a pot in a greenhouse		AF095779
C.r.2	*C. rumphii* Miq.	Specimen grown in a pot in a greenhouse		AF095772
C.r.3a	*C. rumphii* Miq.	Specimen grown outdoor in the botanical garden	Different samples from a single coralloid root	
C.r.3b	*C. rumphii* Miq.	Specimen grown outdoor in the botanical garden		AF095773
C.r.3c	*C. rumphii* Miq.	Specimen grown outdoor in the botanical garden		
C.r.3d	*C. rumphii* Miq.	Specimen grown outdoor in the botanical garden		
E.l.4	*Encephalartos lebomboensis* I. Verd.	Specimen grown outdoor in the botanical garden		AF095775
E.v.5	*E. villosus* Lem.	Specimen grown in a pot in a greenhouse		AF095778
E.v.6a	*E. villosus* Lem.	Specimen grown outdoor in the botanical garden	Different coralloid roots from a single cycad individual	AF095776
E.v.6b	*E. villosus* Lem.	Specimen grown outdoor in the botanical garden		AF095777
Z.p.7	*Zamia pumila* L.	Specimen grown outdoor in the botanical garden		AF095774

Fig. 1. PCR-generated DNA fragments of the tRNA^{Leu}(UAA) intron from sample C.c.1 analyzed by agarose gel electrophoresis. The effects of either washing the sample with SDS (10%) in combination with a dilution series or only a dilution series before the amplifications are demonstrated. Size markers (100-bp ladder) are shown on both sides and a negative PCR control (no cyanobacterial cells) is included in the gel.

included in the protocol. The results (Fig. 1) demonstrate that the initial PCRs were inhibited by the biological material from the coralloid root. This problem can be solved by cleaning and diluting the biological material.

3.2. Nested PCR

Using nested PCR, a single DNA fragment was consistently amplified from the different biological samples (Fig. 2). Each of the single DNA fragments was sequenced. The obtained DNA fragments ranged in size from 287 to 329 bp. Size variation was not observed in samples from several different parts of the single coralloid root of *Cycas rumphii* (sample C.r.3).

3.3. Genetic diversity

Fig. 3 is constructed based on the complete sequences from all cyanobionts analyzed. The subsamples from sample C.r.3 showed the same sequence. Different intron sequences were observed when analyzing different specimens, with different growth conditions, of both *Cycas rumphii* and *Encephalartos villosus*. Moreover, different coralloid roots from a single *E. villosus* specimen may harbor different cyanobacteria. In addition, cyanobionts in coralloid roots of both *Encephalartos lebomboensis* and *E. villosus* possess the same intron sequence indicating that the same cyanobiont is present in two different cycad species. The intron sequences from the cycad cyanobionts show high similarities to the corresponding sequences of the free-living strains *Nostoc* sp. strain PCC 73102 (originally isolated from the cycad *Macrozamia*), *Nostoc muscorum*, and to intron sequences in several lichen cyanobionts [14,15]. However, differences occur in two regions, variable region 1 and 2 respectively (Fig. 3). The differences in variable region 1 cause the observed size variations of the cyanobacterial tRNA^{Leu}(UAA) introns. The second variable region, corresponding to bases 219–222 in the *Anabaena* sp. strain PCC 7120 intron sequence [17] is longer in the cyanobionts examined. However, they are all different compared to the *Anabaena* sp. strain PCC 7120 intron sequence. The stability of the secondary struc-

