

Pseudanabaena foetida sp. nov. and *P. subfoetida* sp. nov. (Cyanophyta/ Cyanobacteria) producing 2-methylisoborneol from Japan

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Abstract: Nasty smell of tap water supplied from Lake Biwa caused a great trouble. Then many researches have been conducted from the point of view of water supply management or water quality in Japan. The matter of this bad smell was identified as 2-methylisoborneol (2-MIB) and the source organism of this bad odor was then reported as *Phormidium tenue* and later two different cultured strains were established. One of these strains shows green color, produce 2-MIB, and is marked PTG. The other shows brown color, does not produce 2-MIB and is marked PTB. However their nomenclatural description has not been done yet and, in fact, they have morphological characters of genus *Pseudanabaena* rather than *Phormidium*. *Pseudanabaena* species are also observed in Lake Kasumigaura. PS1306 produces 2-MIB and other strain PS1303 has no smell. This study focuses on morphological and genetical (16S rRNA) comparison of strains from both lakes. In addition, the ultrastructure of cells of PTB and PTG are demonstrated. On the basis of this comparison we propose description of two new planktic species producing 2-MIB: *Pseudanabaena foetida* NIIYAMA, TUJI et ICHISE sp. nov. and *P. subfoetida* NIIYAMA et TUJI sp. nov.

Key words: Lake Biwa, Lake Kasumigaura, 2-methylisoborneol (2-MIB), *Phormidium tenue*, *Pseudanabaena foetida*, *Pseudanabaena subfoetida*, Pseudanabaenaceae, PTB, PTG, 16S rRNA gene

INTRODUCTION

In 1969, nasty smell of tap water supplied from Lake Biwa (Fig. 1), which is the largest lake in Japan, caused a great trouble. The source organism of this trouble was investigated afterward and became known to be a thin filamentous cyanobacteria. This cyanobacteria was reported then as *Phormidium tenue* (MENEGH.) GOMONT and the cultured strains were established. The substance causing the musty odor was identified as 2-methylisoborneol (2-MIB) (YAGI 1983; YAMADA et al. 1985, 1986). Later, it was apparent that two kinds of cyanobacteria species were contained in the above mentioned cultured strains. One shows green color, produces 2-MIB and has musty odor, and is called PTG, the other shows brown color, does not produce 2-MIB and is called PTB.

PTG and PTB have morphological characteristics of genus *Pseudanabaena* as follows; trichomes are solitary or in small clusters, straight or slightly curved, with conspicuous constrictions at cross-walls, less than 2 µm wide and without sheath; apical cells are not differentiated and without calyptra; cells are cy-

lindrical, longer than wide. Although many researches about these strains had been conducted in Japan from the point of view of water supply management or water quality, nomenclatural description has not been done yet. The species name *Phormidium tenue* is still used for both strains and similar cyanobacteria producing 2-MIB especially in the field of applied biology in Japan.

In Japan, it is reported that several species and cultured strains of bloom-forming cyanobacterial algae, especially *Microcystis* and *Dolichospermum* (*Anabaena*) produce the toxin as microcystin, anatoxin or cylindrospermopsin (WATANABE et al. 1994). Although a red colonial *Pseudanabaena rutilus-viridis* from Canadian large lakes is indicated to have the ability to produce the toxin microcystin (KLING et al. 2012), no species of the family Pseudanabaenaceae is reported to produce the toxin in Japan. The matter of concern in Japan is water quality or smell of drinking water supplied from reservoirs and bad smell of fish and shellfish caused by thin filamentous algae, so called *Phormidium tenue* like species. The substances causing the musty odor are identified as 2-methylisoborneol (2-MIB) or geosmin (YAGI 1983), and troubles caused by the musty

odor producing cyanobacteria are reported from several lakes, reservoirs or ponds in Japan (cf. MORII et al. 1982; YAMADA et al. 1985, 1986; OIKAWA et al. 2000; OIKAWA & ISHIBASHI 2004), but there is almost no systematic study about the organisms causing the above mentioned troubles. Recently, the species names in Japan are even more confusing because several researchers observe the light-microscopic morphology of odor producing cyanobacteria and use species names quoted from the new systematics of Oscillatoriales (KOMÁREK & ANAGNOSTIDIS 2005) and others still use name *Phormidium tenue*. NGUYEN et al. (2012) describe *Annamia toxica* gen. et sp. nov. based on the ultrastructural and phylogenetic study. This species is first identified and reported as *Pseudanabaena* cf. *moniliformis* (NGUYEN et al. 2007). DVOŘÁK et al. (2015) point out the importance of consideration of cryptic diversity in cyanobacteria and describe a new monospecific genus *Pinocchia* with *Pseudanabaena*-like morphology using molecular, ecological and morphological data. Therefore systematic study being composed of detail morphological description, ecological observation and phylogenetic analysis is needed to clarify the Japanese species producing 2-MIB.

Then we began to analyze the morphology and 16S rRNA gene sequences of PTB and PTG strains. Comparison of the ultrastructure in cells, especially the arrangement of thylakoids in cells are so useful for taxonomic evaluations of cyanophyta (WHITTON 1972; ANAGNOSTIDIS & KOMÁREK 1988; KOMÁREK & ČASLAVSKÁ 1991; KOMÁREK & KAŠTOVSKÝ 2003;

KOMÁREK & ANAGNOSTIDIS 2005). Three main kinds of thylakoid arrangements correspond with three families in Oscillatoriales; parietal, radial and irregular arrangement of thylakoids are typical of *Pseudanabaenaceae*, *Phormidiaceae* and *Oscillatoriaceae*, respectively. Light and electron microscopic observations of PTB and PTG are conducted in this research to confirm their taxonomic position.

