CRYPTIC DIVERSITY OF CYANOBACTERIA

DOCTORAL THESIS FOR A DOCTORAL DEGREE

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You can't even begin to understand biology,
you can't understand life, unless you understand what
it's all there for, how it arose-and that means
evolution.
Richard Dawkins

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DECLARATION

I declare that this Ph.D. thesis has been written solely by myself. All the sources quoted in this work are listed in the Reference section. All published results included in this thesis have been approved with the help of mentioned co-authors.

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ABSTRACT

Cyanobacteria emerged on Earth approximately 3.5 billion years ago. They are the major contributor to global biogeochemical cycles and are ancestors of today's chloroplast of higher plants. Cyanobacteria thrive across many habitats. However, due to their enormous diversity, taxonomy remains in chaos and most taxa are uncharacterised. In this thesis, I have investigated the morphological and molecular features of three new cyanobacterial genera from tropical areas (*Pinocchia*, *Onodrimia*, and *Elainella*). Moreover, I aimed to identify gaps in cyanobacterial systematics and cooperated on a book chapter focused on cyanobacterial diversity.

Recent investigations revealed that *Leptolyngbya* and *Pseudanabaena* are extensively polyphyletic cyanobacterial taxa. Both species-rich genera have very simple morphology, create thin filaments and occur mostly in aquatic and terrestrial habitats. However, due to polyphyly, *Leptolyngbya* was divided into several genera such as *Oculatella* and *Nodosilinea*. *Pseudanabaena* still waits for its revision. I identified two unknown genera similar to *Leptolyngbya* and *Pseudanabaena*. They were named *Onodrimia* and *Pinocchia*. Both of them are simple filamentous cyanobacteria with tropical origin. The phylogenetic study of 16S rRNA clearly distinguished *Pinocchia* and *Onodrimia* as monophyletic clades distant from *Pseudanabaena* and *Leptolygbya sensu stricto*. I endeavoured to find the unique apomorphic character which would strictly define newly described genera. *Pinocchia* is a cryptic genus but some trichomes possess prolongated, pointed and sometimes conical apical cell. For confirmation of this morphological apomorphy, I applied nutrient experiment. The results did not show any significant difference between standard and experimental cultures. On the other hand, *Onodrimia* possess a peculiar type of reproduction (it reproduces by tree-like hormogonia tufts). This character could be considered as apomorphy.

Another new tropical cyanobacterium *Elainella* is morphologically very similar to *Pseudophormidium*. *Pseudophormidium* is poorly studied lineage, with unclear morphology. For confirmation of this new genus, I used 16S rRNA phylogeny, morphological data and also whole genome assessment. The total length of the draft genome was 8 702 141 bp. *Elainella* genome contains genes for atmospheric nitrogen fixation and also 10 potentially biosynthetic genes clusters, but only 2 were assigned as already known genes with the ability to produced cytotoxic metabolites. A dated phylogeny

of 69 orthologous genes revealed the origin of *Elainella* and its sister's clade *Leptolyngbya* sp. JSC-1. They diverged probably 2.24–2.47 BYA.

Gene for the small subunit of the prokaryotic ribosome (16S rRNA) is a widely used marker for bacterial phylogeny. There are hundreds of thousands of sequences of this gene in molecular databases. But it is widely known that morphological characters do not perfectly correspond with molecular phylogeny. Therefore, I wanted to describe this evident paradox of increasing data yet poor phylogenetic resolutions by PTP (Poisson Tree Process) algorithm. I aligned 10037 sequences of 16S rRNA and I used PTP delimitation to a defined quantity of species in GenBank database. PTP identified just 2741 PTP-defined species, but 51% PTP-defined species were assigned to uncultured samples. I also proposed possible suggestions which could improve this difficult situation.

ABSTRAKT

Sinice (Cyanobacteria) se na Zemi poprvé objevily asi před 3,5 miliardami let. Patří mezi hlavní organismy ovlivňující světové biogeochemické cykly a jsou prapředky chloroplastů dnešních vyšších rostlin. Sinice jsou schopny přežít v nejrůznějších prostředích. Díky jejich obrovské rozmanitosti zůstává velká většina taxonů systematicky nezařazena. V této práci jsem studovala morfologické i molekulární znaky tří nových rodů sinic z tropických oblastí (*Pinocchia, Onodrimia* a *Elainella*), také jsem se zaměřila na mezery v systematice sinic. Spolupracovala jsem na knižní kapitole pojednávající o diverzitě cyanobakterií.

Recentní studie odhalily, že rody jako je *Leptolyngbya* nebo *Pseudanabaena* jsou výrazně polyfyletické. Oba druhově bohaté rody se značí jednoduchou morfologií, vytvářejí tenká vlákna a obývají jak vodní, tak i terestrické habitaty. Nicméně, na základě polyfyletického původu byla *Leptolyngbya* rozdělena do několika nových linií, jako je *Oculatella* nebo *Nodosilinea*. *Pseudanabaena* na svou revizi stále čeká. Podobně jsem i já popsala dva neznámé rody odvozené z rodů *Leptolyngbya* a *Pseudanabaena*. *Pinocchia* a *Onodrimia* jsou jednoduché vláknité cyanobakterie tropického původu. Fylogeneze založená na genu 16S rRNA jednoznačně odlišila monofyletické linie rodu *Pinocchia* a *Onodrimia* od *Pseudanabaena* a *Leptolyngbya sensu stricto*. Snažila jsem se najít morfologickou apomorfii, která by striktně definovala nově popsané rody. V případě rodu *Pinocchia* se jedná pravděpodobně o kryptický rod, avšak některé z trichomů měly výrazně prodlouženou, špičatou, někdy kuželovitou apikální buňku. Pro ověření, zda se skutečně jedná o apomorfii, jsem použila nutriční experiment. Nebyl však nalezen signifikantní rozdíl, který by odlišoval kontrolní a experimentální kulturu. Na druhou stranu, *Onodrimia* je charakteristická zvláštním typem reprodukce (produkuje hormogonia v takzvaných stromečkovitých formacích). Tento charakteristický znak lez považovat za apomorfii.

Další nově popsaná tropická sinice *Elainella* je velice podobná sinici rodu *Pseudophormidium*. *Pseudophormidium* je málo prostudovaný taxon s nejasnou morfologií. Pro definici tohoto nového rodu jsem použila jak fylogenezi na základě 16S rRNA genu a morfologické informace, tak i celo-genomový přístup. Celková délka genomu byla 8 702 141 párů bází. V genomu rodu *Elainella* jsem identifikovala geny sloužící k fixaci vzdušného dusíku a deset potencionálně biosyntetických genových klastrů, pouze dva z nich zřejmě produkující cytotoxické metabolity. Z datované fylogeneze na základě 69 ortologních genů jsme se pokusila odhalit vznik rodu *Elainella* a

kmene *Leptolyngbya* sp. JSC-1. Tento klád se pravděpodobně diversifikoval asi před 2,24–2,47 miliardami let.

Gen kódující malou podjednotku ribozomu (16S rRNA gen) je hojně užívaný marker pro fylogenezi bakterií. V databázích můžeme najít tisíce sekvencí tohoto genu. Na druhou stranu, je obecně známo, že morfologická charakteristika neodpovídá molekulární fylogenezi. Proto jsem chtěla tento zjevný paradox, neustále se zvětšujícího množství dat a nesprávného fylogenetického určení, prostudovat pomocí algoritmu PTP (Poisson Tree Process). Alignovala jsem 10 037 sekvencí genu 16S rRNA a pomocí PTP algoritmu jsem delimitovala počet druhů sinic v databázi GenBank. PTP identifikovalo pouze 2741 PTP-definovaných druhů, avšak 51 % PTP-druhů bylo nekultivovatelných. Také jsem se zaměřila na faktory, které by mohly tuto nelehkou situaci taxonomie sinic zlepšit (např. nově popsané taxony by měly být vždy charakterizovány autapomorfií, metagenomická data by měla být vyhodnocována pečlivěji, revize by měly být prováděny pouze s robustním a správným výběrem taxonů).

CONTENTS

Part 1

GENERAL INTRODUCTION

1

GENERAL INTRODUCTION

Cyanobacteria (Cyanoprokaryota, Cyanophyceae, Blue-green algae) are an ancient group of autotrophic prokaryotes with the ability to produce oxygen by photosynthesis. They colonize a myriad of ecosystems (e.g. marine, freshwater, terrestrial, etc.), they occur in extreme biotopes such as deserts, polar regions or hot springs. The origin of cyanobacteria is dated to a period of Archean 2.7–3.5 (3.8) billion years ago (BYA) (Sleep 2010; Blank & Sanchez-Baracaldo 2010; Schopf 2001). The oldest cyanobacterial fossil record was found in Apex Cherts (Western Australia), from 3.3–3.5 BYA (Schopf & Packer 1987). According to molecular, physiological, paleontological and geochemical data, Tomitani et al. (2006) estimated the origin of akinetes and heterocytes to 2.45 to 2.1 BYA. As primary producers, cyanobacteria have enormous influence on the global ecosystem (marine cyanobacteria produce 25% of total oxygen) and they can also affect the evolution of other organisms (Flombaum et al. 2013). The Great Oxidation Event occurred between 2.45 and 2.47 BYA (Kopp et al. 2005), during this period cyanobacteria started to produce a tremendous amount of oxygen. It was probably one of the first global disasters which led to mass extinction of many organisms. Possessing the ability to fix atmospheric nitrogen, cyanobacteria create symbiotic iterations with eukaryotic algae, lichens, and plants (Whitton & Potts 2000). Moreover, the chloroplast of plants and algae evolved by the engulfing of a cyanobacterial ancestor by ancient eukaryotic cell (McFadden 1999). The dominant photosynthetic pigment is chlorophyll-a, some cyanobacteria (*Prochlorococcus*) also possess divinyl-chlorophyll-b (Partensky et al. 1999), or chlorophyll-d (*Acaryochloris*) (Miyashita et al. 2003). Chlorophyll is mostly accompanied by xanthophylls, carotenoids, and phycobilisomes (phycoerythrin, phycocyanin, and allophycocyanin) (Bryant 1982).

