Phylogenetic analyses of nitrogen-fixing cyanobacteria from the Baltic Sea reveal sequence anomalies in the phycocyanin operon

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The examination of molecular phylogenies of cyanobacteria and other micro-organisms is increasing dramatically. The use of a single locus in these studies leaves the resulting phylogenies unconfirmed. In this study, the partial sequences of two loci containing segments of protein-encoding genes, the *hetR* and the phycocyanin locus (PC-IGS), were examined. Laboratory strains and natural populations of the heterocyst-forming cyanobacteria *Anabaena*, *Aphanizomenon* and *Nodularia* from the Baltic Sea were used, in total 41 sequences were determined and their phylogenies were analysed with maximum-likelihood methods. The *hetR* phylogenies suggested that the planktonic *Aphanizomenon* and *Nodularia* each comprise one species, while there were numerous *Anabaena* species present in the Baltic Sea. In the case of *Nodularia*, the PC-IGS phylogenies were incongruent with this and suggested that several lineages of *Nodularia* plankton species existed. In the *hetR* phylogeny, the floating and nodularin-producing strains of *Nodularia* were grouped together. For both the *hetR* and PC-IGS loci of cultured species of *Nodularia* their molecular phylogeny did not correspond well with the affiliation suggested by morphology. In sequences derived from species of *Anabaena* and *Aphanizomenon* the PC-IGS and *hetR* phylogenies were congruent, suggesting that *Aphanizomenon* sp. from the Baltic Sea is genetically distinct from both *Aphanizomenon flos-aquae* from lakes and *Aphanizomenon* sp. TR183 from the Baltic Sea. In both *Nodularia* and *Anabaena/Aphanizomenon*, the PC-IGS sequences showed a significant degree of either recombination events or selection, while none was detected within the *hetR* sequences. This is the first study comprising the phylogenies of multiple loci from all heterocystous cyanobacteria from the Baltic Sea and shows that earlier results using the PC-IGS locus should be interpreted cautiously in the absence of a confirmation using a second locus.

Keywords: *Anabaena*, *Aphanizomenon*, *hetR*, *Nodularia*, phycocyanin

INTRODUCTION

The cyanobacteria are diverse in habitat and mor-

Abbreviations: PCC, Pasteur Culture Collection; KAC, Kalmar Algae Collection; BC Nod, Bristol Collection *Nodularia*; PC-IGS, the intergenic transcribed spacer between, and flanking regions of *cpcB* and *cpcA*.

A table of the taxa and accession numbers used in this study is available as supplementary data in IJSEM Online (<http://ijs.sgmjournals.org>).

The GenBank accession numbers for the *hetR* sequences determined in this study are AF320004–AF320008 and AF364336–AF364338. The GenBank accession numbers for the sequences of PC-IGS determined in this study are AF364339–AF364343, AY036900, AY036901 and AF193249.

phology but the genetic diversity does not always conform to the phenotypic diversity. Nitrogen fixation by filamentous cyanobacteria in the oceans plays an important role in the global nitrogen budget (Capone *et al.*, 1997; Carpenter *et al.*, 1999). In the Baltic Sea, species of the heterocyst-forming cyanobacteria *Aphanizomenon* Morren, *Nodularia* Mertens and *Anabaena* Bory become the dominant nitrogen-fixing cyanobacteria and fix N₂ in the order of 10⁵ tonnes N per year (Leppänen *et al.*, 1988; Rahm *et al.*, 2000). There are several unresolved taxonomic questions regarding the Baltic Sea cyanobacteria. The *Aphani-*