In the course of the study of PTB and PTG, we observed *Pseudanabaena* species almost throughout the year in Lake Kasumigaura (Fig. 1), which is the second largest lake in Japan. We could establish several *Pseudanabaena* strains from Lake Kasumigaura. Strains originated from Lake Kasumigaura resemble PTG and PTB in their morphology when they are observed under the light microscope of low magnification. One strain from Lake Kasumigaura produces 2-MIB and has same extreme musty smell as PTG (TUJI et al. unpublished data).

This study focuses on comparison of the morphology and genetic characteristics of four cultured strains from both lakes. Moreover, morphological data of other nature and fixed materials and ultrastructure of PTB and PTG strains are demonstrated.

MATERIALS AND METHODS

Cultured strains. The strains PTG and PTB were collected from Lake Biwa during the summer in 1985. The area of Lake Biwa is ca. 670 km² and its maximum depth is 103.58m and mean depth is 41.2 m. The strain PS1303 and the strain PS1306 were collected in March and June, respectively, from Lake Kasumigaura in 2013. The area of Lake Kasumigaura is ca. 220 km² and its maximum depth is 7.1 m and mean depth is 4.0 m. Isolation was done by the pipette washing method under a binocular or agar plate method (TUJI & NIYAMA 2014) with d medium (modified WC medium: TUJI 2000). 10 ml of culture medium contained in a test tube was used for maintenance of strains. CT medium (ICHIMURA & WATANABE 1977) or M-11 medium (YAGI et al. 1979) was used for strains PTG and PTB, and modified C medium (ICHIMURA & WATANABE 1977, NIYAMA et al. 2011) was used for strains originated from Lake Kasumigaura and PTG and PTB. The cultures were illuminated by cool-white fluorescent lamps, with photon flux density of ca. 20 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, a photoperiod of 8 hours light and 16 hours dark, and a temperature of 18 °C or 20 °C. Morphological observation was performed for these cultured strains under the light microscope (BH-2, Olympus Corporation, Tokyo, Japan). Microphotographs were taken with a Canon digital camera EOS Kiss X5 (Canon Inc., Tokyo, Japan). Fifty measurements were conducted for calculating the cell width and length of each strains. Cultured strains PTB and PTG are maintained in the Lake Biwa Environmental Research Institute, and strains PS1303 and PS1306 are maintained in the Department of Botany, National Museum of Nature and Science in Tsukuba, Japan. The specimens (TNS-AL in TNS) are kept in the herbarium of the Department of Botany, National Museum of Nature and Science (Table 1). To compare with the European strain, the morphology and 16S rRNA gene of cultured strain



Fig. 1. Site map of Lake Biwa and Lake Kasumigaura in Japan.

NIVA–CYA 276/6 originating from Sweden were also analyzed. Modified C medium and above mentioned illumination, temperature and photoperiod conditions were used for NIVA–CYA 276/6.

Fixed material. Photographs of trichomes of *Pseudanabaena* were taken, which were in a sample collected from Lake Biwa in 1980 and fixed with formaldehyde. The trichomes in this sample, called Biwa1980 in this paper, had been identified as *Phormidium tenue* green-type with bad smell. The cell width and length of Biwa1980 were measured for comparing with those of PTG.

Ultrastructure (Transmission Electron Microscopy). The laboratory-cultured cyanobacterial cells were harvested by centrifugation (PTG: 12,000 rpm, 10 min, 15 °C. PTB: 3,000 rpm, 10 min, 15 °C). After washing with a cacodylate buffer 0.1M (7.4 pH), the pelleted cells were prefixed with 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd) in the cacodylate buffer at room temperature for 30 min. After washing with the buffer, the material was postfixed with 1 % osmium tetroxide (TAAB Laboratories Equipment Ltd) in the buffer for 30 min at room temperature. Fixed cells were rinsed again and dehydrated by ethanol and embedded in Epon 812 resin (TAAB Laboratories Equipment Ltd). The samples were sectioned using an ultramicrotome (Reichert–Nissei), and mounted on EM grids. Sections (80 nm) for the ultrastructural analyses were stained for 15 min with uranyl acetate followed by 5 min in lead citrate. Transmission electron microscope (TEM) images were acquired by using JEOL JEM 1400 TEM operating at 80 kV accelerating voltage.

Genomic DNA extraction, PCR amplification and sequencing. 1.5ml of fresh culture material was centrifuged at 10,000 rpm for 5 min at room temperature. The supernatant was removed and the cell pellets were kept in freezer at 4 °C until extraction. Total genomic DNA was extracted using the extraction kit (PP–207, Jena bioscience) following the instructions of the manufacturer. 16S rRNA, 16S–23S internal transcribed spacer (ITS) and partial 23S rRNA gene were amplified by using the two primers sets, set A: universal primer 27f (5′–AGA GTT TGA TCM TGG CTC AG–3′ : LANE 1991) and *Pseudanabaena* specific primer designed in this study, PS16s600r (5′–TCC TGC CCC TAT CTC TCT C–3′: designed in this study) and set B: *Pseudanabaena* specific primer, PS16s440f (5′–ACC TCT TTT GTT AGG GAA GAT AAT G–3′: designed in this study) and cyano-specific primer, pitsE–cyanR (5′–CTC TGT GTG CCA AGG TAT C–3′ : ERNST et al. 2003). PCR was performed on a Thermal Cycler (iCycler, BioRad), using 10µl KAPA plant PCR buffer, 0.1µl KAPA3G Plant DNA polymerase, 0.04µl KAPA Plant PCR Enhancer (KAPA Biosystems), 7.46µl sterile deionized water, 0.7µl each of 10pM concentration of forward and reverse primers, and 1µl DNA template. The temperature cycling program was used the following conditions: 95 °C for 4 min; 40 cycles of 95 °C for 20 sec, 54 °C for 15 sec, 72 °C for 40 sec; the final elongation step was 72 °C for 7 min. The concentration of the amplified products was verified on a 1% agarose gel. Direct sequencing of PCR products, with primers 27f and PS16s600r for set A, and PS16s440f, F3L (5′–GTC CCG CAA CGA GCG CAA C–3′) and pitsE–cyanR for set B, were undertaken using Big Dye Terminator Chemistry and an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). The Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) was utilized for locating strains/

