A new tropical cyanobacterium *Pinocchia polymorpha* gen. et sp. nov. derived from the genus *Pseudanabaena*

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Abstract: Tropical cyanobacteria are an enigmatic group, often overlooked due to undersampling, yet expected to yield tremendous biodiversity. Many recent taxonomical studies have reported the existence of polyphyletic genera complexes in cyanobacteria (cryptogenera), where morphological coherent groups (often hardly distinguishable) have polyphyletic origins. In this paper, we employed a combined genetic and phenotypical approach to describe some newly isolated *Pseudanabaena*–like cyanobacteria from a lake in Vietnam. We found that two studied strains belonged to the monophyletic clade outside of the *Pseudanabaena sensu stricto*, thus it may be designed as a new genus, which has been called *Pinocchia*. However, there are only minor morphological differences from the other *Pseudanabaena* species. Thus, it may be considered as example of the cryptic genus. Moreover, it is additional evidence for a polyphyletic origin of the genus *Pseudanabaena.*

Key words: 16S rRNA, 16S–23S ITS, cryptogenus, new species

Introduction

Cyanobacteria are one of the most important and the oldest primary producers capable of oxygenic photosynthesis, which can be found in nearly all environments from polar to tropical areas (Whitton & Potts 2000).

The biodiversity of cyanobacteria has been studied more extensively in temperate zones, which may be demonstrated by the number of published papers involved in cyanobacterial diversity. Web of Knowledge database contains 9066 papers involved in diversity of cyanobacteria (database searched 26th November 2014), from which only 280 papers investigated tropical cyanobacterial diversity. Moreover, new cyanobacterial isolates retrieved from tropical habitats often lead to description of new taxa (e.g. Fiore et al. 2007; Hašler et al. 2014; Vaccarino & Johansen 2011 and many others). Thus, the tropical biodiversity seems to be largely underestimated. A reason for that may lie in undersampling and very high, yet enigmatic, cyanobacterial diversity in tropical habitats.

The taxonomy and systematics of cyanobacteria has been undergoing substantial changes due to an employment of molecular markers (mainly 16S rRNA). One of the chief concerns arises from the important problem of polyphyletic genera. For example, it has been noted that almost all Geitlerian genera (after Geitler 1932) have been confirmed using molecular markers. On the other hand, most of genera appear to be polyphyletic (Komárek 2010). Such a polyphyly might be extreme. For instance, Dvořák et al. (2014a) noted 12 lineages within the genus *Synechococcus sensu lato*, which suggest frequent convergence in cyanobacteria. What might be the reasons for such entangled evolutionary relationships? Dvořák et al. (2014a) suggested that homologous recombination and horizontal gene transfer within local gene pools *sensu* Polz et al. (2013) provide a space for convergence of phenotypes, and therefore an existence of polyphyletic groups.

The genus *Pseudanabaena* (Lauterborn 1915) is widely distributed. According to Komárek & Anagnostidis (2005), *Pseudanabaena* represents filamentous, non–heterocytous, sheathless, cyanobacteria, usually with thin trichomes (up to 3.5 mm), and often with cells connected by hyaline bridges. Members of the genus *Pseudanabaena* differ in the shape of apical cells and the presence of aerotopes, dividing the genus into three subgenera (*Ilyonema*, *Skujanema*, *Pseudanabaena*). There are likely several polyphyletic lineages within this morphotype, and likely more masked by cryptic diversity (Acinas et al. 2009; Dvořák et al. 2014a). However, no extensive revision has been performed.

16S–23S internal transcribed spacer (ITS) is commonly used molecular marker as an addition to 16S rRNA sequence, because it offers higher resolution

under the species level. It might be used for phylogeny reconstruction or for an estimation of secondary structures of several semi–conservative helices (e.g. BOYER et al. 2001, 2002).

In this paper, we will present a new genus of *Pseudanabaena*–like cyanobacteria from periphyton and plankton of the lake Hồ Đâu Co in Vietnam using combination of molecular, ecological and morphological data.

