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Sequence types were identified from lichen-forming *Nostoc* strains and other cyanobacteria using multivariate analyses of tRNA^{Leu}(UAA) intron sequences. The nucleotide sequences were first incorporated into a large alignment spanning a wide diversity of filamentous cyanobacteria and including all *Nostoc* sequences available in GenBank. After reductions the data matrix was analysed with ordination methods. In the resulting ordinations, most Nostocalean tRNA^{Leu}(UAA) intron sequences grouped away from those of non-Nostocalean cyanobacteria. Furthermore, most *Nostoc* sequences were well separated from those of other Nostocalean genera. Three main sequence types, the *Muscorum*-, *Commune*- and *Punctiformis*-type, were delimited from the main cluster of *Nostoc* intron sequences. All sequences so far amplified from lichens have belonged to the latter two types. Several subgroups existed within the main intron types, but due to inadequate sampling, only a few were discussed in any detail. While the sequence types offer a heuristic rather than a formal classification, they are not in conflict with previous phylogenetic classifications based on the 16S rRNA gene and/or the conserved parts of the tRNA^{Leu}(UAA) intron. The groups also seem to broadly correspond with classical *Nostoc* species recognised on the basis of morphological characters and life-history traits. Hence, the types could be useful for distinguishing between ecological, geographic and/or taxonomic entities within the genus.

Key words: symbiosis, specificity, ordination, NMS.

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Introduction

Species of the cyanobacterial genus *Nostoc* are well known for their ability to participate in symbioses, either serving as a source of fixed carbon and nitrogen, as in many cyanolichens, or solely as a source of nitrogen, as in most other associations. Strains of *Nostoc* are by far the most common cyanobionts in lichens, especially in association with lichen-forming fungi of the Lecanorales (Ascomycota). Many recent studies have shown that lichen mycobionts are highly selective with respect to their *Nostoc* photobionts. Only a few closely related strains typically serve as the appropriate symbiotic partners for each fungal species. On the other hand, many different fungi, sometimes from distantly related lineages, may house identical cyanobiont strains (Rikkinen 2002, 2003).

Generic delimitations in the Nostocales (Subsection IV in the bacteriological system) are not clear and several classifications with different taxonomic concepts are in use (Komárek & Anagnostidis 1989, Castenhotz 2001, Gugger & Hoffman 2004). The order consists of filamentous heterocystous cyanobacteria dividing in a plane at right angles to the long axis of the trichome and therefore uniseriate and without true branching. Komárek & Anagnostidis (1989) recognised four

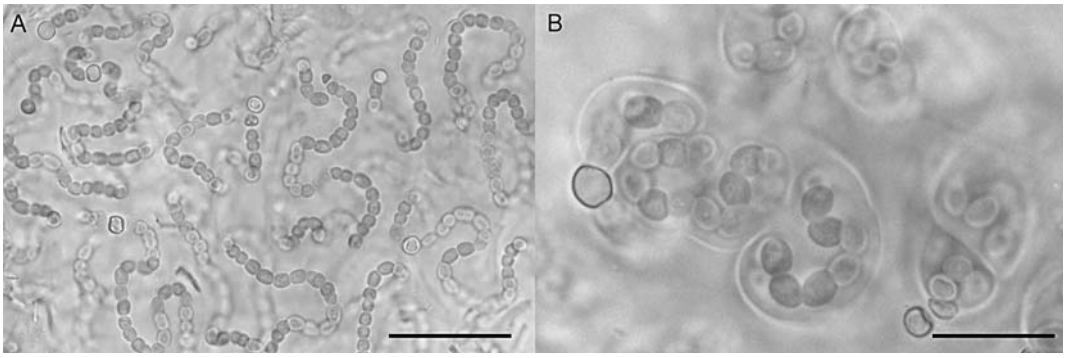

Figure 1. Lichen-forming *Nostoc* strain in culture. A. Curved filaments with heterocysts. Bar 40 µm. B. Proliferation gives rise to slowly expanding clusters of daughter-colonies. Bar 20 µm.

families and 32 genera in the order. Typical *Nostoc* species produce isopolar trichomes with no evidence of branching or meristematic zones and with cylindrical or spherical cells (Fig. 1A). Also the characteristic life-cycle, with morphologically distinct, motile hormogonia and with vegetative filaments exhibiting different degrees of coiling is shown by many strains in culture (Mollenhauer 1988, Potts 2000, Paulsrud 2001). However, some symbiotic strains do not produce hormogonia under common growth conditions. Their trichomes may form pearl-like colonies eventually giving rise to grape-like clusters (Fig. 1B). Many botanical names have been used for morphologically distinct, colony-forming *Nostoc*. Most lichen-forming strains have been called *Nostoc punctiforme*. Also *Nostoc commune*, *N. microscopicum*, *N. muscorum*, and *N. sphaericum* have been mentioned to occur in lichens (Degelius 1954, Tschermak-Woess 1988).

The shotgun phase of sequencing the genome of one symbiotic *Nostoc* strain, *N. punctiforme* Pasteur Culture Collection (PCC) 73102 [synonym American Type Culture Collection (ATCC) 29133] has been completed and a preliminary analysis of the genome has been published (Meeks et al. 2001). This strain was originally isolated from the roots of a cycad (*Macrozamia*), but it can also establish a symbiotic association with the hornwort *Anthoceros* in the laboratory. It has been widely used as a model strain to define molecular and physiological properties of symbiotic *Nostoc* (Meeks et al. 2002, Wong & Meeks 2002). However, our studies have indicated that sequence identical *Nostoc* strains are not commonly found in thalloid bryophytes in the field (Costa et al. 2001, Rikkinen & Virtanen unpubl.). Neither have they been found from lichens.

We have previously used 16S rRNA gene sequences to study phylogenetic relationships between lichen-forming *Nostoc* strains (Rikkinen et al. 2002; Lohtander et al. 2003; Oksanen et al. 2004a, 2004b). The results have confirmed that there is considerable genetic variation among the symbiotic strains. The lichenized strains have grouped together with free-living strains, forming a well supported monophyletic group among the Nostocalean cyanobacteria. All true *Nostoc* strains so far sampled have been divided into two main groups, one of which has mainly included cyanobionts of epiphytic cyanolichens. The second group has been genetically more diverse and included cyanobionts of various, predominately terricolous cyanolichens, bryophytes and cycads, along with some colony-forming, free-living strains.

Diversity and specificity among lichen-forming *Nostoc* has also been examined by using nucleotide sequences of the $tRNA^{Leu}$(UAA) intron as a genetic marker (Paulsrud 2001). This group I intron is found in most cyanobacterial lineages and in the plastids of many eukaryotic algae and all green plants. The distribution and characteristics of the intron are consistent with an ancient origin