sequences similar to our species. We used 5 new sequences obtained in this study, and 18 sequences from NCBI after performing BLAST. The short sequences from NCBI, were delimited from our analysis. Phylogenetic and molecular evolutionary analyses of the obtained sequences were conducted using the MEGA 5 computer program (TAMURA et al. 2011). Alignments were computed using MUSCLE in MEGA 5, and checked manually. A maximum likelihood (ML) and Neighbour-joining (NJ) trees were calculated using MEGA software with the best fit model determined by Akaike Information Criterion (AIC) corrected scores, and the substitution nucleotide matrix parameters were calculated by the software. A sequence for *Pseudanabaena* sp. 1a–03 (FR798944.1) was used for an out-group. One thousand bootstrap replicates were performed for NJ and ML analysis. The DNA sequences obtained in this study have been deposited in the DDBJ under the GenBank accession numbers as follows (see Table 1); PTB: LC016774, PTG: LC016773, PS1303: LC016775, PS1306: LC016779, and NIVA–CYA276/6: LC016776.

Secondary structure models of ITS regions. The putative secondary structures of the 16S–23S rRNA ITS region were predicted with the Mfold Web server using version 2.3 (ZUKER 2003), with temperature set at 20 °C, but all other settings at default followed by SIEGESMUND et al. (2008).

RESULTS

The parietal arrangement of thylakoids in cells was observed for both PTB (Fig. 2) and PTG (Fig. 3). Because the parietal pattern of thylakoid arrangement is characteristic in the family Pseudanabaenaceae, PTB and PTG are considered to be a member of Pseudanabaenaceae, thus the name *Phormidium tenue* is wrong. The morphology of trichomes and cells of Japanese strains PTG, PTB, PS1303 and PS1306 and Swedish one NIVA–CYA 276/6 is seemingly similar under the light microscope of low magnification (Figs. 4, 6, 8, 10, 12), but the cell size measured under the light microscope of high magnification and other characters such as trichome color, odor, etc. of each strain are different as shown in Tables 1 and 2.

Then differences of cell width and cell length among PTG, PS1306 and Biwa1980 were evaluated using analysis of variance (ANOVA). As the calculated F values are higher than the $F_{111}^2(0.01) = 4.802$, there is a significant difference among PTG, PS1306 and Biwa1980. The differences of cell width and cell length between PTB and PS1303 were evaluated by t-test. There are significant differences between the cell width and the cell length between PTB and PS1303 ($p < 0.001$).

The trichome color of PTB is brownish green but those of PTG, PS1303, PS1306 and NIVA–CYA 276/6 are bright blue–green (Table 2). Trichomes of every strains have no sheath at the beginning of cultivation. Trichomes of PTG, PS1306 and NIVA–CYA 276/6 are growing separately but trichomes of PTB and

PS1303 are sticky and sometimes grow close together and form small colonies (Figs. 4, 8). Only the old trichomes of PS1303 have very thin sheath and they stick together to form a thin membranous colony, but other cultured strains have no sheath even in the late growing stage.

The cell color of PTB is pale blue–green to olive green. The cell color of PTG, PS1303, PS1306 and NIVA–CYA 276/6 is bright blue–green. Cells of PTB and PTG are long cylindrical and clearly longer than wide. The mean value of L/W of PTB and PTG are larger than other strains (Table 1). The cells of PS1303 are short to long cylindrical, and those of PS1306 and

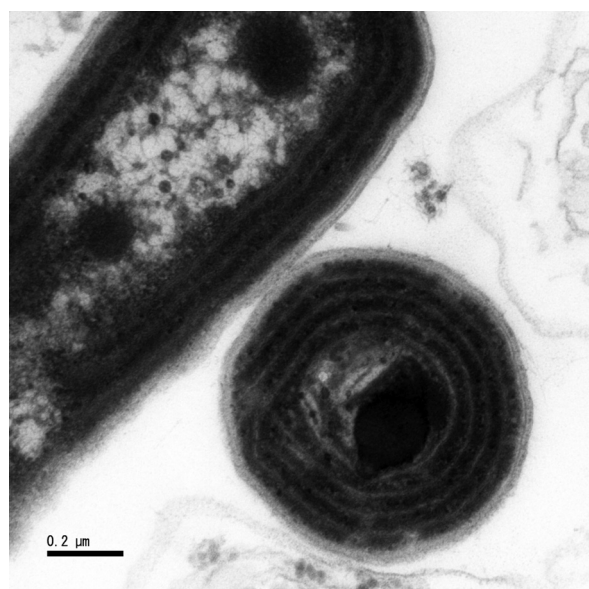


Fig. 2. TEM photo of cells of PTB, *Pseudanabaena limnetica*. Scale bar 0.2 μm .