MATERIALS AND METHODS

Strain isolation. Samples were collected from plankton and periphyton of a lake Hồ Đâu Co, province Đồng Nai, Vietnam (GPS: 11° 28.336'N, 107° 20.462'E, conductivity: 44 µS.cm^{-1}, temperature 28 °C and pH 5.47) on 10th September 2010. Strains were isolated from fresh samples using standard isolation techniques. Two cultures (E5 and E10) were maintained in 90 mm Petri dishes under the following conditions: temperature 26±1 °C, illumination 20 µmol.m^{-2}.s^{-1}, light regime 12h light/12h dark, and liquid Zehnder medium (Z medium; STAUB 1961).

Morphological assessment. Morphology of the strains was analyzed using a light microscope Zeiss AxioImager (objectives EC Plan–Neofluar 40×/1.3 N.A., oil immersion, DIC; Plan–Apochromat 100×/1.4 N.A., oil immersion, DIC) with a high resolution camera (AxioCam HRc 13MPx). During morphological evaluation strains, the following characters were assessed: cell shape, terminal cells, cell dimensions, reproduction, sheaths, and granulation of cells.

Strain E5 was cultivated under different physical–chemical parameters in order to describe morphological variability. The strain was cultivated at 16 °C and 26 °C for 14 days in conditions as stated above. For each temperature, four media were prepared: standard Z medium as a control, nitrogen free (N–NO_3^-) medium, phosphorus free (P–HPO_4^{2-}) medium, and both nitrogen and phosphorus free medium. Measurements were performed on first five cells of 30 filaments from each combination of culture conditions (temperature and nutrients). Analysis of variance (one–way ANOVA) with Tukey's pairwise comparison was performed in PAST 3 (HAMMER et al. 2001).

PCR amplification and sequencing. Genomic DNA was extracted from approximately 50 mg of fresh biomass using an UltraClean Microbial DNA Isolation Kit (MOBIO, Carlsbad, CA, USA) following the manufacturer's manual. DNA quality and consistence was checked on GelRed (Biotinum Inc., Hayward, CA, USA) stained 1.5% agarose gel. DNA was quantified using the NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

The partial 16S rRNA and the whole 16S–23S ITS were obtained using PCR amplification with primers: forward P2 (5'–GGGGAATTTTCCGCAATGGG–3'), and reverse P1 (5'– CTCTGTGTGCCTAGGTATCC–3') previously described in BOYER et al. (2002). The PCR reaction, with a total volume of 40 µl, contained: 17 µl of sterile water, 1 µl of each primer (0.01 mM concentration), 20 µl FastStart PCR Master (Roche Diagnostics GmbH, Mannheim, Germany), and 1 µl of template DNA (50 ng.**µl**$^{-1}$). PCR amplification was performed with the conditions used before in DVOŘÁK et al. (2012). PCR products were cloned using StrataClone PCR Cloning kit (Agilent Technologies, Stratagene Product Division, La Jolla, CA, USA) following manufactures manual with modification described in DVOŘÁK et al. (2012).

Plasmids were commercially sequenced using primers M13f and M13r. Moreover, two additional internal sequencing primers were added P5 (5'–TGTACACACCGCCCGTC–3'), and P8 (5'–AAGGAGGTGATCCAGCCACA–3') (BOYER et al. 2001, 2002). Sequences were assembled and proofread in Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/), accession numbers: KP640604 – KP640613.

