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Phenotypic and genetic diversification of *Pseudanabaena* spp. (cyanobacteria)

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*Pseudanabaena* species are poorly known filamentous bloom-forming cyanobacteria closely related to *Limnothrix*. We isolated 28 *Pseudanabaena* strains from the Baltic Sea (BS) and the Albufera de Valencia (AV; Spain). By combining phenotypic and genotypic approaches, the phylogeny, diversity and evolutionary diversification of these isolates were explored. Analysis of the *in vivo* absorption spectra of the *Pseudanabaena* strains revealed two coexisting pigmentation phenotypes: (i) phycocyanin-rich (PC-rich) strains and (ii) strains containing both PC and phycoerythrin (PE). Strains of the latter phenotype were all capable of complementary chromatic adaptation (CCA). About 65 kb of the *Pseudanabaena* genomes were sequenced through a multilocus sequencing approach including the sequencing of the 16 and 23S rRNA genes, the ribosomal intergenic spacer (IGS), internal transcribed spacer 1 (ITS-1), the *cpcBA* operon encoding PC and the IGS between *cpcA* and *cpcB*. In addition, the presence of *nifH*, one of the structural genes of nitrogenase, was investigated. Sequence analysis of ITS and *cpcBA*-IGS allowed the differentiation between *Pseudanabaena* isolates exhibiting high levels of microdiversity. This multilocus sequencing approach revealed specific clusters for the BS, the AV and a mixed cluster with strains from both ecosystems. The latter comprised exclusively CCA phenotypes. The phylogenies of the 16 and 23S rRNA genes are consistent, but analysis of other loci indicated the loss of substructure, suggesting that the recombination between these loci has occurred. Our preliminary results on population genetic analyses of the PC genes suggest an evolutionary diversification of *Pseudanabaena* through purifying selection.

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Keywords: *Pseudanabaena*; cyanobacteria; complementary chromatic adaptation; microbial diversity; phylogeny; evolutionary diversification

Introduction

Cyanobacteria are the dominant component of the phytoplankton in many freshwater and marine environments where they may form nuisance blooms (Chorus and Bartram, 1999; Huisman et al., 2005; Granéli and Turner, 2006). The attention is usually toward the larger species that form aggregates and possess gas vesicles that make them buoyant and therefore accumulate at the surface. These species fix N\(_2\) and may be toxic and may cause serious environmental and socioeconomic problems. However, often it is not recognized that smaller cyanobacteria exceed the larger species in terms of biomass and activity and therefore play a major role in the ecosystem dynamics. Several reports mentioned the occurrence of the tiny filamentous *Pseudanabaena* in cyanobacterial blooms in brackish and freshwater ecosystems (Vasconcelos and Pereira, 2001; Stal et al., 2003; Gkelis et al., 2005; Zwart et al., 2005; Kim et al., 2006; Willame et al., 2006). Nevertheless, *Pseudanabaena* spp. has only had little attention in the scientific community where limited data exist about their phenotypic and genetic characteristics.

*Pseudanabaena* species are nonheterocystous cyanobacteria belonging to the order of Oscillatoriaceae. The family of *Pseudanabaenaceae* is characterized by simple trichomes with a width less than 4 \(\mu\)m. The cells are longer than wide, possess parietal thylakoids, contain polar gas vesicles and the cross walls are conspicuously constricted (Castenholz et al., 2001; Komárek, 2003). Some strains display complementary chromatic adaptation (CCA). This process allows these organisms to regulate the ratio
of the accessory photosynthetic pigments phycocyanin (PC) and phycoerythrin (PE), which helps them to adapt to the prevailing light spectrum (reviewed by Kehoe and Gutu (2006)) thereby favoring their persistence in competition against other species (Stomp et al., 2004, 2008). Most cultured strains reveal gliding motility and some are capable of anaerobic N₂ fixation (Rippka and Herdman, 1992).

Morphologically, Pseudanabaena resembles Limnothrix making their identification difficult. The main differences are the somewhat wider cells (1–6 µm) and the less distinct constriction of the cross walls in Limnothrix (Castenholz et al., 2001). Although they are rarely recognized as dominant organisms, Pseudanabaena as well as Limnothrix species occur and form blooms in eutrophic water bodies and occasionally dominate the phytoplankton (Mayer et al., 1997; Rücker et al., 1997; Zwart et al., 2005). Limnothrix is typically found in meso- to eutrophic freshwater ecosystems, whereas Pseudanabaena is more widely distributed and occurs in diverse aquatic as well as in benthic environments (Castenholz et al., 2001; Zwart et al., 2005; Diez et al., 2007). On the basis of the 16S rRNA gene, Pseudanabaena and Limnothrix form a monophyletic cluster within the cyanobacteria (Zwart et al., 2005; Willame et al., 2006). However, the available molecular data of the Pseudanabaena/Limnothrix group is scarce and consists of a limited number of environmental sequences and a few isolates. This limited data does not resolve the phylogeny of the Pseudanabaena/Limnothrix group.

Here, we present a multiphasic phenotypic and genotypic approach to explore the diversity, phylogeny and evolutionary diversification of Pseudanabaena strains isolated from two distant geographical regions in Europe, the BS, a large brackish basin in the North and Albufera de Valencia (AV), a coastal lagoon in the South. The BS is one of the largest bodies of brackish water in the world. It is a eutrophic system that develops blooms of the conspicuous N₂-fixing heterocystous cyanobacteria Aphaniizomenon and Nodularia, of which the latter is toxic (Stal et al., 2003). However, the dominant component of the cyanobacterial community in the BS consists of a colorful mixture of unicellular picocyanobacteria of the Synechococcus group and the tiny filamentous Pseudanabaena (Stal et al., 2003; Stomp et al., 2007; Haverkamp et al., 2008). The AV is a highly eutrophic coastal freshwater lagoon in Spain that is fed by streams, rivers and irrigation channels carrying fertilizer from the surrounding rice fields. The Albufera is characterized by dense water blooms of cyanobacteria among which Pseudanabaena spp. is a dominant group of organisms (Romo and Miracle, 1994; Villena and Romo, 2003). This report describes the phenotypic and genetic diversification found within Pseudanabaena from two geographical locations and it is thereby a first step toward an understanding of the ecology of this interesting but poorly known filamentous bloom-forming cyanobacteria.