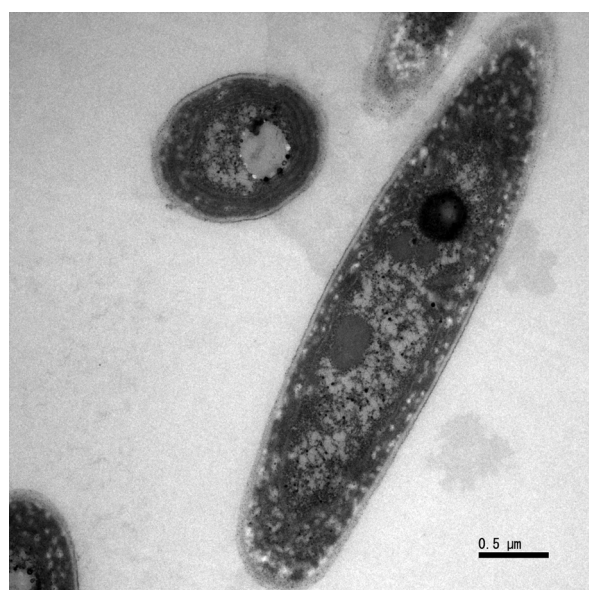


Fig. 3. TEM photo of cells of PTG, *Pseudanabaena foetida* sp. nov. Scale bar 0.5 μm .

NIVA–CYA 276/6 are nearly isodiametric to longer than width and with rounded ends. The ends of cells of PTG, PS1306 and NIVA–CYA 276/6 are rounded and there is a clear aerotope at the both ends of each cell of these three strains. The aerotopes of PTG, PS1306 and NIVA–CYA 276/6 are colorless and transparent, and their trichomes are intensely constricted at the cross-walls, then each cell seems to be connected with bright shining papillae and trichomes seem to have small conical protrusions at the apical cells when observed under the light microscope of lower magnification. PTB and PS1303 have an apical small aerotope at the both ends of each cell and the cells of their trichomes also seem to be connected with small bright papillae under the light microscope of lower magnification. Cell content of PS1306, NIVA–CYA 276/6 and sometimes PTG are differentiated between centropalasmic and chromatoplasmic regions. There are several small granules in the cells of PS1306. Cell content of PTB and PS1303 seems to be almost homogeneous.

Phylogenetic analysis of our four strains (PTB, PTG, PS1303, PS1306), NIVA–CYA 276/6 and related 18 taxa, is shown in Fig. 16. PTB and PS1303 are included in the same cluster with high bootstrap values (96 for NJ and 92 for ML) and cluster with *Oscillatoria limnetica* (synonym of *Pseudanabaena limnetica*). PTG and PS1306 are included in another cluster with high bootstrap values (92 for NJ and 93 for ML). PTG and PS1306 differ 12 base positions (0.8% of 1462bp) in 16S rRNA gene region and 26 base positions (4.9% of 534bp) excluding 45 bp gaps in ITS region. The difference of the secondary structures of the 16S–23S rRNA ITS region of PTG and PS1306 was also found (Figures S1 and S2), and it suggests the species level difference for both strains.

As a result of phylogenetic and morphological analysis, PTB and PS1303 are concluded to be *Pseudanabaena limnetica*. On the other hand, both PTG and PS1306 have very unique character producing 2–MIB which has strong musty odor but their trichome width, cell morphology and phylogenetic result are different from each other, so we propose two new species, *Pseudanabaena foetida* NIIYAMA, TUJI et ICHISE for PTG and *Pseudanabaena subfoetida* NIIYAMA et TUJI for PS1306 as follows.

***Pseudanabaena foetida* NIIYAMA, TUJI et ICHISE sp. nov. (Figs 6, 7)**

Description: Trichomes solitary, straight or slightly curved, bright blue–green colored, with conspicuous constrictions at cross-walls, 1.0–1.7 (–2.2) μm wide, without mucilage or sheath, not attenuated nor differentiated at the ends, without calyptra, infrequently move. Cells long cylindrical with rounded ends, bright blue–green, longer than wide, 3.4–11.0 μm long, ratio of width to length ca.1.7–8.5, with aerotopes at both ends of cells, differentiated in centro- and chromatoplasmic regions. Cell division perpendicular to

the longitudinal axis of a trichome. Trichomes separate between two neighboring cells or by fragmentation without necridic cells. Hormogonia trembling. Heterocytes are not known. Thallus has the extreme musty odor that comes from 2-methylisoborneol.

Holotype: A formalin fixed specimen, TNS–AL–57781 in TNS (Department of Botany, National Museum of Nature and Science), from cultured strain PTG.

Type strain: PTG maintained in the Lake Biwa Environmental Research Institute, Japan.

Iconotype: Fig. 14.

Type locality: Lake Biwa, Shiga Pref., Japan.

Habitat: Plankton in lakes.

Etymology: the epithet name *foetida* reflects the bad smelling of this species.

***Pseudanabaena subfoetida* NIYAMA et TUJI sp. nov. (Figs 10, 11)**

Description: Trichomes solitary, straight, bright blue-green colored, with conspicuous constrictions at cross-walls, 2.1–2.9 μm wide, without mucilage or sheath, not attenuated nor differentiated at the ends, without calyptra, infrequently move. Cells isodiametric to longer than wide with rounded ends, bright blue-green, 2.5–8.5 μm long, ratio of width to length ca.0.9–4.0, with aerotopes at both ends of cells, sometimes with several small granules, clearly differentiated in centro- and chromatoplasmic regions. Cell division perpendicular to the longitudinal axis of a trichome. Trichomes separate between two neighboring cells or by fragmentation without necridic cells. Hormogonia trembling. Heterocytes are not known. Thallus has the extreme musty odor that comes from 2-methylisoborneol as same as *Pseudanabaena foetida*.

