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A molecular approach to cyanobacterial diversity in a rock-pool community involving gelatinous lichens and free-living *Nostoc* colonies

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The cyanobacterial symbionts of *Collema multipartitum* and free-living *Nostoc* strains were studied by using the nucleotide sequence of the cyanobacterial $tRNA^{Leu}$(UAA) intron as a genetic marker. Biological specimens were collected from a series of interconnected depressions on a limestone pavement in western Ireland. In this material, free-living and lichenised *Nostoc* strains could be distinguished on the basis of the first variable region of the $tRNA^{Leu}$(UAA) intron. All the variable stem-loops had a similar heptanucleotide repeat motif, but lichenised and free-living strains differed in the number of repeats and in the presence/absence of additional sequences interrupting a single repeat. The results indicate that some filamentous cyanobacteria in rock-pool communities can be rapidly identified by using this molecular marker. The method could prove useful in ecological and environmental studies.

Keywords: *Collema*, cyanobacteria, lichen, *Nostoc*, symbiosis, $tRNA^{Leu}$ intron

Introduction

Small, temporarily water-filled rock-pools are found wherever bare rock is exposed. Such depressions are subjected to drastic environmental changes, involving rapid changes in the extremes of irradiation, temperature, moisture, and nutrient availability. Accordingly, they have received considerable interest from ecologists and other biologists. For example, several studies have shown strong correlations between the distribution of saxicolous lichens and the duration of flooding events within limestone depressions (Wessels & Büdel 1989, Ott *et al*. 1996, 1997a, 1997b). Gelatinous lichens and large, gelatinous colonies of free-living *Nostoc* are frequently encountered in rock-pool communities in Karst regions (Degelius 1954, Potts 2000). Both are known for their ability to absorb large quantities of water. However, even in deep limestone hollows gelatinous lichens and *Nostoc* colonies are likely to experience repeated cycles of wetting and drying interspersed with variable periods of complete desiccation.

In this study we examine possibilities of using the $tRNA^{Leu}$(UAA) intron as a genetic marker for identifying lichenised and free-living *Nostoc* strains in rock-pool environments. Our main goal is to determine whether or not the cyanobacterial photobionts of the gelatinous lichen *Collema multipartitum* (Lecanorales, Ascomycota) and neighbouring *Nostoc* colonies belong to the same strain. In previous studies, we have studied genetic diversity of cyanobacteria, particularly *Nostoc*, in different lichens (Paulsrud & Lindblad 1998, Paulsrud *et al*. 1998, 2000, 2001). Using nested PCR, we have specifically amplified, cloned and sequenced cyanobacterial introns from field specimens containing *Nostoc* strains, but also fungal tissue, green algae and contaminating bacteria. We have found that individual thalli of most cyanolichens house only one *Nostoc* strain, this being in contrast with higher levels of cyanobiont diversity in most other *Nostoc* symbioses (West & Adams 1997, Costa *et al*. 2000, 2001). One notable exception is *Peltigera venosa*, in which different *Nostoc* colonies from single thalli were commonly found to contain different intron sequences. This may have reflected an ability to exhibit different degrees of lichenisation with different *Nostoc* strains, ranging from loosely associated colonies to well corticated cephalodia (Paulsrud *et al*. 2000).

Little is still known about cyanobacterial diversity in *Collema* and other gelatinous lichens. The cyanobionts of *Collema* clearly belong to the *Nostoc* type, i.e., they are filamentous, heterocystous and produce isopolar trichomes with more or less spherical cells and exhibit no evidence of branching (Geitler 1932, Rippka *et al*. 1979, Mollenhauer 1988, Komárek & Anagnostidis 1989, Castenholz 2001, Wright *et al*. 2001). Also the typical life-cycle of *Nostoc*, with motile hormogonia and with vegetative filaments exhibiting different degrees of coiling is expressed by most cyanobacteria isolated from gelatinous macrolichens (Degelius 1954). The phenotypic characteristics of lichen-forming *Nostoc* strains vary and several names have been used for morphologically different *Collema* cyanobionts. At least *Nostoc commune*, *N. microscopicum*, *N. muscorum*, *N. punctiforme*, and *N. sphaericum* have been mentioned as possible cyanobionts of different *Collema* species (Degelius 1954).

