



Characterisation of *Pseudanabaena amphigranulata* (Synechococcales) isolated from a man-made pond, Malaysia: a polyphasic approach

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Abstract

A filamentous benthic cyanobacteria strain isolated from a tropical man-made pond in Malaysia was characterised using combined phenotypic and genetic approaches. Morphological and ultrastructural observations were performed together with growth measurements. Cell dimensions, thylakoid arrangement and apical cell shape with aerotopes were consistent with the description of *Pseudanabaena amphigranulata* (Goor) Anagnostidis. Molecular characterisation of the 16S rRNA gene gave 94% pairwise sequence identity with *Pseudanabaena* sp. PCC 6802, which corresponds to the genus identification threshold value while also suggesting that the strain is distinctly different to the species of *Pseudanabaena* currently represented in available databases. The strain showed identical 16S-23S ITS configuration with other strains of *Pseudanabaena* apart from having a larger spacer region. Cultures of the strain were exposed to various temperature and photoperiod treatments and harvested at exponential phase in order to examine phenotypic plasticity. Significant relationships between environmental conditions and morphological characteristics (cell dimensions and shape) were identified for the first time within the genus *Pseudanabaena*. The maximum cell length ($5.7 \pm 0.07 \mu\text{m}$) was observed at 25 °C under 12:12 light to dark, while the greatest cell width ($3.2 \pm 0.11 \mu\text{m}$) was also observed at 25 °C but under 16:8 light to dark. The strain showed high plasticity in cell dimensions and shape under different temperature and photoperiod treatments, with 25 °C under 12:12 light to dark providing the optimal conditions for its growth.

Keywords Cyanobacterium · Morphological plasticity · 16S rRNA · tRNA · Tropics

Introduction

The non-heterocystous cyanobacterial genus *Pseudanabaena* was first established by Lauterborn in 1915 (Geitler 1932). However, it has often been overlooked because of difficulties in observation related to relatively small cell/filament sizes. The genus includes a group of microscopic and simple members of Synechococcales, comprising more than 33 species

(Kling and Watson 2003; Komárek and Anagnostidis 2005). The family Pseudanabaenaceae is characterised by simple trichomes with a width less than 4 μm (Acinas et al. 2009). Members of the genus play key roles in ecosystem dynamics (Bertos-Fortis et al. 2016) and have been reported to be sources of antibacterial and antifungal compounds (Oufdou et al. 2001). Therefore, increasing knowledge of the ecology, morphological variability and phylogenetic status of members of this key genus is of particular interest.

Pseudanabaena has undergone several taxonomic and systematic revisions (Yu et al. 2015), but current taxonomic knowledge remains unsatisfactory due to the typically superficial identification of strains of simple cyanobacteria (Komárek and Anagnostidis 2005). Additionally, the presence of apparently identical morphotypes under different ecological conditions, or different morphotypes under the same ecological conditions, leads to further taxonomic uncertainty (Kling and Watson 2003). While in classical taxonomy,

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morphological stability is often considered a key feature in taxonomic studies, it has been reported that filamentous cyanobacteria can show high morphological variability under different growth conditions, with only a few characters such as the shape of the apical cell being truly diagnostic (Lyra et al. 2001; Gugger et al. 2002; Gupta and Agrawal 2006). Temperature is one of the most important environmental variables affecting the growth and morphology of phytoplankton (Coles and Jones 2000), making identification of species based on morphology alone unreliable. Taxonomic information, such as ultrastructural features, pigment composition, DNA sequence data and robust phylogenetic analyses of most *Pseudanabaena* species, remains incomplete (Yu et al. 2015).

Cyanobacterial classification has undergone important taxonomic and systematic changes in recent years (Komárek et al. 2014). Furthermore, sequence data in GenBank are unsatisfactory due to a general lack of integrated information on morphology that leads to incorrect recognition of strains. Komárek and Anagnostidis (2005) proposed a simplified generic classification based on morphological features. Advances in molecular technologies will increasingly play a critical role in prokaryote identification and characterisation (Emerson et al. 2008), especially when integrated with electron microscopy (EM) techniques and biochemical analyses (Castenholz 2001; Komárek 2003; Komárek and Anagnostidis 2005).

The species *Pseudanabaena amphigranulata* (Goor) Anagnostidis is distributed worldwide and is often very common in fresh water (Anagnostidis 2001). It is benthic or planktic and has been reported from shallow eutrophic lakes and other water bodies with muddy sediments, together with purple and colourless sulphur bacteria (Anagnostidis 2001). Hence, we expected the species to occur in the freshwater pond Tasik Harapan (Penang Malaysia), which is shallow and polluted from the untreated effluent discharged from domestic sewage (Salih et al. 2013).