Holotype: A formalin fixed specimen, TNS–AL–58650 in TNS (Department of Botany, National Museum of Nature and Science), from cultured strain PS1306.

Type strain: PS1306 maintained in the Department of Botany, National Museum of Nature and Science, Japan.

Iconotype: Fig. 15.

Type locality: Lake Kasumigaura, Ibaraki Pref., Japan.

Habitat: Plankton in lakes.

Etymology: the epithet name *subfoetida* reflects the same bad smelling as and different cell morphology from *Pseudanabaena foetida*.

DISCUSSION

The cultured strains PTB and PTG have been cultivated for about 30 years, so their morphology may have been changed, for example their trichome width may have been thinner than the initial stage. Unfortunately, we have no data of trichome width or cell size of PTB and PTG at the beginning of their cultivation. As far as the photographs and descriptions of so called musty

odor producing strains established at the same period of PTG (MORII et al. 1982; YAMADA et al. 1986) are compare to PTG, trichome or cell width of those strains are 1.4 to 1.8 μm and somewhat wider than those of PTG. The cell width of Biwa1980 is discontinuously wider than those of PTG (Table 1, Fig. 17). Takemoto et al. (2012) point out that PTB has thin mucilaginous sheath but PTG has not any sheath when observed under the low temperature/low vacuum SEM, and that the cell width of PTG is 1.0–1.7 μm (mean value 1.35 μm) and is 1.3 times larger than that of PTB. It is unclear that the trichome width of PTG have become smaller or not after the 30 years cultivation, but PTG has kept producing 2-MIB for 30 years.

YAMADA et al. (1985) report that the optimum temperature, pH and light intensity for growth and 2-MIB production of the strain are 20–25 $^{\circ}\text{C}$, 8–9 and 1000–2000 lux, respectively, and its production rate of 2-MIB is directly proportional to its growth rate. IWASE & ABE (2010) also report that 2-MIB synthesis increases as cells grow in strain NIES-512. IWASE and ABE (2010) also point out that cells grow between 10–35 $^{\circ}\text{C}$ and the concentration of 2-MIB is highest at 25 $^{\circ}\text{C}$ but 2-MIB is significantly synthesized even at 10 $^{\circ}\text{C}$ and furthermore the concentration of 2-MIB is considerably high at exponential growth phase when cells are incubated under phosphate- and light-limiting conditions. Therefore the production of 2-MIB is considered to be one of the taxonomic characters for genus *Pseudanabaena*.

