ELSEVIER

FEMS Microbiology Ecology 49 (2004) 481–487

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Sequence based data supports a single *Nostoc* strain in individual coralloid roots of cycads

José-Luis Costa [a,*], Esperanza Martínez Romero [b], Peter Lindblad [a]

[a] *Department of Physiological Botany, Evolutionary Biology Centre, Uppsala University, Villavägen 6, SE-752 36 Uppsala, Sweden*
[b] *Centro de Investigacion sobre Fijación de Nitrógeno, UNAM, Ap. Postal 565-A, Cuernavaca, Mexico*

Received 22 March 2004; received in revised form 30 April 2004; accepted 4 May 2004

First published online 18 May 2004

Abstract

The genetic diversity of cyanobacteria associated with cycads was examined using the $tRNA^{Leu}$ (UAA) intron as a genetic marker. Coralloid roots of both natural populations of the cycad *Macrozamia riedlei* (Fischer ex Gaudichaud-Beaupré) C.A. Gardner growing in Perth, Australia and cycads growing in greenhouses, also in Perth, were used and their respective cyanobionts analyzed. Several *Nostoc* strains were found to be involved in this symbiosis, both in natural populations and greenhouse-originated cycads. However, only one strain was present in individual coralloid roots and in individual plants, even when analyzing different coralloid roots from the same plant. Moreover, when examining plants growing close to each other (female plants and their respective offspring) the same cyanobacterium was consistently present in the different coralloid roots. Whether this reflects a selective mechanisms or merely the availability of *Nostoc* strains remains to be ascertained. The high cyanobacterial diversity in coralloid roots of cycads revealed by PCR fingerprinting is, therefore, contested. In this study, the potential problems of using different methods (e.g., PCR fingerprinting) to study the genetic diversity of symbiotic cyanobacteria, is also addressed.

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

1. Introduction

Photosynthesizing and N_2-fixing cyanobacteria participate in a wide range of symbiotic associations with hosts from different taxonomic groups. Among plants, endophytic symbiotic associations with representatives from bryophytes (mosses, hornworts and liverworts), pteridophytes (the water fern *Azolla*), gymnosperms (cycads) and angiosperms (*Gunnera*) are formed [1,2]. In addition to these plant associations, the association with fungi in the formation of lichens is an example of another symbiosis with great ecological importance [1–4].

In all plant associations, the initial structure in which *Nostoc* is housed is formed in the absence of a symbiont. The symbiotic plant structure is then colonized by hormogonia, which subsequently develop into mature filaments with differentiated heterocysts (specialized cell type where nitrogen fixation occurs). In association with plants it is the capacity of *Nostoc* to fix atmospheric nitrogen, which is of profit for the photosynthesizing host. Accordingly, the heterocyst frequencies in the symbiotic condition are highly elevated as compared to the free-living state. The *Nostoc* symbiont is believed to obtain a carbon source from the host in exchange for the product of N_2-fixation [1].

Cycads are an ancient group of seed plants that first appeared in the Pennsylvanian period and so have existed for approximately 300 million years. Today they are naturally occurring on every continent except Europe and Antarctica, but are restricted to small populations in the tropics and subtropics of both hemispheres. Many species are facing possible extinction in nature. Cycads produce three types of roots: (i) a taproot that is equivalent to the primary root system found in most types of plants (a special type of root restricted

* Corresponding author. Tel.: +46-18-4712814; fax: +46-18-4712826.
E-mail address: jose.costa@ebc.uu.se (J.-L. Costa).

0168-6496/$22.00
doi:10.1016/j.femsec.2004.05.001

to the genus *Cycas*, which is adventitious, arises from the lower side of trunk offsets and grow downwards in close proximity to the trunk), (ii) lateral roots, and (iii) a highly specialized type of lateral root usually termed 'coralloid roots' in which the symbiotic cyanobacteria are found [5,6]. Instead of a downward growth pattern, these roots show a marked negative geotropism and grow laterally and upward toward the surface of the soil. When infected, the cyanobacteria are found in a specific cortical layer inside the root, the cyanobacterial zone. This layer is transversed by elongated cycad cells interconnecting the two adjacent cortical layers. It has been suggested that these elongated cells are specialized cells responsible for the transfer of metabolites between the partners [7]. The presence of cyanobacteria inside the root induces irreversible modifications of the growth and development of the root. The process of infection is still unclear. Invasion of filamentous cyanobacteria may occur at any stage of development of the root, but the precise time and location of the invasion is unpredictable [6,8].