Here, we report the first application of apolyphasic phenotypic and genotypic approach to characterise a strain of *Pseudanabaena* obtained from Tasik Harapan using a combination of morphological characterisation, electron microscopy, 16SrRNA and 16S-23S internal transcribed spacer (ITS) sequencing. We also examined the morphological plasticity of this strain under different temperature and photoperiod treatments to examine the stability of the morphological features that are of highest taxonomic significance.

Materials and methods

Sample collection and isolation

Pond sediments were collected from Tasik Harapan, Universiti Sains Malaysia, Penang (5°21' N 100°18'0" E). A

Pseudanabaena strain (strain USMAC18) was isolated by streaking and micropipette picking (Andersen and Kawachi 2005). The isolated strain was established in both BG-11 liquid and 1% agarised media (Rippka et al. 1979) at 25 ± 2 °C, under a light intensity of $27 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and photoperiod of 12L:12D. Cycloheximide at a concentration of $200 \mu\text{g mL}^{-1}$ was added to both media to eliminate green algae contamination.

Morphological identification

The strain was examined morphologically using an Olympus light microscope (Model BX53F, Olympus, Japan) equipped with digital camera (Olympus, Japan). Individual trichomes were observed at 1000× magnification. The images were analysed concurrently with cell measurement software (Cell Sens Standard Version 1.4.1). Cell length and cell width of 30 individual mature trichomes were measured.

Morphological plasticity in response to environmental variation

To study morphological plasticity, 100 mL of BG-11 were added to 250-mL Erlenmeyer flasks, and then 10 mL aliquots containing 2×10^6 cells mL^{-1} density of cyanobacteria culture were inoculated into the flasks. Flasks were exposed to 12 different combinations of temperature and photoperiod in a cross-gradient design (Fig. 1) for 21 days. The temperature ranged from 4 to 25 °C, and photoperiod from 0L:24D to 24L:0D (L: light and D: dark), with illumination provided by a cool white fluorescent lamp (Philips), with a light intensity of $27 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Observations were made using an Olympus BX53F microscope at 1000× magnification, and length and width of apical cells from 30 mature trichomes were measured under each treatment combination. Growth rates were measured by the cell count method using a haemocytometer. Cell counts were completed under a light microscope each day until day 21.

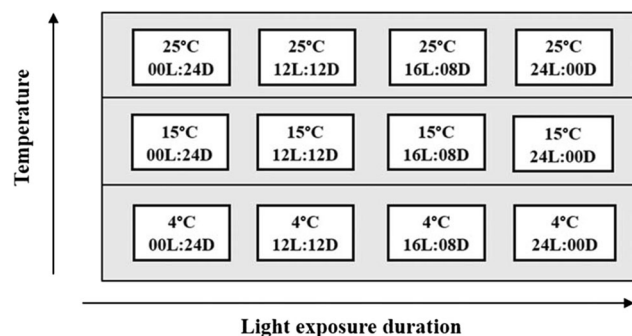


Fig. 1 Experimental design for estimation of morphological variability of *Pseudanabaena amphigranulata* in relation to temperature and light exposure duration (L: light and D: dark)

Statistical analyses

The mean values of growth rates and measured morphological parameters were compared by using a 3×3 factorial experimental design with a two-way analysis of variance (ANOVA) in SPSS v20.0 software, with temperature and photoperiod as fixed factors. Duncan's post hoc analysis was used to test multiple comparisons, when the main treatment effect was significant at $P < 0.05$. All of the experiments were conducted in triplicate. All data are presented as mean \pm standard error.

TEM (transmission electron microscopy)

Cell ultrastructure was analysed using TEM. Samples were prepared from actively growing cultures and fixed at room temperature with McDowell-Trump fixative prepared in 0.1 M phosphate buffer (pH 7.2). After buffer washing, samples were fixed with osmium tetroxide, dehydrated, embedded in Spurr's resin and sectioned at $< 0.1 \mu\text{m}$ thickness using an ultramicrotome (Power Tome XL, USA). Samples were stained with uranyl acetate and lead citrate before viewing under TEM (EFTEM Libra 120). Microphotographs were generated with a digital camera using Olympus, SIS iTem version 5.0, software.

DNA isolation

Genomic DNA was extracted from cells harvested during the exponential growth phase using the Tiangen DNA secure plant kit (China). The presence of DNA in the extracts was confirmed using 1% agarose gel electrophoresis. Extracted DNA was quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA).