zomenon sp. in the Baltic Sea is often referred to as *Aphanizomenon flos-aquae* but ultrastructural observations have challenged this view (Janson *et al.*, 1994). Several planktonic species of *Nodularia* from the Baltic Sea have been described based on ultrastructure and morphology, including *N. baltica*, *N. litorea* and *N. spumigena* (Komárek *et al.*, 1993). In a study using both phenotypic and genetic analysis of species of *Nodularia* it was suggested that only one species exists in the Baltic Sea (Barker *et al.*, 1999). These genera have been subjected to intense phylogenetic studies using gene sequences, RFLP and fingerprinting methods (Neilan *et al.*, 1995; Bolch *et al.*, 1999; Barker *et al.*, 2000a; Lehtimäki *et al.*, 2000; Lyra *et al.*, 2001; Moffitt *et al.*, 2001). None of these studies used gene sequences from more than one locus to be able to confirm the observed phylogenies and in the absence of confirmation, the resulting phylogenies are open to question.

The assessment of the phylogeny of organisms through gene sequence analysis has increased dramatically since the advent of PCR and automated sequencing. Most studies have used the 16S rRNA gene for molecular phylogenies and inference of bacterial evolution. The variation of the 16S rDNA sequences between closely related species of *Bacillus* have been found to be insufficient to separate them (Fox *et al.*, 1992). In filamentous planktonic cyanobacteria, the 16S rDNA has been found to vary less than protein-encoding gene sequences (Janson *et al.*, 1999a; Barker *et al.*, 2000a, b). Two loci comprising partial protein-encoding genes that have been used to infer phylogenies of cyanobacteria are the *hetR* gene (Janson *et al.*, 1999a, b) and the region including the intergenic space and flanking regions of *cpcB* and *cpcA* genes (PC-IGS) (Bolch *et al.*, 1999; Barker *et al.*, 2000a; Robertson *et al.*, 2001; Tillett *et al.*, 2001). The *hetR* gene encodes a serine-type of protease with a regulatory role in the differentiation process of heterocysts (Zhou *et al.*, 1998; Buikema & Haselkorn, 2001). The *hetR* gene has only been detected in filamentous cyanobacteria (Buikema & Haselkorn, 1991; Janson *et al.*, 1998), which facilitates the analysis of samples containing other micro-organisms. The phycocyanin genes *cpcB* and *cpcA* encode proteins making up the accessory pigments of photosystem II (Gantt, 1994). The PC-IGS locus is found in some photosynthetic organisms including cyanobacteria, red algae and cryptophytes. Since the PC-IGS locus contains both coding and non-coding regions one could expect that different parts of the sequence would give different phylogenies, i.e. different evolutionary constraints exists for non-coding and coding regions (Li & Grauer, 1991). Despite this, many phylogenetic analyses have been performed using PC-IGS, without addressing the possibility of sequence anomalies within this locus.

In this study, datasets including two loci from identical or comparable strains were used. The phylogeny and sequence anomalies of the *hetR* and PC-IGS loci from heterocyst-forming cyanobacteria from the Baltic Sea

were analysed. The results showed that the phylogenies of these two loci were to some extent incongruent, and sequence anomalies were detected in the PC-IGS locus but not in the *hetR* locus.

METHODS

Organisms. The cyanobacterial isolates that were examined by sequencing in this study are all maintained in the Kalmar Algae Collection (KAC) at the University of Kalmar, Sweden, except *Aphanizomenon* sp. TR183, which was obtained from Dr K. Sivonen, Department of Applied Chemistry and Microbiology, Helsinki University, Finland. Strains *N. spumigena* KAC 10 through 17, *Anabaena lemmermannii* KAC 16 and *Aphanizomenon* sp. KAC 15 were isolated from the sound between Öland and the mainland of Sweden. *Nodularia* spp. KAC 64 through 68 and *Aphanizomenon* sp. KAC 60 were isolated from the Landsort deep in the Northern Baltic proper station number BY 31 (58° 35' N, 18° 14' E). All cultures were maintained at 16 °C, under cool fluorescent light tubes giving a light intensity of 30 µmol s⁻¹ m⁻², and grown in a Baltic Sea water-based f/2 medium with silicate omitted (Rippka, 1988). The natural population of *Aphanizomenon* sp. A1, A5 and the water used in mesocosm experiments where the *Anabaena* spp. were collected originated from an area 6 nautical miles east of Öland in the central part of the Baltic proper. *Aphanizomenon* sp. NB1 and NB5 were sampled at station BY 31. The natural population of *Aphanizomenon flos-aquae* was collected in lake Bysjön, Scania, Sweden, and the one of *Aphanizomenon flos-aquae* var. *klebahnii* originated from a park lake, Lilla Pildammen, Malmö, Sweden.