Materials and methods

Isolation, cultivation and strain collection

The Pseudanabaena strains used in this study were isolated between 1995 and 2004 (Table 1). Strain CCY9508 was isolated in 1995 from the BS (Bornholm Sea). The Spanish strains were isolated in 1997 from AV. Although we used the same basic medium for the isolation of the cyanobacteria, we varied the source of nitrogen and applied two different approaches: (i) size fractionation filtrations and (ii) dilution to extinction. For the isolation of these strains, water was prefiltered through 2 µm mesh plankton net. Subsequently, the filtrate was spread onto 0.7% agarose medium in Petri dishes. Strain CCY9508 was isolated on a mixture consisting of 1/3 volume ASNIII + 2/3 volume BG11 medium with a salinity of 12.2‰ (Rippka et al., 1979). The Spanish isolates were isolated on the freshwater BG11 medium using nitrate as nitrogen source and incubation at 14°C. Single colonies were picked from the agarose plates and repeatedly transferred until axenic monoclonal strains were obtained. The other BS strains were isolated from samples collected at various stations in the Gulf of Finland (from 59.1°N 22.2°E to 60.0°N 26.2°E) during a research cruise in July 2004. Water samples were collected from defined depths using a rosette sampler. Pseudanabaena strains were isolated using two different approaches. In one approach, water samples were fractionated, first using one layer and subsequently two layers of plankton net (20 µm mesh) under gentle vacuum. This filtrate was successively filtered through 5 µm, 1 µm and 0.45 µm membrane filters. Finally, the 0.45 µm filter was transferred to a sterile 10 cm Petri dish filled with a mixture of 4/5 parts BG11 and 1/5 parts ASNIII medium, containing NH₄Cl (0.05 g l⁻¹) as the nitrogen source. In the other approach, water was filtered through 20 µm plankton net and subsequently diluted to extinction in 96 deep-well microtiterplates (Nunc Inc., Thermo Fischer Scientific, Langenselbold, Germany) containing a mixture of 4/5 parts BG11 and 1/5 parts ASNIII medium containing NH₄Cl (0.05 g l⁻¹). In both approaches, the cells were first grown under a light regime of 10 µmol photons m⁻² s⁻¹ for 2 weeks at 20°C. Subsequently, the light intensity was increased to 20 µmol photons m⁻² s⁻¹ and the cultures were incubated for another 6 weeks at the same temperature. Trichomes growing on the filters or at the surface of the wells were picked and transferred to solid media. To obtain monoclonal axenic strains, trichomes were repeatedly transferred. Once obtained, monoclonal axenic strains were maintained in their specific growth medium in the Culture Collection Yerseke (CCY; Table 1).
Table 1 Characteristics of *Pseudanabaena* strains used in this study, year and location of isolation and PCR amplification for *nifH* genes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolation details</th>
<th>Growth</th>
<th>Morphological characteristics</th>
<th>Motility</th>
<th>CCA</th>
<th>Absorption ratio</th>
<th>nifH PCR Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Origin</td>
<td>Year</td>
<td>Medium*</td>
<td>Cell size (μm)^b</td>
<td>PGV</td>
<td>570/625^d</td>
</tr>
<tr>
<td>CCY9701</td>
<td>Albufera de Valencia</td>
<td>1997</td>
<td>BG 11</td>
<td>6.1 × 1.5</td>
<td>+</td>
<td>−</td>
<td>0.75</td>
</tr>
<tr>
<td>CCY9702</td>
<td>Albufera de Valencia</td>
<td>1997</td>
<td>BG 11</td>
<td>6.7 × 1.4</td>
<td>+</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>CCY9703</td>
<td>Albufera de Valencia</td>
<td>1997</td>
<td>BG 11</td>
<td>5.5 × 1.5</td>
<td>+</td>
<td>+</td>
<td>1.07</td>
</tr>
<tr>
<td>CCY9704</td>
<td>Albufera de Valencia</td>
<td>1997</td>
<td>BG 11</td>
<td>6.0 × 1.6</td>
<td>+</td>
<td>−</td>
<td>1.02</td>
</tr>
<tr>
<td>CCY9705</td>
<td>Albufera de Valencia</td>
<td>1997</td>
<td>BG 11</td>
<td>4.3 × 1.6</td>
<td>+</td>
<td>+</td>
<td>1.18</td>
</tr>
<tr>
<td>CCY9709</td>
<td>Albufera de Valencia</td>
<td>1997</td>
<td>BG 11</td>
<td>4.7 × 1.5</td>
<td>+</td>
<td>+</td>
<td>1.06</td>
</tr>
<tr>
<td>CCY9710</td>
<td>Albufera de Valencia</td>
<td>1997</td>
<td>BG 11</td>
<td>3.5 × 1.4</td>
<td>+</td>
<td>−</td>
<td>0.82</td>
</tr>
<tr>
<td>CCY9712</td>
<td>Albufera de Valencia</td>
<td>1997</td>
<td>BG 11</td>
<td>4.9 × 1.5</td>
<td>+</td>
<td>+</td>
<td>0.67</td>
</tr>
<tr>
<td>CCY9714</td>
<td>Albufera de Valencia</td>
<td>1997</td>
<td>BG 11</td>
<td>4.9 × 2.0</td>
<td>+</td>
<td>+</td>
<td>0.69</td>
</tr>
<tr>
<td>CCY9715</td>
<td>Albufera de Valencia</td>
<td>1997</td>
<td>BG 11</td>
<td>4.9 × 1.4</td>
<td>+</td>
<td>−</td>
<td>1.18</td>
</tr>
<tr>
<td>CCY9508</td>
<td>Baltic Sea</td>
<td>1995</td>
<td>1/3A+2/3B</td>
<td>6.1 × 1.5</td>
<td>+</td>
<td>+</td>
<td>0.67</td>
</tr>
<tr>
<td>CCY0471</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>3.2 × 1.4</td>
<td>+</td>
<td>+</td>
<td>1.04</td>
</tr>
<tr>
<td>CCY0472</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>5.2 × 1.2</td>
<td>+</td>
<td>+</td>
<td>1.17</td>
</tr>
<tr>
<td>CCY0473</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.5 × 1.5</td>
<td>+</td>
<td>+</td>
<td>1.28</td>
</tr>
<tr>
<td>CCY0474</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.8 × 1.5</td>
<td>+</td>
<td>+</td>
<td>1.49</td>
</tr>
<tr>
<td>CCY0475</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.9 × 2.0</td>
<td>+</td>
<td>+</td>
<td>1.52</td>
</tr>
<tr>
<td>CCY0476</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.2 × 1.6</td>
<td>+</td>
<td>+</td>
<td>0.62</td>
</tr>
<tr>
<td>CCY0477</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.6 × 2.0</td>
<td>+</td>
<td>+</td>
<td>1.29</td>
</tr>
<tr>
<td>CCY0478</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.7 × 1.6</td>
<td>+</td>
<td>+</td>
<td>1.21</td>
</tr>
<tr>
<td>CCY0479</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.6 × 1.9</td>
<td>+</td>
<td>+</td>
<td>1.4</td>
</tr>
<tr>
<td>CCY0480</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.4 × 1.3</td>
<td>+</td>
<td>+</td>
<td>0.74</td>
</tr>
<tr>
<td>CCY0481</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.1 × 1.9</td>
<td>+</td>
<td>+</td>
<td>1.52</td>
</tr>
<tr>
<td>CCY0482</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.3 × 1.2</td>
<td>+</td>
<td>+</td>
<td>0.55</td>
</tr>
<tr>
<td>CCY0483</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.4 × 1.9</td>
<td>+</td>
<td>+</td>
<td>1.46</td>
</tr>
<tr>
<td>CCY0484</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.4 × 1.9</td>
<td>+</td>
<td>+</td>
<td>0.6</td>
</tr>
<tr>
<td>CCY0485</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.3 × 1.3</td>
<td>+</td>
<td>−</td>
<td>0.59</td>
</tr>
<tr>
<td>CCY0486</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>2.6 × 1.5</td>
<td>+</td>
<td>+</td>
<td>1.17</td>
</tr>
<tr>
<td>CCY0488</td>
<td>Baltic Sea</td>
<td>2004</td>
<td>1/5A+4/5B</td>
<td>3.0 × 1.3</td>
<td>+</td>
<td>+</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Abbreviations: CCA, complementary chromatic adaptation; Chl, chlorophyll; ND, not determined; PGV, polar gas vesicles.