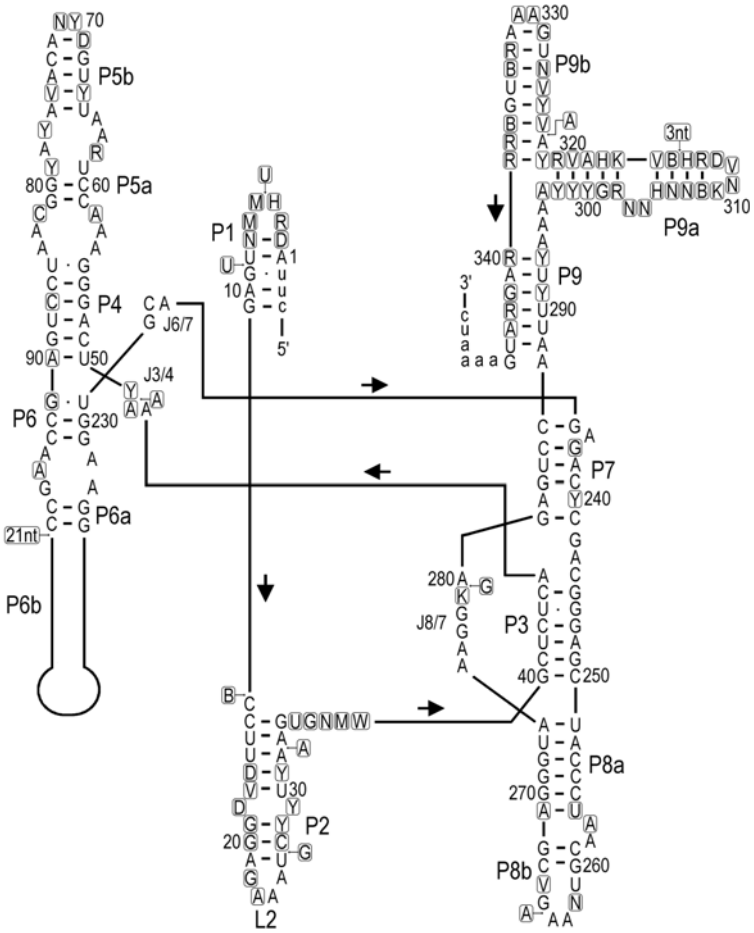

Figure 2. Variable positions (boxed) in *Nostoc* tRNA-^{Leu}(UAA) intron sequences as visualized in the predicted secondary structure of the transcribed intron (Cech et al. 1994). Deviating positions that were found in only one sequence are shown according to the consensus of all *Nostoc* sequences (n = 552); all other positions are marked by ambiguous codes. The numbering of positions follows that in the longest *Nostoc* sequences (346 nt).

and vertical transmission, supporting the view that it was obtained by eukaryotic organisms in the primary plastid endosymbiosis. The intron has subsequently been lost at various occasions, resulting in its present scattered distribution. Over half of all modern cyanobacteria may lack the intron (Besendahl et al. 2000, Fewer 2001, Simon et al. 2003).

As shown by Costa et al. (2002) length variation in cyanobacterial tRNA^{Leu}(UAA) introns is mainly caused by processes other than random mutations. Single nucleotide differences are strongly restricted by the secondary and tertiary structure of the transcribed intron. Thus, all *Nostoc* sequences tend to be similar, except in one stem-loop (P6b) where both sequence and size variation is considerable (Fig. 2). This stem-loop 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 (Costa et al. 2002).

Conserved elements in the tRNA^{Leu}(UAA) intron have been used for the identification of *Nostoc* at the genus level, while more variable regions have been used to identify specific strains. Cyanobacterial tRNA^{Leu}(UAA) intron sequences have not been widely used in phylogenetic reconstruction, mainly due to the restricted number of in-

formative nucleotide positions in the conserved parts of the intron. Phylogenetic groupings based on the conserved parts of the intron (without stem-loops P6b, P9a and parts of P5) have corresponded with 16S rRNA gene phylogenies, but typically have had weak support. As a rule, the P6b stem-loop should be excluded from phylogenetic analyses as similarities in this region may lead to wrong phylogenies. For example, comparisons with 16S rRNA gene sequences have indicated that cyanobacterial strains with the same repeat class in the P6b stem-loop are not always more closely related to each other than to strains with other motifs (Oksanen et al. 2004a).

Studies of cyanolichens in Europe (Paulsrud & Lindblad 1998; Paulsrud et al. 1998, 2001; Oksanen et al. 2002; Rikkinen et al. 2002, unpubl.; Linke et al. 2003), western North America (Paulsrud et al. 2000), East Asia (Rikkinen et al. 2002, unpubl.), New Zealand (Summerfield et al. 2002), and Antarctica (Wirtz et al. 2003) have shown that lichen-forming *Nostoc* strains are a rich source of different tRNA^{Leu}(UAA) intron sequences. Further diversity has been found from hornwort, liverwort and cycad symbioses (Costa et al. 1999; 2001, Rikkinen & Virtanen unpubl.), and from non-symbiotic cyanobacteria (Xu et al. 1990, Rudi et al. 1997, Rudi & Jacobsen 1999, Wright et al. 2001, Rikkinen unpubl.). All these investigations have contributed to the accumulation of a large data set on the distribution and frequency of tRNA^{Leu}(UAA) introns in various cyanobacteria, geographical areas and ecological settings. In the present study I analyse these data with multivariate methods, as a step towards delimiting tRNA-^{Leu}(UAA) intron types in lichen-forming *Nostoc* strains. Identification of such entities could offer useful means of distinguishing ecological, geographic and/or taxonomic groups within the genus, and aid in the rapid placement of newly acquired sequences into a meaningful context.

Material and methods

All tRNA^{Leu}(UAA) intron sequences used in this study are cited in the Results. Due to many difficulties in delimiting Nostocalean taxa, identification of all cyanobacterial species and some genera should be considered very tentative (Baker et al. 2003). For the newly acquired intron sequences amplification and sequencing were performed as previously described (Rikkinen et al. 2002). The obtained sequences were aligned manually on the basis of sequence similarity and a secondary structure model of the transcribed introns. The model followed the conventions for cyanobacterial tRNA-^{Leu}(UAA) introns (Michel & Westhof 1990, Cech et al. 1994, Costa et al. 2002). Variable stem-loop structural elements were individually folded online using the *mfold* version 3.1 web server (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/, Zuker 2003).

The tRNA^{Leu}(UAA) intron sequences were first incorporated into a large alignment spanning the full range of filamentous cyanobacteria and including all *Nostoc* sequences available in GenBank on April 1, 2004. Accession numbers for the GenBank sequences are listed in the Results. Characters in the variable positions of the aligned sequences were transformed into columns in a raw data matrix. Only data from well aligned regions were used, i.e. the P6b stem-loop (corresponding to nucleotide positions 99–224 in the longest *Nostoc* sequences) was always excluded and when analysing the full taxonomic range of filamentous cyanobacteria also the P9a stem-loop (corresponding to positions 300–319 in the longest *Nostoc* sequences) was removed (Fig. 2). The raw data matrix including sequences of all filamentous cyanobacteria was 606 sequences × 162 characters, while the raw data matrix of *Nostoc* sequences was 552 sequences × 167 characters.

Prior to multivariate analyses, all incomplete sequences were removed and only one representative from each group of identical sequences was saved. In order to further reduce noise, also all columns that had fewer than two non-zero values were deleted. Finally, the columns 2A, 2G, 7C, 334T, and 340A were removed in order to de-emphasise clustering based on co-varying base pairs. In the base pairs 2:7, 327:334, and 293:340 a change in one position was almost invariably accompanied by a change in the base pairing strand, presumably in order to retain the structure needed

for autocatalysis of the transcribed intron (Fig. 2). In the analysis of the *Nostoc* data matrix also some sequences with very unusual combinations of characters were a matter of concern because of their large effects on the outcomes of descriptive ordinations. Such sequences were identified by using the Outlier Analysis of PC-ORD. It calculated the average distance from each sequence to every other sequence and indicated those sequences that fell greater than two standard deviations above the mean for average distance. These outliers were usually removed from the data sets and analysed separately. After all reductions the data matrix for all filamentous cyanobacteria was 194 sequences × 99 characters, while the data matrix for *Nostoc* strains was 147 sequences × 82 characters.