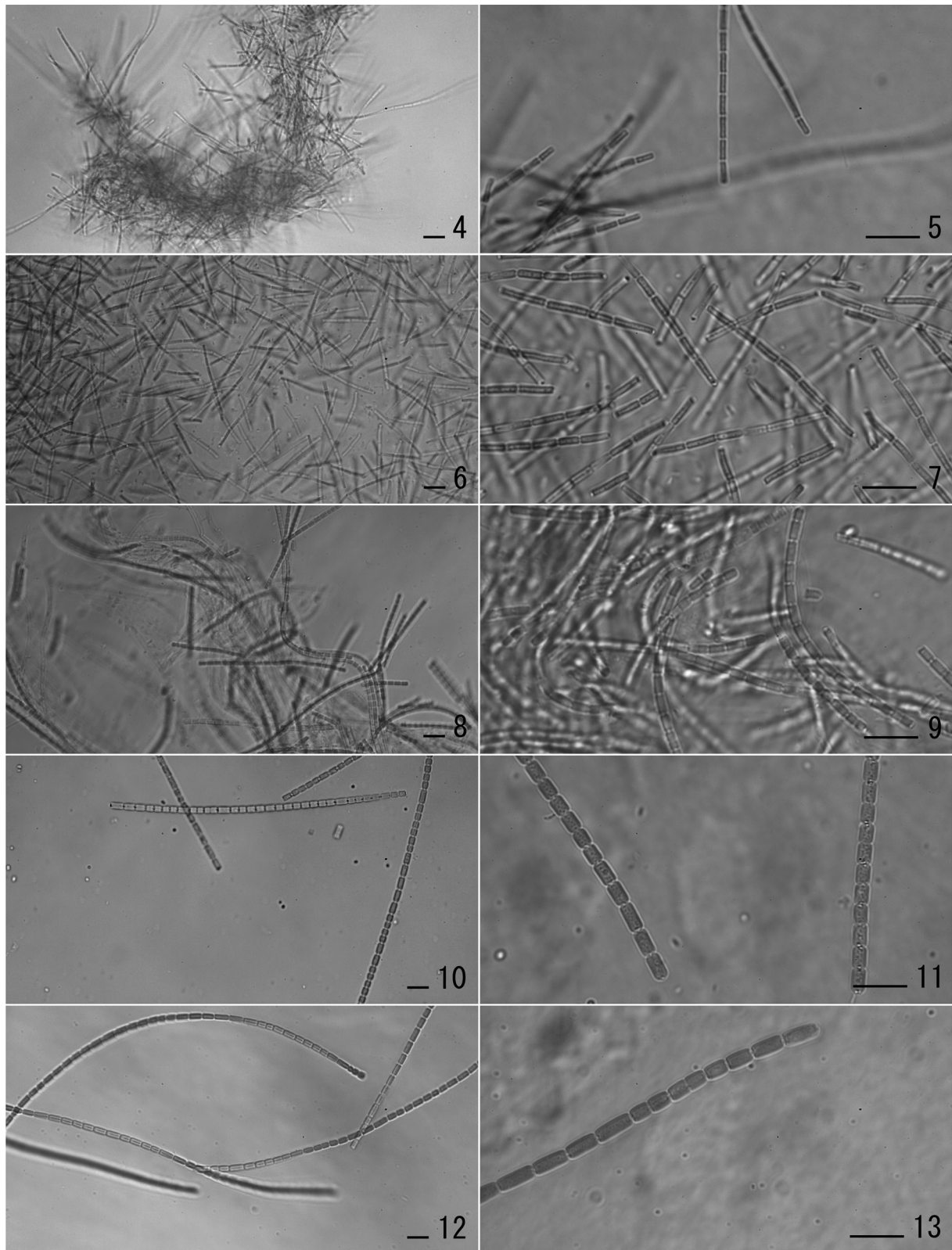
There are aerotopes at both ends of cells of *Pseudanabaena foetida* and *P. subfoetida*. Then the apical part of the terminal cells and the cross-walls of each cell seem to shine under the light microscope. The shape of aerotopes of *P. foetida* and *P. subfoetida* are helmet-shape to hemispherical as those of *P. galeata* BÖCHER (BÖCHER 1949). Cell content of *P. subfoetida* sometimes has several small granules and the cell content of trichomes seems to be separated with centro- and chromatoplasmic regions as *P. biceps* (BÖCHER 1946). The morphology of *P. foetida* (Figs. 6, 7, 14) looks like *P. galeata* and that of *P. subfoetida* (Figs. 10, 11, 15) looks like *P. biceps*, although the apical cells of *P. subfoetida* are rounded and not rounded-pointed at the ends. *P. biceps* and *P. galeata* are benthic in small and shallow brackish lakes with muddy bottom (BÖCHER 1946, 1949) or *P. galeata* is epiphytic or endogloeic and solitary trichomes rarely secondary in plankton (KOMÁREK & ANAGNOSTIDIS 2005). *P. foetida* and *P. subfoetida* are planktic in relatively large freshwater lakes. *P. biceps* shows vivacious creeping or gliding movement (BÖCHER 1946) and *P. galeata* also shows movement. On the other hand, *P. foetida* and *P. subfoetida* rarely show motility, and the trichomes infrequently slowly move forwards. Shorter trichomes consisting of two to several cells (hormogonia) sometimes tremble or move forwards faster than the longer ones but they do not glide or creep as species of

Table 1. Cell size of cultured strains PTB, PTG, PS1303, PS1306 and NIVA-CYA276/6, and that of Biwa1980 [(L/W) cell length/cell width; (std) standard deviation; n=14 in Biwa1980 and n = 50 in others].

strain	cell width		cell length		L/W		specimen no. TNS-AL	Accession number 16S rRNA
	min-max (μm)	mean μm (std.)	min-max (μm)	mean μm (std.)	min-max	mean (std.)		
PTB	0.9–1.3	1.03 (0.131)	2.5–7.1	4.47 (1.276)	2.5–7.0	4.39 (1.269)	57780	LC016774
PS1303	1.1–1.5	1.38 (0.113)	1.5–5.0	3.20 (0.947)	1.1–4.2	2.34 (0.735)	58649	LC016775
PTG	1.0–1.5	1.28 (0.153)	3.9–11.0	6.18 (1.803)	2.7–8.5	4.90 (1.477)	57781	LC016773
PS1306	2.1–2.9	2.42 (0.204)	2.5–8.5	4.61 (1.391)	0.9–4.0	1.94 (0.664)	58650	LC016779
Biwa1980	1.6–2.2	1.89 (0.175)	3.4–10.4	6.06 (1.968)	1.7–5.2	3.22 (0.994)	–	–
NIVA-CYA276/6	2.0–3.0	2.52 (0.282)	3.0–8.0	5.35 (1.489)	1.0–3.6	2.15 (0.666)	58651	LC016776

Table 2. Morphological characteristics of PTB, PTG, PS1303, PS1306 and NIVA-CYA276/6.

	PTB	PTG	PS1303	PS1306	NIVA-CYA276/6
trichome color	pale brownish green	bright blue-green	bright blue-green	bright blue-green	bright blue-green
sheath	–	–	+	–	–
motility of trichome	sometimes trembling	rarely trembling	sometimes slowly go forward	rarely trembling or slowly go forward	rarely slowly go forward
cell color	pale blue-green to olive blue-green	bright blue-green	bright blue-green	bright blue-green	bright blue-green
cell morphology	long and thin cylindrical	long and thin cylindrical	long and thin cylindrical	isodiametric to longer than wide	isodiametric to longer than wide
apical cell	long cylindrical with rounded end	long cylindrical with rounded end	long cylindrical with rounded end	isodiametric to cylindrical with rounded end	isodiametric to cylindrical with rounded end
poles aerotopes	+	+	+	+	+
constriction at cross-wall	+	+	+	+	+
musty odor	–	+	–	+	–



Figs. 4–13. Photos of five *Pseudanabaena* strains, PTB, PTG, PS1303, PS1306 and NIVA–CYA 276/6: (4, 5) PTB, *Pseudanabaena limnetica*; (6, 7) PTG, *P. foetida* sp. nov.; (8, 9) PS1303, *Pseudanabaena limnetica*; (10, 11) PS1306, *P. subfoetida* sp nov.; (12, 13) NIVA–CYA 276/6. Scale bars 10 μm.