Cyanobacteria can produce a wide range of secondary metabolites, some of them are toxic. Toxic effects are discussed mainly for their influence on human health. The vast majority of bloom-forming cyanobacteria produce cyanotoxins (Carmichael 1992) which have different biological activity e.g. antibacterial, antiviral and anticancer. These materials could be potentially used in the pharmaceutical industry (Singh et al. 2011).

In the 18th and 19th century, phycologists focused their attention mostly on temperate zones (Dvořák et al. 2015a). Researchers investigated predominantly areas that were close to their homes and universities and also research institutes were mostly situated in temperate zone. Recently, the focus has turned into freezing Arctic and Antarctic regions (Taton et al. 2003; Casamatta et al. 2005). However, the diversity of cyanobacteria in tropical areas remained poorly studied and evidently underestimated. As an evidence for such a statement, the recent descriptions of new genera could be used: *Brasilonema* (Fiore et al. 2007), *Jacksonvillea* (Hašler at al. 2017), *Ammassolinea* (Hašler et al. 2014b), *Elainella* (Jahodářová et al. 2018), *Pinocchia* (Dvořák et al. 2015a), *Onodrimia* (Jahodářová et al. 2017), *Chamaethrix* (Dvořák et al. 2017b, etc.).

1.1. TAXONOMY

The diversity of cyanobacteria started to be studied in the 19th century. For example Agardh (1812, 1817), Kützing (1849), Thuret (1875), Bornet & Flahault (1887, 1888), Gomont (1892) and many other phycologist were pioneers and wrote the first taxonomic monographs about algae and cyanobacteria. The traditional phenotypic classification was based on a filament or cell morphology, cell dimensions, presence of sheath or envelope, type of cell division, color, and type of branching (e.g. Bornet & Flahuatl 1887; Gomont 1892; Geitler 1932; Komárek & Anagnostidis 1998; Komárek & Anagnostidis 2005; Komárek 2013). The late 20th century was a revolutionary time for cyanobacterial taxonomy. A revision of cyanobacterial system based on ecology, ultrastructural properties, molecular markers (especially 16S rRNA) and also morphological data led to establishment of a new framework for cyanobacterial classification called "polyphasic approach". It has become a respected method of cyanobacterial taxonomy and determination widely used by phycologists (Johansen & Casamatta 2005; Siegesmund et al. 2008; Komárek 2010; Hašler et al. 2014b; Dvořák 2017; Dvořák et al. 2017a; Jahodářová et al. 2018 etc.). However, reconstruction of evolutionary history does not necessarily correspond with morphological pattern, as demonstrated by phylogenetic reconstructions. This led to a proposal of cryptic taxa which are phenotypically indistinguishable. They could be only identified by molecular markers from one gene or whole genome (16S rRNA, ITS region etc.) (Dadheech et al. 2014; Komárek et al. 2014; Dvořák et al. 2015a; Jahodářová et al. 2018). Furthermore, unrelated taxa share similar morphological characters and therefore create polyphyletic clusters caused by morphological convergence.

In morphologically simple cyanobacteria such as *Synechococcus* or *Leptolyngbya,* phenomenon was named by Dvořák et al. (2014) as a "Model of serial convergence." Globally distributed *Synechococcus* contains at least 12 polyphyletic lineages, each of these lineages could be determined as a separate genus, other examples of tangled relationship are 10 separate lineages in *Leptolyngbya* (Jahodářová et al. 2017). It is absolutely necessary to make revisions of the great majority of genera and species quickly enough. A possible explanation comes from unclear evolutionary relationships of cyanobacteria (cryptic taxa, horizontal gene transfer, and homologous recombination). Dvořák et al. (2014) suggest that horizontal (lateral) gene transfer (HGT) and homologous recombination (HR) are the essential evolutionary factors which frequently exchange genes within local gene pools (Polz et al. 2013). In fact, as an example of convergent evolution, new genera were derived from the genus *Leptolyngbya*: *Onodrimia* (Jahodářová et al. 2017), *Nodisilinea* (Perkerson et al. 2011), *Oculatella* (Zammit et al. 2012) and *Stenomitos* (Miscoe et al. 2016). Komárek et al. (2014) suggest that the majority of genera described using morphological criteria are polyphyletic.

In last 20 years, higher taxonomic units have undergone considerable changes. The latest version of the cyanobacterial system was published by Komárek et al. (2014) who split cyanobacteria to 8 sections: Gloeobacterales, Synechococcales, Spirulinales, Chroococcales, Pleurocapsales, Chroococcidiopsidales, Oscillatoriales, and Nostocales.

Estimated number of described cyanobacterial species varies from 2783 (Nabout et al. 2013) to 4484 (Guiry & Guiry 2015). The real number of extant species is likely much higher. Nabout et al. (2013) estimated 6280 species and Guiry (2012) expected about 8000 species of cyanobacteria. These estimates are based on traditional morphological methods. A precise species number estimation is obstructed by problematic culturing (with using of standard laboratory techniques) of a majority of prokaryotes (Amann et al. 1995). It seems to be impossible to uncover the real biodiversity. However, metagenomics is a possible way how to easily sequence uncultured organisms from environmental samples, but metagenomics has also its limits. Previous considerable handicap of this approach was an insufficient length of barcode sequences (only 300–500 bp, on the other hand new sequencing technology developed by Oxford Nanopore and Pacific Bioscience are capable of producing fragments of 10,000–100,000 bp), the impossibility of cross-validation between morphological and molecular data or limited photo documentation.

As the prokaryotic organism, cyanobacteria are described under two different nomenclature codes: International Code for Nomenclature of Prokaryotes (ICNP) and

International Code of Nomenclature for Algae, Fungi, and Plants (ICN), this fact substantially complicates cyanobacterial systematics. ICN makes a provision for the description of new taxa purpose on type material stored as dried biomass, unlike ICNP which requires viable axenic culture as nomenclatural type material (Oren 2011). Names described under ICNP are automatically accepted by ICN, but they are not reciprocal. Moreover, *Candidatus* species concept has been proposed under the International Code for Nomenclature of Prokaryotes (INCP), as a result of the impossibility of growing and maintaining selected taxa in cultures (Konstantinidis & Rosselló-Móra 2015). Also, some groups of cyanobacteria are more studied than the other ones (it is caused by problems with DNA amplification, some are more important for human. g. *Microcystis*) (Poulíčková et al. 2008; 2014).

1.2. MOLECULAR MARKERS USED IN CYANOBACTERIAL TAXONOMY

The most common approach to obtain data for phylogenetic inference and evolution understanding is analysis of DNA (protein) sequences. The first bacteria were sequenced in 1977 (Woese & Fox 1977). The most universal and widely used bacterial marker for phylogenetic reconstructions is the 16S rRNA gene (Rajendhran & Gunasekaran 2011), which encodes small ribosomal subunits (SSU). It is very conservative sequence among bacteria and is usable to delimit intergeneric relationships. Currently, the GenBank database contains thousands of sequences of the cyanobacterial 16S rRNA gene (Dvořák et al. 2018).

Bacterial ribosomal genes are arranged in an operon: 16S rRNA–23S rRNA–5S rRNA. 16S rRNA and 23S rRNA are followed by an Internal Transcribed Spacer (ITS region) (Srivastava & Schlessinger 1990; Iteman et al. 2000). 16S–23S rRNA ITS region is also utilized in cyanobacterial taxonomy. In addition, this region is more convenient to use for recognizing taxa on the species level (i.e. further use in population genetics), because it possesses adequate variability in length and also in base frequencies. The ITS region contains several semiconservative structures which are important for post-transcriptional processing (D1-D1' helix, V2, Box-B, V3). These structures (loops and stems) and ITS sequences themselves are used to reveal an evolutionary relationship (Iteman et al. 2000; Boyer et al. 2001; Boyer et al. 2002; Siegesmund et al. 2008; Dvořák et al. 2015a).

Additional molecular markers have been proposed for phylogenetic reconstruction, such as multilocus sequencing (MLST), *rbcl* gene, *nif* genes, phycocyanin encoding locus and RNA polymerase genes (Giovannoni et al. 1988; Ludwig & Schleifer 1994; Lee et al. 1996;

Nelissen et al. 1996; Zehr et al. 1997; Maiden et al. 1998; Honda et al. 1999; Neilan et al. 2002; Hartmann & Barnum 2010; Mishra et al. 2013; Singh et al. 2013). Recently, DNA barcoding (metagenomics) appeared to be a right way for practical and quick identification of bacteria. But barcoding gaps in the cyanobacterial (also in other taxonomical groups) determination are significant for correct taxa determination on species level (Eckert et al. 2015).

Recent research has shown that 16S rRNA is not sufficient for recognition of different species among the species clusters within defined genus. Additional data should be used for detailed identification, for instance, ITS region or another gene sequence (Osario-Santos et al. 2014). On the other hand, in practical "daily" identification for applied phycology, ecology and genetics, morphological observations are still easiest to use for cyanobacterial determination. But with an increasing number of newly described taxa, moreover, without any morphological apomorphy, the situation will be more complicated.