Several markers and techniques including nucleotide sequence, RFLP, DGGE and fingerprinting methods have been applied to the study of the genetic identity and diversity of cyanobacteria [9]. The marker most extensively used for symbiotic systems is the $tRNA^{Leu}$ (UAA) intron [3,10–19].

Southern hybridizations using probes against conserved genes and genomic cyanobacterial DNA from natural populations of different cycad species revealed the presence of several cyanobionts in a single cycad species [20]. However, in order to get sufficient amounts of genomic DNA, samples from many coralloid roots were pooled for each sample being analyzed, so no conclusion can be drawn concerning whether a single plant or coralloid root, hosts multiple cyanobionts. In a previous work we analyzed the genetic diversity of cyanobacteria associated with cycads growing at the Fairchild Botanical Garden, USA. It was the first attempt to address the genetic diversity of cyanobacteria associated with individual coralloid roots of a plant, using molecular methods. We demonstrated that individual coralloid roots contain a single *Nostoc* strain, indicating a relative high specificity of the association. Furthermore, different species of cycads could harbor the same cyanobacterial strain. However, since it was based on cycads growing at a Botanical garden, we cannot ascertain when the coralloid roots were infected. Some may have been infected in their natural habitats whereas others might have been infected in the botanical garden. Also, we do not know if the same events take place in natural populations as in cycads growing in a botanical garden [10]. Recently, using cycads growing in a field of a National Forest Park in Fuzhou (China) it was showed by PCR fingerprinting that a mixture of strains could coexist in a single coralloid root [21]. With the present study we further address questions related to the genetic diversity of the cycad – *Nostoc* association using natural populations of the cycad *Macrozamia riedlei*, as an example, and discuss the potential problems of PCR fingerprinting when examining the specificity and diversity of the cycad – *Nostoc* association.

2. Material and methods

2.1. Biological material

The coralloid roots used in the present study were collected in August 2001 in Perth, Western Australia. The collections performed for this study were made from natural populations of the cycad *Macrozamia riedlei* (Fischer ex Gaudichaud-Beaupré) C.A. Gardner growing at the Kings Park and from different cycad species growing in green-houses at the campus of The University of Western Australia (Table 1). All biological material was collected with written permission from the Australian authorities. The coralloid roots were sectioned with sterile scalpels and the cyanobiont(s) from each section was/were washed and collected in 50 μL of sterile water [10]. Cyanobacterial colonies were used directly as template for the PCRs.

2.2. $tRNA^{Leu}$ (UAA) intron amplification and sequencing

Primers were used to specifically amplify the $tRNA^{Leu}$ (UAA) intron from the cyanobionts using nested PCR. The primers used were A/C and TL25/TL23, respectively [17]. These primers allowed direct sequencing of the obtained PCR fragments without prior cloning. Obtained PCR fragments were purified with the "Wizard© PCR Preps – DNA purification system" (Promega) and sequenced using the "BIGDYE™ Terminator Cycle Sequencing Ready Reaction Kit" (Perkin–Elmer). The sequencing reactions were run and analyzed on a 373A automated DNA sequencer (Applied Biosystems). Computer assisted sequence analyses and comparisons were performed at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

2.3. Cyanobacterial PCR fingerprinting

The primer used was the STTR*mod* (5′-GCGCCCCAATCC-3′), based on the tandemly repeated repetitive sequence STRR 1 (5′-CCCCART-3′). This primer was previously used to analyze the diversity of cyanobacteria inside coralloid roots of cycads growing at a National Forest Park in Fuzhou, China [21]. However, some modifications (no addition of either BSA or DMSO) were made in order to consistently obtain amplification products. The PCRs were carried out in 25 μL solution