Molecular characterisation

The 16SrRNA gene sequences and 16S-23S rRNA internal transcribed spacer (ITS) sequences of strain USMAC18 were amplified from the genomic DNA with cyanobacteria-specific primers (Boyer et al. 2001). For PCR, 10 ng of extracted DNA was used in 20 μL reactions consisting of 1 μL of each forward and reverse primer, 2 μL of MgCl_2 buffer, 2 μL dNTP mixture and 0.5 units Ex *Taq* DNA polymerase (all obtained from intron iTaq plus, Intron Biotechnology, South Korea). PCR was carried out in a Bio-Rad Thermal Cycler with parameters set as follows: 94 °C for 1 min, 56 °C for 1 min, 72 °C for 4 min (35 cycles), followed by 10 min extension at 72 °C. The products were confirmed on 1% agarose gels and sequenced commercially (Sanger sequencing, MyTACG Bioscience Enterprise, Malaysia). The 16s rRNA sequence (1285 bp) and 16S-23S ITS region (654 bp) were analysed by using the BLAST nucleotide search function of GenBank at the NCBI online site and the Seq Match tool of the

Ribosomal Database Project II (<http://www.ncbi.nlm.nih.gov/> and <http://rdp.cme.msu.edu/>, respectively). Strain USMAC18 16SrRNA and 16S-23S ITS sequences are deposited in GenBank under accession numbers KU216231 and MF754077, respectively.

Phylogenetic analyses were conducted using the two sets of sequence data (16S rRNA and 16S-23S ITS) separately. The first set included all 16S rRNA gene sequences of *Pseudanabaena* deposited in GenBank. Homologous sequences were identified using a MegaBlast search of the NCBI database in which only closely related sequences were selected to build the phylogenetic tree. These sequences were aligned using the CLUSTAL W program (European Bioinformatics Institute; <http://www.ebi.ac.uk/>). Phylogenetic analysis was carried out using MEGA version 6 (Tamura et al. 2013) and Mr. Bayes version 3.2 (Ronquist and Huelsenbeck 2003). Maximum likelihood (ML) analysis was implemented in MEGA version 6 (Tamura et al. 2013). The evolutionary model selected was the K2+gamma model, selected on the basis of the BIC (Bayesian Information Criterion) model using model test in MEGA version 6 (Tamura et al. 2013). Bootstrap re-sampling was performed using 1000 replications. Bayesian Inference was implemented in Mr. Bayes and analysis was performed using a mixed model with parameters set to two replicates of eight chains each for 1000,000 generations, with trees sampled every 100 generations. The first 1000 trees were discarded as burn-in. Parameter 220 stability was estimated by plotting log-likelihood values against generation time, and a consensus tree with posterior probabilities was then generated.

The second set included the 16S-23S ITS sequence of strain USMAC18 and its closest match, identified through MegaBlast search and compared directly without tree building analysis. Presence and absence of tRNAs, sequence lengths and spacers were obtained by using the tRNA scan-SE Search Server (Lowe and Chan 2016). *Limnothrix redekei* was selected as an outgroup as it is often confused with *Pseudanabaena* due to close morphological resemblance making identification more difficult (Acinas et al. 2009).

Results

Morphology

Trichomes were motile, straight, few slightly bent, without branching, 20–40 (50) μm long and 8–14 celled, rarely 20 celled. Cells dark blue-green, arranged in uniseriate row, cylindrical, up to $2 \times$ longer than wide, 1.2–2 (2.5) μm wide and 2–5 (6) μm long, mostly slowly motile, distinctly constricted at cross walls with aerotopes on both sides of septa. Cell content was granulated and differentiated into centropylasm and chromatoplasm. The apical cells were rounded with polar

aerotopes but without calyptas, no terminal attenuation, heterocytes and akinetes absent (Fig. 2). Reproduction was by hormogonia.

In liquid medium, the isolate grew as dark blue-green filaments suspended in the medium, and older cultures formed a thin layer on the walls and bottom of the culture vessel. On solid medium, the filaments aggregated into colonies and did not show any colonial gliding motility or phototaxis even after a long duration of light exposure.

Remarks The specimens examined are consistent with the description of *P. amphigranulata* (Komarek and Anagnostidis 2005).

Growth rates

Growth rates were evaluated under various culture temperatures and photoperiods over a period of 21 days (Fig. 3). Growth rates differed significantly between treatment combinations ($P < 0.05$, $F_{11,24} = 281.7$). The lowest growth rate ($0.008 \pm 0.03 \text{ day}^{-1}$) was observed at $4 \pm 2 \text{ }^\circ\text{C}$ and 0L:24D.



Fig. 2 Light micrograph of *Pseudanabaena amphigranulata* trichome (scale bar 5 μm)

Progressively higher growth rates were obtained at 15 ± 2 and $25 \pm 2 \text{ }^\circ\text{C}$ with 12L:12D photoperiod, with the highest growth rate being $1.84 \pm 0.04 \text{ day}^{-1}$.