The data matrices were analysed using the statistical package PC-ORD (McCune & Medford 1995). Non-metric multidimensional scaling (NMS) and Bray-Curtis ordination were used to produce graphical depictions of clustering based on similarities in the tRNA^{Leu}(UAA) intron sequences (sequences in nucleotide space). NMS is a non-parametric ordination technique and well suited to data that are non-normal, are on discontinuous scales, and contain a large proportion of zero values. The NMS ordinations were performed using the quantitative version of Sørenson's distance measure, 100 iterations, and random starting co-ordinates. The program was run numerous times with different starting configurations to ensure that the obtained minimum stress was not a local minimum. A two dimensional ordination of the data matrix was produced after determining that higher dimensional solutions did not substantially reduce stress. Also Bray-Curtis ordination has empirically shown to be an effective way of reducing data sets that contain a large proportion of zero values (McCune & Medford 1995). Two dimensional ordinations of the data matrix were performed using the quantitative version of Sørenson's distance measure and the variance-regression method of selecting endpoints. For visual clarity, the resulting ordinations were rigidly rotated. The distribution of different repeat motifs in the P6b regions of tRNA^{Leu}(UAA) intron sequences were overlaid on the resulting ordinations. Also other variables were visualised in this manner.

Statistical significance of differences between sequence groups were estimated using multi-response permutation procedures (MRPP). MRPP is a non-parametric method for testing the hypothesis of no difference between two or more *a priori* groups (McCune & Medford 1995). The quantitative version of Sørenson's distance measure was used as the measure of dissimilarity. Signature characters indicating specific groups of tRNA^{Leu}(UAA) intron sequences were identified by calculating the faithfulness of their occurrence in each group. The statistical significance was evaluated by a Monte Carlo method. Sequences were randomly reassigned to groups 1000 times and each time the highest proportional frequency for a given character across groups was calculated. The probability of type I error was the proportion of times that the highest value from the randomized data set equalled or exceeded the value from the actual data set. The null hypothesis was that the highest value was no larger than would be expected by chance, i.e. that the character had no signature value. The relative frequency of signature characters in given groups was given as percentage of perfect indication.

All analysed tRNA^{Leu}(UAA) intron sequences are listed in the Results. Note that the lists also include incomplete sequences that were not used in multivariate analyses, but were grouped according to their overall similarity with complete sequences. Their placement within lists mainly reflects signature characters in the conserved parts of the intron, i.e. the P6b and P9a stem-loops were only compared within groups. Sequences that were included in multivariate analyses are indicated by an asterisk (*). Identical sequences from different sources are separated by commas, while sequences differing in at least one nucleotide position are separated from each other by periods.

Results

Non-Nostoc sequences

NMS ordination of all cyanobacterial tRNA^{Leu}(UAA) intron sequences (P6b and P9a regions

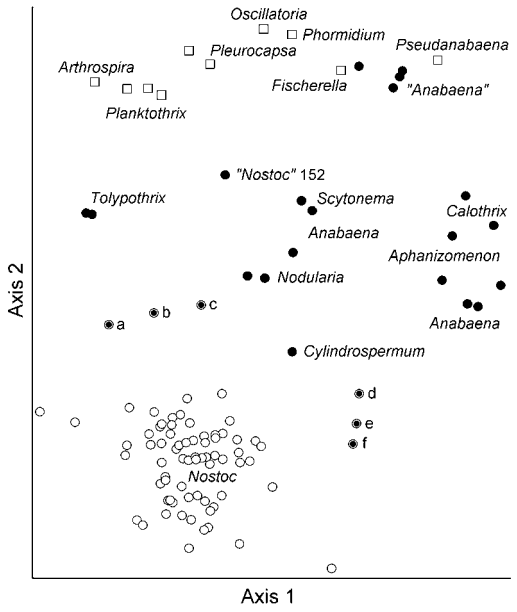

Figure 3. NMS ordination diagram based on nucleotide data from the conserved parts of tRNA^{Leu}(UAA) intron sequences (variable loops P6b and P9a excluded; 194 sequences/99 characters). Typical *Nostoc* sequences (open circles) cluster away from those of other Nostocalean cyanobacteria (filled circles) and non-Nostocalean cyanobacteria (open squares). Sequences a-f are outliers, i.e. they originate from misidentified strains or represent inadequately sampled intron types within the genus *Nostoc*.

excluded) grouped *Nostoc* sequences away from those of other filamentous cyanobacteria (Fig. 3). This reflected statistically significant differences between the *Nostoc* sequences and other sequences (MRPP, T = –79.45, P < .0001). Most sequences that had reportedly been amplified from *Nostoc* clustered closely together, but a few grouped closer to sequences of other Nostocalean cyanobacteria (e.g. *Cylindrospermum* and *Nodularia*). Most Nostocalean sequences were well separated from those of non-Nostocalean cyanobacteria, but some (including the sequences from '*Anabaena*/*Nostoc*' PCC 7120) grouped close to sequences of *Fischerella* (Stigonematales) and *Pseudanabaena* (Oscillatoriales). Differences between the three main groups of sequences (*Nostoc*, other Nostocalean genera, and non-Nostocalean genera) were highly significant (T = –59.68, P < .0001). Widespread signature characters (P < .0001) that helped to identify non-*Nostoc* sequences were (relative frequency in non-*Nostoc*/*Nostoc* sequences): 28T (73/1), 250T (74/4), 333G (76/4), 322C (78/8), 297G (70/0), and 325T (73/2).

SEQUENCES EXAMINED (54). **Non-Nostocalean cyanobacteria (Pleurocapsales, Oscillatoriales and Stigonematales)**. *AJ228714 *Arthrospira fusiformis*; *U83258 *Fischerella ambigua*; *U83255 *Oscillatoria* sp.; *AJ228704 *Phormidium* sp. *AJ228702 *Planktothrix agardhii*; AJ228701 *P. agardhii*; AJ228700 *Planktothrix mougeotii*; *AJ228703 *Planktothrix prolifica*; *AJ571703 *Planktothrix* sp.; *AJ571702 *Planktothrix* sp.; *AJ228713 *Pleurocapsa minor*; *U83253 *Pseudanabaena* sp.; *AF509431 Cyanobacterium sp.; **Nostocalean cyanobacteria (excluding *Nostoc* s.str.)**. *AJ228705 '*Anabaena*' sp.; *M38691 *Anabaena azollae*; *AJ571714 *Anabaena crassa*; *U83251 *Anabaena cylindrica*; *AJ571713 *Anabaena spiroides*; *M38692 '*Anabaena*' PCC 7120; *AP003597 '*Nostoc*' PCC 7120; *AJ571711 *Aphanizomenon* sp.; *AJ571712 *Aphanizomenon flos-aquae* var. *klebahnii*; *AJ228707 *Aphanizomenon flos-aquae*; *AJ228706 *Aphanizomenon gracile*; *U83252 *Calothrix desertica*; *AJ571704 *Calothrix* sp.; *AJ571715 *Cylindrospermum* sp.; *U83250 *Cylindrospermum* sp.; *AJ571707 *Nodularia spumigena*; *AJ571708 *Nodularia* sp.; *AJ571710 *Nodularia* sp.; *AJ571706 *Nodularia* sp.; *AJ571709 *Nodularia* sp.; AF204067 '*Nostoc commune*'; AF204083 '*Nostoc commune*'; AF204065 '*Nostoc*' sp.; *AF095773 '*Nostoc*' sp. from *Cycas rumphii,* outlier (b) in Fig. 3; *AJ571719 '*Nostoc*' 152; *M61164 *Scytonema* sp.; *AJ571705 cf. *Tolypothrix* sp.; AF509428 Cyanobacterium sp.; AF509429 Cyanobacterium sp.; *AF509430 Cyanobacterium sp.; JRA1 Cyanobacterium sp.; JRA4 Cyanobacterium sp.; JRA7 Cyanobacterium sp.; *JRA8 Cyanobacterium sp.; JR50N21 Cyanobacterium sp.; JR88K2 Cyanobacterium sp.; JR89K2 Cyanobacterium sp.; AY304267 Cyanobacterium sp. from *Placopsis parellina*; AY304276 Cyanobacterium sp. from *Placopsis parellina*; AY304270 Cyanobacterium sp. from *Placopsis parellina*; AY304279 Cyanobacterium sp. from *Placopsis parellina*.