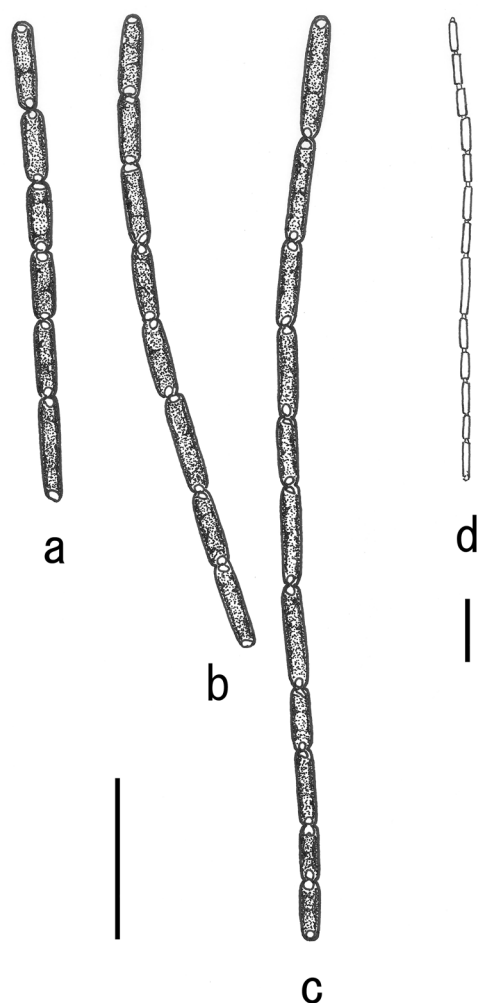


Fig. 14. Drawings of *P. foetida* sp. nov. Scale bars 10 μ m.

Geitlerinema or *Phormidium*. And *P. foetida* and *P. subfoetida* live in solitary and do not form fine mats nor cluster in small groups. These characteristics are also different from those of *P. galeata* or *P. biceps* and other members of subgen. *Illyonema* species (KOMÁREK & ANAGNOSTIDIS 2005). BÖCHER (1946, 1949) points out that *P. biceps* and *P. galeata* live on the mud which smells strongly of sulphureted hydrogen but he does not describe the smell of themselves.

From the result of our phylogenetic analysis, *P. foetida* and *P. subfoetida* are in a different clade of *P. galeata* and seem to be closely related to *Pseudanabaena* sp. dph15 and NIVA-CYA 276/6 (Fig. 16). The Chinese strain collected from Lake Dongqian, *Pseudanabaena* sp. dph15, is in the same clade of *P. foetida* and *P. subfoetida* (Fig. 16) and reported to have 2-MIB synthesis associated operon (HQ830028.1). NIVA-CYA 276/6 seems to be misidentified as *Pseudanabaena limnetica*, as it is morphologically and genetically different from *P. limnetica* (Figs. 12, 13 and 16). Although the morphology of NIVA-CYA 276/6 is very similar to that of *P. subfoetida*, NIVA-CYA 276/6 has no smell and is considered to be a distinct species



Fig. 15. Drawings of *P. subfoetida* sp. nov. Scale bars 10 μ m.

from *P. foetida* and *P. subfoetida*.

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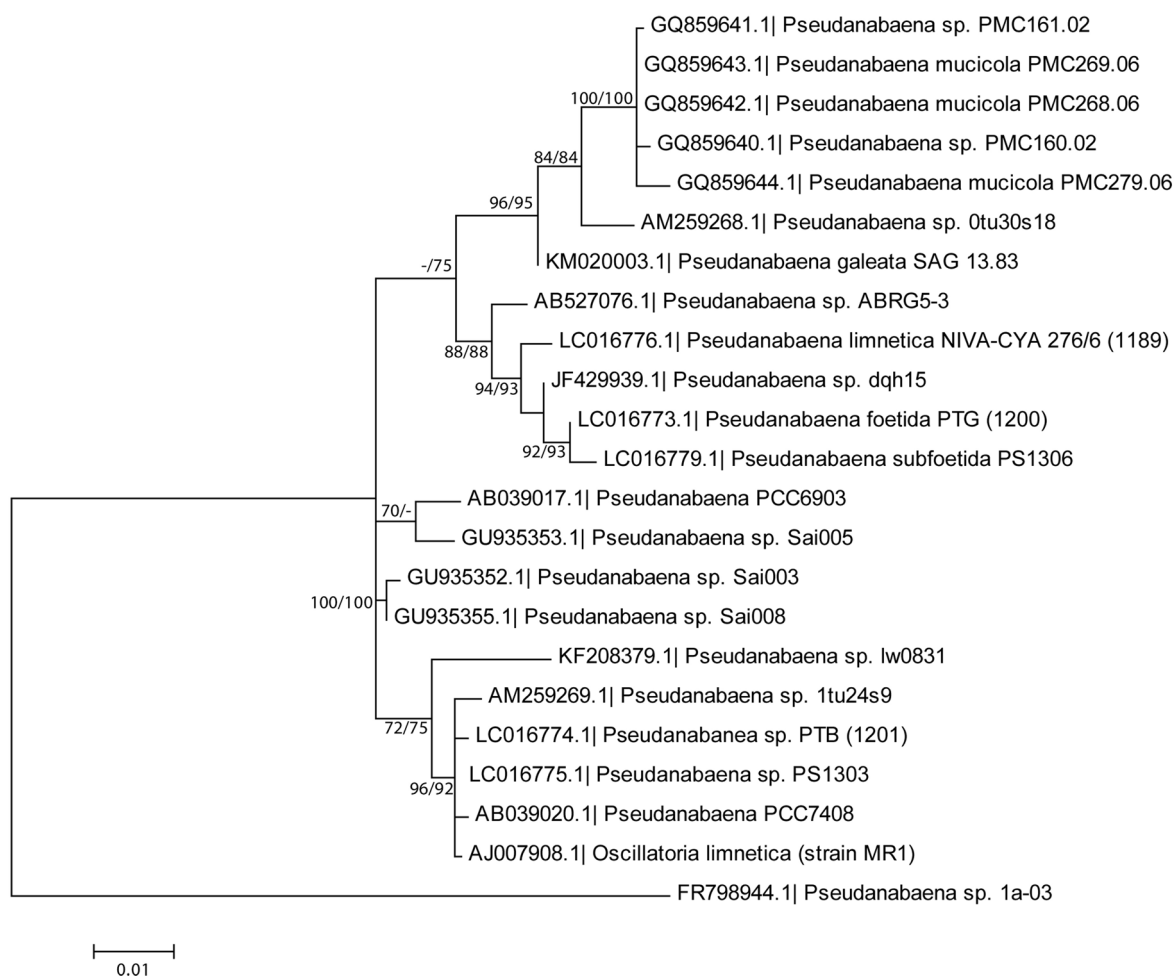