Table 1
Individual cycads from which coralloid roots where collected, and symbiotic cyanobacteria freshly isolated and used in the present study

Sample	Biological material		GenBank Accession No.
	Cycad species	Specimen	
M.r.1	*Macrozamia riedlei* (Fisch. ex Gaudich.) C.A. Gardner	Plant growing at the Kings Park	AY452281
M.r.2	*Macrozamia riedlei*	Growing 10 cm apart from M.r.1	AY452282
M.r.3	*Macrozamia riedlei*	Growing 10 m from M.r.1 and 1 m to M.r.4	AY452283
M.r.4	*Macrozamia riedlei*	Growing 10 m from M.r.1 and 1 m to M.r.3	AY452284
M.r.5	*Macrozamia riedlei*	Growing 10 m from M.r.1 and 5 cm to M.r.4	AY452285
M.r.6	*Macrozamia riedlei*	Growing 25 m from M.r.5 and 5 cm to M.r.7	AY452286
M.r.7	*Macrozamia riedlei*	Growing 25 m from M.r.5 and 5 cm to M.r.6	AY452287
M.r.8	*Macrozamia riedlei*	Growing 20 m from M.r.5	AY452288
M.r.9	*Macrozamia riedlei*	Plant growing at the Kings Park	AY452289
M.r.10	*Macrozamia riedlei*	Growing 10 m from M.r.9	AY452290
M.r.11	*Macrozamia riedlei*	Growing 50 m from M.r.9	AY452291
M.r.12	*Macrozamia riedlei*	Plant growing at the Kings Park	AY452292
M.r.13	*Macrozamia riedlei*	Growing 2 m from M.r.14	AY452293
M.r.14	*Macrozamia riedlei*	Growing 2 m from M.r.13	AY452294
M.r.15	*Macrozamia riedlei*	Growing 10 m from M.r.13 and M.r.14	AY452295
M.r.16	*Macrozamia riedlei*	Green-house plant in a pot	AY452296
M.r.17	*Macrozamia riedlei*	Green-house plant in a pot	AY452297
M.r.18	*Macrozamia riedlei*	Green-house plant in a pot	AY452298
M.r.19	*Macrozamia riedlei*	Green-house plant in a pot	AY452299
M.r.20	*Macrozamia riedlei*	Green-house plant in a pot	AY452300
M.r.21	*Macrozamia riedlei*	Plant in pot outdoors	AY452301
M.r.22	*Macrozamia riedlei*	Same plant as M.r.21 but a different cluster	AY452302
B.s.1	*Bowenia spectabilis* Hook. ex Hook.	Green-house plant in a pot	AY452303
C.b.1	*Cycas basaltica* C.A. Gardner	Green-house plant in a pot	AY452304
C.m.1	*Cycas media* R. Br.	Green-house plant in a pot	AY452305
C.rev.1	*Cycas revoluta* Thunb.	Green-house plant in a pot	AY452306
C.rev.2	*Cycas revoluta*	Same plant as C.rev.1 but a different cluster	AY452307
C.rev.3	*Cycas revoluta*	Green-house plant in a pot	AY452308
C.rev.4	*Cycas revoluta*	Green-house plant in a pot	AY452309
E.a.1	*Encephalartos altensteinii* Lehm.	Green-house plant in a pot	AY452310
E.a.2	*Encephalartos altensteinii*	Same plant as E.a.1 but a different cluster	AY452311
L.p.1	*Lepidozamia peroffskyana* (Regel) Miq.	Green-house plant in a pot	AY452312
Z.f.1	*Zamia furfuracea* L.	Green-house plant in a pot	AY452313
Z.i.1	*Zamia integrifolia* L.	Green-house plant in a pot	AY452314

containing 1 μL (50 pmol) primer, 2.5 μL of 2 mM dNTPs, 1 U Taq DNA polymerase (Amersham Biosciences), 2.5 μL of the buffer supplied with the enzyme and 1 μL template sample. The PCR cycles were: 95 °C for 4 min; 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 4 min; an extension step at 72 °C for 10 min and a final step at 4 °C. After the completion of the reaction, 5 μL of the amplified products were loaded onto a 1.5% agarose gel, and run under constant voltage in TBE buffer, as described [21].