*Growth media description can be found in Rippka et al., 1979 (1/3A+2/3B: 1 part ASNIII medium+2 parts BG11 medium).

*Cell size description can be found in the average length × width of at least 40 cells.

*Motility on agarose plates.

*Ratio between absorption at 570 nm (phycoerythrin) and 625 nm (phycocyanin).

*Ratio between absorption at 680 nm (Chl) and 570 nm (phycoerythrin).

*Ratio between absorption at 680 nm (Chl) and 625 nm (phycocyanin).
Morphology and microscopy
Cells were collected from exponential or stationary liquid cultures and were fixed in a mixture of 1% (w/v) formaldehyde and 0.05% (w/v) glutaraldehyde and subsequently stored at –80°C until they were analyzed (Biegala et al., 2003). Microscope slides were prepared by covering them with a thin layer of 1% (w/v) molten agarose (50°C; Sigma-Aldrich, Zwijndrecht, The Netherlands) that was allowed to solidify shortly before cells were applied. The slides were examined using a Zeiss Axiophot microscope equipped with a ProgRes C10 plus digital imaging system (JENOPTIK Laser, Optik, Systeme GmbH). The images were subsequently processed using ProgRes CapturePro2.0 software (JENOPTIK Laser, Optik, Systeme GmbH). From at least 30 cells in each culture, the width and length were measured.

In vivo absorption spectra
Exponential or stationary liquid cultures grown under white light (20 μmol photons per m² per second) were used for the determination of the in vivo absorption spectra. Spectra were measured from 400 to 750 nm using a Varian Cary 100 Bio equipped with an integrating sphere DRA-CA-3300. Distilled water was used as reference.

Determination of CCA
To test for the capacity of CCA, the strains were cultured on solid (agarose) media. Each strain was inoculated in two Petri dishes (Greiner Bio-One) that were incubated under a different color of light. Green light was obtained using Lee filter no. 124 (dark green) and red light through Lee filter no. 26 (red). The incident white light intensity was 100 μmol photons per m² per second. To document CCA, the cultures were photographed after 2 weeks of growth under monochromatic light. Subsequently, the cultures were changed to the other color of monochromatic light and incubated another 2 weeks after which they were documented again. A change from green to reddish/black phenotype and vice versa, was taken as evidence for CCA.

DNA isolation
For DNA extraction, cells were collected from exponentially growing or stationary phase cultures. Briefly, two milliliters of culture was centrifuged in a tabletop centrifuge (Eppendorf type 5424) at 10000 r.p.m. for 1 min at room temperature. The supernatant was removed and the cell pellet was resuspended in lysis buffer provided by the Power-soil DNA extraction kit (MoBio Laboratories, Inc., Carlsbad, CA, USA). DNA extraction was performed following the instructions of the manufacturer. DNA quantity and quality were checked by running agarose gels as well as spectrophotometrically (Nanodrop ND1000).

PCR and sequencing
PCR reactions were performed using the PCR primers listed in Table 2. The B1055F and PitsEcyanR were used to amplify the end of the 16S rRNA gene plus the internal transcribed spacer 1 (ITS-1). The 23S rRNA gene was amplified with 129F and 2241R primers and the PC operon (cpcBA) with cpcAR and cpcBF primers. Finally, nifH PCR amplification were performed by the set of primers

Table 2 List of the primers used for PCR and sequencing in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Target</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bact1055F²</td>
<td>16S rRNA gene</td>
<td>AATGGCTGTGTCCGTCGCTCGT</td>
<td>García-Martínez et al. (1999)</td>
</tr>
<tr>
<td>PitsEcyanR¹</td>
<td>23S rRNA gene</td>
<td>CTCTGCTGTGGCAAGGATGC</td>
<td>Ernst et al. (2003)</td>
</tr>
<tr>
<td>129F⁴</td>
<td>23S rRNA gene</td>
<td>CYGAAATGCGGVRAGAC</td>
<td>Hunt et al. (2006)</td>
</tr>
<tr>
<td>2241R⁵</td>
<td>23S rRNA gene</td>
<td>ACCGCCCAGTHAAACT</td>
<td>Lane (1991)</td>
</tr>
<tr>
<td>CpcAR⁶</td>
<td>Phycocyanic operon</td>
<td>TACGCAAAACCAACAAAGCTCG</td>
<td>Robertson et al. (2001)</td>
</tr>
<tr>
<td>CpcBF⁷</td>
<td>Phycocyanic operon</td>
<td>TACGGAGAAAAACGCTATGGAACCTAGG</td>
<td>Robertson et al. (2001)</td>
</tr>
<tr>
<td>nifH¹</td>
<td>nifH</td>
<td>GGYGAYCCAAGGNGCA</td>
<td>Zani et al. (2000)</td>
</tr>
<tr>
<td>nifH2</td>
<td>nifH</td>
<td>ADNGCCATCATYTCNCA</td>
<td>Zani et al. (2000)</td>
</tr>
<tr>
<td>nifH³</td>
<td>nifH (internal primer)</td>
<td>ATRTTTRTNGGCRCRTA</td>
<td>Zani et al. (2000)</td>
</tr>
<tr>
<td>nifH⁴</td>
<td>nifH (internal primer)</td>
<td>TTYTAYGCGAARGNNGG</td>
<td>Zani et al. (2000)</td>
</tr>
</tbody>
</table>