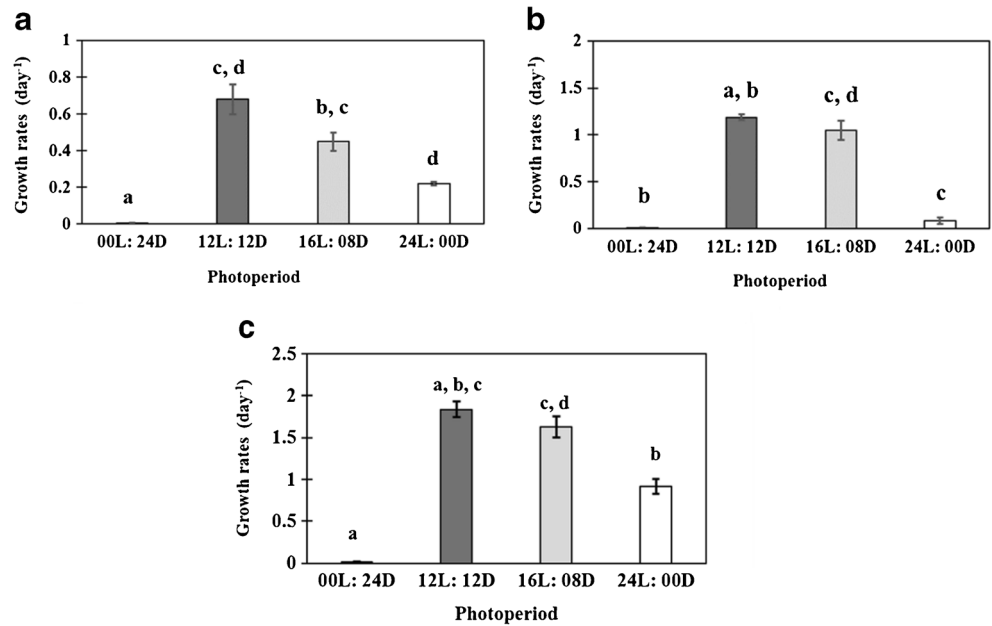
Morphological plasticity under varying growth conditions

Morphological plasticity was examined under various temperature and photoperiod treatments to investigate the stability of the measured diacritical characteristics. In stock culture under $25 \pm 2 \text{ }^\circ\text{C}$ and 12L:12D, the cells were cylindrical, 2x longer than wide, 2–5 (6) μm long and 1.2–2 (2.5) μm in width, and apical cells were rounded with polar aerotopes and clear constrictions at the cross walls, consistent with the previous description of *Anagnostidis* (2001). Morphological features mainly related to size and appearance differed between treatments (Table 1). Individual effects of temperature and photoperiod on cell dimensions were insignificant, while significant effects of combined treatments were observed. Cells growing at $4 \pm 2 \text{ }^\circ\text{C}$ with 0L:24D treatment had the smallest cell length ($0.95 \pm 0.05 \text{ } \mu\text{m}$, $P < 0.05$) and width ($1.02 \pm 0.08 \text{ } \mu\text{m}$, $P < 0.05$) amongst all the treatments, and were smaller than the range previously described by *Anagnostidis* (2001). Duncan's test revealed that cell dimensions differed significantly between all photoperiods ($P < 0.05$) at $4 \pm 2 \text{ }^\circ\text{C}$. Cells were mostly isodiametric with aerotopes. Trichomes were narrower with unclear constrictions at the cross walls. Under the $25 \pm 2 \text{ }^\circ\text{C}$ treatment with 12L:12D where tropical aquatic conditions were imitated, there were no differences apparent in cell shape and cell length from those reported by *Anagnostidis* (2001). The cell shape remained consistent throughout, which was cylindrical, longer than wide with polar aerotopes and distinctly constricted cross walls. Culture under this specific treatment led to cell length ($5.7 \pm 0.07 \text{ } \mu\text{m}$) in the range previously described by *Anagnostidis* (2001), and the longest amongst all treatments. However, the widest cells ($3.2 \pm 0.11 \text{ } \mu\text{m}$), observed at $25 \text{ }^\circ\text{C}$ under 16L:8D h photoperiod, exceeded the range (1.2–2.5 μm) reported by *Anagnostidis* (2001).

Transmission electron microscopy

TEM analysis (Fig. 4) clearly showed the presence of cell wall constrictions and the uniseriate row of connected cells. *Pseudanabaena amphigranulata* has six to eight peripheral thylakoids (T) parietally arranged in the peripheral cytoplasm and concentrically arranged around the long axis of the trichome. The cell wall (Cw) and cross walls (Cr) are simple, similar to other *Pseudanabaena* species, without prominent mucilaginous envelopes or sheaths. Carboxylate (C) and cyanophycin granules (Cy) were present. TEM analysis was consistent with the description of *P. amphigranulata*

Fig. 3 Specific growth rate (mean ± s.e) of *Pseudanabaena amphigranulata* under 4 °C (a), 15 °C (b) and 25 °C (c). The values presented are the mean of three replicates and standard errors. Means were compared using the multiple range test of Duncan ($\alpha = 0.05$) ($F_{11, 24} = 281.7$)



ultrastructure as previously described by Komárek and Anagnostidis (2005).