1.3. BACTERIAL GENOME

In the last decade, a new trend of sequencing and analyzing of whole bacterial genomes started to be more popular and more affordable. In 1995, the first bacterial genome was completely sequenced by shotgun sequencing method (Fleischmann et al. 1995). Some researchers argue that inconspicuous evolution events driven by environmental diversification are capturable only by whole genome sequencing approach (Kopac et al. 2014; Olsen et al. 2015). Disadvantages of phylogenomic approach are: analysis of genomes (i.e. biggest obstacle is difficult differentiation of paralog or ortholog genes) and insufficient number of sequenced genomes. GenBank database contains about 1500 available cyanobacterial genomes (15.5.2019). Furthermore, non-negligible part of these genomes is the marine picoplanktonic *Prochlorococcus* and *Synechococcus* (Larsson et al. 2011). Bacterial DNA is organized mostly to a single circular chromosome, which can have many copies (polyploidy). For instance, *Synechocystis* sp. PCC 6803 contained 258 chromosome copies in a cell (Griese et al. 2011). There are three types of gene groups in the bacterial genome based on their distribution among prokaryotes (Koonin & Wolf 2008). First and the smallest group contains conservative set of housekeeping genes which are core part of a genome (about 70 genes) and thus present in all prokaryotes. The second group (shell genes) is moderately common (approximately 5700 genes). Finally, so called “cloud” genes are the least common (probably 24 000 genes). These numbers will change with availability of new genome sequences, especially “cloud” genes will be found more.

Tettelin et al. (2005) introduced the term pan-genom, which includes all genes present within a species.

A number of protein-coding genes correlates with genome size in prokaryotes (0.8–1.2 gene by 1 kb). Bacterial genome size fluctuates mostly from 2 to 5 Mb (Koonin & Wolf 2008). The largest cyanobacterial genome has been identified in *Mastigocoleus testarum* BC008 with size 12.7 Mb and the smallest cyanobacterial genome was sequenced in *Candidatus Atelocyanobacterium thalassa* (marine symbiotic cyanobacterium UCYN-A), only 1.44 Mb. Photobiont *Acaryochloris marina* (cyanobacterium containing chlorophyll-d) has the largest genome among unicellular cyanobacteria (8.36 Mb). Genome streamlining (adaptive genome size reduction) was observed in some prokaryotic lineages (Partensky & Garczarek 2010). Gene loss tends to compensate HGT or duplications. Streamlined genomes are typically characterized by small size, a high number of conservative core genes and only a few pseudogenes, low proportion of intergenic spacer DNA to coding DNA (Batut et al. 2014; Giovannoni et al. 2014). One possible explanation for a genome streamlining was called Black Queen hypothesis. In contrast with the Red Queen hypothesis when ecological antagonists have to evolve as quickly as possible to survive. The Black Queen hypothesis describes the mutualistic and commensal relationship between species. Some microorganisms are able to cover the need of the other microorganisms, therefore they become dependent on each other and coexisting together. Thanks to that microorganisms could reduce the number of genes in a genome. The Black Queen hypothesis was applied on planktic cyanobacterium *Prochlorococcus* which possess small genome size. Reductive genome evolution is common in endosymbiotic bacteria, therefore was surprising in free-living organisms (Morris et al. 2012).

1.4. HORIZONTAL GENE TRANSFER AND HOMOLOGOUS RECOMBINATION

Horizontal gene transfer and homologous recombination are important forces in cyanobacterial speciation. Bacteria lack sexual reproduction, but HGT and HR are the way how to gain new genes with variable functions. HGT does not take place randomly in a genome, but is concentrated in heterogeneous genome regions, called genomic islands (Dobrindt et al. 2004). Horizontal gene transfer is more frequent among phylogenetically closed taxa (Beiko et al. 2005; Popa et al. 2011) and decreases with geographical distance. Approximately 50% of gene families are associated with HGT in cyanobacteria (Zhaxybayeva et al. 2006).

There are three ways how could bacteria incorporate a foreign fragment of DNA to its cell: natural transformation, conjugation, and transduction. Extraneous DNA from surrounding environment is incorporated to the bacterial chromosome by homologous recombination during transformation. This type of gene sharing is the most common in unicellular cyanobacteria (transformation has been observed in *Synechococcus* PCC 7002, PCC 7942 and *Synechocystis*) (Shestakov & Khyes 1970; Stevens & Porter 1980; Grigorieva & Shestakov 1982; Lightfoot et al. 1988).

During conjugation, mobile elements (for instance plasmids) are transferred from one bacterial cell to another one. Conjugation was observed in unicellular and also in filamentous cyanobacteria. Moreover, even a phylogenetically distant organism could conjugate (i.e. *Anabaena* and *Escherichia coli*, *Synechococcus* and Proteobacteria (Wolk et al. 1984; Delaye et al. 2011).

Transduction via bacteriophage (virus) mediator has not been observed in cyanobacteria yet. Although, the exchange of genes between cyanobacteria and their phages occurred (genes for photosystem II protein was found in bacteriophages of picoplantic *Synechococcus*) (Mann et al. 2003). About the bacteriophage-cyanobacterium interaction testifies CRISPR-Cas system (Clustered regularly interspaced short palindromic repeats-CRISPR associate proteins). This important system serves as a bacterial cell immune system (Bhaya et al. 2011).

There are two ways how to identify HGT, using parametric and phylogenetic methods. Parametric methods investigate G+C content variation (McLean et al. 1998), nucleotide composition, oligonucleotide frequencies (Lawrence & Ochman 1998) or structural characters of genomes (Worning et al. 2000). Explicit phylogenetic methods utilize conflict tree topology in phylogenetic reconstruction for detection of HGT. Implicit phylogenetic methods using sequence similarities and evolutionary distances (Haggerty et al. 2009; Ravenhall et al. 2015).

1.5. PROBLEMATIC GENERA IN THIS STUDY

Molecular methods allow us to provide a greater taxonomic resolution (Johansen & Casamatta 2005) and to describe new taxa. Likewise, the onset of molecular methods has an impact on cyanobacterial taxonomy. Advance in molecular phylogeny uncovers the real relationship in cyanobacterial taxonomy and showed polyphyletic origin of the vast majority of cyanobacterial genera. Morphological features do not reflect phylogenetic relationships. Morphologically defined genera with polyphyletic origin were disintegrated to new separate genera. Some of the newly created taxa are cryptic, visually indiscernible from "mother" taxon. Some problematic genera are listed below.

1.5.1. *Pseudanabaena* Lauterborn 1915

Pseudanabaena is widely distributed cyanobacterium. The genus contains 36 species. This thin filamentous cyanobacterium occurs in the plankton and benthos of freshwater or brackish waters, it often forms fine mats. Trichomes are straight or curved, usually constricted at the cross-walls, cells occasionally connected by a hyaline bridge, thin up to 3.5 μm wide, isodiametric to longer than wide (up to 3×), cell content divided into parietal chromatoplasm and central nucleoplasm, gas vesicles (aerotopes) occasionally present, diffluent sheath rarely present (Komárek & Anagnostidis 2005).

Recent phylogenetic analyses supported polyphyletic origin of *Pseudanabaena* (Acinas et al. 2009; Dvořák et al. 2015a; Yu et al. 2015). Although, *Limnothrix* and *Pseudanabaena* are evolutionary related taxa, they are phenotypically different (Suda et al. 2002). *Limnothrix* trichomes are about 3 μm wide, with less considerable constriction at the cross-walls. Komárek & Anagnostidis (2005) divided genus *Pseudanabaena* to thee subgenera: *Skujanema* (species with pointed ends), *Ilyonema* (species with polar gas vesicles) and *Pseudanabaena* (species with cylindrical and rounded terminal cells) (Komárek & Anagnostidis 2005). The cryptic genus *Pinocchia* was separated from *Pseudanabaena* (Dvořák et al. 2015a).

1.5.2. *Leptolyngbya* Anagsnotidis & Komárek 1988

Leptolyngbya contains probably the highest number of species across filamentous genera. Komárek & Anagnostidis (2005) mentioned about 91 species and around 25 unrevised species of its genus. *Leptolyngbya* forms very thin filaments (<2 μm wide) with lack of morphological characters. Trichomes are surrounded by a sheath, unbranched, occasional pseudobranched (Anagnostidis & Komárek 1988; Komárek & Anagnostidis 2005; Dvořák

et al. 2017a). This genus inhabits benthic, aerophytic, periphitic, subaerophytic and also aerophytic habitats. Simple morphology with improper phenotypic determination leads to extensive polyphyly of *Leptolyngbya* (Casamatta et al. 2005; Jahodářová et al. 2017). Based on 16S rRNA phylogeny, *Leptolyngbya* created 10 genetically separate clusters (Jahodářová et al. 2017). Moreover, some lineages of *Leptolyngbya* and *Synechococcus* reported analogous phylogenetic relationships (Dvořák et al. 2014, 2015b).

1.5.3. *Pseudophormidium* (Forti) Anagnostidis & Komárek 1988

Pseudophormidium was originally described as of the section of the *Plectonema* genus and it contained only one species *Plectonema phormidioides*. Afterward, this section was elevated to the genus level. *Pseudophormidium* is filamentous, falsely branched, mostly with sheath. Trichomes are comparatively wide (up to 10 μm), mostly constricted at the cross-walls. The apical cell is rounded. *Pseudophormidium* is reproduced by hormogonia. The most majority of species grow periphytically on stones, soils and on other submersed substrates (Anagnostidis & Komárek 1988; Komárek & Anagnostidis 2005; Anagnostidis 2001). *Phormidium* differs from the *Pseudophormidium* by the absence of false branching (Anagnostidis & Komárek 1988). *Plectonema* has wider trichomes (8–25 μm) than *Pseudophormidium*. But morphological descriptions of *Plectonema* and *Pseudophormidium* largely overlap (e.g. thallus formation, filaments branching and hormogonia formations) (Komárek & Anagnostidis 2005). Delimitation of both genera is well supported by 16S rRNA gene analysis (Jahodářová et al. 2017).