The online version of this paper (at <http://ijs.sgmjournals.org>) contains a supplementary table of the taxa and accession numbers used in this study. The assignments of morpho-species were done according to the descriptions in Geitler (1932).

PCR amplification and sequencing. Filaments or colonies of filaments, from natural populations were transferred to PCR tubes in a volume of five µl of environmental water. DNA was extracted using the procedure described earlier (Janson *et al.*, 1999a), briefly the cells are lysed by lysozyme, SDS and heat treatment. The DNA was then extracted from the crude lysate with cetyl tri-methyl ammonium bromide (CTAB). The 448-bp fragment of *hetR*, corresponding to numbers 159–606 in *Nostoc* sp. PCC 7120 (Buikema & Haselkorn, 1991), was amplified from extracted DNA or intact filaments using primers *hetr1* and *hetr2* (Janson *et al.*, 1998). The PC-IGS region was amplified using primers PCBF and PCAR (Neilan *et al.*, 1995). The PCR reaction mix was supplemented with 2.5% (v/v) final concentration of DMSO (Sigma) in order to lower the annealing temperature and improve the permeability of the cells. The reaction mix was also supplemented with *Taq* DNA polymerase buffer (Amersham Pharmacia Biotech), 200 µM dNTP (Amersham Pharmacia Biotech) and 1.5 U *Taq* DNA polymerase. The PCR was run for 30 cycles with 60 s at 94 °C, 30 s at 45 °C and 30 s at 72 °C, followed by an extension of 7 min at 72 °C. The PCR products used in sequencing were purified with QIAquick PCR purification kit (Qiagen). The purified PCR products were sequenced bidirectionally using an ABI model 373A automated sequencer (Applied Biosystems) and dye-terminator chemistry, using the same primers, *hetr1*, *hetr2*, PCBF and PCAR. When needed, sequencing was repeated on independent

PCR products to exclude errors incorporated by *Taq* polymerase or in the sequencing reaction (von Wintzingerode *et al.*, 1997).

Sequence analysis. The GenBank accession numbers for the sequences determined in this study and those used for phylogenetic analyses are listed in the online version of this paper (at <http://ijs.sgmjournals.org>). Alignments of sequences were produced manually with Seaview (Galtier *et al.*, 1996). The trees were constructed using the PhyloWin package (Galtier *et al.*, 1996) and PHYLIP 3.6 [<http://evolution.genetics.washington.edu/phylip.html>]. The sequences from *Nostoc* sp. PCC 7120 were used as outgroups in all trees. When using distance matrices to generate the trees the correction of Jukes & Cantor (1969) was used. When using maximum-likelihood in PhyloWin the fastDNAm1 (Olsen *et al.*, 1994) was implemented, which takes into account the transition/transversion ratio but does not allow for different base frequencies. When using PHYLIP the DNAML program was used (Felsenstein, 1981). The sequences were tested for recombination events or selection with a likelihood method implemented by PLATO (Grassly & Holmes, 1997). In PLATO, the sequence alignment is scanned and each position is tested for the likelihood of producing the observed phylogenetic tree. Regions with sequence anomalies are given a score, the Z-value, where values over 3.5 are significant. A significant value of a certain region means that this region does not follow the phylogeny of the complete sequence, which could mean that a recombination event or unusually high selection pressure has occurred.