Material and methods

Thalli of *Collema multipartitum* and large free-living colonies of *Nostoc* were collected from the Burren region of County Clare, western Ireland in September 2000. All specimens were taken from a series of interconnected depressions on a flat limestone pavement (Fig. 1). Two types of depressions were sampled: shallow concavities with well developed *Collema* thalli (A1–A4) and deeper depressions with large *Nostoc* colonies (B1–B4). The depressions were prone to flooding during rain and routes for the flow of surface water are shown by arrows in Fig. 1. Generally, the shallow depressions with *Collema* were likely to dry out quickly, while the deeper concavities with *Nostoc* were likely to stay flooded for extended periods.

In the laboratory the field specimens were hydrated and washed with sterile ultra-pure water (MQ, Millipore). After a couple of hours nostocalean cyanobacteria were isolated under

the microscope. Samples were taken for direct amplification and amplification of extracted DNA. Many fragments of lichen thalli and cyanobacterial colonies were also grown in sterile MQ-water and/or on Z8-agarose medium without nitrogen (Kotai 1972) at room temperature. Growing cyanobacterial filaments were harvested after several weeks.

The total DNA from specimens was extracted using the Dneasy Tissue Kit (Qiagen) according to the manufacturer's instructions with slight modifications described in Lohtander *et al*. (2000). The presence of fungal DNA in the field samples was determined by using fungal specific primers for a region consisting of the internal transcribed spacers ITS1 and ITS2, and the 5.8S rDNA. PCR-reactions were performed using PHARMACIA Biotech Inc. Ready To Go PCR beads following the procedure described in Lohtander *et al*. (2000). The primers used in PCR were ITS1-F (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990), in a 30-cycle reaction with a PCR profile of 60 sec at 95 °C (denaturation), 60 sec at 60 °C (annealing), and 60 sec at 72 °C (extension). The amplified DNA fragments were separated by loading 4 µl of each product on a 2% agarose gel containing ethidium bromide. Pharmacia Biotech Inc.'s 100 Bp ladder was used as a DNA molecular weight standard. The presence or absence of DNA bands in the agarose gel was observed on a UV-transilluminator. If a visible DNA band was present in the gel after electrophoresis, there was fungal DNA present in the specimen. The fungal origin of the DNA was confirmed by sequencing.

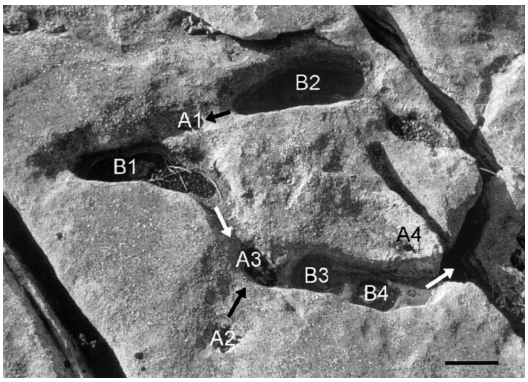


Fig. 1. Rock-pool morphology and position of sampling points. Small depressions in the rock housed well developed *Collema* thalli (A1–A4). Deeper concavities had macroscopic colonies of free-living cyanobacteria, growing on shallow deposits of detritus and fine grit (B1–B4). All depressions were prone to flooding during rain. Obvious routes for the flow of surface water are shown by arrows. Scale bar 20 cm.