¹These primers were used to obtain the complete ITS-1 sequence.
²These primers were used to amplify the complete 16S rRNA gene from the Pseudanabaena strains.
³This primer was revised recently by Hunt et al., 2006.[50]
⁴These primers were used to amplify the subunits β and α of the phycocyanin operon plus the intergenic spacer (IGS) between both genes.
⁵These primers were used to amplify the complete 23S rRNA gene from the Pseudanabaena strains.
described in Table 2. Each reaction contained 0.2 mM of each dNTP, 2 mM MgCl₂, 5 or 10 pmol of each primer, 1 μl template DNA (5–10 ng μl⁻¹), 1 x PCR buffer and 1 U HotStarTaq (Qiagen GmbH, Hilden, Germany). MQ-grade H₂O was added to a final volume of 30 μl. The PCR reactions were carried out in a GeneAmp System 2700 thermocycler (Applied Biosystems, Foster City, CA, USA). The PCR program was used to amplify the 16S rRNA-ITS-1 region, the 23S rRNA gene and the cpcBA operon were as follows: a hot start at 94 °C of 15 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. Following the last cycle, an elongation step of 10 min at 72 °C was applied. The PCR program for amplification of the nifH gene was according to the nested PCR protocol as described by Zani et al. (2000) with modifications. In brief, the first PCR using primers nifH 1 and 2 was started with a hot start at 15 min at 96 °C and followed by 35 cycles of 1 min at 94 °C, 1 min at 57 °C, 1 min at 72 °C, followed by 10 min of elongation at 72 °C. One microliter of the PCR product was then used in the second PCR using the primers nifH 3 and 4. The PCR program started with a 15 min hot start at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 54 °C, 1 min at 72 °C, followed by 10 min of elongation at 72 °C. The DNA clean & concentrator-5 kit (Zymogram, Zymo Research, Orange, CA, USA) was used to remove primer dimers from the PCR reactions following the instructions of the manufacturer. DNA concentration of the purified PCR products was checked spectrophotometrically (Nanodrop, ND1000). For sequencing reactions, 3.5 μl of the purified and concentrated PCR product served as template using 10 μl of the sequencing primer and the Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems) following the manufacturer’s instructions. Sequencing primers were the same forward and the reverse primers as were used for PCR amplification (Table 2). Sequence products were analyzed using a 3130 Genetic Analyzer (Applied Biosystems). Sequences were edited manually using ChromasPro V 1.41 (Technelysium Pty Ltd, Tewantin, Queensland, Australia) and manually checked for errors in base calling. Only high-quality sequences were included in the final data set. The sequences were deposited in GenBank under the following accession numbers: 16S rRNA (EU025781-EU025806), ITS-1 region (EU119301-EU119325), 23S-129F (EU025807-EU025831), 23S-2241R (EU025756-EU025780) and the cpcBA operon (EU119326-EU119352).

Phylogenetic analysis
All Pseudanabaena sequences obtained from this study were aligned using CLUSTALW integrated into the package BioEdit (Hall, 1999). These sequences were aligned against sequences closely related to Pseudanabaena/Limnothrix group previously identified by BLASTN comparison from GenBank and other references identified in previous published studies. Sequence comparison and phylogenetic analyses of the partial sequencing of 16S rRNA, 23S rRNA, ITS-1, cpcBA and intergenic spacer (IGS) were performed using the software MEGA3.1 (Kumar et al., 2004). Neighbor-joining with Jukes–Cantor correction and 1000 bootstraps was used to build the corresponding phylogenetic trees. Partial amino-acid sequences of the coding region of the cpcBA operon were also used in the phylogenetic analyses performed with the neighbor-joining method as well as with maximum parsimony. Maximum parsimony was used with the close-neighbor-interchange search algorithm with random tree addition using 100 bootstraps.

Population genetic analysis
The protein coding nucleotide sequences from the cpcBA locus aligned by ClustalW were analyzed using DnaSP version 4.0. (Rozas et al., 2003) to calculate the following parameters: (i) synonymous and nonsynonymous polymorphic sites, (ii) estimation of Ka/Ks divergence ratio, (iii) The McDonald–Kreitman test (McDonald and Kreitman, 1991) to detect positive or purifying selection and the HKA test (Hudson, Kreitman, and Aguadé) on the basis of neutral theory of molecular evolution (Hudson et al., 1987), (iv) degree of genetic differentiation between populations, estimated by Fst (Hudson et al., 1992), (v) estimation of the recombination parameter (R) and minimum number of recombination events (Hudson et al., 1987). Finally, Selecton version 2.2 (http://selecton.bioinfo.tau.ac.il) was used to identify positive and purifying selection at each of the amino acids using a Bayesian inference approach (Stern et al., 2007). The Selecton server automatically calculates the ratio between Ka and Ks (ω) at each codon site using a maximum likelihood approach. The value of ω at each site is translated to a discrete color scale projected onto one of the homologous sequences for each sequence clusters. Colors 1 to 2 (dark and light yellow) indicate ω > 1 and stand for sites with positive selection, whereas the shades of white through magenta (colors 3 through 7) indicated various levels of ω ≤ 1 where Selecton results can be accurately used to infer sites undergoing purifying selection.

Results
Phenotypic traits: morphological characteristics and photosynthetic pigment composition
A total of 28 strains were isolated from two geographically distant locations: AV, Spain (10 strains isolated in 1997) and the BS (1 strain isolated in 1995, 17 strains in 2004). The isolates were assigned to Pseudanabaena on the basis of their morphological characteristics, such as cell size, motility on agarose plates and the presence of polar
gas vesicles (Table 1). The isolates displayed different cell dimensions ranging from 2.1 × 1.9 to 6.7 × 1.4 μm (length × width) (Supplementary Figure 1SM). While cell width remained within a narrow range, cell length varied considerably. All strains, except CCY9710 from AV, possessed polar gas vesicles (Table 1). Gliding motility was observed in most of the strains except for four isolates from AV (Table 1).