Molecular characterisation

The newly sequenced *P. amphigranulata* strain USMAC18 had pairwise sequence identity in the range of 93–94% with 61 strains of *Pseudanabaena* currently available in GenBank. The best matches were 94% pairwise sequence identity with *Pseudanabaena* sp. PCC 6802 (accession no. AB039016.1) from Japan (query cover 79%) and *P. mucicola* KLL-C016 (KP726258.1) from Israel (query cover 71%). A sister relationship between *P. amphigranulata* USMAC18 and *Pseudanabaena* sp. PCC 6802 is supported by bootstrap per-

centage (81%) and BI (0.99). However, *P. amphigranulata* is clearly distant from *P. mucicola* KLL-C016 (Fig. 5).

We obtained a single band of 16S-23S ITS PCR from *P. amphigranulata* USMAC18, indicating the presence of a single rRNA operon. The ITS sequence of *P. amphigranulata* strain USMAC18 was compared with other ITS sequences of members of *Pseudanabaena* retrieved from GenBank. There was variability in the size of the ITS region across the species examined. This took the form of variation in the ITS sequence length, with *P. amphigranulata* having two inserts, a tRNA^{Ile} and tRNA^{Ala}, otherwise showing similar ITS composition to the other strains of *Pseudanabaena* retrieved from GenBank except *Pseudanabaena* sp. CCY9705, which lacked tRNA^{Ala} (Table 2). *Pseudanabaena. amphigranulata* had the

Table 1 Morphological characteristics of *Pseudanabaena amphigranulata* under various temperature and photoperiod duration treatments (L and D indicate light and dark hours, respectively)

Treatments	Cell length (µm)	Cell width (µm)	Cell shape	Apical cell shape
4 °C; 00L: 24D	0.95 ± 0.05 A	1.02 ± 0.08 A	Cylindrical, mostly isodiametric	Rounded with aerotopes
4 °C; 12L:12D	2.1 ± 0.13 B	1.54 ± 0.1 B	Cylindrical, some longer than wide	Rounded with aerotopes
4 °C; 16L:08D	2.85 ± 0.1 C	1.73 ± 0.65 B, C	Cylindrical, mostly longer than wide	Rounded with aerotopes
4 °C; 24L:00D	2.4 ± 1.11 C	1.8 ± 0.05 C, D	Cylindrical, mostly longer than wide	Rounded with aerotopes
15 °C; 00L: 24D	2.74 ± 0.1 C	1.7 ± 0.05 B, C	Cylindrical, mostly isodiametric	Rounded with aerotopes
15 °C; 12L:12D	3.4 ± 0.12 D	2 ± 0.05 D	Cylindrical, mostly longer than wide	Rounded with aerotopes
15 °C; 16L:08D	1.9 ± 0.12 C	1.99 ± 0.06 D	Cylindrical, mostly longer than wide	Rounded with aerotopes
15 °C; 24L:00D	2.97 ± 0.09 C	3.01 ± 0.06 B, C	Cylindrical, mostly longer than wide	Rounded with aerotopes
25 °C; 00L:24D	2.22 ± 0.08 B	1.59 ± 0.06 B, C	Cylindrical, mostly longer than wide	Rounded with aerotopes
25 °C; 12L:12D	5.7 ± 0.07 E	1.79 ± 0.05 C, D	Cylindrical, mostly longer than wide	Rounded with aerotopes
25 °C; 16L:08D	3.04 ± 0.1 C	3.2 ± 0.11 E	Cylindrical, mostly longer than wide	Rounded with aerotopes
25 °C; 24L:00D	2.82 ± 0.08 C	1.73 ± 0.04 B, C	Cylindrical, mostly isodiametric	Rounded with aerotopes

The values presented are the mean of three replicates and standard errors. Means were compared using the multiple range test of Duncan ($\alpha = 0.05$) (for length $F_{11,24} = 34.37$ and width $F_{11,24} = 27.52$); differences are non-significant for groups within the cell length or cell width columns with the same letter

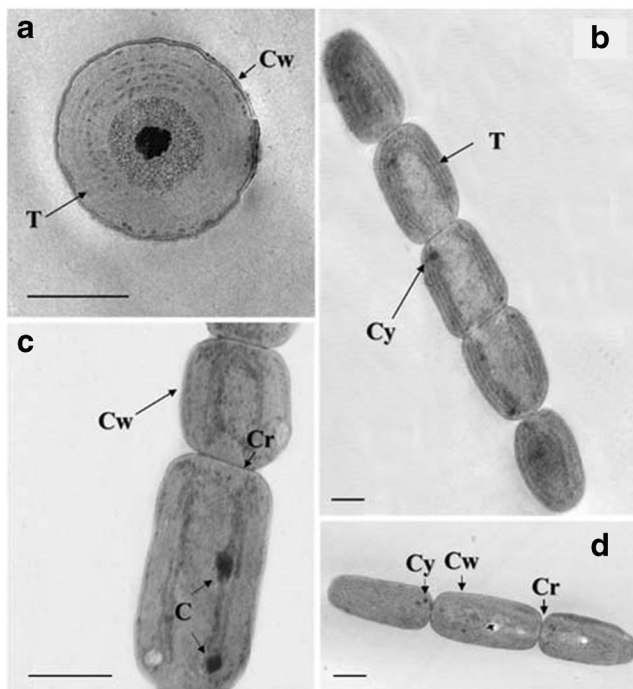


Fig. 4 Ultrastructure of *Pseudanabaena amphigranulata*. Thylakoids are marked with T, cell wall is marked as Cw, cross walls as Cr, cyanophycin granules are marked with Cy and carboxysomes are marked as C. Scale bar 1 μ m