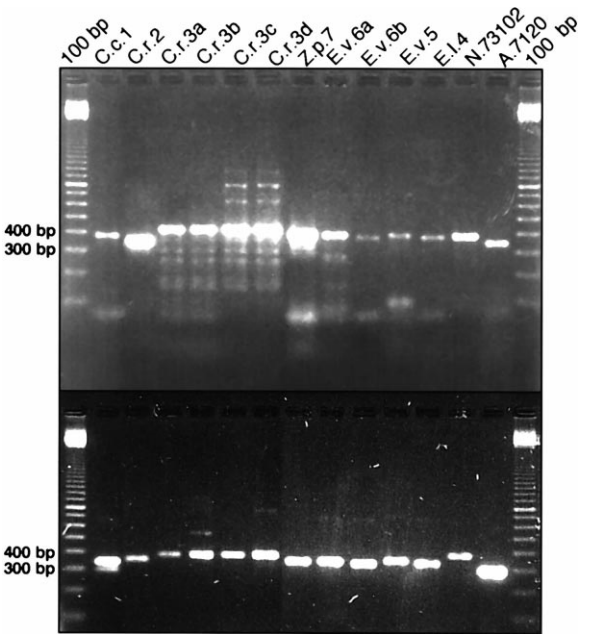

Fig. 2. PCR-generated DNA fragments of the tRNA^{Leu}(UAA) intron from symbiotic cyanobacteria in different cycad samples (for abbreviations see Table 1) and from the free-living strains *Nostoc* sp. strain PCC 73102 (N.73102) and *Anabaena* sp. strain PCC 7120 (A.7120) analyzed by agarose gel electrophoresis. Upper panel: Amplification products after the first PCR using the outer primer pair. Lower panel: Amplification products after the second PCR using the inner primer pair. Size markers (100-bp ladder) are shown on both sides.