The presence of the major light-harvesting pigments chlorophyll \textit{a} (Chl\textit{a}), PC and PE was determined by \textit{in vivo} absorption spectra, from which the ratios PE:PC, Chl\textit{a}:PE and Chl\textit{a}:PC were calculated (Table 1). \textit{Pseudanabaena} isolates were divided into strains that have both PE and PC pigments (63\% of all strains) and those with only PC (37\%) as their major pigment (Figure 1). All strains possessing both PE and PC pigments were capable of CCA. PC-rich strains reveal an absorption peak at ∼625 nm and, hence, harvest orange-red light effectively. The proportion of strains capable of CCA that were isolated from the BS was 66\%, slightly higher than the 55\% for the AV. The majority of strains that were positive for CCA possessed a ratio of the absorption at 570 and 625 nm of approximately one or more when incubated in white light. When incubated under red or green light, cultures changed pigmentation toward green and red, respectively (Figures 1b and c, Supplementary Figure 2SM). Chlorophyll \textit{a} showed absorption peaks at 440 nm (Soret band) and 680 nm. The absorption peaks of the three major light harvesting pigments were at the same wavelengths in all isolates but their relative heights varied substantially (Table 1).

**The phylogeny of \textit{Pseudanabaena} revealed from their 16- and 23S rRNA genes**

To determine the phylogenetic relationships of the \textit{Pseudanabaena} isolates, the 16- and 23S rRNA genes were partly sequenced and analyzed. Because the 23S rRNA gene offers a higher phylogenetic resolution, the start and end of the 23S rRNA gene were both sequenced using the forward 129F and reverse 2241R primers, respectively (Table 2). This resulted in two products with a sequence length of 440 bp (129F) and 527 bp (2241R), respectively. The sequences obtained by using these primers gave similar phylogenetic relationships, although a larger number of polymorphisms were observed when using primer 129F (data not shown). Figure 2 depicts the phylogeny on the basis of neighbor-joining analysis and compares the 23S rRNA (Figure 2a) with the 16S rRNA gene tree topologies (Figure 2b) obtained using the primers 129F (440 bp) and Bact1055F (400 bp), respectively. In both trees, all isolates grouped into a single 99\% similarity cluster indicating congruency in the phylogeny of the 16- and 23S rRNA genes. Moreover, the 23S rRNA gene displayed more polymorphisms than the 16S rRNA gene, which was virtually identical in virtually all isolates. The phylogenetic analysis of the 16S rRNA gene confirmed that all isolates belong to the \textit{Pseudanabaena}/\textit{Limnothrix} group with 99\% cluster similarity (Zwart \textit{et al.}, 2005; Willame \textit{et al.}, 2006). From BLAST searches against the GenBank database, we observed that 42\% of the isolates were 100\% identical to \textit{Pseudanabaena} sp. PCC6903 (AM709632). The other strains possess high similarity (99\%) to \textit{Pseudanabaena} sp. 1tu24s9 (AM259269), which originated from the Finnish freshwater Lake Tuusulanjarvi (Supplementary Table 1SM). Only strain CCY9709 from AV exhibited a higher divergence...
Figure 2 Neighbor-joining tree based on comparison of the partial sequences of the 23S rRNA (a; 129F primer) and 16S rRNA (b; Bact1055F primer) genes of the *Pseudanabaena* strains collected in this study. All *Pseudanabaena* strains grouped in a single 99% cluster, for both the 23- and the 16S rRNA genes (shadowed area).
on the basis of the 23S rRNA sequence (Figure 2a) showing a 94% similarity with *Pseudanabaena* sp. PCC6903 (Supplementary Table 1SM).

**Microdiversity within *Pseudanabaena* strains: ITS sequencing analysis**

The partial sequencing of the 16S- and 23S rRNA genes was not sufficient to resolve the phylogeny of the *Pseudanabaena/Limnothrix* group. Therefore, the ITS region located between the 16S- and 23S rRNA genes was sequenced. All *Pseudanabaena* ITS sequences revealed the same structure and contained two tRNA genes, tRNA*\_Ile* and tRNA*\_Ala* (data not shown). However, there was a high nucleotide divergence and length variability among the ITS sequences. The phylogenetic analysis of the ITS revealed a higher level of differentiation within the *Pseudanabaena/Limnothrix* group (Figure 3), which constrained most of the strains in three major 99% clusters ('microdiversity clusters'). Hence, ITS exhibited a higher degree of microdiversity compared to the 16S or 23S rRNA gene sequences where all sequences clustered together in one single 99% similarity cluster (Figure 2). Unfortunately, no ITS sequences related to *Pseudanabaena* species have been published to date, and therefore our sequences clustered with the two known *Limnothrix* ITS sequences.

The ITS analysis uncovered different patterns with clusters containing isolates specific for the BS, isolates specific for the AV, and a mixed cluster containing isolates from both locations. Most of the BS strains grouped in the BS cluster (BSC) with 99.6% similarity. BSC contained 12 isolates from the BS but also one strain from the AV (CCY9710) and *Limnothrix* sp. MR1 (isolated from Lake Loosdrecht, The Netherlands (Zwart et al., 2005)). The mixed Albufera and BSC (MABSC) contained five identical sequences retrieved from isolates from both locations. The fact that strains originating from such distant locations and with 7 years between their isolation possess identical ITS sequences is at least surprising, since it is well-known that ITS is highly variable (in length and/or sequence) and therefore changes were expected. The AV (AVC) comprised six isolates from AV possessing 99.2% similarity. Strain CCY9709 grouped in a fourth cluster together with *Limnothrix redekei* Culture Collection of Algae and Protozoa (CCAP) 1443/1. Furthermore, unique

![Figure 3](image_url)

**Figure 3** Microdiversity of *Pseudanabaena* spp. as revealed by ITS-1 analysis. The ITS allowed further differentiation showing geographical patterns, with clusters specific for the Baltic Sea (cluster I) and Albufera de Valencia, Spain (cluster III), but also a cluster with representatives of both locations (cluster II). Strain CCY9710 from the Albufera de Valencia represents the only exception and clustered with the Baltic Sea strains. ITS, internal transcribed spacer.
insertion sequences were found in some clusters. A unique 36 bp insertion sequence was found in the MABSC. In CCY9709, three specific insertion sequences of 23, 6 and 5 bp were found. The position of the isolates in the ITS phylogenetic tree showed a close relationship with cell length (Figure 4). The cell length of all BSC strains, including CCY9710 from AV, ranged from 2.1 to 3.5 \( \text{m} \). The MABSC cluster was characterized by cells that are slightly longer, ranging from 3.2 to 6.1 \( \text{m} \). Finally, the AV cluster comprised the strains with the longest cells, ranging from 4.3 to 6.7 \( \text{m} \). Strain CCY9709 possessed cells of 4.9 \( \text{m} \) long and grouped in an independent monophyletic cluster with \( L. \ redekei \) CCAP 1443/1 which, however, has considerably longer cells (6–10 \( \text{m} \); JG Day, personal communication with the CCAP).