RESULTS AND DISCUSSION

Sequencing and morphological observations

In total, 31 nucleotide sequences from species of *Nodularia* were determined in this study. The nucleotide sequence of the partial (448 bp) *hetR* gene was determined for 16 *Nodularia* spp. strains. Out of these 16 strains 13 were also subjected to PCR and se-

quencing of the PC-IGS (518 bp). For the remaining three strains (BC Nod 9401, BC Nod 9402 and BC Nod 9427) the PC-IGS sequence already existed (Hayes & Barker, 1997). The *hetR* gene from nine strains of *Aphanizomenon* spp. and PC-IGS sequences from two *Aphanizomenon* spp. and *A. lemmermanni* KAC 16 were determined. The strains of *Nodularia* could be divided into two main groups based on their buoyancy. The *N. harveyana* KAC 34, *N. harveyana* PCC 7804, *N. spumigena* KAC 32 and *N. spumigena* KAC 33 strains were all negatively buoyant. This group will be referred to as the non-floating group of strains. All the other *Nodularia* strains were floating. The *hetR* sequences from *Aphanizomenon* sp. originating from the Baltic Sea were compared with *hetR* sequences from the fresh-water strains of *Aphanizomenon* and the relatively closely related *Anabaena* spp. The morphology of *Nodularia* strains in culture varied between different strains and between different occasions of sampling of the identical strains (data not shown). The variation in morphology was such that no meaningful description of the strains in culture was possible. It has been shown that the morphology of *Nodularia* spp. is influenced by environmental and culturing conditions (Barker *et al.*, 1999; Bolch *et al.*, 1999; Lehtimäki *et al.*, 2000). Indeed, the morphological characters of the strains presented here varied far more than the genetic distances would suggest. On the other hand, the sequences from *N. spumigena* isolates KAC 32 and KAC 33 were different from all other *N. spumigena* isolates. These differences could be due to inadequate identification, and highlight the problem of using morphology alone as a taxonomic criterion on cultured strains. Instead, a polyphasic approach including the organism's ecology, biochemical features and/or molecular phylogeny using mul-

Fig. 1. Distance trees of sequences from two loci in *Nodularia*. The correction of the distance was that of Jukes & Cantor (1969). The numbers at the branches are the bootstrap support as percentage of 500 replicates. The scales represent 10% sequence divergence. In (a), the tree was based on 448 positions of *hetR* sequences determined in this study, except pcc7120 and nodbc9408. In (b), the tree was inferred from 497 positions of PC-IGS sequences from the same strains as in (a). Note the different topologies between the two trees.

Fig. 2. Comparison of maximum-likelihood phylogenies generated with various datasets of *hetR* and PC-IGS sequences from species of *Nodularia*. The numbers at branches and scales are as in Fig. 1, except that in (b) and (d) the scale represents 1% sequence divergence. In (a), the complete sequence was used and analysed with maximum-likelihood in PHYLIP using the observed transition/transversion ratio and base frequencies. In (b), the third position in the protein encoding frame has been removed from the analysis using PhyloWin. In (c), the complete sequence was used. In (d), the third position and the IGS region has been removed and (e) shows the tree based on the IGS region. In (f), the tree was based on the nucleotides 127–131 (422–426 in *Nostoc* sp. PCC 7120) that showed a significant sequence anomaly. The trees in (a) and (c) were generated with maximum-likelihood in PHYLIP using the observed transition/transversion ratio and base frequencies, all other trees were generated with PhyloWin.

tiple loci can better resolve species relationships among bacteria (Castenholtz, 1992; Ward, 1998).