Part 2

AIMS

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AIMS

The principal goal of this thesis was to investigate new filamentous cyanobacteria isolated from different habitats using polyphasic approach with focus on cryptic lineages.

2.1. SPECIFIC AIMS

- To identify and describe new lineages from families Leptolygbyaceae and Pseudanabaenaceae.
- To date evolution of a new genus *Elainella* using whole genome data.
- To evaluate a reliability of sequence data of cyanobacteria in GenBank database for taxonomy and metagenomics.

Part 3

MATERIAL AND METHODS

3 MATERIAL AND METHODS

3.1. TAXONOMICAL PART

Strain isolation

Cyanobacterial samples were collected from plankton and periphyton of a lake Hồ Đâu Co in Vietnam (strains E5, E10) and from plankton of ephemeral waterbodies in the forest of the National Park Cat Tien in Vietnam (strains E1, E8, E11), and from submersed bark of tree branches, near Tamanjayae, Ujung Kulon NP, West Java (strains E27, E28, E30). Cultures were maintained in 90 mm Petri dishes under the laboratory conditions as follows: temperature 26±1°C, illumination 20 μmol photons m^{-2} s^{-1}, light regime 12h light:12h dark, and liquid Zehnder medium (Z medium) (Staub 1961).

Morphological assessment

Strain morphology was studied using light microscope Zeiss AsioImager (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 D512 12MPx). Cultures were used for studying and evaluation of morphology (see Dvořák et al. 2015a; Jahodářová et al. 2017; Jahodářová et al. 2018).

Nutrient and temperature experiment with Pinocchia culture

To find morphological apomorphy for direct recognition of *Pinocchia* from *Pseudanabaena*, I used modified Z medium (Zehnder) without nitrogen, medium without phosphorus and medium without both of elements. I cultured strains in two different temperatures, at 16 °C and 26 °C. The difference was found among all cultures without nitrogen, phosphorus or both elements maintained at 26 °C.

PCR amplification and sequencing

Genomic DNA was extracted from ca 50 mg of fresh biomass using an UltraClean Microbial DNA Isolation Kit (MOBIO, Carlsbad, California, USA). DNA quality and consistence was checked on GelRed (Biotinum Inc., Hayward, California, USA) stained

1.5% agarose gel. DNA was quantified using the NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, Delaware, USA). A partial 16S rRNA sequence and the whole 16S–23S rRNA ITS sequence were obtained using PCR amplification. PCR products were cloned using StrataClone PCR Cloning kit (Agilent Technologies, Stratagene Product Division, La Jolla, California, USA).

Phylogenetic analyses

The most similar sequences of 16S rRNA were retrieved from the NCBI database and identified using nucleotide BLAST. Multiple sequence alignment was performed in MEGA 6 (Tamura et al. 2013) using Muscle algorithm (Edgar 2004) or in Mafft with E-INS-I algorithm (Katoh et al. 2002). The phylogenetic tree was rooted using *Gloeobacter violaceus* as the outgroup. The most appropriate model for Bayesian inference was determined by jModelTest 0.1.1 (Posada 2008) based on both the Bayesian and the Akaike Information Criterion. Bayesian inference majority consensus tree was constructed in MrBayes 3.2.3 (Ronquist & Huelsenbeck 2003). Maximum likelihood analysis was performed in RaxML 8.0.2 (Stamatakis 2006). Maximum parsimony analyses were performed in MEGA version 6.0. (Tamura et al. 2013) or in PAUP*4.0b10 (Swofford 2002).

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).

3.2. GENOMIC PART

In the case of *Elainella saxicolla* I used phylogenomics assessment.

De novo genome sequencing

Genomic DNA was extracted from wet biomass using UltraClean Microbial DNA Isolation Kit (MOBIO, Carlsbad, CA, USA). DNA quality and consistence was checked on GelRed (Biotinum Inc., Hayward, California, USA) strained 1.5% agarose gel. The quantification of DNA was performed by NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

The library for sequencing was prepared by TruSeq Nano DNA kit (Illumina Inc, San Diego, CA, USA) using 200 ng DNA according to manufacturer's instruction, but DNA was digested by Bioruptor Plus (Diagenode, Liege, Belgium) and size selection was

modified to achieve insert size about 1,000 bp. The insert size of libraries was defined by Agilent High Sensitivity DNA Kit (Agilent Technologies, Inc.) and concentration of library was assessed by KAPA Library Quantification Kit for Illumina (Kapa Biosystems, Woburn, MA, USA). MiSeq Reagent Kit v3 (Illumina Inc, San Diego, CA, USA).

A total of 2,880,274 pair ended reads with an average length of 226 bp were assembled *de novo* using the MIRA 4 assembler. I used a following procedure of contaminant contigs removal. I ran BLASTN with all contigs against complete bacterial genomes.

The de novo assembled genome resulted in 284 contigs (>500 bp) with an N50 73 085 bp, and a theoretical coverage of 38× based on the estimation of a length of 8.7 megabases.

Rapid Annotation using the Subsystems Technology (RAST) pipeline (Aziz et al. 2008) was used for annotating; tRNA was predicted using tRNAscan-SE 1.21 (Lowe & Eddy 1997). CRISPRs (clustered regularly interspaced short palindromic repeats) and CRISPR spacers were identified using CRISPRfinder (Grissa et al. 2007). Putative secondary metabolite gene clusters and molecule structures were predicted by antiSMASH 3.0 (Weber et al. 2015). Average nucleotide identity (ANI) was determined by Jspecies (Richter & Rosselló-Móro 2009). A visual representation of the BLAST searched genome similarities of *Elainella* with *Leptolyngbya* sp. JSC-1 was performed in BRIG (Alikhan et al. 2011).

A total of 129 available and annotated genomes of cyanobacteria were acquired from the ftp server of GenBank. Other genomes of cyanobacteria from GenBank were added to cover the broad evolutionary array of this group, representing most major niches/ habitats. Genomes of *Leptolyngbya boryana* PCC 6306, *Geitlerinema* sp. PCC 7105, *Spirulina subsalsa* PCC 9445 and *Nodosilinea nodulosa* PCC 7104 were re-annotated using RAST due to lack of annotation in the GenBank database.

The super alignment of 69 protein sequences for a subsequent phylogenomic reconstruction of a cyanobacterial species tree was obtained using phylogenomic Perl pipeline Hal (Robbertse et al. 2001) with options described in Dvořák et al. (2014). The phylogenomic reconstruction based on a resulting super alignment with a total of 15 141 amino acids was performed in RAxML 8.1.15 (Stamatakis 2006). The most suitable substitution matrix was identified in ProtTest 3.3 (Abascal et al. 2005). *Gloeobacter violaceus* and *Gloeobacter* sp. were used as outgroup taxa. The dating of the phylogenomic reconstruction has been performed using calibration points and settings of penalized likelihood (Sanderson 2002), as previously used by Dvořák et al. (2014) in r8s (Sanderson 2012).

For clarification of mentioned methods and workflow see Fig. 1.

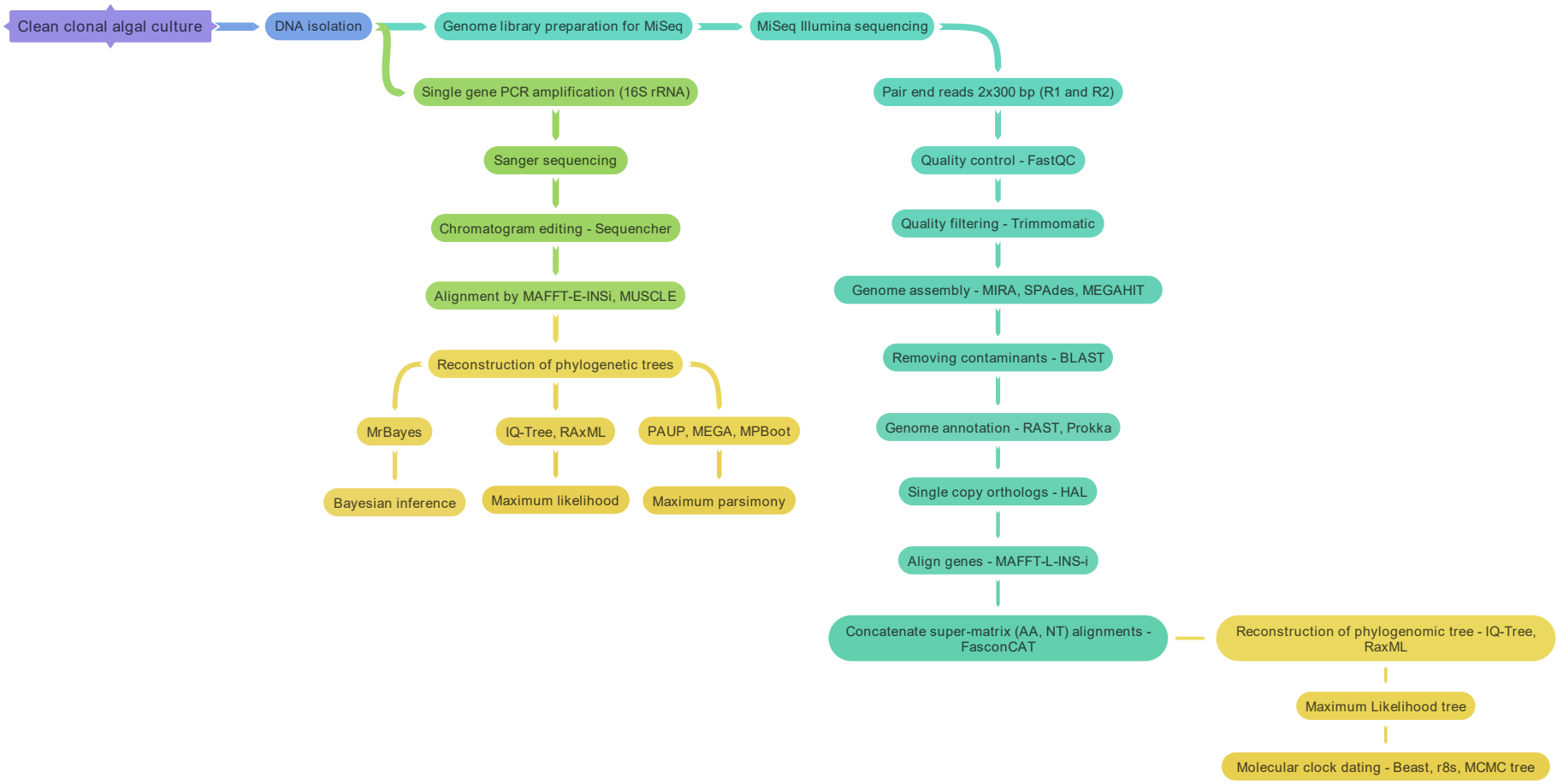

Fig.1. The diagram illustrating the whole process from the isolation of the clonal cyanobacterial culture to the dating phylogenetic tree (created by: https://coggle.it).