Muscorum-type sequences

Bray-Curtis ordination of the *Nostoc* tRNA-^{Leu}(UAA) intron sequences (P6b region excluded) resulted in the separation of two unequal groups (Fig. 4A). The smaller subgroup included the sequence from a laboratory strain of *Nostoc musco-*

rum and several sequences from *Nostoc* strains from the symbiotic roots of cycads. This group of sequences (*Muscorum*-type), which differed significantly from other *Nostoc* sequences (MRPP, T = –23.96, P < .0001), has not yet been amplified from bryophyte or lichen symbioses. Useful signature characters (P < .0001) for identifying the group included (relative frequency in *Muscorum*-type/other *Nostoc* sequences): 262A (100/8), 299T (100/3), 303A (100/6), 304G (100/5), 305C (60/2), 310G (60/1), and 320A (100/1).

SEQUENCES EXAMINED (11). *AF019925 *Nostoc muscorum*; *AY452304 from *Cycas basaltica*; *AY452306, -307 two sequences from *Cycas revoluta*; *AY452309 from *Cycas revoluata*; *AF095772 from *Cycas revoluta*; *AY452308 from *Cycas revoluta*; *AF095776 from *Encephalartos villosus*; *AY452314 from *Zamia integrifolia*; *AY452313 from *Zamia furfuracea*; *AF095774 from *Zamia pumila*.

Commune-type sequences

Bray-Curtis ordination of the remaining *Nostoc* $tRNA^{Leu}$(UAA) intron sequences (P6b region excluded) divided them into two distinct groups (Fig. 4B). The smaller subgroup included many sequences from free-living strains corresponding to the form species *Nostoc commune*. Similar sequences have also been found from cycad and lichen symbioses, particularly from terricolous gelatinous lichens. *Commune*-type sequences differed significantly from other *Nostoc* sequences (MRPP, T = –64.12, P < .0001). Widespread signature characters (P < .0001) that helped to distinguish them from other *Nostoc* sequences included (relative frequency in *Commune*-type/other *Nostoc* sequences): 303C (68/0), 306T (98/2), 311A (100/1), and 312A (100/3).

SEQUENCES EXAMINED (77). *AF204077 *Nostoc commune* (UK); Nine sequences from colony-forming strains: *AF204075 *Nostoc commune* (Switzerland), AF204076 *N. commune* (Virginia), AF204087 *N. commune* var. *flagelliforme* (Mexico), AF204088 *N. pellucidum* (Italy), AF204092 *N. commune* (Romania), AF204094 *N. commune* (Indonesia), AF204096 *N. commune* (Germany?), AF204101 *N. sphaericum* (China), and AF204106 *N. commune* var. *flagelliforme* (Texas); *AF204097 *Nostoc commune* (Germany?); *AF204095 *Nostoc commune* (Indiana); *AF204100 *Nostoc commune* (Italy); *AF204089 *Nostoc commune* (China); *AF204080 *Nostoc commune* (New Zealand); *AF204103 *Nostoc commune* (Tennessee); *AF204072 *Nostoc commune* (Antarctica); *AF204070 *Nostoc* sp. (Antarctica); *AF491918, -919 two sequences from *Nostoc* sp. (Ireland); *AF204102 *Nostoc commune* (Switzerland); *AF204084 *Nostoc commune* (Romania); *AF204104 *Nostoc commune* (South Carolina); *AF204073 *Nostoc commune* (Antarctica); *AF204085 *Nostoc commune* (Uruguay); AF204091 *Nostoc commune* (China); *AF204098 *Nostoc commune* (UK?); *AY452297 from *Macrozamia riedlei* (Australia); *AF204093 *Nostoc commune* (Germany); *AJ421996 from *Pseudocyphellaria crocata*, probably epiphytic (New Zealand); *AF204068 *Nostoc commune* var. *flagelliforme* (Aldabra Atoll); *AF204069 *Nostoc commune* var. *flagelliforme* (Aldabra Atoll); *AF204090 *Nostoc commune* (China); *AF176597 from *Peltigera venosa* (Oregon); *AF176598 from *Peltigera venosa* (Oregon); *AY452290 from *Macrozamia riedlei* (Australia); *AY452289 from *Macrozamia riedlei* (Australia); *AF204074 *Nostoc commune* (Virginia); *AF204071 *Nostoc commune* (Antarctica); *AF204082 *Nostoc* sp. (Aldabra Atoll); AF204066 *Nostoc* sp. (Aldabra Atoll); *AY452281, -282 two sequences from *Macrozamia riedlei*; *AY452294 from *Macrozamia riedlei*; *AF204078 *Nostoc commune* (UK); *AF204079 *Nostoc commune* (UK); *AF204086 *Nostoc commune* (Massachusetts); AF204105 *Nostoc commune* (Romania); *AF176596 from *Peltigera vensosa* (Oregon); AY304254 *Nostoc* sp. (Antarctica); JRGC02 from *Leptochidium albociliatum* (Gran Canaria); JRGC10 from *Leptochidium albociliatum* (Gran Canaria); JRGC08 from *Leptochidium albociliatum* (Gran Canaria); JRGC13 from *Polychidium muscicola* (Gran Canaria); *JRGC14 from *Polychidium muscicola* (Gran Canaria); *JRGC15 *Leptochidium albociliatum* (Gran Canaria); JRGC17 *L. albociliatum* (Gran Canaria); *JRGC16 from gelatinous macrolichen (Gran Canaria); *AF491915 from *Collema* sp. (Ireland); *AF491916 from *Collema* sp. (Ireland); *AF491912-914 three sequences from *Collema* sp. (Ireland); *JR003566 from *Leptogium sessile* (China); AJ422009 from *Pseudocyphellaria murrayi* (New Zealand); AY170133 from *Peltigera malacea*; *AF176617 two sequences from *Peltigera venosa* (Washington); *AF019920 two sequences from *Peltigera aphthosa* (Sweden); AF055655-656 six sequences from both morphs of *Peltigera aphthosa* fotosymbiodeme (Finland); *AY170132 from *Peltigera malacea*.