3. Results and discussion

Cyanobacteria can form symbiotic associations with different plants [1,2]. Several methods have been used to ascertain the genetic identity of the cyanobacterium [9]. In this work we characterize the symbiotic cyanobacteria in association with cycads grown under natural conditions, in comparison to greenhouse, using the $tRNA^{Leu}$ (UAA) intron sequences. We also address the potential problems of using PCR fingerprinting in such a complex association for determining diversity.

3.1. $tRNA^{Leu}$ (UAA) intron analyses

From the sequences generated in this study it was observed that different *Nostoc* strains are encountered in coralloid roots of cycads under natural conditions. When aligning and comparing the different sequences it is clear that all strains share a highly conserved sequence with the exception of the specific sequence localized to the structural element P6b (Fig. 1). This is in agreement with our previous studies [10,11,14–17,22]. Most of the variability within the intron is located in non base-pairing positions and when it is on base-pairing positions compensatory mutations occur so that the base pairing is not lost (Fig. 1). This is of great importance since it is believed that the overall structure of the intron is crucial for its autocatalytic activity [23]. The structural

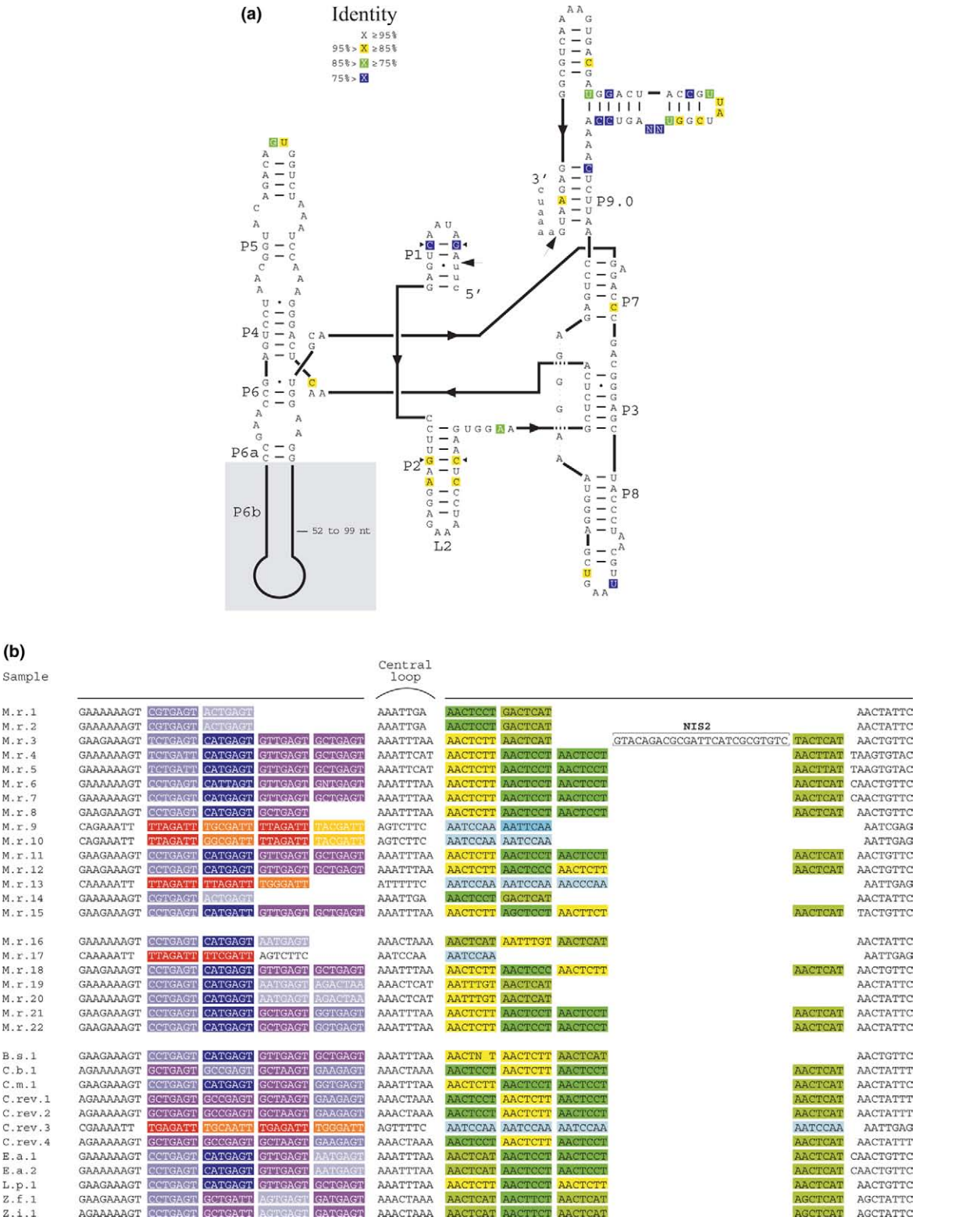

Fig. 1. Cyanobacterial $tRNA^{Leu}$ (UAA) intron sequences obtained and analyzed in the present study. (a) Variability of the consensus sequence of the intron visualized in the secondary structure predictions. Intron sequence is shown in upper case letters and the tRNA in lower case. Arrows mark the intron–exon boundaries. The colors over the nucleotides reflect how large a fraction of the different sequences supports the consensus sequence. Arrowheads indicate positions where variation between differing sequences still retain the base pairing structure. The structural element P6b is represented as a schematic hairpin due to size variation. (b) Alignment of the first variable region (structural element P6b) of the intron sequences. Groups and subgroups of the different heptanucleotide repeats are identified according to Costa et al. [22]. One sequence that does not follow the heptanucleotide repeat pattern is identified as NIS-2 (*N*ostoc *I*terated *S*equence).