3.3. GAPS IN CYANOBACTERIAL TAXONOMY

I obtained a comprehensive dataset of 16S rRNA sequences of cyanobacteria from GenBank using search query to make sure I congregate sufficiently long sequences for analyses. Multiple sequence alignment was created using MAFFT (Katoh et al. 2002). Identical sequences were removed from alignment. I also could not employ shorter reads (<899 bp) for MSA because these fragments might not overlap. The ITS regions were included.

A phylogenetic reconstruction was performed using maximum likelihood criterion in RaxML 8.0.0. (Stamatakis 2014). A phylogenetic reconstruction using neighbor joining optimality criterion was performed in MEGA 6 (Tamura et al. 2013). Species were delimited in the Python program package PTP (Poisson Tree Process) (Zhang et al. 2013). A list of phylogenetically identified species (created by PTP) so called operational taxonomic units (OTUs). Analyses exploit and evaluate taxa with cultured strains if they were identified as an existing and validly described species under ICN or the ICNP.

I used the mentioned procedure to identify which sequences employed in the GenBank dataset corresponded to definitely named and reliably identified PTP-defined species. I also considered all identical sequences, which were subsequently assigned to PTP-defined species. All sequences of "uncultured cyanobacteria" and sequences without epithet or with unsure species epithet were removed from the list of definitively named and reliably identified species. I also did an extensive search in GenBank and literature to confirm whether a particular PTP-defined species contains described species valid under either the ICN or the ICNP. The PTP-defined species containing at least one definitively named and reliably species were included in the procedure, whereas the species with *Candidatus* status were excluded from this analysis.

PART 4

CONCLUSIONS

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CONCLUSIONS

4.1. BOOK CHAPTER

4.1.1. Diversity of the Cyanobacteria

DVOŘÁK P, CASAMATTA DA, HAŠLER P, JAHODÁŘOVÁ E, NORWICH AR, POULÍČKOVÁ A (2017) Diversity of the Cyanobacteria. In: Modern topics in the phototrophic Prokaryotes: Environmental and Applied Aspects. (Ed. by Hallenbeck PC), Springer International Publishing, Switzerland. pp 3–46.

This chapter is focused on evolutionary history, speciation and taxonomy of cyanobacteria. The introduction describes the origin of cyanobacteria, their impact on the global ecosystem, other organisms or humans. It contains information about morphology, different views on cyanobacterial classification and cyanobacterial phylogeny. Subsequent part is focused on a cyanobacterial speciation and on problems of modern taxonomy as well. The largest part of the chapter discusses the species taxonomy and it is divided according to the latest cyanobacterial system proposed by Komárek et al. (2014) (Synechoccoccales, Chroococcales, Chroococcidiopsidales, Pleurocapsales, Oscillatoriales, Spirulinales, Nostocales). Each section describes the most important members of the individual order. The whole chapter is enriched by phylogenetic and phylogenomic trees, drawings, and photographs.

4.2. INDIVIDUAL PAPERS

4.2.1. Polyphasic characterization of *Pinocchia* gen. and sp. nov.

DVOŘÁK P, JAHODÁŘOVÁ E, HAŠLER P, GUSEV E, POULÍČKOVÁ A (2015) A new tropical cyanobacterium *Pinocchia polymorpha* gen. et sp. nov. Derivated from genus *Pseudanabaena*. Fottea 15: 113–120.

Benthic, planktic and mataphytic representatives of the genus *Pseudanabaena* occur in the sea, freshwater and also terrestrial habitats (Komárek & Anagnostidis 2005). This genus was found to be composed of polyphyletic or cryptic lineages (Komárek et al. 2014). Paper I is focused on a polyphasic characterization of new *Pseudanabaena*-like cyanobacterium named *Pinocchia polymorpha*. *Pinocchia* was isolated from plankton and periphyton of the lake Hồ Đâu Co, province Đồng Nai, Vietnam. Morphology of the trichomes was an important factor which was considered. *Pinocchia* was morphologically very similar to *Pseudanabaena*, particularly to *Ilyonema galeata* or *P. catenata* (species with polar gas vesicles and specific shape of a terminal cell are ranked to subgenus *Ilyonema* in Komárek & Anagnostidis 2005). *Pinocchia* differs from *I. galeata* by high variability of cell length (this is characteristic for *P. catenata*). Furthermore, *Pinocchia* possess prolongated, pointed and sometimes conical apical cell which is unusual in *I. galeata* and *P. catenata*. Phylogenetic analysis of 16S rRNA revealed the real position of *Pinocchia* as a significantly supported monophyletic clade. Furthermore, *Pseudanabaena sensu stricto* was very distant from *Pinocchia*. I found high variability of ITS region in particular strains even in clones. I discover three types of D1-D1´ helix and B-boxes. It might suggest potential existence of two cryptic species in *Pinocchia* genus. I applied nutrient (different concentration of nitrogen and phosphorus) and temperature (cultivation in 16 °C and 26 °C) experiment to find morphological apomorphy. It has been shown many times that nitrogen, phosphorus or it rations affect the intensity of cell division (Poulíčková et al. 2001; Hašler et al. 2003; Hašler & Poulíčková 2010). Obviously, a higher temperature (26 °C) was more convenient for *Pinocchia* growing, probably due to the isolation from the tropical lake. Taken together, *Pinocchia* represents cryptic lineage derivate from polyphyletic *Pseudanabaena*. Its monophyly confirms the tree topology, secondary structures of ITS region and also tropic origin.

4.2.2. Polyphasic characterization of *Onodrimia* gen. and sp. nov.

JAHODÁŘOVÁ E, DVOŘÁK P, HAŠLER P, POULÍČKOVÁ A (2017) Revealing hidden diversity among tropical cyanobacteria: the new genus *Onodrimia* (Synechococcales, Cyanoabcteria) described using the polyphasic approach. Phytotaxa 326: 28–40.

Polyphyletic genus *Leptolyngbya* contains more than 100 species (Komárek & Anagnostidis 2005; Perkerson et al. 2011; Osario-Santos et al. 2014). All *Leptolyngbya*-like species have thin filaments and simple morphology (Komárek & Anagnostidis 2005). Paper II describes new taxon *Onodrimia javanensis* derived from *Leplogynbya*. Strains were isolated from the submersing bark of tree branches which fell into hot water spring in the rainforest, near Tamanjayae, Ujung Kulon NP, West Java. *Onodrimia* possesses morphological autapomorphy and differs from other Leptolyngbyaceae genera. The peculiar form of reproduction represents hormocytes and hormogonia stuck by sheath on mother trichome and create tree-like tuft structures. Moreover, the phylogeny of 16S rRNA gene confirmed the strong polyphyletic origin of *Leptolyngbya* (*Leptolyngbya* created 10 separate lineages, more than previously reported). *Onodrimia* is clearly separated from other Leptolyngbyace genera and encompass clade with significantly support. Moreover, *Onodrimia* shares 96.2% sequence similarity with Uncultured bacterium TG-102 (JQ769612) and 94% sequence similarity with Uncultured bacterium TG-104 (JQ769614). Above that, *Leptolyngbya sensu stricto* creates a clade on the base of the tree and is not closely related to *Onodrimia*, which clustered with *Phormidesmis*, *Stenomitos*, *Pantanalinema*, and *Neosynechococcus*. On the other hand, all these taxa are morphologically distant from *Onodrimia*. *Leptolyngbya corticola* is the only species characterized by similar ecology (tree bark) but was found in temperature forest (Johansen et al. 2011). The D1-D1´ helix and B-box were identical among all studied strains, as all *Onodrimia* strains have no variability in ITS structure. I compared ITS secondary structures of *Onodrimia* to other Leptolyngbyace members. *L. appalachiana* keeps extremely long D1-D1´ helix as *Onodrimia*. *Onodrimia javanensis* possess only minor morphological difference from other taxa in Leptolyngbyaceae family, but it could be distinguished by morphological autapomorphy (tree-like hormogonial tufts), phylogenetic position in the tree, secondary structures, low sequence similarity and unique ecology.

4.2.3. The complex genomic approach in a description of *Elainella*

JAHODÁŘOVÁ E, DVOŘÁK P, HAŠLER P, HOLUŠOVÁ K, POULÍČKOVÁ A (2018) *Elainella* gen. nov.: a new tropical cyanobacterium characterized using a complex genomic approach. European Journal of Phycology 53: 39–51.