Punctiformis-type sequences

The larger subgroup in Figure 4B included the $tRNA^{Leu}$(UAA) intron sequence from the laboratory strain *Nostoc punctiforme* PCC 73102 and

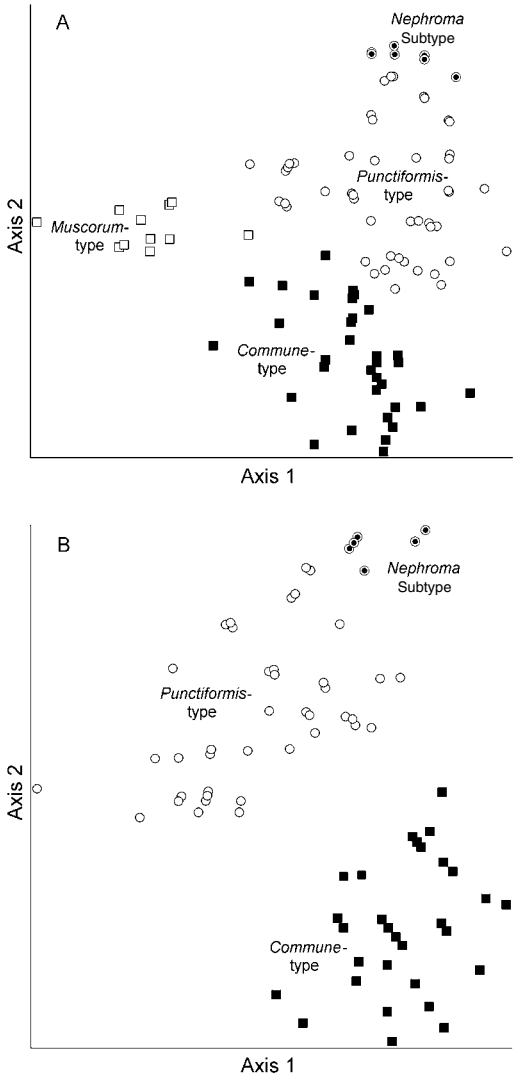

Figure 4. Bray-Curtis ordination diagrams based on nucleotide data from the conserved parts of Nostoc tRNA-^{Leu}(UAA) intron sequences (variable loop P6b excluded). A. *Muscorum*-type sequences cluster away from other *Nostoc* sequences (data set: 147 sequences / 82 characters). B. *Commune*-type sequences cluster away from *Punctiformis*-type sequences (data set: 135 sequences / 75 characters).

similar sequences from many symbiotic *Nostoc* strains, including lichen-forming strains and cyanobionts of bryophytes and cycads. As a group, the *Punctiformis*-type sequences differed significantly from all other *Nostoc* sequences (MRPP, T = –64.86, P < .0001). However, as they were quite variable and included representatives from many inadequately sampled subgroups, no single signature character could be used to reliably distinguish *Punctiformis*-type sequences from all other *Nostoc* sequences. While it is beyond the scope of this study to describe nucleotide variation in the *Punctiformis*-type sequences in any detail, three subgroups are briefly described below.

Nephroma subtype. One characteristic subgroup among the *Punctiformis*-type sequences were those amplified from bipartite *Nephroma* species and many other epiphytic cyanolichens (*Nephroma* guild, Rikkinen et al. 2002). This group of sequences appears to be restricted to lichen-forming *Nostoc* strains, as similar sequences have not yet been amplified from bryophyte or cycad symbioses. The sequences clustered closely together in all analyses (Fig. 4) and differed significantly from other *Nostoc* sequences (MRPP, T = –45.69, P < .0001). The most convenient signature character (P < .0001) for distinguishing them from other *Nostoc* sequences was (relative frequency in *Nephroma*-type/other *Nostoc* sequences): 71T (100/2).

SEQUENCES EXAMINED (89). **P6b region without inserted elements**. *AF509392-396 three sequences from *Nephroma parile*, one from *N. resupinatum*, and one from *Parmeliella triptophylla* (Finland); *AJ437321-325 five sequences from *Pseudocyphellaria neglecta* (New Zealand); *AF509354-388 12 sequences from *Parmeliella triptophylla*, 11 from *Nephroma bellum*, five from *N. resupinatum*, one from *N. parile*, one from *Lobaria pulmonaria*, and five from cultured *Nostoc* strains (Finland); *AF509389-391 three sequences from *Nephroma parile* and one from *Parmeliella triptophylla* (Finland); *JR001260 from *Leptogium asiaticum*, JR004478 from *L. azureum*, and JR991122, JR010658 two sequences from *Leptogium cyanescens* (China); *JR010552 from *Leptogium cyanescens* (China); *JR001328 *Leptogium cyanescens* (China); *JR010015 *Leptogium denticulatum* (China); *JR990905 from *Leptogium burnetiae* var. *hirsutum* (China); *JR000760 from *Leptogium* sp.; JR991150 from *Leptogium azureum* (China); *JR003552, JR010925, JR991175 three sequences from *Leptogium burnetiae* var. *hirsutum*, and one JR990994 from *L. pedicellatum* (China); *JR000130 from *Leptogium burnetiae* var. *hirsutum* (China); *AF509405 from *Nephroma resupinatum* (Oregon); *AF509406 from *Pseudocyphellaria anomala* (Oregon). **P6b region with**

24 nt insertion. *AJ421998, AJ422001-003 four sequences from *Pseudocyphellaria crocata* (New Zealand); *AJ421999, AJ421997 two sequences from *Pseudocyphellaria crocata* (New Zealand); *AJ422004-006 three sequences from *Pseudocyphellaria maculata* (New Zealand); *AF509397-399 three sequences from *Nephroma resupinatum*, AF055660 from *Parmeliella triptophylla* (Finland); *AJ422000 from *Pseudocyphellaria crocata* (New Zealand). **P6b region with 24 + 21 nt insertion**. *AF509400-403 two sequences from *Lobaria pulmonaria*, one from *Nephroma helveticum*, and one from *N. laevigatum* (Oregon); *AF509404 from *Pseudocyphellaria anthraspis* (Oregon). **P6b region with 24 + 24 nt insertion**. *AF509407-408 from *Nephroma helveticum* and *Lobaria retigera* (China); JR990803 from *Leptogium burnetiae* var. *burnetiae* (China); JR990961 from *Leptogium burnetiae* var. *burnetiae* (China); JR010647 from *Leptogium burnetiae* var. *burnetiae* (China).

Placopsis subtype. Another characteristic subgroup among the *Punctiformis*-type intron sequences were those amplified from the cephalodia of many tripartite cyanolichens and some terricolous bipartite species. Also these sequences may be restricted to lichen-forming *Nostoc* strains as they have not yet been amplified from bryophyte or cycad cyanobionts. This group seems to predominate in the *Nostoc* cyanobionts of lichens in maritime Antarctica (Wirtz el al. 2003), but they have also been amplified from some Northern Hemisphere lichens. *Placopis*-type sequences differed significantly from other *Nostoc* sequences (MRPP, T = –10.73, P < .0001). The most widespread signature character (P < .0001) for distinguishing them from other *Nostoc* sequences was (relative frequency in *Placopsis*-type/other *Nostoc* sequences): 70C (67/4). Other useful signature characters (P = .001) included: 6C (25/1), 237C (17/0), and 292G (25/1).