**Phylogeny of the phycocyanin operon: correlation of the cpcBA gene clusters with light absorption spectra**

A major limitation of the use of ribosomal genes as molecular markers is the impossibility to attribute ecophysiological traits to these genes. Therefore, we also sequenced the cpcBA. CpcBA encodes the two subunits of PC, which is part of the phycobilisome, the major light-harvesting complex of cyanobacteria. The main goal was to assign sequence clusters of cpcBA to the in vivo light absorption spectra of the isolated strains to reveal ecologically different populations (ecotypes) within \( Pseudanabaena \) (Figure 5). The cpcBA locus has been widely used for the study of cyanobacterial diversity and phylogeny, and it is therefore suitable for our purpose (Ivanikova et al., 2007; Six et al., 2007; Haverkamp et al., 2008). In addition, the IGS between cpcB and cpcA (cpcBA-IGS) was sequenced (Supplementary Figure 3SM and Figure 5b) to explore whether coding and noncoding regions of the cpcBA operon display different evolutionary rates resulting in different phylogenies.

Phylogenetic analysis of the partial sequences of cpcBA encoding for 150 amino acids, displayed two well-supported clusters (Figure 5a) with similarities higher than 99% (‘microdiversity clusters’). Cluster I comprises sequences with 99.9% similarity and grouped 20 isolates from both locations. Half of these isolates were rich in PC (PC-rich) and unable to perform CCA, whereas the other 10 strains were capable of CCA. This cluster is closely related to \( Pseudanabaena \) sp. PCC7409 that is capable of CCA. Cluster II contained only strains capable of CCA, both from the BS and the AV and possess 100% sequence similarity. Half of the strains of cluster II investigated for nifH PCR amplification possessed nifH encoding for dinitrogenase reductase, which is a component of nitrogenase. Among cluster I, there was only one strain (CCY0477) that possessed nifH in a total of 20 isolates. NifH was found in strains originating from both environments. CpcBA cluster II in Figure 5 corresponds to the MABSC cluster of ITS sequences in Figure 4. This hints to the presence of a conserved and coherent lineage within \( Pseudanabaena \). Again, the CCA positive strain CCY9709 displayed the most divergent position in the cpcBA phylogeny.

The phylogeny of the concatenated cpcBA-IGS sequences revealed geographical patterns that were not shown in the cpcBA phylogeny alone (Figure 5b). The tree topology of the concatenated cpcBA-IGS phylogeny was consistent with the topology of the cpcBA phylogeny without the IGS.
region. Moreover, the heterogeneity and length variability of the IGS sequences allowed a higher level of differentiation and revealed different subclusters from specific geographic locations (‘geotypes’). Four geotypes were discerned within cluster I (PC-rich/CCA), two of them originated from the BS and the other two from Albufera de Valencia (Figure 5b). In all geotypes, specific base-pair signatures were assigned (Supplementary Figure 3SM). Complete IGS sequence length ranged from 104 to 212 bp. The strains of cluster I (PC-rich/CCA) possessed an IGS of 153 bp, whereas the IGS sequences of cluster II (only CCA) were shorter and possessed only 104 bp. Strain CCY9709 had the longest (212 bp) and most divergent IGS (Supplementary Figure 3SM). Moreover, cluster II (only CCA) comprised different IGS sequences compared to cluster I-IGS and contained several deletions (in total 49 bp). Nonetheless, cluster II-IGS sequences were conserved and only a few specific signatures were found. For example, the T at position 120 or the C at position 132 are representative for two of the three BS strains that possess nifH (Supplementary Figure 3SM).

Evolutionary forces operating on the phycocyanin operon genes

On the basis of the analysis of the cpcBA operon, the evolutionary processes shaping the PC genes among Pseudanabaena spp. were investigated. Partial sequencing of 450 nucleotides constrained 26 strains in two major lineages, clusters I (20 isolates) and II (six isolates; Figure 5a). The number of polymorphisms within and between both lineages was examined. A total of 16 single nucleotide polymorphisms were found between both lineages and therefore the nucleotide divergence observed between both clusters was 3.82%. The number of synonymous (silent) nucleotide substitutions per synonymous site (Ks = 0.0809) was 12.3-fold higher than the nonsynonymous substitutions (amino-acid change) per nonsynonymous site (Ka = 0.00655). The ratio of nonsynonymous and synonymous nucleotide substitutions in protein-coding genes (Ka/Ks) may give important clues about the selection pressure on and evolution of the protein-coding genes (McDonald and Kreitman, 1991). The ratio of Ka/Ks observed for cpcBA was 0.074. Ratios of Ka/Ks<1 indicate that purifying selection takes...
place. Hence, this is the case for the cpcBA clusters I and II. Purifying selection means that nonsynonymous vs synonymous mutations is favored in the direction of the latter. As a consequence, over time, slightly deleterious nonsynonymous mutations are continuously removed from the population leaving only synonymous mutations. In addition, the possibility to detect different types of selection (positive or purifying) on specific amino acids within the cpcBA operon was explored (Stern et al., 2007). Positive selection was not detected in any of the amino acids, which indicates that all amino acids must have been under different levels of purifying selection (Figure 6). Furthermore, the degree of genetic differentiation between both clusters (Pseudanabaena subpopulations) was also explored by estimating the fixation index $F_{st}$, which indicates the amount of gene flow between populations (Hudson et al., 1992). Values of $F_{st}$ range between 0 and 1. The value of 0 indicates that the populations share the same alleles, whereas the value of 1 shows that the populations are fixed for different alleles. The $F_{st}$ value for the cpcBA loci was 0.886, which indicated high gene flow compared with other described bacterial populations (Whitaker et al., 2003; Miller et al., 2006).

Finally, the number of amino-acid changes (nonsynonymous sites) was assessed for the 150 codons analyzed. Only two amino-acid changes were detected, yielding a 1.4% amino-acid divergence. In both cases, the identity of the amino acids differed systematically between clusters I and II of the Pseudanabaena cpcBA lineages. At position 42 at the end of the beta subunit (cpcB), a serine (S, alcohol polar R-group) found in cluster I was exchanged in cluster II by an alanine (A, aliphatic R-group, a hydrophobic molecule). Moreover, at position 100, at the beginning of the $\alpha$-subunit (cpcA) a threonine (T, a hydrophilic and hydroxyl-containing amino acid) in cluster I was replaced by alanine (A) in cluster II. However, neither of these two amino acids were found to be under positive selection (white color in Figure 6).