Phylogenetic analyses. The most similar sequences of 16S rRNA were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/) and identified using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Reference sequences of *Pseudanabaena* were added. Multiple sequence alignment was performed in MEGA 6 (TAMURA et al. 2013) using Muscle algorithm (EDGAR 2004). The tree was rooted to the outgroup *Gloeobacter violaceus*. The most appropriate model for Bayesian inference was determined in jModelTest 0.1.1 (POSADA 2008) based on both the Bayesian and the Akaike Information Criterion as following: F81 model with gamma distributed variation across sites. 50% majority consensus tree was constructed in MrBayes 3.2.3 (RONQUIST & HUELSENBECK 2003). Two separate runs were performed, each with 3 heated and 1 cold chains for 8,000,000 generations. The sampling frequency was each 1000th generation. 25% trees were discarded as burn–in. Maximum likelihood analysis was performed in RaxML 8.0.2 (STAMATAKIS 2006) with a GTRGAMMA model. Maximum parsimony analyses were performed in PAUP* 4.0b10 (SWOFFORD 2002), gaps were treated as missing data. All analyses were tested using bootstrapping with 1000 replicates.

The secondary structures of D1–D1´ helix and Box–B helix ITS regions were predicted with the Mfold web server version 3.5 (ZUCKER 2003) with temperature set to default (37 °C).

RESULTS

***Pinocchia* DVOŘÁK, JAHODÁŘOVÁ et HAŠLER gen. nov.**
Description: Trichomes solitary or in colony (mats), sheath thin, colourless and facultative. Trichomes straight or bent, constricted at cross–walls, motile, 2 to 34 cells. Cells with distinctive centro– and chromatoplasma, cell length significantly varies within filament, cells connected with hyaline bridges, cell content homogenous or with small granules. Terminal cell often elongated and differentiated. Reproduction by disintegration into short filaments (hormogonia) without help of necridic cells.

Etymology: Generic epithet refers to the elongated cells, especially to terminal cells. Pinocchio is a popular character from an Italian fairy tale (by Carlo Collodi), who had longer nose when telling lies.
Type species: *Pinocchia polymorpha* DVOŘÁK, JAHODÁŘOVÁ et HAŠLER

***Pinocchia polymorpha* DVOŘÁK, JAHODÁŘOVÁ et HAŠLER sp. nov.**
Description: Trichomes solitary or in colony (mats), sheath thin, colourless and facultative. Trichomes straight or bent, constricted at cross–walls, motile, maximally 75 µm long (2 to 34 cells). Cells blue–green, with distinctive centro– and chromatoplasma, facultative polar aerotopes, cells connected with hyaline bridges, cell content homogenous or with small granules, 1.09–2.86 µm wide and 1.28–8.63 (12) µm long, cell length significantly varies within filament. Terminal cell often elongated up to 12 µm, pointed, conical, or rounded. Reproduction by disintegration into short filaments (hormogonia) without help of necridic cells.

Etymology: A species name refers to the fact that a cell length is highly polymorphic in the filaments.
Type locality: Lake Hồ Đâu Co, province Đồng Nai, Vietnam (GPS 11° 28.336'N, 107° 20.462'E), *coll.* E. S. Gusev 10th September 2010.
Habitat: Plankton and periphyton of freshwater tropical lake.
Iconotype: Fig. 1a.
Holotype: Holotype OLM Botany 24: Lichenes and others No. 9219, dried sample is deposited in Regional Museum in Olomouc, Czech Republic. Type strain:

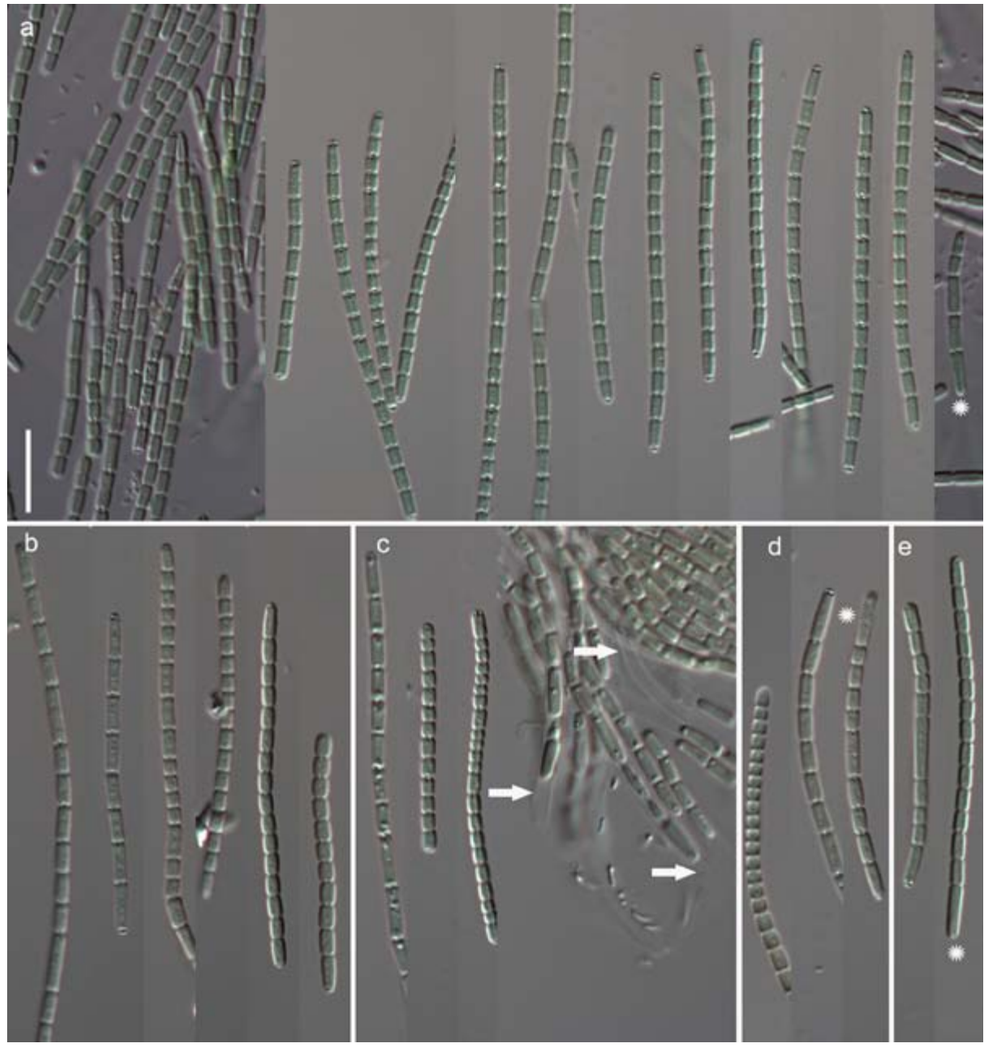

Fig. 1. *Pinocchia polymorpha* sp. nov.: (a) strain E 5 in full Z medium and 26 °C; (b) the same in medium without nitrogen; (c) the same in medium without phosphorus; (d) the same strain in medium withouth both N and P; (e) the same strain in full Z medium and 16 °C. Scale bar 10 µm, asterix show elongated terminal cells, arrow show colourless sheath.

UPOC 62–P/2013, deposited at the culture collection of Department of Botany, Palacký University in Olomouc,Czech Republic.

Morphological observations

Cell length and width varied significantly among filaments measured in different culture conditions. The maximum cell length (8.63 µm) was observed in medium without nitrogen in 26 °C. On the other hand, the shortest cell (1.28 µm) was observed in medium without phosphorus in 16 °C. The widest cell (2.86 µm) in medium without nitrogen and the narrowest cells (1.09 µm) occurred in standard Z medium (in 26 °C), and without phosphorus (in 26 °C) respectively. An ANOVA test revealed significant differences ($p < 0.01$) only among cultures maintained in 26°C (Table 1). Tukey's test showed significant difference ($p < 0.01$) in variance of cell length/width ratio among all compared measurements except control culture versus culture without phosphorus, and control versus culture without both nitrogen and phosphorus. Polymorphism in cell length is demonstrated in Fig. 1. Filaments with short cells and with both short and long cells were frequent particularly in cultures limited by nutrients and low temperature (Fig. 1b).