This paper aimed at a description of new genus and species *Elainella saxicola*. *Elainella* is filamentous cyanobacterium, with simple morphology. Strains were collected from plankton of ephemeral waterbody in the forest of the National Park Cat Tien, province Đồng Nai, Vietnam. In Paper III, I used polyphasic approach for the new taxa description. Polyphyly can also be observed in *Pseudophormidum* (Taton et al. 2006; Alwathnani & Johansen 2011; Osario-Santos et al. 2014) which is morphologically very similar to *Elainella*. All selected taxa from *Plectonema* and *Leptolyngbya* have overlapping descriptions with *Elainella* (especially Gardner´s taxa from Puerto Rico, later classify to unclear species of *Pseudophormidium* and *Leptolyngbya*) (Komárek & Anagnostidis 2005; Anagnostidis & Komárek 1988). *Pseudophormidium* and *Plectonema* overlap each other in some morphological characters (for instance thallus formation, branching of filaments and formation of hormogonia) (Komárek & Anagnostidis 2005). Their only distinguishing feature is the width of the trichome. *Plectonema* possesses wide trichomes (8–25, up to 72 μm), on the other hand, *Pseudophormidium* creates narrowed trichomes (less than 10 μm). Bayesian phylogeny of 16S rRNA gene exposed monophyletic origin of *Elainella saxicola* despite of other filamentous lineages as *Pseudanabaena*, *Trichocoleus*, *Nodosilinea*, *Oculatella*, *Symploca*, and *Spirulina*. The most phylogenetically related cyanobacterium to *E. saxicola* is *Leptolyngbya* sp. D1C10 (KJ654308). *Leptolyngbya* sp. D1C10 is probably a species of *Elainella*. Unfortunately, additional information about this strain cannot be acquired. Therefore, I was not able to revise these taxa. I could not add the type species of *Pseudophormidium* (*P. phormidioides*) and also Gartner´s taxa to the phylogeny, because it has not been sequenced yet. *Elainella* was found to form a highly supported clade distant from *Leptolyngbya sensu stricto*, *Pseudophormidium*, and *Plectonema* in 16S rRNA phylogeny.

The total length of the *Elainella* draft genome was 8 702 141 bp, G+C content was 47.6%. RAST annotation detects a total of 8472 coding sequences and 102 RNAs. Based on known proteins with biological function, 51.3% of coding sequences were annotated; 48% of them were identified as potential proteins. Genome of *Elainella* contains genes for fixation of atmospheric nitrogen (*nif*B, *nif*S, *nif*U, *nif*H, *nif*D, *nif*K). The gene composition is similar

as in other non-heterocytous, nitrogen-fixing cyanobacteria, for instance, *Cyanothece* sp. PCC 7425 (Bothe et al. 2010). However, the real activity of the nitrogenase should be experimentally tested. Moreover, the genome contains 24 confirmed CRISPRs with 223 CRISPR spacers. Cyanobacteria are able to produce an inexhaustible quantity of secondary metabolites, very often in high concentrations. Some of these substances have a negative impact on human health (cyanotoxins), they can also affect the function of an ecosystem (Leflaive & Ten-Hage 2007). Ten potential biosynthetic gene clusters was detected *in silico* analysis using AntiSMASH, but only two of them are assigned to already known gene: puwainaphycins and cryptophycin. Both puwainaphycin and cryptophycin have exhibit cytotoxic activity (Hrouzek et al. 2012; Weiss et al. 2013). Overleaf, the genome of *Elainella* lacks crucial genes for Far-Red Light Photoacclimation or abbreviated FaRLip (e.g. ApcA2, ApcB2) (Gan et al. 2014). These genes were found in phylogenetically closes cyanobacterium *Leptolyngbya* sp. JSC-1. *Leptoloyngbya* sp. JSC-1 is the first discovered cyanobacterium with this mechanism. FaRLip ensures an advantage in low light conditions in an environment such as mast, soil or stromatolites (Gan et al. 2014). Hypothetically, *Elainella* should be better adapted to the higher light intensity. I attempted to reveal the origin of *Elainella* using dated 69-gene phylogeny to put it in the context of all cyanobacteria. *Elainella* is sister to *Leptolyngbya* sp. JSC-1, these taxa are likely to diverge 2.24–2.47 BYA, before the Great Oxygenation Event (Kopp et al. 2005). In this phylogenomic analysis, they created a clade with 100% support, but in 16S rRNA phylogeny their cluster scored only low node support. I was able to compare morphological characters of both taxa using data from Brown et al. (2010). *Leptolyngbya* sp. JSC-1 creates two types of cells (short and narrow) and *Elainella* possess pseudobranching. Moreover, *Leptolyngbya* sp. JSC-1 is unrelated to *Leptolyngbya sensu stricto* in 16S rRNA phylogeny, its taxonomic status should be revised in the future. Based on Komárek et al. (2014) *Elainella* would be classified to order *Synechococcales*, family Leptolyngbyaceae (pursuant on the phylogeny of 16S rRNA gene). Significant differences in ANI, 16S rRNA, different genome sizes and different autecology (*Leptolyngbya* sp. JSC-1 was found in hot spring in Great Yellowstone area) give us enough evidence to distinguish these two taxa.
Elainella represents evolutionary new lineages among cyanobacteria and the first *Pseudophormidium*-like cyanobacterium with whole genome data.

4.2.4. Difference without distinction? Gaps in cyanobacterial systematics

DVOŘÁK P, JAHODÁŘOVÁ E, CASAMATTA D, HAŠLER P, POULÍČKOVÁ A (2018) Difference without distinction? Gaps in cyanobacterial taxonomy: where more is just too much. Fottea 18: 130–136.

The most widely used marker in cyanobacterial phylogeny is 16S rRNA gene. But it is generally known that some morphological features do not necessarily correspond with molecular phylogenetic reconstructions. Nowadays, the majority of cyanobacterial genera are polyphyletic (Komárek et al. 2014). The mentioned problem makes cyanobacterial systematic very unclear. It is obvious to ask questions: how many cyanobacterial species do exist, how many species may be expected among sequences collected from metagenomics data without culturing or how many taxa have been described using phylogenetic species complex (Dvořák et al. 2018). In Paper IV I tried to find answers to these questions and elucidate the real situation as well as I tried to quantify an overlap between described species and available 16S rRNA sequences from the GenBank database. The final alignment of the 16S rRNA gene comprised 10037 sequences (but only 4983 sequences were unique). From this amount, PTP delimited only 2741 PTP-defined cyanobacterial species. It is interesting, that 51% of PTP-defined species were assigned to uncultured environmental cultures. Though, scientists estimated that only about 1% of prokaryotes are maintained in cultures. These extreme differences between number of sequences in database and number of delimit species are probably caused by the length of sequences used in the analysis, only sequences longer than 899 bp were used (metagenomics analysis exploit sequences under 300–500 bp). I excluded shorter fragments than 900 bp from the alignment. There are two ways to explain this situation, the cultivation of most cyanobacteria is very unsuccessful or that habitats with a high amount of uncultured cyanobacteria are lacking in metagenomics data. Cyanobacterial and algal members of planktic habitats are prominently studied, on the other hand, freshwater sediments are neglected (Hašler et al. 2008, Poulíčková et al. 2014). Despite that fact Mann et al. (2008) and Poulíčková et al. (2014) thought that epipelon communities are very complex and diverse. For example, Hašler & Poulíčková (2010) described a new species of epipelic *Komvophoron* or Hašler et al. (2014a) determinated new genus *Johanseninema* from epipelon. Moreover, there are multiple areas that are unexplored, especially tropical areas or polar regions, in sharp contrast with temperate zones where the vast majority of phycologists do their research (Dvořák et al. 2017b).

I discover that only 571 PTP-defined species (20.9%) may be assigned to a certain name and reliably identified species under ICN or ICNP. The number of PTP-defined species represent only 12.7–21.2% of all describes species under ICN in either CyanoDB or AlgeaBase databases (Guiry & Guiry 2015, 4484 species; Nabout et al. 2013, 2698 species). PTP-defined species are delimited only phylogenetically (Nixon & Wheeler 1990). New taxa under ICN and ICNP are described using different species concept. Hence, the total number of species may be varied depending on the applied species concept.

In conclusion, I have shown that taxonomic and genetic databases may not to reflect accurately the real diversity of cyanobacteria. There are several ideas on how to improve this situation: 1. Phycologists should do the revisions of polyphyletic taxa, newly described genera should always be characterized by autapomorphy. 2. Metagenomic data should be assessed more carefully. 3. Revisions should be undertaken only with robust and proper taxon sampling.

PART 5

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REFERENCES

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PART 6

LIST OF PUBLICATIONS IN THIS THESIS

6

LIST OF PUBLICATIONS INCLUDED IN THIS THESIS

BOOK CHAPTER

DVOŘÁK P, CASAMATTA DA, HAŠLER P, **JAHODÁŘOVÁ E**, NORWICH AR, POULÍČKOVÁ A (2017) Diversity of the cyanobacteria. In: Modern Topics in the Phototrophic Prokaryotes: Environmental and Applied Aspects. (Ed. by Hallenbeck PC), Springer International Publishing, Switzerland. pp 3–46.

PAPER I.

DVOŘÁK P, **JAHODÁŘOVÁ E**, HAŠLER P, POLÍČKOVÁ A (2015) A new tropical cyanobacterium *Pinocchia polymorpha* gen. et sp. nov. derivated from the genus *Pseudanabaena*. Fottea 15: 113–120.

PAPER II.

JAHODÁŘOVÁ E, DVOŘÁK P, HAŠLER P, POULÍČKOVÁ A (2017) Revealing the hidden tropical diversity among cyanobacterial using polyphasic approach, new genus *Onodrimia* (Synechococcales, Cyanobacteria). Phytotaxa 326: 25–40.

PAPER III.