element P6b, where the major intron differences are located corresponds to a hairpin previously described to consist of heptanucleotide repeats [22]. These repeats based on their specific sequences, fall into two different classes [22]. The element P6b from the sequences retrieved in the present study is also mainly made up by heptanucleotide repeats and both previously identified classes are represented (see samples M.r.1 and M.r.8, respectively). Interrupting the heptanucleotides, the symbiotic cyanobacteria in sample M.r.3 contain an extra stretch of DNA not having any repeats. This stretch of DNA corresponds to a putative hairpin structure within the element P6b [22]. The sequence represented in Fig. 1(b) has the signature designated NIS-2 (*N*ostoc *I*terated *S*equence, data not shown, manuscript under preparation).

3.2. Genetic diversity/specificity

Although several strains were found to be involved in the association, only a single *Nostoc* strain was consistently found in each individual coralloid root. Furthermore, when examining different coralloid roots from the same individual plant, only one strain was found (e.g., M.r.21-22; C.rev.1-2 and E.a.1-2). Cycads are dioecious plants meaning that have the two sexes in separate individuals. When analyzing the natural populations of *M. riedlei* and by looking at their spatial distribution, an interesting observation was made. Plants that are growing close together (up to 1 m) share the same *Nostoc* strain. In fact in this study the plants found close to each other correspond to females and respective descendants, so to state that the selectivity of the plant towards the *Nostoc* strain is genetically transmitted is very appealing. However this observation could merely correspond to the availability of *Nostoc* strains in sandy soil. When comparing the *Nostoc* sequences described in this study with existing sequences in GenBank, highly similar sequences were found, with few nucleotide differences located on the variable regions of the intron. These sequences were also obtained from symbiotic strains of *Nostoc* associated with different organisms, as fungi [13,15], bryophytes [11] and also cycads [10]. It is relevant that the same intron sequence could be found in cyanobacteria from cycads growing in as separate places as Florida, USA (GenBank No. AF095778) and Perth, Australia (e.g., M.r.12), since this has biogeographic and population implications.