Phylogeny

16S rRNA phylogeny based on Bayesian inference revealed that strains of *Pinocchia polymorpha* form a monophyletic lineage among other filamentous cyanobacteria of genera *Leptolyngbya*, *Trichocoleus*, *Pseudanabaena*, and *Nodosilinea* (Fig. 2). The *Pinocchia* clade had 100 bootstrap and posterior probability support. Five closest relatives identified by BLAST were *Leptolyngbya* sp. Kovacik 1990/37 (EU528671), and four strains of *Trichocoleus desertorum* (described by MÜHLSTEINOVÁ et al. 2014). However, none of these strain belonged to the same monophyletic cluster of *Pinocchia*.

The *Pseudanabaena sensu stricto* formed a monophyletic clade near a root of the phylogenetic tree (Fig. 2), thus distant to *Pinocchia*. There was also noticeable variability within the clade of *Pinocchia*. Clones were divided into two groups, however ambiguous, with low bootstrap support.

Table 1. Results of ANOVA analysis and Tukey's test for both studied temperatures. Right upper triangle represents probabilities and lower left triangle represents Studentize Range Statistics Q [(C) control medium, (N) medium without nitrogen, (P) medium without phosphorus, and (NP) medium without phosphorus and nitrogen].

Tukey's test 16 °C	C	N	P	NP
C		0.4404	0.6064	0.6723
N	2.114		0.9936	0.04327
P	1.742	0.3719		0.08497
NP	1.595	3.709	3.337	

ANOVA: p = 0.0333, F = 2.924

Tukey's test 26 °C	C	N	P	NP
C		$7.72e^{-6}$	0.3865	0.6924
N	9.776		$8.23e^{-6}$	$7.72e^{-6}$
P	2.243	7.532		0.03684
NP	1.549	11.32	3.792	

ANOVA: p = $2.33e^{-15}$, F = 25.2

Secondary structures of 16S–23S ITS

The strain E5 contained likely two ribosomal operons. Although it contained no tRNAs, two of five clones contained identical inserts at position 553 to 567. The topology of D1–D1' and Box–B helices were identical among all sequenced clones (Fig. 3). Strain E10 had at least two ribosomal operons, one containing tRNA coding isoleucine and one with missing tRNA. Estimated secondary structures of the ITS of *Pinocchia* strain E10 exhibited considerable variability and significantly differed among clones (Fig. 3). However, a pattern of D1–D1' as well as Box–B helices was congruent within the clones that contained and missing tRNA. An operon with tRNA had the D1–D1' significantly longer (118 bp) than the operon without tRNA with size of 53 bp. They also exhibited significantly different topology (Fig. 3). Moreover, D1–D1' helices were largely dissimilar between studied strains considering both length and shape. On the other hand, Box–B helices were similar among clones and also between strains.

DISCUSSION

Molecular techniques have allowed researchers to uncover reticulate evolutionary histories among cyanobacteria (see KOMÁREK 2010 for review). Most of the traditional Geitlerian genera have been shown polyphyletic, resulting in an assumption of the existence of cryptogenera (KOMÁREK et al. 2014). Cryptogenera are evolutionary lineages among cyanobacteria which possess similar (often unrecognizable) morphology, but they are usually polyphyletic based on analysis of phylogeny usually of 16S rRNA. It has been suggested that this phenomenon is connected with frequent convergent evolutionary events among cyanobacteria (DVOŘÁK et al. 2014a).

Such an example of a cryptogenus is presented in this paper. The newly established genus *Pinocchia* would be identified to the genus *Pseudanabaena* based solely on morphology as reviewed in KOMÁREK & ANAGNOSTIDIS (2005). However, phylogenic reconstruction using 16S rRNA sequence data showed *Pseudanabaena* to be polyphyletic. A clade of *Pinocchia* is far from *Pseudanabaena sensu stricto* clade (with type species *P. catenata*) situated near the root of the tree (Fig. 2).