Nested PCR was used to amplify the cyanobacterial tRNA^{Leu}(UAA) intron directly from field specimens, cultured cyanobacteria, or from extracted DNA (dilution 1:10 vol.). Most intron sequences were obtained from crushed field specimens or from cultured cyanobacteria; only in two cases total DNA was first extracted using the Dneasy Tissue Kit (Qiagen) as mentioned above (Table 1). The tRNA^{Leu}(UAA) intron was amplified with two sets of primers, i.e., the cyanobacterial specific primers A & C (Paulsrud & Lindblad

Table 1. Abbreviations of obtained tRNA^{Leu}(UAA) intron sequences and corresponding GenBank accession numbers. The presence/absence of fungal DNA is also shown. The abbreviations of the intron sequences refer to the sampling points in Fig. 1.

Abbreviation	Origin of tRNA^{Leu}(UAA) intron sequence	GenBank accession number	Fungal DNA
A1	*Collema* (DNA extracted from field specimen)	AF491912	+
A2	*Collema* (field specimen)	AF491913	+
A3a	*Collema* (field specimen)	AF491914	+
A3b	*Collema* (culture)	AF491915	+
A4	*Collema* (DNA extracted from field specimen)	AF491916	+
B1a	*Nostoc* colony (culture)	AF491917	–
B1b	*Nostoc* colony (culture)	AF491918	–
B2a	*Nostoc* colony (field specimen)	AF492487	–
B2b	*Nostoc* colony (culture)	AF491919	–
B4	*Nostoc* colony (culture)	AF491920	–

1998), and TL25 & TL23 (Biniszkiewicz *et al*. 1994) under conditions described in Paulsrud and Lindblad (1998). PCR products were purified with the PCR Purification Kit (Qiagen). 100–200 ng of the double-stranded, amplified DNA was directly sequenced with the primers TL25 and TL23 using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Sequence reactions were analysed on an ABI Prism 377 automated sequencer or on an ABI Prism 310 genetic analyzer (PE Biosystems). The sequences are deposited in GenBank (accession numbers in Table 1).

Results

Eight different cyanobacterial tRNA^{Leu}(UAA) intron sequences were amplified from the biological material. Three different intron sequences were obtained from samples of *Collema multipartitum* (Table 1). One intron type was amplified three times: once from extracted DNA (A1), and twice directly from thallus fragments (A2 and A3a). A cultured *Nostoc* strain from one of the above thallus fragments produced a second, slightly different intron sequence. A third, unique intron sequence was obtained from DNA extracted from an additional *Collema* specimen (A4). Fungal DNA of approximately 600 bp was obtained from specimens A1–A4, confirming that these specimens were indeed lichenised (Table 1).

Five different intron sequences were obtained from free-living *Nostoc* colonies (Table 1). No fungal PCR product was obtained from any of these specimens. Only one intron sequence was obtained by direct amplification from a field specimen of *Nostoc* (B2a). The other sequences were amplified from cultures, and in two cases different sequences were obtained from two cultures originating from a single field specimen (Table 1). Most samples from extremely mucilaginous free-living *Nostoc* colonies failed in PCR despite attempts to remove polysaccharides from the template using the CTAB-based method for cyanobacterial cells (Wilson 1994) and extra cleaning of the extracted DNA using Prep-A-Gene DNA Purification Systems (Bio-Rad) combined with dilution series. Thus, we were unable to amplify any intron sequences from field specimen B3, even though nostocalean cyanobacteria were clearly present.