3.3. Comparison of the molecular tools

Different methods may render different results when analyzing bacterial diversity. Based on sequencing the $tRNA^{Leu}$ (UAA) intron from the cyanobacteria inside the coralloid roots, we demonstrated that a single strain of *Nostoc* is present in an individual coralloid root. In contrast, using a different molecular method based not on sequence similarities/differences but instead on banding patterns (fingerprinting) generated using PCRs and a single primer, it was concluded that several different cyanobacterial strains are present inside individual coralloid roots [21]. The primer used was based on a repeated DNA element (STTR1) that is known to be present in several, but not in all, cyanobacterial genomes [24]. This marker, as a genetic tool for DNA fingerprinting, has been examined using purified DNA from selected cyanobacterial strains [24,25].

However, fingerprinting methods have limitations. To be able to compare the different results a high level of standardization is required. Artifactual variation represents a potential problem in surveys of genetic variation in natural populations and must be discriminated from true polymorphism. In a previous work, we demonstrated that the cyanobacteria filaments inside the coralloid roots are embedded in very complex chemical mucilage that had to be removed in order to consistently obtain PCR generated DNA products. In addition, it is known that the cyanobacterial cells are in different physiological states along the coralloid root [26]. Using a single cyanobacterial strain, *Nostoc* sp. PCC 73102, but by varying the PCR conditions we further analyzed the method developed and used by Zheng et al. [21]. In these PCR assays the same primer as Zheng et al. [21] was used but the amplification conditions varied, either by means of using purified DNA versus whole cells, cells grown under different conditions or by using cells in different physical state. Interestingly, this resulted in different banding patterns, even though the same cyanobacterial strain was always used (Fig. 2). This clearly demonstrates that fingerprinting methods can not be used without caution on biological material arising from such complex chemical and physical environment as, e.g., the conditions inside the coralloid roots of cycads. Furthermore, the primer used was based on a genomic element never tested on cycad DNA. As shown in Fig. 2, this element is indeed present in cycad DNA. When collecting the cyanobacterium it seems unavoidable to disrupt the elongated cycad cells that exist in the cyanobacterial zone inside the coralloid root. So at least, this plant DNA will be present in the sample and may be a template generating DNA fragments. Thus, since this method is based only on comparing obtained DNA banding patterns one cannot, e.g., distinguish the source of template DNA. PCR fingerprinting has also been used to reveal the genetic diversity of the cyanobiont(s) of *Gunnera tinctoria*. In this association the symbiotic conditions where the cyanobacteria are harbored are noteworthy different from the cycad–cyanobacteria association [1,2]. In the study a different primer based on the STTR1 sequence was used. The analyses revealed an important genetic diversity of the cyanobionts among host plants, although, a single fingerprint was obtained

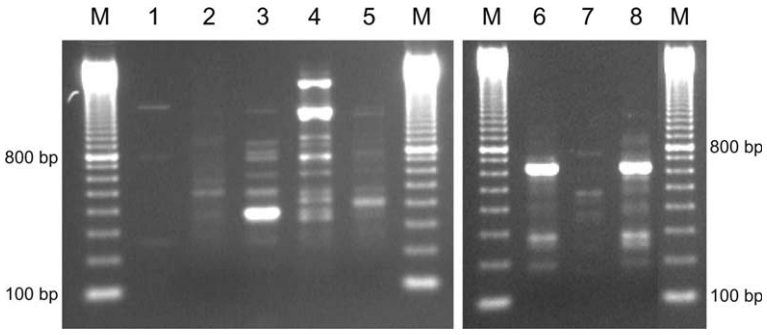

Fig. 2. PCR generated DNA fingerprinting patterns obtained using the cyanobacterium *Nostoc* sp. strain PCC 73102 and the cycad *Cycas media*. Lane M, 100 base-pair ladder; lane 1, *Nostoc* cells grown under non-nitrogen fixing conditions; lane 2, purified *Nostoc* DNA with the addition of *Escherichia coli* cells as template in the PCR reaction; lane 3, purified *Nostoc* DNA; lane 4, *Nostoc* cells after being crushed at 1600 psi using a French Press; lane 5, same as in 4 but with two cycles of pressurizing the cells at 1600 psi; lane 6, purified *Cycas* DNA; lane 7, purified *Nostoc* DNA (same as lane 3 but in different amount); and lane 8, a mixture of DNA purified from *Nostoc* and from *Cycas*.

from the cyanobacteria of individual plants. In this study, the primer used in the fingerprinting did not amplify DNA samples from plant leaves [27].