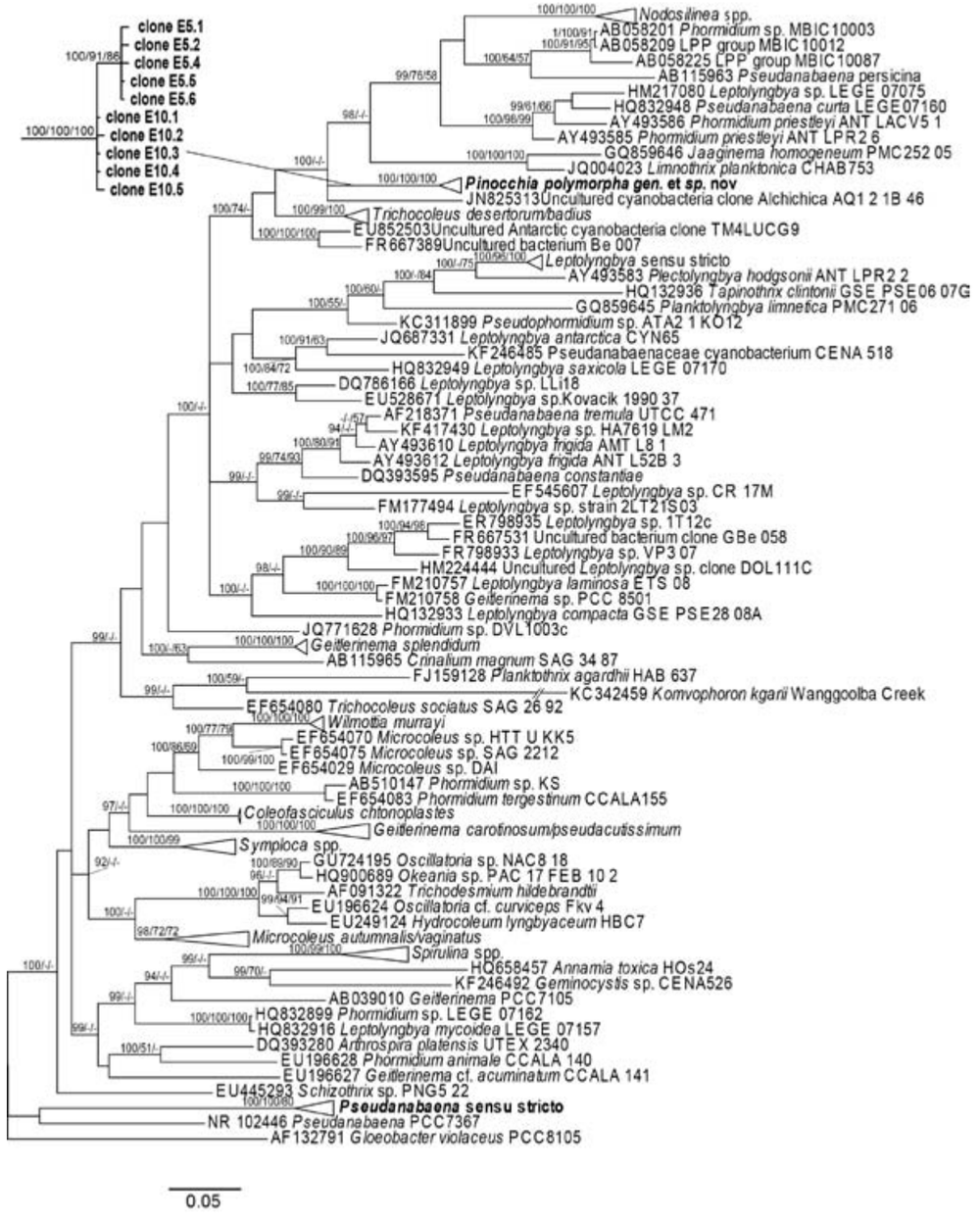

Fig. 2. A phylogenetic reconstruction based on 16S rRNA using Bayesian inference. Studied strains and *Pseudanabaena sensu stricto* are in bold. Supports at the nodes (Bayesian inference/maximum likelihood/maximum parsimony) represent only bootstrap values >50 and posterior probabilities >90. A collapsed cluster of *Pinocchia* is unfolded aside of the tree.