**Discussion**

**Pseudanabaena/Limnothrix group: agreement of phenotype with genetic data**

Pseudanabaena is morphologically similar to Limnothrix. The large variation of cell lengths observed among the strains isolated in this study suggests that Pseudanabaena shows a high level of plasticity. Accordingly, cell length is not very useful for distinguishing Pseudanabaena from Limnothrix.

On the basis of 16S rRNA gene sequences, earlier studies showed that Pseudanabaena and some strains of Limnothrix cluster with Pseudanabaena spp., including one isolate assigned to L. redekei. This cluster is commonly referred to as the Pseudanabaena/Limnothrix group (Gkelis et al., 2005; Willame et al., 2006). This group also comprised several environmental sequences as well as other isolates including the type strain Pseudanabaena PCC7408, strains belonging to L. redekei (Van Goor, Meffer) (Limnothrix sp. MR1 from Lake Loosdrecht, L. redekei CCAP 1443/1 and L. redekei CCAP 227/1) and several other isolates from Lake Loosdrecht, The Netherlands (Zwart et al., 2005; Willame et al., 2006). A second cluster, comprising only L. redekei
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strains isolated from Lake Kastoria (Greece), has also been observed (Gkelis et al., 2005; Willame et al., 2006). On the basis of partial sequencing of the 16- and 23S rRNA genes, all Pseudanabaena strains are isolated in this study cluster with the Pseudanabaena/Limnothrix group. Neither 16- nor 23S rRNA gene analysis could distinguish between Pseudanabaena and Limnothrix, confirming previous reports (Zwart et al., 2005; Willame et al., 2006). However, ITS analysis of the Pseudanabaena isolates shows a high nucleotide divergence and size variability revealing a higher level of differentiation in this group. For instance, strains belonging to the AVC and strain CCY9709 all possessed cells that were almost 3 times longer than wide (Figure 4), a characteristic that is considered typical for Limnothrix. This could indicate that these strains are indeed closely related to L. redekei. Nonetheless, in order to confirm these observations, it is necessary to increase the number of ITS sequences of L. redekei and Pseuadanabaena. Moreover, it will be necessary to re-evaluate the morphological basis on which the genera Limnothrix and Pseudanabaena are separated.

Distribution and abundance of Pseudanabaena

The distribution of Pseudanabaena is widespread, although their actual abundance is still to be determined. Microscopic observations confirm their occurrence as a major fraction of the summer cyanobacterial blooms in the BS and AV. Remarkably, environmental clone libraries from the BS targeting the 16S rRNA–ITS and the cpcBA and cpeBA genes encoding the phycocyanin and phycoerythrin operons, respectively did not recover any Pseudanabaena sequences (Haverkamp et al., 2008). However, it is a well-known fact that the environmental sequences are strikingly different from the organisms isolated from most microbial communities (Suzuki et al., 1997; Eilers et al., 2000, 2001; Stevens et al., 2005; Donachie et al., 2007). We cannot exclude the possibility that Pseudanabaena filaments were retained by the 20 μm nylon mesh filter that was used as a prefiltration step and that this was the reason that we did not recover their sequences. Nevertheless, Pseudanabaena strains were isolated from the <20 μm fraction. Moreover, Denaturing Gradient Gel Electrophoresis (DGGE) fingerprinting using cyanobacteria-specific 16S rRNA primers detected Pseudanabaena in the BS (B Diez, Stockholm University, personal communication and unpublished results). As we used general bacterial primers for the construction of our environmental clone libraries, we may have missed out Pseudanabaena because of the far more abundant unicellular picocyanobacteria.

Members of Pseudanabaena have been reported from a variety of different environments including freshwater lakes (Gkelis et al., 2005; Zwart et al., 2005; Kim et al., 2006; Willame et al., 2006), brackish environments (Stal et al., 2003), hot springs (Castenholz et al., 2001) as well as from epilithic cyanobacterial communities of beach rock (Heron Island, Great Barrier Reef) (Diez et al., 2007). Our study revealed several lineages of Pseudanabaena from the BS and from the AV, on the basis of a multilocus sequence typing approach using five loci of 28 isolates. The average genetic divergence of each of the markers varied from 1% in the small and large subunit ribosomal RNA genes to 3.5% in ITS-1 and 3.82% in cpcBA. The BS is geographically distant from the AV. Moreover, they represent quite different habitats (i.e., brackish vs freshwater) and it is, therefore, not surprising to find different Pseudanabaena genotypes at these two locations. Endemic clusters (geotypes) were detected in the BSC and AVC on the basis of the analysis of ITS sequences (Figure 3). The analysis of the cpcBA-IGS operon revealed an even better differentiation of these geotypes (Figures 5b, Supplementary Figure 3SM).

Yet, the BS is connected to the North Sea, and through the Atlantic Ocean and the Mediterranean Sea, it ultimately links to AV. This connection could allow the long-range dispersal of Pseudanabaena genotypes, although other mechanisms for long-range dispersal could also be envisioned (such as transport by birds). Salinity differences between the BS and the AV are minor, and organisms might adapt quickly to somewhat higher or lower salt levels. Indeed, a coherent and ‘cosmopolitan’ monophyletic cluster, comprising members from both locations was found for several loci (MABSC by ITS and cluster II by cpcBA genes or cpcBA-IGS analysis) suggesting a conserved and homogeneous lineage within Pseudanabaena (Figures 3 and 5). This lineage clustered the strains isolated from the two distant locations, even though the isolation at the two locations was separated by 7 years. This could point to the global dispersal of these Pseudanabaena strains. However, more Pseudanabaena strains from other environments and locations should be included to confirm the possibility of global dispersal. On the basis of the analysis of the genes of the PC operon and the light absorption spectra associated with them, it is proposed that this lineage or subpopulation of Pseudanabaena (MABSC by ITS and cluster II by cpcBA genes or cpcBA-IGS analysis) represents an ecotype that possesses the ability of CCA. Interestingly, this ecotype also possesses the nifH gene in at least half of their members, and therefore might be capable of N2 fixation (Figure 5). This ecotype proposed with the name of Pseudanabaena Complementary Chromatic Adaptation (PCCA) is probably widely distributed, and the ability of CCA might provide it with a selective advantage.