JAHODÁŘOVÁ E, DVOŘÁK P, HAŠLER P, HOLUŠOVÁ K, POULÍČKOVÁ A (2018) *Elainella* gen. nov.: a new tropical cyanobacterium characterized using a complex genomic approach. European Journal of Phycology 53: 39–51.

PAPER VI.

DVOŘÁK P, **JAHODÁŘOVÁ E**, CASAMATTA D, HAŠLER P, POULÍČKOVÁ A (2018) Difference without distinction? Gaps in cyanobacterial taxonomy: where more is just too much. Fottea 18: 130–136.

BOOK CHAPTER

Diversity of the Cyanobacteria

Petr Dvořák, Dale A. Casamatta, Petr Hašler, Eva Jahodářová, Alyson R. Norwich, and Aloisie Poulíčková

Abstract The cyanobacteria are an ancient lineage of photo-oxygenic bacteria. Globally responsible for much of the primary productivity and nitrogen fixation, they are also evolutionarily significant as the photosynthetic members of serial endosymbiotic events leading to the establishment of chloroplasts. Traditionally classified based on morphological characters, recent research revealed an abundance of cryptic diversity evidenced by molecular analyses, most notably the 16S rDNA gene sequence. Explorations of seldom sampled habitats, such as tropics environments, aerophytic habitats, soil crusts, etc., have also revealed a tremendous new diversity of taxa. This increase in the alpha-level diversity, coupled with new molecular techniques, has greatly altered our perceptions of the evolutionary relationships within this clade. Many of the traditional genera have proven to be polyphyletic, but revisions are underway.

Keywords Cyanobacteria • Phylogeny • Taxonomy • Biodiversity • Morphology

Introduction

Cyanobacteria (Cyanophytes, Cyanoprokaryotes, blue-green algae) are an ancient group of prokaryotic microorganisms with the ability to undertake oxygenic photosynthesis. The earliest estimates of their origin lie at the beginning of Archean 3.5–3.8 BYA (reviewed in Sleep 2010 and Schopf 2001), while more conservative estimates suggest a later appearance at the end of Archean 2.7 BYA (reviewed in Blank and Sanchez-Baracaldo 2010). Cyanobacteria have greatly impacted global ecosystems, as their photosynthetic activity provided much of the oxygen necessary for the proliferation of aerobic life forms (Bekker et al. 2004). Moreover, cyanobacteria are among

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the most abundant and potent primary producers on Earth. They occur in freshwater, marine, cold (e.g., polar), hot (e.g., thermal springs), and terrestrial habitats. Cyanobacteria are also commonly encountered in symbiotic relationships with plants, fungi (lichens), and eukaryotic algae (Whitton and Potts 2000). Through the process of endosymbiosis, ancient organisms (likely protistans) engulfed a cyanobacterium, resulting in the origin of the chloroplast of algae and plants (McFadden 1999). The current number of cyanobacterial species is subject to debate, with estimates ranging from 2783 (Nabout et al. 2013) to 4484 (Guiry and Guiry 2015). However, the expected total number of species may reach 8000 (Guiry 2012).

Besides primary metabolism, cyanobacteria produce myriad secondary metabolites, or bioactive compounds (chemically, mostly alkaloids and oligopeptides), which are toxic to the environment and humans (collectively referred to as cyanotoxins; e.g., Carmichael 1992; Dittmann et al. 2013). However, a number of these compounds have been isolated and show promise as drugs for the treatment of cancer and other diseases due to their biological activity, e.g., anti-viral, anti-protistan, and anti-bacterial properties (comprehensively reviewed in Singh et al. 2011). A variety of cyanobacterial strains also promise further advances in biotechnology. Cyanobacteria have been postulated as an alternative source of energy, they may be used in wastewater plants for utilization of macronutrients, for degradation of oil, for fertilization in agriculture, and as food for humans and animals (reviewed in Abed et al. 2008).

On the other hand, cyanobacteria are often noted for their ability to form blooms, which results from an overabundance of planktic forms. This typically occurs in eutrophic habitats (those with elevated nutrient levels, typically nitrogen and/or phosphorus), such as freshwater lakes. Freshwater harmful algal blooms are often accompanied by unfavorable phenomena such as extreme pH fluctuations, anaerobic conditions, and aforementioned toxins (reviewed in Oliver and Ganf 2000). Thus, cyanobacteria may have a large negative impact on both the environment and human endeavors such as fisheries, potable water production, recreational usage of aquatic habitats, etc.

The purpose of this chapter is to provide an overview of recent advances in the taxonomy, phylogeny, and diversity of cyanobacteria. We will focus on taxonomic revisions and new taxa in light of current problems of cyanobacterial species concepts and definitions. Furthermore, we will show that significant gaps persist in our knowledge of cyanobacterial biodiversity. We will discuss geographical regions and habitats where our knowledge of the diversity and ecology of cyanobacteria is most limited.

How to Distinguish Cyanobacteria?

Morphology

Cyanobacteria exhibit a relatively high degree of morphological features compared to other prokaryotes. Until recently, cyanobacteria were identified and categorized using morphological traits such as cell dimensions, shape, color, type of branching, sheath characteristics, and cell contents (summarized in Komárek and Anagnostidis 1998, 2005; Komárek 2013). Cyanobacteria may be unicellular, colonial, or filamentous.

Colonies of unicellular cyanobacteria may have regular (e.g., *Merismopedia*) or irregular distribution of cells (e.g., *Microcystis*). The Pleurocapsales possess relatively complex colony formation, which may resemble filaments, branching, and cells may be heteropolar (see Komárek and Anagnostidis 1998 for review and Fig. 1). The number of cells may vary from two to several thousand per colony.

Filamentous cyanobacteria may exhibit both false and true branching. False branching is present in all orders of filamentous cyanobacteria, while true branching has been observed only in members of Nostocales (see details of cyanobacterial systematics for further details). Similarly, multiseriate growth of trichomes (a parallel succession of multiple trichomes) evolved only in some members of the Nostocales, specifically in the order formerly referred to as the Stigonematales (Komárek 2013).

While cyanobacteria reproduce via binary fission, they may not be considered as fully clonal organisms due to horizontal gene flow and recombination, as seen in other prokaryotes (for detailed discussion see, e.g., Cohan 2001, 2002; Cohan and Perry 2007). However, cyanobacteria have evolved some interesting reproductive strategies. For instance, some unicellular cyanobacteria may produce baeocytes and exocytes, which are differentiated from the mother cell by size, shape, and successive multiple fission with subsequent release to the environment (see details in Komárek and Anagnostidis 1998). Filamentous cyanobacteria may produce short, often motile filaments called hormogonia. Furthermore, Nostocalean cyanobacteria may produce long-term or overwintering reproductive cells called akinetes (see further).

Besides vegetative cells (those cells dedicated to photosynthetic processes), filamentous cyanobacteria of the order Nostocales may produce two types of differentiated cells: heterocytes and akinetes. Heterocytes do not possess functioning photosynthetic apparatus because their primary function is anaerobic fixation of atmospheric nitrogen using the enzyme nitrogenase (Meeks et al. 2002), also developed in some soil bacteria. Heterocytes may be distinguished from vegetative cells by the former's homogenous content and the presence of polar pores, and heterocytes may be situated intercalary or terminally in filaments (Kumar et al. 2010; Komárek 2013). Akinetes are usually larger than vegetative cells, and with large amounts of stored nutrients visible as granules. Akinetes are reproductive cells, which may lie quiescent in the environment (e.g., lake sediments, soils, etc.) during unfavorable conditions (drought, low temperatures, fall turnover in dimictic lakes, etc.; Kaplan-Levy et al. 2010). Olsson-Francis et al. (2009) suggested that akinetes of *Anabaena cylindrica* may survive the environment on Mars.

Cell ultrastructures are typically visualized by transmission electron microscopy (TEM) and may exhibit significant variability among cyanobacteria. The cell walls of cyanobacteria have similar composition to other gram-negative bacteria. Furthermore, some cyanobacteria possess S-layers, a crystalline, proteinaceous layer covering the entire surface of the cell. These S-layers appear to be an important structure for filament motility (Hoiczyk and Hansel 2000). Cells often produce mucilaginous sheaths composed of exopolysaccharides, which range from <1 μm to several times the filament thickness. Sheaths are often environmentally inducible and provide several putative functions, such as protection from UV radiation, desiccation,