Phylogenetic trees of *Nodularia* sequences

The overall relationship between the *Nodularia* strains and their *hetR* and PC-IGS sequences was displayed by inferring a distance tree (Fig. 1). The *hetR* phylogeny of the *Nodularia* spp. isolates clearly distinguished two main groups. All the floating strains had identical *hetR* sequences, which is in agreement with the suggestion that the pelagic population of *Nodularia* is represented by one species, *N. spumigena* (Barker *et al.*, 1999). In the sister group, were the *hetR* sequences from the non-floating strains PCC 7804, KAC 32, KAC 33 and KAC 34, where KAC 32 and KAC 33 were identical. In contrast, the tree of the PC-IGS sequences showed a different topology with some of the floating strains clustered together with non-floating strains (Fig. 1). The phylogenetic relationships was studied in more detail using a smaller dataset only taking into account those sequences that were different and using the maximum-likelihood approach to build the trees. The topology of the *hetR* tree using all positions gave the same topology as the first tree (Fig. 2a). In general, the third position in the derived reading frame of protein-encoding genes is more variable than the first and second. Reducing the dataset by using

only the first and second positions of the sequences resulted in the same topology but the branch length between *kac32* and *kac34* became zero, i.e. they were identical (Fig. 2b). In the PC-IGS tree (Fig. 2c), as with the *hetR* tree, the topology of the tree did not change compared with the distance tree (Fig. 1b). The topology of the tree was the same when only the first and second positions were used, floating strains were still paraphyletic, but the branches became shorter (Fig. 2d). When only the intergenic spacer was used, in total 77 positions without gaps, the tree topology changed. The ancestral sequence was that of *kac13-pc*, as compared to *kac34-pc* in the previous trees, and the branch lengths became shorter and it was zero between *kac10-pc* and *bcnod9427-pc* (Fig. 2e).

Even though buoyancy and toxin production can be lost under culturing conditions, all the floating *Nodularia* KAC-isolates are capable of producing the cyclic pentapeptide nodularin, a hepatotoxin that can also promote tumours (W. Stolte and E. Granéli, unpublished data; Dahlmann *et al.*, 2001). The non-floating *N. harveyana* PCC 7804 is capable of producing a nodularin of a different type, [L-Har²]nodularin, than those produced by *N. spumigena* (Beattie *et al.*, 2000). The PC-IGS phylogeny observed here and previously (Bolch *et al.*, 1999) is not in accordance with the nodularin production as some of the sequences

Fig. 3. Distance trees of sequences from two loci in a variety of *Anabaena* and *Aphanizomenon* species. The correction of the distance was that of Jukes & Cantor (1969). The numbers at the branches are the bootstrap support as percentage of 500 replicates. The scales represent 10% sequence divergence. In (a), the tree was based on 448 positions of *hetR* sequences and in (b), on 343 positions of the PC-IGS locus.

retrieved from the floating and toxin producing isolates clustered with sequences from the group of non-floating isolates. A positive correlation with toxin production and 16S rDNA sequences of *N. spumigena* has been reported previously (Lehtimäki *et al.*, 2000; Moffit *et al.*, 2001). These congruent results of 16S rDNA and *hetR* suggest that the *hetR*, but not the PC-IGS phylogeny, was consistent with the organism phylogeny. The reason for the discrepancy between the relationships revealed by PC-IGS locus and the *hetR*/16S rDNA loci could result from gene transfer, as this has been suggested to occur among the different genotypes of *N. spumigena* in the Baltic Sea (Barker *et al.*, 1999, 2000b).