Genetic diversification of Pseudanabaena populations

Correlation of the cpcBA gene clusters with the light absorption spectra hinted at the coexistence of two Pseudanabaena populations with a niche differen-
tiation along the light spectrum (Figure 5, clusters I and II). Cluster II or the ‘PCCA ecotype’ occurs as a group with 99% similarity (‘microdiversity cluster’), at a variety of different loci (ITS, cpcBA gene and cpcBA-IGS analysis). Previous studies indicate that such microdiversity clusters could represent important units of differentiation as ecotypes in natural populations of bacteria (Palys et al., 1997; Moore et al., 1998; Rocap et al., 2003; Konstantinidis and Tiedje, 2005; Lopez-Lopez et al., 2005; Thompson et al., 2005; Cohan, 2006; Polz et al., 2006; Cohan and Perry, 2007), and are often observed in environmental clone libraries (Field et al., 1997; Acinas et al., 2004; Morris et al., 2005; Johnson et al., 2006; Pommier et al., 2007). The microdiversity clusters identified here are correlated with morphological and ecophysiological traits such as cell length and the capacity to perform CCA (Figures 4 and 5), providing further support for the designation as an ecotype (Ahlgren and Rocap, 2006; Johnson et al., 2006; Polz et al., 2006; Ward et al., 2006). Although this genetic pattern agrees with the ecotype model for bacterial species (Palys et al., 1997; Cohan, 2002; Gevers et al., 2005; Cohan and Perry, 2007), other mechanisms causing the genetic diversification of Pseudanabaena populations cannot be excluded.

Indeed, the loss of substructure in the tree topology occurred when different loci were compared (e.g., ITS vs cpcBA-IGS). For instance, strain CCY9710 from AV fell into the BSC on the basis of its ITS sequence and it shared a similar cell length with all strains. However, this strain grouped with other isolates from AV when considering the phylogeny on the basis of cpcBA-IGS sequences. Similarly, the MABSC was an independent branch in the phylogeny of the cpcBA locus, whereas it was sister to the BSC using ITS. Moreover, at least 12 recombination events (Rm) were detected at the cpcBA locus, emphasizing the importance of homologous recombination and that this process should be taken into account.

By using the ratio of nonsynonymous vs synonymous fixation as a measure of the level of selective pressure on the cpcBA, it was concluded that purifying selection is involved in the evolutionary diversification of Pseudanabaena populations. Other population genetic analyses such Tajima’s D and the Mc Donald–Kreitman (MK) tests for selection were not significant (data not shown), further supporting our finding that the mutations at cpcBA do not deviate from those expected from neutrality and, hence, are not under positive selection. The results show that divergence of the cpcBA in clusters I and II is promoted by purifying selection in both populations. Evidence for purifying selection in cyanobacteria exist, for instance, for hetR of Trichodesmium, nifH of Cylindrospermopsis raceborskii and rpoC1 of Anabaena lemmermannii (Mes and Stal, 2005; Mes et al., 2006). Our results extend this list and provide the first report on evolutionary diversification in Pseudanabaena genus confirming similar evolutionary trends as described previously for other cyanobacterial taxa. However, this should not be generalized as other studies detected positive selection for PE genes of Prochlorococcus and Synechococcus (Qin et al., 2005; Zhao and Qin, 2007) and for other functional genes such kaIC of Microcoleus chthonoplastes and rbcX of Anabaena and Aphaniizomenon sp. (Mes et al., 2006). Moreover, it is also known that hetR of different lineages of Trichodesmium (Mes and Stal, 2005) or the kai genes, family of Nostoc linckia, (Dvornyk et al., 2002) undergo different selective forces and that similar genes like sasA and kai genes in Synechococcus are subject to different selective constraints (Dvornyk et al., 2004).

Recent laboratory experiments investigated the role of CCA in the competition of Pseudanabaena against red and green Synechococcus strains (Stomp et al., 2008). The competition experiments showed that Pseudanabaena was a strong competitor in fluctuating light environments, provided that it had sufficient time to adjust its pigment composition to the prevailing light spectrum. Pseudanabaena can change its pigmentation from red to green, and vice versa, within ~7 days. Thus, Pseudanabaena benefited from CCA only if fluctuations in underwater light color were slow compared with the time required for CCA, corresponding to slow mixing processes or infrequent storms in their natural habitat (Stomp et al., 2008). We hypothesize that PC-rich strains in cluster I have lost the capacity of CCA recently by the loss or presence of dysfunctional genes required to synthesize the PE disks in the phycobilisome, or the genes like rcaE which is needed for the control of CCA (Terauchi et al., 2004; Kehoe and Gutu, 2006). Knockout experiments targeting the rcaE gene showed that this gene is needed for responsiveness to both red and green light under CCA (Terauchi et al., 2004). The loss or the presence of a dysfunctional copy of rcaE or other genes involved in the phycobilisomes or CCA may be caused by deleterious mutations, resulting in strains that have lost PE and are only able to use PC and Chla as their main light harvesting pigments. PC absorbs photons in the orange-red part of the light spectrum. Accordingly, loss of CCA is likely to be advantageous in moderately turbid waters where orange-red light predominates (Stomp et al., 2007), and during storm periods with rapid mixing when CCA is too slow to track changes in the underwater light spectrum experienced by the entrained Pseudanabaena filaments (Stomp et al., 2008). Variation in the underwater light spectrum at a range of different time scales could thus have induced genetic divergence between PC-rich and CCA strains because of differences in fitness. The proposed mechanism of selective sweeps could enable such genetic diversification (Cohan, 2002, 2006; Gevers et al., 2005; Cohan and Perry, 2007).

Analyses of other functional genes such as those coding for PE will provide further insights into the
mechanisms of diversification within *Pseudanabaena*. Also, it would be interesting to explore with competition experiments the fitness adaptation to recognize any correlation between these two traits and establish their ecological implications.

In summary, multilocus sequencing of five independent loci revealed the existence of several lineages or subpopulations within *Pseudanabaena*. The phylogenies of the 16- and 23S rRNA genes are consistent, but analysis of the other loci indicated loss of substructure, suggesting the recombination between these loci. *Pseudanabaena* isolates exhibited high levels of microdiversity unveiling different patterns with both local as well as more globally dispersed populations. A conserved *Pseudanabaena* lineage proposed as PCGA ecotype was characterized by the capacity of chromatic adaptation and possibility for N₂ fixation. Population genetic analyses of the PC genes suggest an evolutionary diversification of *Pseudanabaena* through purifying selection.

The isolation of additional *Pseudanabaena/Limnothrix* strains from a variety of different environments is required to further elucidate of the ecology, biogeography and evolution of this understudied group of bloom-forming cyanobacteria.

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The isolation of additional *Pseudanabaena/Limnothrix* strains from a variety of different environments is required to further elucidate the ecology, biogeography and evolution of this understudied group of bloom-forming cyanobacteria.

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)