Our 16S rRNA tree largely corresponds to other recently published phylogenies (e.g. PERKERSON et al. 2011; DAGAN et al. 2013; KOMÁREK et al. 2014) in a sense that there are formed same monophyletic clusters representing genera (e.g. *Wilmottia*, *Nodosilinea*, and *Pseudanabaena sensu stricto*). A position of higher

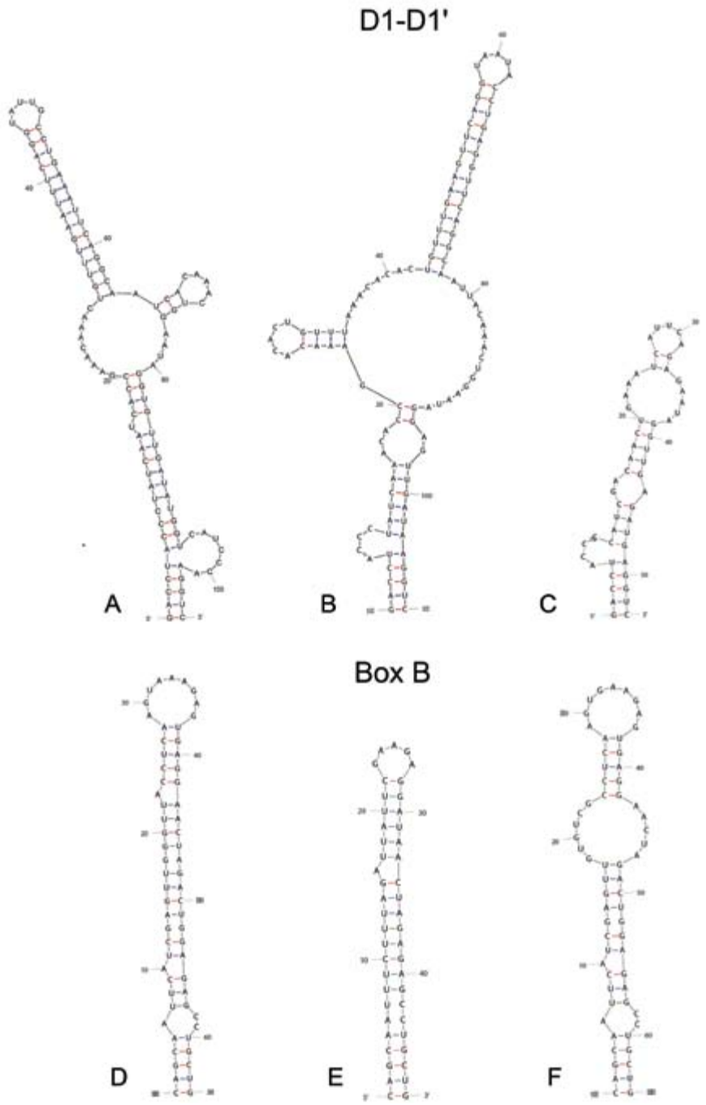

Fig. 3. Estimated 16S–23S ITS secondary structures D1–D1' and Box–B helices of *Pinocchia*: (A, D) for clones E5.1, E5.2, E5.4, E5.5, E5.6; (B, E) for clones E10.1, E10.3; (C, F) for clones E10.2, E10.4, E10.5

taxonomical groups seems to be more reticulate. For instance, the family Gomontiellaceae *sensu* KOMÁREK et al. (2014) is not monophyletic in our phylogenetic analysis (Fig. 2) because *Crinalium* is not in the same clade as *Komvophoron*. Further, the order Synechococcales also appears to be polyphyletic too (Fig. 2), because members of family Pseudanabaenaceae (e.g. *Pseudanabaena*) and Leptolyngbyaceae (e.g. *Nodosilinea*) do not form related clusters. However, none of these studies (including ours) had complete taxon sampling 16S rRNA sequences, thus they cannot present complete view on cyanobacterial evolution.