SEQUENCES EXAMINED (64). *AJ571717 from cultured *Nostoc* sp. (Finland); *AF176603 four sequences from *Peltigera britannica* (Oregon) and *AF176604 one from *P. venosa* (Oregon); *AF019918 two sequences from *Nephroma arcticum* (Sweden); *AF019912 three sequences from *Nephroma arcticum* (Sweden); AY304247, -250 two sequences from *Massalongia carnosa* (Antarctica); AY304246 from *Massalongia carnosa* (Antarctica); AY304249 from *Massalongia carnosa* (Antarctica); AY304284, -288, -291, -292 four sequences from *Psoroma cinnamomeum* (Antarctica); AY304278 from *Placopsis parellina* (Antarctica); AY304283 from *Placopsis parellina* (Antarctica); AY304281 from *Placopsis parellina* (Antarctica); AY304251, -252 two sequences from *Massalongia carnosa*, AY304273 one from *Placopsis parellina* (Antarctica); AY304248 from *Massalongia carnosa* (Antarctica); AY304235 from *Leptogium puberulum* (Antarctica); *AY304236 from *Leptogium puberulum* (Antarctica); AY304237, -239 two sequences from *Leptogium puberulum* (Antarctica); AY304240 from *Leptogium puberulum* (Antarctica); AY304241 from *Leptogium puberulum* (Antarctica); AY304243 from *Leptogium puberulum* (Antarctica); AY304244 from *Leptogium puberulum* (Antarctica); AY304263 from *Placopsis contortuplicata* (Antarctica); AY304264 from *Placopsis parellina* (Antarctica); *AY304272 from *Placopsis parellina* (Antarctica); AY304234 from *Psoroma cinnamomeum* (Antarctica); AY304290, -293 two sequences from *Psoroma cinnamomeum* (Antarctica); AY304233, -242, -295 three sequences from *Leptogium puberulum* (Antarctica); AY304256-262 seven sequences from *Placopsis contortuplicata* and AY304238 one from *Leptogium puberulum* (Antarctica); AY304271 from *Placopsis parellina* (Antarctica); AY304274 from *Placopsis parellina* (Antarctica); AY304265 from *Placopsis parellina* (Antarctica); AY304266 from *Placopsis parellina* (Antarctica); AY304269 from *Placopsis parellina* (Antarctica); AY304275 from *Placopsis parellina* (Antarctica); AY304280 from *Placopsis parellina* (Antarctica); AY304282, -296 two sequences from *Placopsis parellina* and AY304285-287, -289 four from *Psoroma cinnamomeum* (Antarctica); AY304294 from *Psoroma cinnamomeum* (Antarctica); *AY304253 from *Nostoc* sp. (Antarctica).

Peltigera subtype. The remaining *Punctiformis*-type sequences included the sequence from *Nostoc punctiforme* PCC 73102 and many similar sequences from symbiotic and free-living *Nostoc* strains. These included both lichen cyanobionts (mainly from *Peltigera* species and other terricolous lichens) and cyanobionts of thalloid bryophytes and cycads. Further subgroups clearly existed within this group, but many of them have been inadequately sampled. Here the sequences are organised into two main groups according to the single signature character 298C/T.

SEQUENCES EXAMINED (341). **P9a region with 298C**. *AJ571718 *Nostoc* sp. (Finland); *AF019913, -915, -919 a total of 86 sequences from *Peltigera aphthosa* (Sweden), AF055657 three from *P. aphthosa* (Finland), AF055658 four from *P. neopolydactyla* (Finland), and AF491917 one from *Nostoc* sp. (Ireland); *AJ422008

from *Pseudocyphellaria murray*, probably epiphytic (New Zealand); *AY170130 from *Peltigera hydrothyria*; *AF509409 *Nostoc* sp. (Finland), AF509410-412 two sequences from *Peltigera praetextata* (Finland), AF176608-610 three sequences from *Peltigera occidentalis* (Oregon), AF019914 two sequences from *Peltigera membranacea* (Sweden), AF176605-606 two sequences from *P. membranacea* (Oregon), and AY170131 one from *Peltigera canina*; AF176605 from *Peltigera membranacea* (Oregon); JR47N21E from *Nostoc* sp. (Finland); *AY170129 *Nostoc sphaericum*; *AF358917 19 sequences from *Blasia pusilla* (Finland); *AF509434 from *Peltigera pruinosa* (China); JR100ABl *Nostoc* sp. (Finland); JR11K1 *Nostoc* sp. (Finland); JR12K1 *Nostoc* sp. (Finland); JR48N21 *Nostoc* sp. (Finland); JR35L1E *Nostoc* sp. (Finland); *AF509433 from *Lobaria hallii* (Oregon); *AF358921 from *Blasia pusilla* (Finland); *AF176611-612 two sequences from *Peltigera neopolydactyla* (Oregon); AF358918 12 sequences from *Blasia pusilla* (Finland); *AF204081 *Nostoc* sp. (Aldabra Atoll); *AF509412-413 two sequences from *Peltigera praetextata* (Finland); *AY304277 from *Placopsis parellina* (Antarctica); *AY304268 from *Placopsis parellina* (Antarctica); *AY452303 from *Bowenia spectabilis*; *AF151777 ten sequences from *Anthoceros fusiformis* (Oregon); *AF358919 two sequences from *Blasia pusilla* (Finland), and JRCavB4 nine sequences from *Cavicularia densa* (Japan); *JRCavB6 from *Cavicularia densa* (Japan); JRCavC4 from *Cavicularia densa* (Japan); JR96S2S2 two sequences from *Blasia pusilla* (Finland); JR108Bla from *Blasia pusilla* (Finland); *AY452288 from *Macrozamia riedlei*; *AY452291 from *Macrozamia riedlei*; *AY452295 from *Macrozamia riedlei*; JR94S2S2 from *Blasia pusilla* (Finland); *AF019916, -917 four sequences from *Nephroma arcticum* (Sweden) and AF055659 two from *N. arcticum* (Finland). **P9a region with 298T**. *AF509432 from *Peltigera collina* (Oregon); *AF509425-426 two sequences from *Nostoc* sp. and AF509427 two sequences cultured from *Lobaria pulmonaria* (Finland); *AJ571716 cf. *Nostoc trichormus* (Finland); *AY304255 *Nostoc* sp. (Antarctica); *AJ422007 from *Pseudocyphellaria maculata*, probably epiphytic (New Zealand); *AF509435 from *Peltigera pruinosa* (China); *AF151780 25 sequences from *Blasia pusilla* (Finland); *AF358920 22 sequences from *Blasia pusilla* (Finland); *AY452287 from *Macrozamia riedlei*; *AF176614 four sequences from both morphs of *Peltigera venosa* (Oregon); *JR010785 and JR010696 two sequences from *Leptogium denticulatum* (China); JR990174 from *Leptogium denticulatum* (China); JR010919 from *Leptogium asiaticum* (China); JR001619B from *Leptogium pseudopapillosum* (China); JR000591 from *Leptogium pseudopapillosum* (China); JR990630 from *Leptogium pseudopapillosum* (China); *AF509424 *Nostoc* sp. (Finland); JR010675 from *Leptogium asiaticum* (China); *AY112694 from *Peltigera venosa* (NW Russia); *AJ571720 *Nostoc* sp. (Finland); *AY452310-311two sequences from *Encephalartos altensteinii*; *AY452284-285 two sequences from *Macrozamia riedlei*; *AY043363 from *Peltigera membranacea* (British Columbia) and AF176613 from *Peltigera britannica* (Washington); AY304245 from *Leptogium puberulum* (Antarctica); *AF055662 from *Peltigera canina* (Finland) and AF019922 two sequences from *P. canina* (Sweden); *AF509423 *Nostoc* sp. (Finland); *AF509416 from *Peltigera praetextata* (Finland); *AF509417-419 three sequences from *Nostoc* sp. (Finland); JR32L1B two sequences from *Blasia pusilla* (Finland); *AY452312 from *Lepidozamia peroffskyana*; *AF358922 from *Peltigera didactyla* (Finland); *AF095778 from *Encephalartos villosus*, AY452292 from *Macrozamia riedlei*; *AY452298 from *Macrozamia riedlei*; *AY452299 from *Macrozamia riedlei*; AJ228708 *Nostoc* sp.; *AJ228709 *"Nostoc commune"*; *AF204099 *"Nostoc commune"*; *U83254 *Nostoc punctiformis* (Australia); JR31L1 *Nostoc* sp. (Finland); *JR55BL *Nostoc* sp. (Finland); JR91CavL *Nostoc* sp. (Japan); *AY452283 from *Macrozamia riedlei*; JR40N22B from *Blasia pusilla* (Finland); *JR990928 from *Leptogium* sp. (China).