Phylogenetic trees of *Anabaena* and *Aphanizomenon* sequences

The *hetR* sequences from *Anabaena* spp. and *Aphanizomenon* spp. were summarized in a distance tree (Fig. 3a). All the strains and natural populations of *Aphanizomenon* sp. from the Baltic Sea had zero branch length and formed a tight cluster with the two variants of *Aphanizomenon flos-aquae* with 100% bootstrap support. In contrast, the sequences from species of *Anabaena* showed multiple forms that suggested that several species exist in off-shore waters of the Baltic Sea. *Aphanizomenon* sp. TR183 was the earliest divergent sequence in this group. The PC-IGS sequences from identical, or morphologically similar, strains were summarized in a distance tree (Fig. 3b). The sequences from two isolates of *Aphanizomenon* sp. from the Baltic Sea had zero branch length, which is in accordance with the finding that the PC-IGS sequence from 25 colonies of *Aphanizomenon* sp. were identical (Barker *et al.*, 2000a), while the *Aphanizomenon* sp. TR183 was identical to *Aphanizomenon* sp. CCAP 1401/1 and was ancestral to the other *Anabaena*/*Aphanizomenon* sequences. The maximum-likelihood analysis of *hetR* sequences gave a tree similar to the distance tree except that the sequence from *Aphanizomenon* sp. TR183 was closest to the sequence of *Anabaena lemmermannii* KAC 16 (Fig. 4a). When the first and second codon positions were used the *Aphanizomenon* sp. TR183 sequence formed a sister group to *Anabaena lemmermannii* KAC 16 (Fig.

4b). The branch length between the other *Aphanizomenon* strains was zero in the same tree. In the PC-IGS tree the topology changed when using maximum-likelihood and the bootstrap support was lower (Fig. 4c). When the third position was removed from the analysis, the PC-IGS tree branching order remained unchanged, but with shorter branch lengths (Fig. 4d). The branching order changed again and the branch lengths were shorter when building the tree with the intergenic space including 71 positions (Fig. 4e). In this tree, the sequences of *Aphanizomenon* sp. from the Baltic Sea and Norman Lake were identical and ancestral to the sequences of *Anabaena lemmermannii* KAC 16, *Aphanizomenon* sp. from Little Crooked Lake, *Aphanizomenon* sp. TR183 and *Aphanizomenon flos-aquae* CCAP1401/1.

Based on ultrastructural observations the *Aphanizomenon* sp. from the Baltic Sea has been suggested to be a separate species from *Aphanizomenon flos-aquae* (Janson *et al.*, 1994). Interestingly, the *hetR* sequence differed by 1.1% between these two organisms, which further suggests that they are not the same species. The sequences from *Aphanizomenon flos-aquae* var. *klebahnii* and *Aphanizomenon* sp. were almost identical, differing in only one nucleotide, which suggests a very close relationship between these two organisms. Surprisingly, the *hetR* sequence from the Baltic Sea isolate *Aphanizomenon* sp. TR183 was very different compared to the other *Aphanizomenon* spp. sequences with more than 11% sequence divergence. This strain has been reported to be inseparable from a cluster of *Anabaena* and *Aphanizomenon* strains based on the 16S rDNA phylogeny (Lyra *et al.*, 2001). For example, the reported sequences of 16S rDNA of *Aphanizomenon* sp. TR183 and *Aphanizomenon* sp. BC Aph 9601 differ by only 0.1%. *Aphanizomenon* sp. TR183 has been used in physiological studies and inferences drawn in relation to the organisms forming blooms in the Baltic Sea (Evans *et al.*, 2000), but it has been shown here that the cultured strain is not identical with the bloom-forming organism. Such data indicate that either the 16S rDNA or the *hetR* sequence evolve at very different rates among different species of cyanobacteria. Consistent with the *hetR* phylogeny, the *Aphanizomenon* sp. TR183 PC-IGS sequence was

Fig. 4. Comparison of maximum-likelihood phylogenies generated with various datasets of *hetR* and PC-IGS sequences from species of *Anabaena* and *Aphanizomenon*. The numbers at branches and scales are as in Fig. 3, except that in (b) and (d) the scale represents 1% sequence divergence. In (a), the complete sequences of the *hetR* alleles have been used and in (b), the third position in the protein-encoding frame has been removed from the analysis. In (c), the complete sequences of the PC-IGS alleles were used and in (d) the third position and the IGS region were removed. In (e), the IGS region was included only and in (f), the tree based on the nucleotides 116–128 (411–423 in *Nostoc* sp. PCC 7120) showed a significant sequence anomaly. The trees in (a) and (c) were generated with maximum-likelihood in PHYLIP using the observed transition/transversion ratio and base frequencies; all other trees were generated with PhyloWin.