longest ITS length (603 bp) and also a significantly larger spacer region than other close relatives retrieved from GenBank. *Limnothrix redekei* CCAP 1443/1, used as an outgroup, had a slightly different ITS configuration with a longer tRNA^{Ala} gene sequence (77 bp).

A summary of diacritical features of morphospecies closely related to *P. amphigranulata* USMAC18, derived from Komárek and Anagnostidis (2005), is presented in Table 3. Cell lengths and widths for *P. amphigranulata* and strains from Genbank with similar ITS composition were mostly in the same ranges, except for *P. minima* which has a somewhat shorter cell length. All these strains of *Pseudanabaena* have isodiametric/longer than wide cells. Apical cells were rounded, but conical rounded for *P. mucicola*.

Discussion

Taxonomic revisions of cyanobacteria at the genus level have been achieved by integrating polyphasic studies during the last decade (Yu et al. 2015). However, considering the complexity of *Pseudanabaena*, Komárek and Anagnostidis (2005) concluded that the genus still needs further taxonomic revision. Modern taxonomic studies on the genus are limited by the lack of reference polyphasic characteristics from the type strain. This background supported the use of microscopy in combination with molecular studies here, since the description of microbial species without genetic support has been

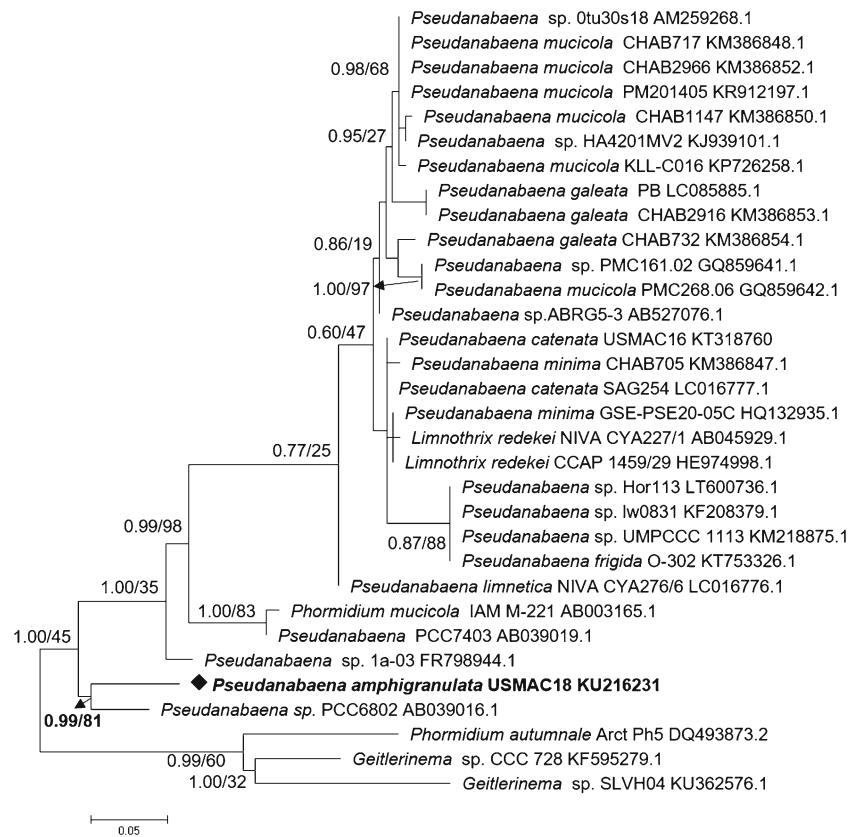
criticised by numerous authors (e.g., Comte et al. 2007; Bruno et al. 2009). Polyphasic approaches are now recognised as crucial to identifying cyanobacterial strains at genus as well as at species level (Marquardt and Palinska 2007).