Fig. 16. Phylogenetic position of *Pseudanabaena foetida*, *P. subfoetida* and related taxa determined by Maximum Likelihood (ML) method using 16S rRNA gene. Accession numbers are followed by taxonomic names. Numbers at branches indicate NJ (Neighbor Joining)/ML bootstrap support values (only values higher than 60 are shown).

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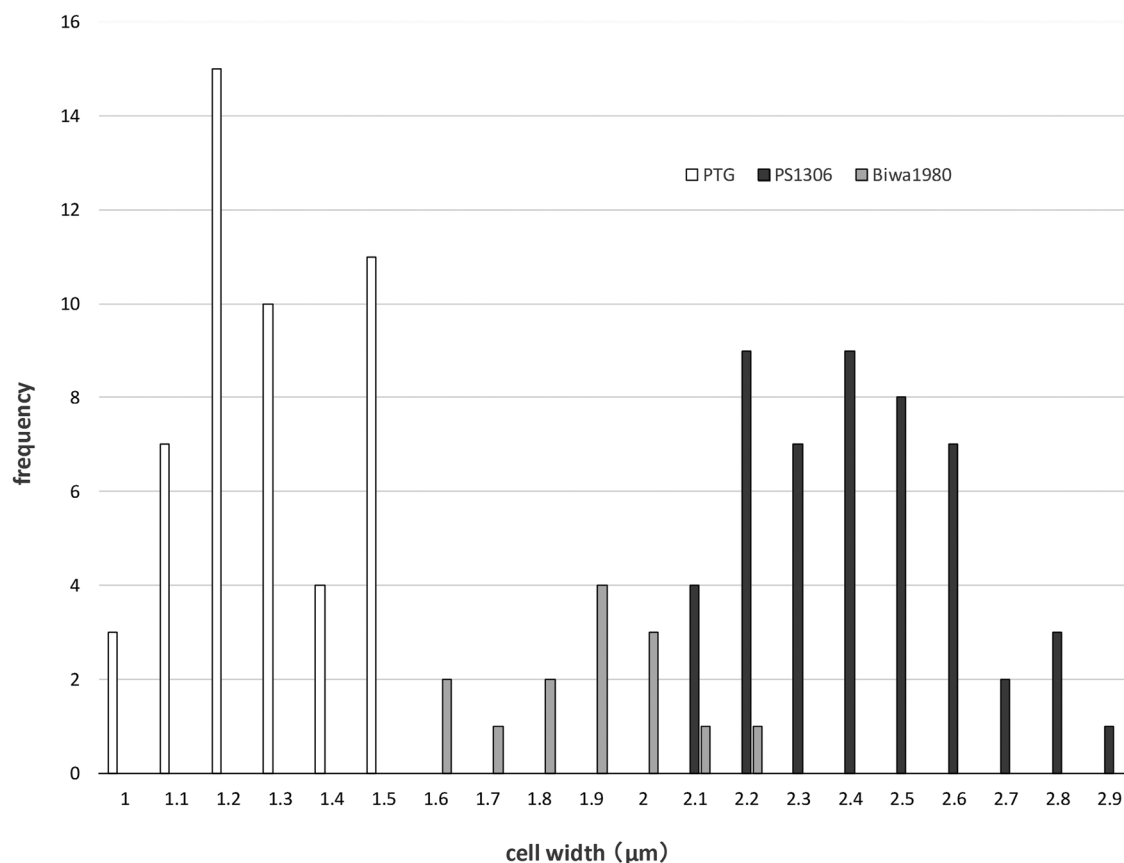


Fig. 17. Comparison of cell width distribution of cultured strains PTG, Biwa1980 and PS1306.

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Supplementary material

the following supplementary material is available for this article:

Fig. S1. Secondary structure of the 16S–23S rRNA ITS region of *P. foetida* (PTG).

Fig. S2. Secondary structure of the 16S–23S rRNA ITS region of *P. subfoetida* (PS1306).

This material is available as part of the online article (<http://fottea.czechphycology.cz/contents>)