Even so, from our results (Fig. 2) we conclude that special caution should be taken when examining genetic diversity using methods based on comparing DNA-patterns/fingerprints, and that direct sequencing of a known genetic marker (e.g., the $tRNA^{Leu}$ (UAA) intron or *rpoC* or *nifH* genes) may be a preferred method.

In conclusion, with this study a further step was taken to understand the genetic diversity of *Nostoc*, in particular, associated with cycads. Based on sequencing the $tRNA^{Leu}$ (UAA) intron from the cyanobacteria inside the coralloid roots, we demonstrate that a single strain of *Nostoc* is present in an individual coralloid root. Furthermore, plants growing within a radius of 1 m share the same cyanobacterial strain. If this is a reflex of a selective mechanism or merely the availability of *Nostoc*s in the soil remains to be ascertain. Also it is of great importance to understand the method one uses when analyzing the diversity of cyanobacteria grown natural conditions.

Acknowledgements

We are thankful to Prof. Craig Atkins (The University of Western Australia) for providing laboratory facilities and to the personal at the Kings Park (Perth, Australia) for the permission to collect the coralloid roots. This study was financially supported by the Elliassons fund and The Royal Swedish Academy of Sciences (to J.-L.C.). Dr. Francisco Vergara Silva is thanked for providing cycad DNA from *Cycas media* (voucher 90-011.02, the National Collection of Cycads; Jardín Botánico Francisco Javier Clavijero, Xalapa, Veracruz, Mexico) obtained from Dr. Andrés Vovides and Dr. Carlos Iglesias. Julio Martínez is acknowledged for technical support (to E.M.R).

References

[1] Rai, A.N., Bergman, B. and Rasmussen, U. (2002) Cyanobacteria in Symbiosis. Kluwer Academic Publishers, Dordrecht, The Netherlands. ISBN 1402007779.
[2] Adams, D.G. (2002) Symbiotic interactions. In: The Ecology of Cyanobacteria (Whitton, B.A.P. and Potts, M., Eds.), pp. 523–561. Kluwer Academic Publishers, Dordrecht, The Netherlands.
[3] Paulsrud, P. (2001) The *Nostoc* symbiont of lichens: diversity, specificity and cellular modifications. Acta Universitatis Upsaliensis 662, ISBN 9155451365.
[4] Rai, A.N., Soderback, E. and Bergman, B. (2000) Cyanobacterium–plant symbioses. New Phytol. 147, 449–481.
[5] Brenner, E.D., Stevenson, D.W. and Twigg, R.W. (2003) Cycads: evolutionary innovations and the role of plant-derived neurotoxins. Trends Plant Sci. 8, 446–452.
[6] Costa, J.L. and Lindblad, P. (2002) Cyanobacteria in symbiosis with cycads. In: Cyanobacteria in Symbiosis (Rai, A.N., Bergman, B. and Rasmussen, U., Eds.), pp. 195–205. Kluwer Academic Publishers, Dordrecht, The Netherlands.
[7] Lindblad, P., Bergman, B., Hofsten, A.V., Hällbom, L. and Nylund, J.E. (1985) The cyanobacterium and *Zamia skinneri* symbiosis: an ultrastructural study. New Phytol. 101, 707–716.
[8] Nathanielsz, C.P. and Staff, I.A. (1975) Mode of entry of blue-green algae into the apogeotropic roots of *Macrozamia communis*. Am. J. Bot. 62, 232–235.
[9] Rasmussen, U. and Nilsson, M. (2002) Cyanobacterial diversity and specificity in plant symbioses. In: Cyanobacteria in Symbiosis (Rai, A.N., Bergman, B. and Rasmussen, U., Eds.), pp. 313–328. Kluwer Academic Publishers, Dordrecht, The Netherlands.
[10] Costa, J.L., Paulsrud, P. and Lindblad, P. (1999) Cyanobiont diversity within coralloid roots of selected cycad species. FEMS Microbiol. Ecol. 28, 85–91.
[11] Costa, J.L., Paulsrud, P., Rikkinen, J. and Lindblad, P. (2001) Genetic diversity of *Nostoc* symbionts endophytically associated with two bryophyte species. Appl. Environ. Microbiol. 67, 4393–4396.
[12] Oksanen, I., Lohtander, K., Paulsrud, P. and Rikkinen, J. (2002) A molecular approach to cyanobacterial diversity in a rock-pool community involving gelatinous lichens and free-living *Nostoc* colonies. Ann. Bot. Fenn. 39, 93–99.
[13] Rikkinen, J., Oksanen, I. and Lohtander, K. (2002) Lichen guilds share related cyanobacterial symbionts. Science 297, 357.
[14] Paulsrud, P., Rikkinen, J. and Lindblad, P. (2001) Field investigations on cyanobacterial specificity in *Peltigera aphthosa*. New Phytol. 152, 117–123.