Growth and morphological plasticity under varying experimental conditions were documented in this study for the first time in the genus *Pseudanabaena*. Temperature changes between 4 ± 2 and 25 ± 2 °C affected growth, with growth rates increasing significantly with temperature and the highest values observed at 25 ± 2 °C. Growth rates were also affected by photoperiod. The highest growth rate was achieved with 12:12 L:D while the lowest was observed in the absence of light. In the absence of light, the ability of a phototroph to grow will clearly be reduced, and cells will eventually die (Dehning and Tilzer 1989). However, growth of the study strain in the absence of light indicates possession of some mixotrophic capacity. Strains cultured under 24:0 L:D experience photoinhibition due to continuous irradiance, which results in a reduction in photosynthetic rate and leads to reduced growth rates (Tyystjarvi and Aro 1996). Previous studies have emphasised the importance of average temperature and light exposure for active metabolic process (Winder and Sommer 2012; Singh and Singh 2015). Temperatures around 25 ± 2 °C with 12:12 L:D photoperiod, which typify many tropical systems, are favourable for the growth of this strain of *P. amphigranulata*.

Temperature and light had substantial effects on morphological features of the strain studied. Under various treatments, *P. amphigranulata* USMAC18 showed significant changes in diacritical characteristics. These changes corroborate the widely accepted observation that cell size changes with temperature (Montagnes and Franklin 2001). Additionally, growth and metabolism are clearly limited at low temperature (*sensu* Graumann and Marahiel 1996), therefore, at 4 ± 2 °C, we assume that metabolic deficiencies were expressed as shorter and thinner cells in trichomes. At 25 ± 2 °C, longer cells were observed with increased metabolic activities. Changing photoperiod alone did not elicit significant effects on the morphological features of strain USMAC18, but in combination with temperature significant responses were obtained. Intermediate photoperiods (12:12 L:D) in the cross-gradient experiments can be considered optimal for maximising cell length and width. An analogous conclusion was drawn by Zapomelova et al. (2008), who reported morphological variation occurring in two strains of cyanobacteria, *Anabaena circinalis* and *Anabaena crassa*, cultivated under various temperature and light conditions.

An important finding of our study is the stability of apical cell shape with polar aerotopes under varied experimental treatments, as originally described by Anagnostidis (2001), supporting the morphological identity of this strain. Thus, the presence of polar aerotopes appears to be a reliable criterion for the identification of *P. amphigranulata* (Anagnostidis 2001), discriminating it from morphospecies with similar cell

Fig. 5 Maximum likelihood (ML) tree showing phylogenetic relationships between *Pseudanabaena amphigranulata* USMAC18 based on 16S rRNA gene sequences with other species of *Pseudanabaena*. The newly isolated strain in this study is shown with a filled diamond. Numbers associated with nodes are maximum likelihood bootstrap percentages/Bayesian posterior probability



dimensions such as *P. mucicola* (Schwabe 1964) and *P. catenata* (Lauterborn 1915).

The genus *Pseudanabaena* is further divided into the subgenera *Skujanema* (species with pointed apical cell), *Pseudanabaena* (species with cylindrical and rounded apical

cell, with or without polar aerotopes) and *Ilyonema* (species with polar aerotopes in cells) (Komárek and Anagnostidis 2005). The cell shape and dimensions of the strain USMAC18 closely resemble those of the group of species with aerotopes classified in the subgenus *Pseudanabaena*.

Table 2 16S-23S ITS configuration of *Pseudanabaena amphigranulata* and other *Pseudanabaena* strains retrieved from GenBank by MegaBlast search. Strains selected from GenBank for comparative purposes are grouped by particular shading

Morphotypes	Acession number	Length of ITS	Spacer	tRNA ^{Ile}	Spacer	tRNA ^{Ala}	Spacer
<i>P. amphigranulata</i> USMAC18	MF754077	603	194	74	96	76	163
<i>Pseudanabaena</i> sp. RK46	KY379894	553	126	74	87	76	190
<i>Pseudanabaena</i> sp. CCY0471	EU119301	536	129	74	88	76	169
<i>Pseudanabaena</i> sp. CCY9705	EU119320	498	129	74	295	-	-
<i>Pseudanabaena</i> sp. CCY9702	EU119317	497	129	74	51	76	167
<i>P. mucicola</i> KLL-C016	KP726258	598	138	74	96	76	214
<i>P. catenata</i> USMAC16	KX668261	599	194	74	51	76	204
<i>P. minima</i> GSE-PSE20-05C	HQ132935	507	139	74	51	73	170
<i>Limnothrix redekei</i> CCAP 1443/1	AJ580007	490	137	74	38	77	164

Table 3 Morphological characteristics of *Pseudanabaena amphigranulata* and other *Pseudanabaena* strains retrieved from GenBank. Subgenus of *Pseudanabaena* are included for comparative purposes are grouped by particular shading