Sequences with unusual character combinations

The last group is an artificial assemblage of *Nostoc*-like $tRNA^{Leu}$(UAA) intron sequences that caused problems in descriptive ordinations due to their unusual character combinations. Many of these sequences were identified as outliers in the Outlier Analysis and removed from the data sets before ordination analyses.

SEQUENCES EXAMINED (24). *AJ228710 *Nostoc flagelliforme*; *AY170128 *Nostoc punctiformis*; *AF491920 *Nostoc* sp. (Ireland); AY112693 *Nostoc* sp. (Finland); JR56LV3B *Nostoc* sp. (Finland); *JR000372 from *Leptogium austroamericanum* (China); *AF151776 from *Anthocerosfusiformis* (Oregon); *AF151778 three sequences from *Anthoceros fusiformis* (Oregon); *AF151779 two sequences from *Anthoceros fusiformis* (Oregon); *AY452286 from *Macrozamia riedlei*; *AY452293 from *Macrozamia riedlei*, outlier (f) in Fig. 3; *AY452305 from *Cycas media*, AY452301,-302 two sequences from *Macrozamia riedlei*; *AY452296, AY452300 from *Macrozamia riedlei*, outliers (d) in Fig. 3; *AY452290 from *Macrozamia riedlei*, outlier (e) in Fig. 3; *AY452289 from *Macrozamiariedlei*, outlier (c) in Fig. 3; *AF204082 *Nostoc* sp., outlier (a) in Fig. 3 (Aldabra Atoll); *AF204066 *Nostoc* sp., outlier (a) in Fig. 3 (Aldabra Atoll); AF204064 *Nostoc* sp. (Aldabra Atoll).

Discussion

The stable secondary and tertiary structure of tRNA^{Leu}(UAA) introns limits possibilities for random mutations in their nucleotide sequences. The low number of informative characters, in turn, does not provide much variation for hierarchical analysis and this restricts phylogenetic group formation. As a result, phylogenetic trees based on cyanobacterial tRNA^{Leu}(UAA) intron sequences have often been unresolved (Paquin et al. 1997, Rudi & Jacobsen 1999, Besendahl et al. 2000, Wright et al. 2001, Linke et al. 2003, Wirtz et al. 2003, Oksanen et al. 2004a). Furthermore, some phylogenetic methods rely on statistical models that should not be used when the possibility of compensatory or convergent nucleotide substitutions cannot be excluded. In tRNA^{Leu}(UAA) intron sequences such substitutions seem to have been instrumental for maintaining specific stem-loop structures that are required for autocatalytic activity of the transcribed intron.

In the present study, clustering of tRNA^{Leu}(UAA) intron sequences in the ordination diagrams reflected relatedness based on sequence similarity. While the sequence types offered a heuristic rather than a formal classification, they were not in obvious conflict with phylogenetic classifications based on the 16S rRNA gene and/or the conserved parts of the tRNA^{Leu}(UAA) intron. For example, *Nostoc* strains of the *Nephroma* subtype also formed a monophyletic group in phylogenetic analyses of 16S rRNA gene sequences. The *Peltigera* subtype sequences, which now grouped closest to the *Nephroma* group had been amplified from the cyanobionts of *Peltigera collina* and *Lobaria pulmonaria* (Fig. 4). These strains were also identified as the closest relatives of the *Nephroma*-group in phylogenetic analyses of 16S rRNA gene sequences (Rikkinen et al. 2002; Lohtander et al. 2003; Oksanen et al. 2004a, 2004b).

The ordination analyses showed that all tRNA^{Leu}(UAA) intron sequences from lichen-forming *Nostoc* strains belonged to two main groups, i.e. the *Punctiformis*- and *Commune*-types (Fig. 5). The third main group of intron sequences, the *Muscorum*-type, has not yet been amplified from the *Nostoc* cyanobionts of lichens or thalloid bryophytes. Interestingly, an overlay showing the distribution of *Nostoc* strains from which also the 16S rRNA gene has been sequenced, indicates that the sampling of 16S rRNA genes has been seriously skewed towards *Nostoc* strains with *Punctiformis*-type tRNA^{Leu}(UAA) intron sequences (Fig. 6). Apparently not a single 16S rRNA gene has been knowingly sequenced from *Nostoc* strains with *Commune*- or *Muscorum*-type intron sequences. This draws into question whether different subgroups within *Nostoc* have been adequately sampled in the presently available 16S rDNA trees.

The data presented here and in earlier studies indicate that tRNA^{Leu}(UAA) intron sequences allow different levels of differentiation to be obtained depending on the region chosen. The P9a stem-loop and informative sites in the conserved stem-loops can be used to assess diversity among distantly related strains and different *Nostoc* species, while the P6b stem-loop should only be used to distinguish closely related strains. Oksanen et al. (2004) studied the utility of intron sequences as a phylogenetic marker among heterocystous cyanobacteria with maximum parsimony, maximum likelihood and Bayesian inference. The analyses involved comparing evolutionary information of the tRNA^{Leu}(UAA) intron to that of the 16S rRNA gene. The main finding was that the P6b stem-loop should not be used in phylogenetic analyses, as trees inferred from the 16S rRNA gene and the distribution of two heptanucleotide repeat classes in the P6b stem-loops of the intron were in conflict. Also the results of the present study confirm that the distribution of the two repeat classes does not always follow classifications based on conserved parts of tRNA^{Leu}(UAA) intron sequences. The evolutionary origins of the complementary repeat classes are not known, but the presence of one of only two repeat motifs in all *Nostoc* sequences indicates that both motifs have been conserved and maintained. Maybe they facilitate the stabilisation of the transcribed intron during its processing by increasing the stability of the RNA stems or fulfil some other functional role, as without any constraints, one would expect to see more variation.

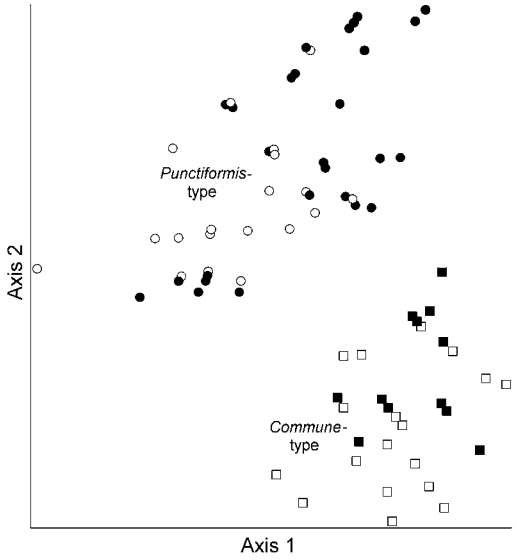

Figure 5. Overlay showing the distribution of presumed lichen symbionts (filled symbols) among *Nostoc* strains with *Punctiformis*- and *Commune*-type tRNA^{Leu}(UAA) introns. The underlying ordination is the same as in Fig. 4B.

The consequences of slipped-strand mispairing of the two strands of the DNA double helix seem to provide a coherent explanation for the length variation in both repetitive motifs (Costa et al. 2002). Slipped-strand mispairing involves local denaturation and displacement of the strands of a DNA duplex followed by mispairing of complementary bases at the site of an existing short tandem repeat. Slipped-strand mispairing, when followed by replication or repair may lead to insertions or deletions of one or several of the short repeat units, the outcome depending on the manner in which the mispaired structure is resolved. As the regions expand, they may become predisposed to interhelical events, such as illegimate recombination (Levison & Gutman 1987, Taylor & Breden 2000, Platas et al. 2003, Cozzolino et al. 2003). Several forces may result in a bias towards retention of moderate sequence lengths in the P6b region of *Nostoc* tRNA^{Leu}(UAA) intron sequences (Rikkinen unpubl.).