clearly separated from the sequence of *Aphanizomenon* sp. KAC 15 in all trees. Moreover, it did not form macrocolonies upon bubbling the culture, as did the *Aphanizomenon* sp. KAC 15 (data not shown). Taken together the simplest way to explain these observed differences between 16S rDNA phylogenies on the one hand and *hetR*/PC-IGS on the other is that 16S rDNA displays an extremely slow evolution in the observed taxa, which makes the 16S rDNA sequence unsuitable for resolving relationships between species of heterocystous cyanobacteria.

Analysis of sequence anomalies

In order to assess the possibility of sequence biases within the loci used in this study, the sequences were tested with PLATO. This test showed that no sequence anomalies were detectable in the *hetR* sequences. In contrast, the PC-IGS sequences from both *Nodularia* spp. and *Anabaena*/*Aphanizomenon* gave significant values in this test. The tree that was tested against the alignment was that in Figs 2(a, c) and 4(a, c), and the parameters given to the program were the model (Hasegawa *et al.*, 1985) and the transition/transversion value of 0.916 and 0.99. The sequences from species of *Nodularia* gave a Z-value of 8.1 for the region between position 239 and 288 (corresponds to the intergenic spacer excluding 36 nucleotides at the

3' end). A Z-value above 3.6 was significant in this analysis. A second region also gave a significant Z-value of 3.7, positions 127–131 (422–426 in *Nostoc* sp. PCC 7120), which is within the *cpcB* coding region. The phylogeny of this region differs from that seen when all positions were used (Fig. 2c, f). The differences also indicate that the amino acid isoleucine 142 in *Nostoc* sp. PCC 7120 is unchanged in *N. harveyana* KAC 34 but had changed to leucine in all the other strains. Significant Z-values in the *Anabaena*/*Aphanizomenon* sequences were those above 3.5. Positions 234–272 and 304–315, which are within the intergenic spacer, gave Z-values of 4.8 and 3.5, respectively. Positions 116–128 in *Anabaena*/*Aphanizomenon* (411–423 in *Nostoc* sp. PCC 7120), which are within the coding region of *cpcB*, gave a Z-value of 4.4. The phylogeny of the *Anabaena*/*Aphanizomenon* group of sequences also differed from the phylogeny seen when using all positions (Fig. 4c, f). Here, the indicated amino acid change was glycine 141 in *Nostoc* sp. PCC 7120, which was unchanged in *Aphanizomenon* sp. KAC 15, *Aphanizomenon* sp. BC Aph 9601 and *Aphanizomenon* sp. Norman Lake derived sequences but which had changed to alanine in all other strains (data not shown).

The complexity of the evolution of the PC-IGS alleles observed in the Baltic Sea has been attributed to

horizontal gene transfer (Barker *et al.*, 2000b). This alone should make this locus less suitable for phylogenetic analysis. In addition, we have shown that in two different groups of heterocystous cyanobacteria, the intergenic space shows less variation than the coding region and that it has a different phylogeny from the coding region. This is surprising because intergenic spaces are generally considered to be more variable than the coding region and this indicates that there has been either selection or recombination events within the PC-IGS region.

Concluding remarks

The phylogeny of the PC-IGS locus must be treated with much care when attempting to deduce organismal phylogenies as the locus displays a complex evolutionary pattern. In contrast, it has been shown by this and previous studies that the *hetR* gene phylogeny seem to closely match the organism phylogeny, and there are no detectable selection biases within the partial *hetR* gene sequences presented here. This study also confirms the suggestions that *N. spumigena* is the only *Nodularia* species in the Baltic Sea and that the Baltic Sea *Aphanizomenon* sp. is not identical with *Aphanizomenon flos-aquae* found in lakes.

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