Komárek et al. (2014) has recently proposed a new classification of higher taxonomical units in cyanobacteria. Based on their criteria and phylogenetic position, *Pinocchia* may be classified to the family Leptolyngbyaceae, order Synechococcales.

Based on morphological criteria *Pinocchia* is similar to the genus *Pseudanabaena*, and particularly, to its subgenus *Ilyonema sensu* KOMÁREK & ANAGNOSTIDIS (2005), which is also characterized by polar aerotopes and shape of terminal cells. The most similar morphotype to *Pinocchia* from the subgenus *Ilyonema* is *I. galeata*, but *Pinocchia* possesses high variability of cell length among cells within a filament, which is rather characteristic for *P. catenata* (KOMÁREK & ANAGNOSTIDIS 2005, Fig. 1). Furthermore, *Pinocchia* slightly differs from *I. galeata* with prolonged, pointed and sometimes conical terminal cell, although *I. galeata* has sometimes rounded–conical terminal cell (KOMÁREK & ANAGNOSTIDIS 2005). *Pinocchia* is also slightly wider (2.84 µm) than *I. galeata* (2.7 µm). Therefore, together with its tropical origin and phylogenetic position, we suggest that *Pinocchia* may be designed as a new monospecific genus.

16S–23S ITS secondary structures are powerful tools for identification of taxa at or below the species level (e.g. BOYER et al. 2001; HAŠLER et al. 2014; OSORIO–SANTOS et al. 2014 and many others). We found high variability among clones and between both isolated strains (Fig. 3). The difference between D1–D1' helices and Box–B helices among two strains was significant (Fig. 3.), which might suggest existence of two cryptic species in *Pinocchia*, because a morphology was congruent between strains. However, we will need to find more strains in future to resolve these enigmatic relationships within *Pinocchia*.

Significant differences were found among cultures of *Pinocchia* maintained in 26 °C in media lacking phosphorus, nitrogen or both. Therefore, nitrogen and phosphorus (or N/P ratio) significantly influence cell length (Table 1), more likely due to influence of intensity of cell division, which has been shown before in other microalgae (LUKAVSKÝ 1973; POULÍČKOVÁ et al. 2001; DŘÍMALOVÁ & POULÍČKOVÁ 2003; HAŠLER et al. 2003; HAŠLER & POULÍČKOVÁ 2010). Higher temperatures seem to be more convenient for *Pinocchia*, probably due to the isolation from the tropical lake. Thus, we suppose that results retrieved from 26 °C resemble more natural conditions. Although these morphological experiments are not at all exhaustive, we do not suppose that future investigation (of pH, light or other parameters) will reveal new morphological feature, which will be able to distinguish *Pinocchia* from the other *Pseudanabaena*–like cyanobacteria more reliably. Such a little morphological plasticity for identification of newly erected genera has been observed also in polyphyletic genera with very simple morphology such as *Synechococcus* (DVOŘÁK et al. 2014b) and *Leptolynbya* (OSORIO–SANTOS et al. 2014).

Taken together, *Pinocchia* represents another example of a cryptogenus derived from polyphyletic *Pseudanabaena*, which seem to be very frequent among cyanobacteria (KOMÁREK et al. 2014) and exemplified in the genus *Synechococcus* (DVOŘÁK et al. 2014a,b). The cryptogenus is very important consideration for taxonomist and ecologist interested in cyanobacteria, because only morphological criteria appear to be unsufficient to recognize distant evolutionary lineages.

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Supplementary material

the following supplementary material is available for this article:

Table S1. Taxa with accession numbers from GenBank database used in the phylogenetic analysis.

Dataset 1. Multiple sequence alignment used in the 16S rRNA phylogenetic analysis in nexus format.

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

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