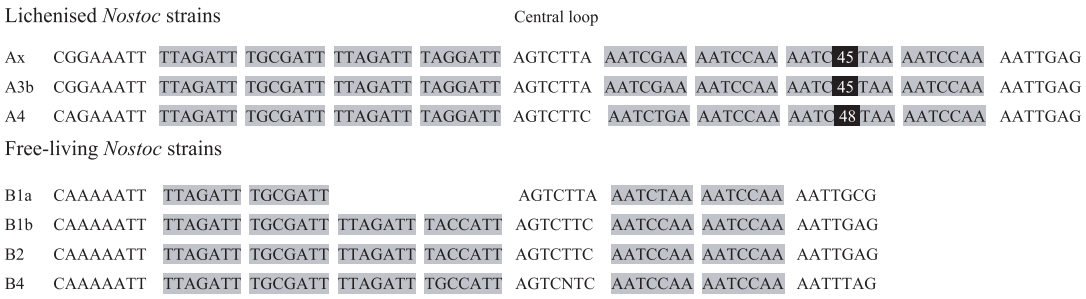
All the tRNA^{Leu}(UAA) intron sequences obtained in this study had a Class 1 heptanucleotide repeat motif (Costa *et al*. 2001) in the first variable stem-loop (Fig. 2). There were consistent size differences in the variable stem-loops, with all free-living *Nostoc*s having fewer copies of heptanucleotide repeats than the lichenised strains. Furthermore, all lichenised *Nostoc* strains had an additional sequence interrupting one copy of the heptanucleotide repeat (Fig. 2). These sequences did not confirm to the heptanucleotide repeat motif, but corresponded both in size and nucleotide sequence with sequences that have previously been found interrupting the variable stem-loops of some other symbiotic *Nostoc* strains (Paulsrud *et al*. 1998, 2000, Costa *et al*. 2001)

Discussion

The tRNA^{Leu}(UAA) intron is found in several cyanobacterial lineages and in the plastids of many eukaryotic algae and all green plants (Paquin *et al*. 1997, Besendahl 2000). The distribution and characteristics of this group 1 intron are consistent with an ancient origin and vertical transmission, supporting the view that it was obtained by eukaryotic organisms in the primary plastid endosymbiosis (Schopf 1993, Paquin *et al*. 1997, Palmer & Delwiche 1998, Brocks *et al*. 1999, Summons *et al*. 1999, Besendahl 2000, Costa *et al*. 2001). The intron has subsequently been lost at a number of occasions, resulting in its present distribution among cyanobacteria and plastids. Some authors (Rudi & Jacobsen 1997, 1999) have suggested a more recent origin for the intron, but this interpretation is problematic (Costa *et al*. 2001). In recent phylogenetic reconstructions based on tRNA^{Leu} intron sequences cyanobacteria have fallen outside the monophyletic group to eukaryotic algae and green plants, and among cyanobacteria the genus *Nostoc* has formed a moderately supported monophyletic group (Besendahl 2000).

We have previously used the tRNA^{Leu}(UAA) intron as a genetic marker for studying the

A. First variable region



B. Sequences not confirming to the heptanucleotide repeat motif (black boxes in A)

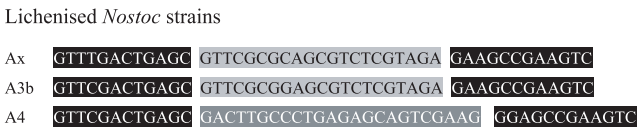


Fig. 2. Alignment of the first variable region of the tRNA^{Leu}(UAA) intron from the *Nostoc* strains. Ax refers to three identical intron sequences amplified from lichen specimens A1, A2 and A3a. The first variable region of the intron is built of degenerate heptanucleotide repeats (grey blocks in A). Sequences that do not follow the heptanucleotide repeat motif are shown as black boxes, with their sizes indicated. The alignment of these deviating sequences is shown in B.