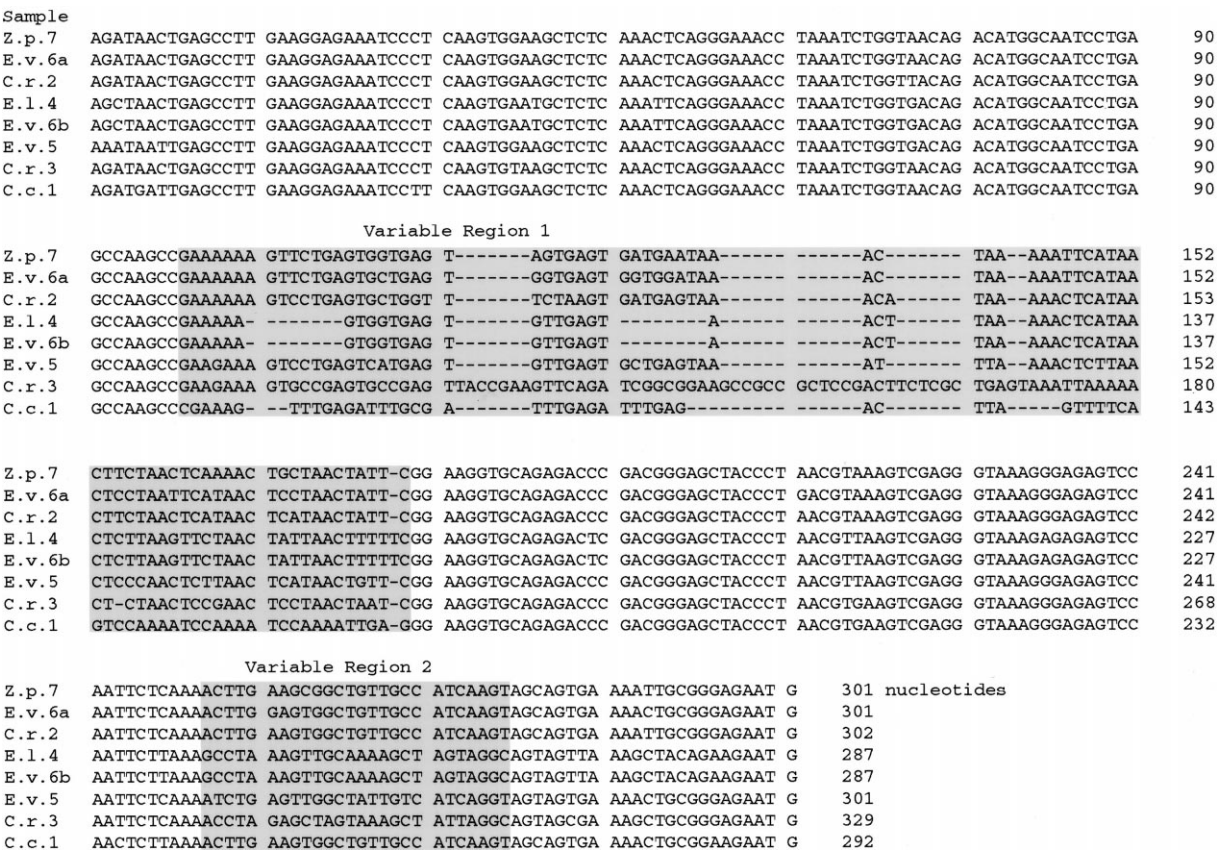

Fig. 3. Nucleotide sequences of the tRNA^{Leu}(UAA) introns amplified from cyanobacterial cycad symbionts.

ture of the introns is often maintained when changes in the sequence have occurred.

4. Discussion

Several studies have focused on the identification of cyanobionts involved in symbiotic associations [11–15,18–23]. However, it is difficult to compare methods as different as, e.g., RFLP and PCR amplification techniques of different segments of the genome. Additionally, several studies analyzed cultured symbionts which, in the case of *Azolla*, has been shown to be problematic since the cyanobiont obtained in culture is not the same organism as the main strain in symbiosis [18]. The methodology presented here makes it possible to easily and specifically amplify the tRNA^{Leu}(UAA) intron from the cyanobacterial symbionts of cycads. The presence of some inhibitory substance(s) in the biological material made it necessary to dilute the samples before the amplification. By direct amplification of the biological material the laborious isolation of the cyanobionts can be avoided. The protocols are kept to a minimum which both saves time and significantly decreases the risk of contamination. Similarly, a direct amplification of cyanobacterial 16S rRNA gene segments has been described [23]. Denaturing gradient gel electrophoresis of the obtained PCR products showed that only one sequence was amplified from the photobionts from the lichens *Collema* cf. *coccophorum* and *Peltula lingulata*, respectively. Moreover, the respective sequences were different [23].

The different tRNA^{Leu}(UAA) introns found demonstrate that different cyanobionts are involved in cycad symbioses. These differences are mainly observed within the highly variable stem loop corre-

sponding to positions 99–143 in the *Anabaena* sp. strain PCC 7120 intron [17]. A search in GenBank using the intron sequences obtained in the present work identifies *Nostoc muscorum*, *Nostoc* sp. strain PCC 73102 and/or lichen cyanobiont intron sequences [14,15] as the most similar intron sequences in the database.

Although different intron sequences were found, no variation was observed within a single coralloid root. This is consistent with infection by a single cyanobiont. That a minor cyanobiont could have avoided detection is still possible but unlikely. However, different coralloid roots from a single *E. villosus* specimen harbor different cyanobacteria. Additionally, cyanobionts in coralloid roots of two different *Encephalartos* species possess the same intron sequence indicating that the same cyanobiont is present in two different cycad species. The present results are in agreement with earlier studies on the diversity and host specificity of cycad cyanobionts [12,13]. Different morphological *Nostoc*s have been isolated from two different cycad species, and the morphological differences were confirmed using zymograms [12].

Using samples from a botanical garden has the advantage that different cycads naturally occurring in both different habitats and geographical regions can be investigated. However, some disadvantages may also be considered. It is not known when the coralloid roots were infected. Some may have been infected in their natural habitat whereas others might have been infected in the botanical garden. In addition, perhaps not the same events take place in natural populations of cycads as in cycads growing in a botanical garden. With the present study the diversity of the cyanobacterial symbioses with cycads has been initiated. In future work one should consider the use of symbiotic cyanobacteria collected in natural populations. Moreover, the genetic diversity of free-living cyanobacteria in the soil surrounding the symbiotic associations deserves additional attention.

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