Many morphological characters that have traditionally been used to delimit cyanobacterial species do not accurately reflect evolutionary relationships (Mollenhauer 1988, Komárek & Anagnostidis 1989, Castenhotz 2001, Gugger & Hoffman 2004, Henson et al. 2004). For example, in many filamentous cyanobacteria the presence and/or structure of a mucilaginous envelope has been seen as a constant strain-specific character. However, the envelope polysaccharides seem to serve as a sink for fixed carbon when C/N metabolism is unbalanced. Hence, in *Nostoc* the properties of the glycocalyx are not constant and the mucilaginous envelope may even be lost under certain growth conditions (Otero & Vincenzini 2004). In the present study, the three main intron types, with *Muscorum*-, *Commune*- and *Punctiformis*-type sequences, seem to broadly correspond with classical *Nostoc* species recognised on the basis of morphological characters and life-history traits. It remains to be seen whether these groups will hold when tRNA^{Leu}(UAA) intron sequences become available from the many poorly sampled subgroups within *Nostoc*. Additional material will also help to determine whether the intron sequences that were now seen as outliers represent unknown species of *Nostoc* or belong to other Nostocalean genera.

In all bipartite cyanolichens studied so far, only one *Nostoc* strain has been detected from each thallus. Also most tripartite lichens have contained the same cyanobiont strain in all cephalodia of individual thalli (Rikkinen 2002). On the other hand, different thalli of one cyanolichen species may contain different strains of *Nostoc* and different cyanolichen species can often share identical cyanobiont strains. Lichen-forming fungi with identical cyanobionts have the potential to form photobiont-mediated guilds (Rikkinen 2003). For example, the mycobionts of many epiphytic cyanolichens are known to depend on *Nostoc* strains with tRNA^{Leu}(UAA) introns of the *Nephroma* subtype. These fungi share a common pool of cyanobionts and form an ecological assemblage, the *Nephroma* guild. Conversely, many predominately terricolous cyanolichens depend on *Nostoc* strains with tRNA-^{Leu}(UAA) introns of the *Peltigera* subtype and form several ecological groups collectively called the *Peltigera* guild (Rikkinen et al. 2002). The my-

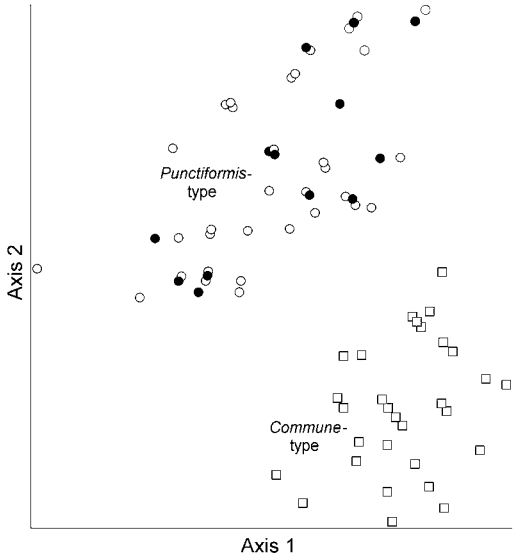

Figure 6. Overlay showing the distribution of *Nostoc* strains from which also the 16S rRNA gene has been sequenced (filled circles). The taxon sampling has been skewed towards *Nostoc* strains with *Punctiformis*-type $tRNA^{Leu}$(UAA) introns. The underlying ordination is the same as in Fig. 4B.

cobionts of many terricolous gelatinous cyanolichens depend on a third group of *Nostoc* strains, these with *Commune*-type $tRNA^{Leu}$(UAA) intron sequences. Also these fungi may form an ecological assemblage, the *Collema* guild. The above-mentioned differences in the substrate spectra of cyanolichen guilds are by no means abrupt nor obligatory. Terricolous, lithophytic and epiphytic habitats form ecological continua, which are strongly influenced by microclimate and other site factors. Thus, in suitable microhabitats members of several cyanolichen guilds can co-occur and form diverse communities. Similar photobiont-mediated guilds most probably influence the ecology of many green algal lichens (Rikkinen 1995; Beck et al. 1998, 2002; Beck 1999; Kroken & Taylor 2000; Dahlkild et al. 2001; Helms et al. 2001; Piercey-Normore & DePriest 2001; Tibell 2001; Romeike et al. 2002; Tibell & Beck 2002).

Guild boundaries have been frequently crossed during evolution. This explains why lichen guilds often include mycobionts from many different genera or even families. Concurrently, even closely related fungi may associate with different photobionts and thus belong to different guilds (Rikkinen 2003). Possible evidence of several guild shifts can be seen in the results of this study. These include for example the conspicuous occurrence of *Punctiformis*- and *Commune*-type $tRNA^{Leu}$(UAA) introns in the *Nostoc* cyanobionts of different *Peltigera aphthosa* thalli. Both types occur in Finland and Sweden: *Punctiformis*-type introns have been amplified from the cephalodia of most tripartite thalli while *Commune*-type introns have been amplified from the cephalodia of some thalli and from both morphs of a *P. aphthosa* fotosymbiodeme (Paulsrud et al. 1998, 2001). A somewhat similar situation exists in *Nephroma arcticum*, which has had *Nostoc* cyanobionts with *Peltigera*- and *Placopsis*-type introns in the cephalodia of different thalli. The variability of *Nostoc* cyanobionts in different *Leptogium* species is also interesting. The *Nostoc* cyanobionts of many epiphytic species from East Asia have had typical *Nephroma* guild introns, while the cyanobionts of others have had *Peltigera*-type introns (Rikkinen et al., unpubl). *Leptogium puberulum* from Antarctica has *Nostoc* cyanobionts with *Placopsis*-type introns (Wirtz et al. 2003).

The ability to form associations with a wide variety of lichen-forming fungi expands the habitat range of symbiotic *Nostoc* strains, but also increases resistance against grazing and environmental change. Different fungi tend to have different defences and thus a spectrum of unrelated mycobionts is directly beneficial for cyanobiont survival. This may have initiated an evolutionary trend towards sharing of photobionts by unrelated fungi. The trend may have been most pronounced in harsh environments, like in the Antarctic, where lichen-forming fungi have been found to exhibit relatively low degrees of photobiont specificity (Romeike et al. 2002, Wirtz et al. 2003).

To conclude, this study opened up a wide range of interesting questions to be explored. For example, are the results typical of all *Nostoc* strains, or only the rather limited number of morphological and ecological groups so far examined? Do *Nostoc* P6b regions posses their two complementary re-

peat motifs as a derived feature or an ancestral one? Analysis of intron sequences across a wider range of Nostocalean cyanobacteria can eventually reveal the extent to which this unusual genetic organization is found. So far, tRNA^{Leu}(UAA) intron sequences from only a minority of Nostocalean genera are represented in GenBank. Also, many interesting groups of *Nostoc*, including all symbiotic strains from the *Gunnera* symbioses, are lacking from the data set. Thus, further studies using a wide range of cyanobacteria from different ecological, geographic and taxonomic settings are warmly encouraged.

Acknowledgements

This contribution is dedicated to Professor Leif Tibell on the occasion of his 60th birthday.

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