[15] Paulsrud, P., Rikkinen, J. and Lindblad, P. (2000) Spatial patterns of photobiont diversity in some *Nostoc*-containing lichens. New Phytol. 146, 291–299.
[16] Paulsrud, P., Rikkinen, J. and Lindblad, P. (1998) Cyanobiont specificity in some *Nostoc*-containing lichens and in a *Peltigera aphthosa* photosymbiodeme. New Phytol. 139, 517–524.
[17] Paulsrud, P. and Lindblad, P. (1998) Sequence variation of the $tRNA^{Leu}$ intron as a marker for genetic diversity and specificity of symbiotic cyanobacteria in some lichens. Appl. Environ. Microbiol. 64, 310–315.
[18] Wirtz, N., Lumbsch, H.T., Green, T.G.A., Turk, R., Pintado, A., Sancho, L. and Schroeter, B. (2003) Lichen fungi have low cyanobiont selectivity in maritime Antarctica. New Phytol. 160, 177–183.
[19] Summerfield, T.C., Galloway, D.J. and Eaton-Rye, J.J. (2002) Species of cyanolichens from *Pseudocyphellaria* with indistinguishable ITS sequences have different photobionts. New Phytol. 155, 121–129.
[20] Lindblad, P., Haselkorn, R., Bergman, B. and Nierzwicki Bauer, S.A. (1989) Comparison of DNA restriction fragment length polymorphisms of *Nostoc* strains in and from cycads. Arch. Microbiol. 152, 20–24.
[21] Zheng, W.W., Song, T.Y., Bao, X.D., Bergman, B. and Rasmussen, U. (2002) High cyanobacterial diversity in coralloid roots of cycads revealed by PCR fingerprinting. FEMS Microbiol. Ecol. 40, 215–222.
[22] Costa, J.L., Paulsrud, P. and Lindblad, P. (2002) The cyanobacterial $tRNA^{Leu}$ (UAA) intron: evolutionary patterns in a genetic marker. Mol. Biol. Evol. 19, 850–857.
[23] Cech, T.R. (1988) Conserved sequences and structures of group I introns: building an active site for RNA catalysis – a review. Gene 73, 259–271.
[24] Robinson, N.J., Robinson, P.J., Gupta, A., Bleasby, A.J., Whitton, B.A. and Morby, A.P. (1995) Singular over-representation of an octameric palindrome, HIP1, in DNA from many cyanobacteria. Nucleic Acids Res. 23, 729–735.
[25] Smith, J.K., Parry, J.D., Day, J.G. and Smith, R.J. (1998) A PCR technique based on the Hip1 interspersed repetitive sequence distinguishes cyanobacterial species and strains. Microbiology 144, 2791–2801.
[26] Lindblad, P. and Bergman, B. (1990) The cycad–cyanobacteria symbiosis. In: Handbook of Symbiotic Cyanobacteria (Rai, A.N., Ed.), pp. 137–159. CRC Press, Boca Raton, FL.
[27] Guevara, R., Armesto, J.J. and Caru, M. (2002) Genetic diversity of *Nostoc* microsymbionts from *Gunnera tinctoria* revealed by PCR-STRR fingerprinting. Microb. Ecol. 44, 127–136.