diversity and specificity of *Nostoc*s symbiotically associated with lichen-forming fungi and bryophytes (Paulsrund & Lindblad 1998, Paulsrud *et al*. 1998, 2000, 2001, Costa *et al*. 2001). Conserved elements in the tRNA^{Leu}(UAA) intron are helpful for the identification of *Nostoc*s at the genus level and more variable regions can be used for the identification of different *Nostoc* strains. Conserved elements in the intron have grouped symbiotic *Nostoc* strains consistent with the corresponding 16S rDNA analyses (Paulsrud 2001; I. Oksanen *et al*. unpubl. data). As shown by Costa *et al*. (2001) sequence variations between different tRNA^{Leu} introns are mainly caused by processes other than random mutations. Single nucleotide differences are strongly restricted by the secondary and tertiary structure of the intron. Thus, all *Nostoc* sequences tend to be quite similar, except in one stem-loop were both sequence and size variation is considerable (Paulsrud & Lindblad 1998, Paulsrud *et al*. 1998, 2000, Costa *et al*. 2001). This variable stem-loop (first variable region, P6b) is built from degenerate heptanucleotide repeats, which fold into a hairpin structure allowing the repeats to base pair. Size differences between variable stem-loops are caused primarily by different numbers of copies of repeats and, in some cases, by insertion of additional sequences not following the heptanucleotide repeat motif. Based on their consensus sequences the variable stem-loops in *Nostoc* can be grouped into two classes. Thus, Class 1 variable regions exhibit the consensus heptanucleotide repeat sequence TDNGATT and its base paring consensus sequence, while Class 2 variable regions have the consensus heptanucleotide repeat sequence NNTGAGT and its base paring consensus sequence (Costa *et al*. 2001).

All the tRNA^{Leu} intron sequences found in this study had a Class 1 heptanucleotide repeat motif in the first variable stem-loop (Fig. 2). There was a consistent size difference between the introns from *Collema* thalli and free-living *Nostoc* colonies, respectively, with the variable stem-loops of free-living *Nostoc*s having fewer copies of heptanucleotide repeats than those of lichenised strains (Fig. 2A). Furthermore, the variable regions of all lichenised *Nostoc* strains had additional sequences interrupting one copy

of the heptanucleotide repeat (Fig. 2B). The inserted sequences did not confirm to the repeat motif, but corresponded with similar interrupting sequences previously found from the variable stem-loops of other symbiotic *Nostoc* strains. Homologous recombination between different loci in the *Nostoc* genome containing the same, or similar, heptanucleotide repeat motifs may have given rise to these interrupting sequences (Costa *et al*. 2001).

Our results clearly demonstrate that several *Nostoc* strains were associated with *Collema* thalli at one field site. Furthermore, consistent differences between $tRNA^{Leu}$ introns amplified from *Collema* thalli and free-living colonies, respectively, indicate that different groups of closely related *Nostoc*s were involved in the two biological systems. Thus, in the restricted rock-pool environment one could distinguish between lichenised and free-living *Nostoc* strains on the basis of the first variable stem-loop of the $tRNA^{Leu}$(UAA) intron. The large *Nostoc* colonies in deep depressions appeared to be genuinely free-living, as no fungal DNA was detected in any of these samples. This is not to say, however, that these *Nostoc*s could not have belonged to potentially lichen-forming strains. This is emphasised by the fact that one intron sequence from a free-living colony (B1a) was identical to a sequence previously obtained from the thallus of *Peltigera neopolydactyla* from Oregon, USA (Paulsrud *et al*. 2000). The fact that several different *Nostoc* strains were often identified from cultures taken from single field specimens emphasises the danger of drawing conclusions from single cyanobacterial isolates from lichens, unless the identity of the *in situ* cyanobiont has first been confirmed.

The contrasting microhabitat preferences of gelatinous lichens and free-living cyanobacteria most probably corresponded to differences in moisture regimes. Shallow limestone depressions provided an ideal habitat for *Collema*. The lichens seemed to avoid convex limestone surfaces, which dry very quickly after rain, but also deep rock pools, which tend to experience prolonged periods of flooding. The high moisture compensation point of gelatinous cyanolichens excludes the use of high atmospheric humidity for reactivation of net photosynthesis and carbon gain. While the lichens thus require hydration with liquid water, due to their high water holding capacity, they can extend their metabolic activities during the desiccation process. However, under prolonged hydration even gelatinous lichens can suffer from suprasaturation depression of net photosynthesis, this leading to substantial reductions in carbon gain (Lange & Kilian 1985, Lange *et al*. 1986, 1988, 1989, Lange 2000).

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