Morphotypes	Cell length (µm)	Cell width (µm)	Shape of apical cell	Aerotopes
<i>P. amphigranulata</i>	2-5 (7)	1.2-2 (2.5)	Rounded	+
<i>P. mucicola</i>	2.2-4 (6)	1.2-2 (2.8)	Conical rounded	-
<i>P. catenata</i>	2-5	1.2-2.2	Rounded	-
<i>P. minima</i>	1.5-4	1.3-2.5	Rounded	-
<i>P. galeata</i>	1.2-7 (10-15)	0.8-2.4 (2.7)	Flat rounded	+
<i>P. tenuis</i>	1-1.5	6-8	Rounded	-
<i>P. frigida</i>	1.6-2.4 (4)	0.8-1.2 (1.8)	Conical rounded	-
<i>P. skujajae</i>	7.5	2.3-2.5	Conically pointed	-
<i>P. curta</i>	1.5-2.4	0.5-0.8	Conical rounded	-
<i>P. biceps</i>	3-7.5	(2.3) 2.7-3	Rounded-pointed	-
<i>P. arcuata</i>	2-14	0.9-1.3	Cylindrical and conical	-
<i>P. starmachii</i>	3-6	2-2.4 (2.8)	Obtuse conical	-
<i>P. limnetica</i>	4-12 (20)	1.2-1.5 (2)	Rounded	-
<i>Limnothrix redekei</i>	(2.5) 6-16	1.2-2.5 (3.5)	Rounded	+
<i>Ilyonema</i> sp.	-	up to 3.3	Cylindrical rounded to pointed	+
<i>Skujanema</i> sp.	-	up to 2	Conically pointed	-

Molecular analysis demonstrated that the strain *P. amphigranulata* USMAC18 had only 94% 16S rRNA sequence similarity with *Pseudanabaena* sp. PCC 6802. Additionally, sequence match analysis of other *Pseudanabaena* 16S rRNA gene sequences of significant length (> 1000 pb) available in GenBank indicated that these strains generally showed similarities close to the limit for genus delimitation (Stackebrandt and Ebers 2006). The best match (94%) sequence similarity of strain USMAC18 with *P. mucicola* KLL-C016 is also close to the limit of genus delimitation, and well below the molecular limits (98.5%) for species identification indicated by Stackebrandt and Ebers (2006). However, *P. amphigranulata* USMAC18 is placed in a single clade with *Pseudanabaena* sp. PCC 6802, with high bootstrap and BI values. Furthermore, *P. mucicola* KLL-C016 is distant from strain USMAC18 and positioned with other *P. mucicola*. The current lack of available sequence data for other strains of *P. amphigranulata*, or other

more closely related species, limits further speculation on the taxonomic position and relationships of this strain.

Limnothrix redekei strains (NIVA CYA 227/1 and CCAP 1459/29), as shown in the phylogenetic tree, are embedded in a cluster with type species of *Pseudanabaena*, suggesting close genetic similarities between these two genera. A similar conclusion was also drawn by Yu et al. (2015) who suggested to transfer the stains of *L. redekei* positioned inside the *Pseudanabaena* cluster to the latter genus. The result is also consistent with Whitton (2011), who used *Pseudanabaena redekei* (Goor 1918) as the synonym of *Limnothrix redekei*. However, differences in the morphology of *Pseudanabaena* and *Limnothrix* are still not resolved. Morphologically, the type species differ by the absence or presence of polar aerotopes. *L. redekei* shows similarities with *P. amphigranulata* in possessing polar aerotopes, but cell dimensions differ widely (Komárek and Anagnostidis 2005).

Pseudanabaena amphigranulata showed nearly identical ITS configuration with other representatives of *Pseudanabaena* available in GenBank (except *Pseudanabaena* sp. CCY9705), apart from possessing a larger spacer region. Multiple rRNA operons have been reported in cyanobacteria, but in most cases the tRNA configuration is identical in both operons. The nucleotide sequences of tRNA^{A1a} and tRNA^{Hc} were identical and highly conserved between species, but the spacer regions surrounding these two tRNA gene sequences were variable (Chen et al. 2000), signifying that ITS regions in the rRNA operon can be helpful in discrimination between species (Boyer et al. 2001). Both the presence and absence of tRNA sequences between multiple copies of the ITS region in the cyanobacterial genome have been reported previously (Sciuto et al. 2012).

The strain USMAC18 differs from *P. mucicola* KLL-C016 and other members of this genus in terms of morphology and molecular sequence data. There are no sequences for this species currently available in GenBank against which to compare and support a precise taxonomic identification of this strain. Therefore, according to the ecological and morphological differences documented here we conclude that strain USMAC 18 is a representative of *P. amphigranulata*. Further molecular studies are necessary to clarify the taxonomic status of this strain.

Conclusions

A polyphasic approach was used to characterise a strain of the cyanobacterial genus *Pseudanabaena* isolated from a tropical pond. Our data indicate that environmental features (temperature, day length) have a clear influence on cell morphology, with longer day length leading to reduced width and length of the cell. A light-dark cycle of 12:12 light to dark allowed the strain to produce the longest and widest cells. The consistent presence of a rounded apical cell under various culture conditions is a reliable criterion for the identification of this strain, which was morphologically consistent with the description of *P. amphigranulata*. 16S rRNA and ITS sequence data confirm the strain is highly distinct at the species level from more than 60 members of the genus *Pseudanabaena* currently available in GenBank.

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