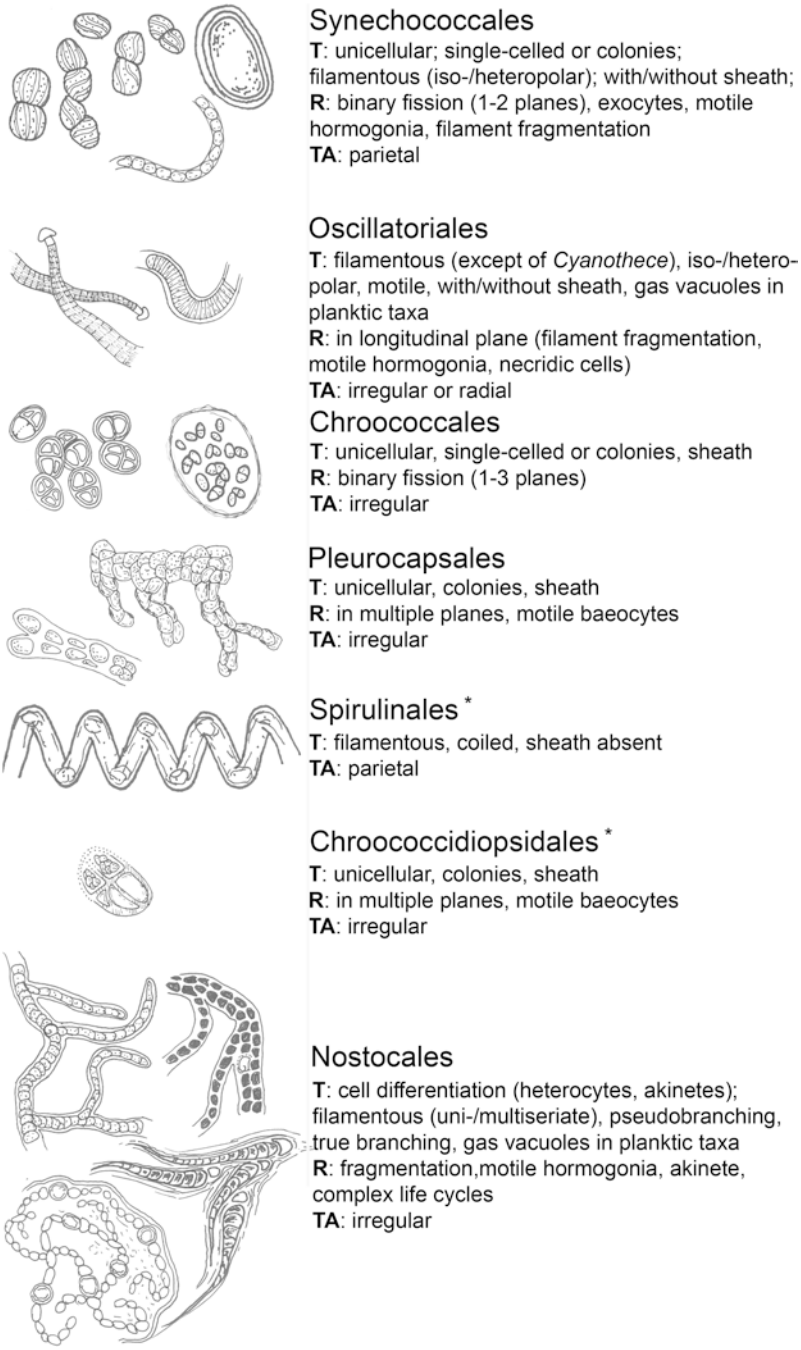

Fig. 1 Ordinal level taxonomic scheme of cyanobacterial taxonomy as proposed by Komárek et al. (2014). The *left side* contains examples of morphotypes for each order, the *right typical features*: T—thallus, R—reproduction type, TA—thylakoid arrangement. *Asterisks* represent the most recently established orders. Note that we did not include the Gloeobacterales, as it is extensively discussed elsewhere and it possesses very simple morphology

and anti-herbivory (Ehling-Schulz and Scherer 1999). Cyanobacteria also possess thylakoids, membrane invaginations used in photosynthetic activity. The placement and arrangement of thylakoids are key taxonomic features. Thylakoids may be located in the cell parietally, radially, or irregularly, where their position is mostly relevant at the family or order level (Fig. 1; Komárek et al. 2014).

Photosynthesis

Cyanobacteria are capable of oxygenic photosynthesis. The main photosynthetic pigment is chlorophyll-*a*, which may be accompanied by carotenoids, xanthophylls, and phycobilisomes, which contain mostly phycoerythrin and phycocyanin. Some cyanobacteria possess divinyl-chlorophyll-*b* (*Prochlorococcus*; Partensky et al. 1999) or chlorophyll-*d* (*Acaryochloris*; Miyashita et al. 2003).

Phylogeny

The advent of molecular biology brought a plethora of tools that have been used to investigate species diversity and evolution. It has allowed researchers to move from a solely phenetic approach to the reconstruction of evolutionary relationships. The most reliable and reproducible approach developed for phylogenetic inference is considered DNA sequencing. Since Woese et al. (1990) have proposed the 16S rDNA gene as the universal marker for all Bacteria, it is the most frequently used gene in phylogenetic reconstructions (Rajendhran and Gunasekaran 2011). However, an expansion of sequenced loci is increasingly inevitable since the 16S itself is too conserved to reflect the true variation of this lineage (Johansen and Casamatta 2005). A combination of various housekeeping and protein-coding loci (usually up to 10), or multilocus sequence typing (MLST; Maiden et al. 1998), has been proposed, as it offers better resolution for recognition of taxa since some of the genes are less conservative than the 16S. Moreover, MLST may provide more robust phylogenetic reconstructions (Maiden et al. 1998; Kämpfer and Glaeser 2012).

Recently, prokaryotic genome sequencing has become relatively inexpensive and widely available to researchers. However, the selection of cyanobacterial taxa to sequence is strongly biased. Most of the sequenced cyanobacteria are marine picoplankton, typically *Synechococcus* and *Prochlorococcus* (Larsson et al. 2011), but efforts to increase genome data coverage of cyanobacterial diversity have been undertaken (Shih et al. 2013). Nevertheless, it should be noted that a number of strains whose genomes have been analyzed are poorly characterized and/or have been in culture collections for extended periods, leading to taxonomic confusion. Fortunately, the taxonomic coverage of genomic sequence data is ever increasing, and thus we anticipate a soon-to-be expansion of whole genome phylogenies for taxonomical purposes outside marine picoplanktic or biotechnologically important taxa.

Challenges of Modern Cyanobacterial Taxonomy

As noted above, the taxonomy of cyanobacteria has traditionally been heavily dependent on morphological data. With a growing number of taxa sequenced, it has become increasingly obvious that the evolutionary relationships of cyanobacteria are more entangled than previously thought (for extensive reviews see Komárek et al. 2014; Dvořák et al. 2015a). First of all, some morphological features (including ultrastructural) appear to be polyphyletic in nature. One of a few exceptions is in the production of specialized, differentiated cells of the Nostocales, which appeared only once, and it is consistently monophyletic in phylogenetic reconstructions based on one or multiple loci. Thus, many (and possibly a majority) of the genera defined using morphology are polyphyletic and therefore need significant taxonomical intervention (Komárek 2010; Komárek et al. 2014). Furthermore, unrelated lineages may be so similar morphologically that it is impossible to distinguish between them. Some authors use the term "cryptogenera" to describe this phenomenon (Komárek et al. 2014). This is also an eminent problem in newly described taxa (Dvořák et al. 2015a). For example, the most enigmatic cyanobacterial genus (measured by number of polyphyletic lineages) is probably *Synechococcus*. Dvořák et al. (2014a) identified 12 polyphyletic lineages within this genus, which all conform to the generic, morphologically derived description of *Synechococcus*. Similar patterns within genera of "cryptic species," or taxa not distinguishable by morphology, may also lead to taxonomic confusion. For example, *Phormidium retzii*, considered the most commonly encountered macro-cyanobacterial taxon in North America (Sheath and Cole 1992), is actually a species complex (Casamatta et al. 2003). This is particularly problematic in the case of morphologically simple cyanobacteria such as *Leptolyngbya* or *Synechococcus* (Osorio-Santos et al. 2014; Dvořák et al. 2015a).

What factors may be responsible for the enigmatic evolutionary relationships among cyanobacteria? Cyanobacterial evolution, as in other prokaryotes, is driven by similar factors (e.g., ecological parameters) to eukaryotes but with different selection pressures. For example, cyanobacteria exhibit vast population sizes, relatively fast generation times, and immense dispersal abilities (reviewed in Achtman and Wagner 2008). Moreover, albeit largely asexual, cyanobacteria horizontally exchange DNA via homologous recombination and horizontal (lateral) gene transfer (e.g., Zhaxybayeva et al. 2006; Fraser et al. 2007; Polz et al. 2013; Dvořák et al. 2014a, and many others). Thus, these complicated evolutionary trajectories lead to an equally complicated species concept with no consensus among scientists (reviewed cf. Achtman and Wagner 2008).

A majority of recent cyanobacterial taxonomical studies use species definitions based on a monophyletic species concept coupled with a "polyphasic" or total evidence approach, which is defined as a combination of morphological, molecular, ecological, and other data (Vandamme et al. 1996; Johansen and Casamatta 2005). Cyanobacterial systematics is further muddled since species descriptions differ between bacteriological and botanical nomenclature, both of which are

appropriate for cyanobacteria. The International Code for Nomenclature of Prokaryotes (ICNP) has very strict requirements compared to the botanical code (International Code for Algae, Fungi and Plants; ICN). For example, the ICNP requires an axenic culture for a valid species description, something exceedingly difficult to achieve with cyanobacteria. This problem is discussed in detail elsewhere (Oren 2011; Pinevich 2015).

Higher taxonomical ranks (e.g., Orders and Families) have undergone drastic changes over the past 20 years due to advances in reconstructions of molecular evolution. A few alternative systems for cyanobacteria have been proposed. Although there is no strict consensus among researchers as to which system is the most reliable, for the sake of clarity and congruence throughout the chapter we will employ the recent scheme advocated by Komárek and collaborators in 2014. Komárek's system relies largely on total evidence reconstructions, and thus should be a good approximation of evolutionary relationships. For example, Fig. 1 is a schematic representation of Komárek's system on a rank of order with the most important morphological features. Figure 2 shows a phylogenetic reconstruction with a designation to orders based on Komárek et al. (2014). However, it should be noted that the system has only recently been proposed and it is far from being generally accepted.

Unfortunately, we unable to capture the entirety of the diversity of all cyanobacteria in a single chapter and thus we will focus on the most important genera with an exception of Nostocales, where we use family level, because we possess a significant amount of data from each family designed by Komárek et al. (2014). Familial designations for the other cyanobacterial lineages (e.g., the Chroococcales) are not as well defined at this time.

Synechococcales

This large group (Fig. 3) contains the most abundant, ecologically significant, and the oldest cyanobacteria, which are characterized by parietal thylakoid arrangement (Komárek et al. 2014). Annual global abundances of marine picoplanktic *Prochlorococcus* sp. and *Synechococcus* sp. in oceans may reach up $2.9 \pm 0.1 \times 10^{27}$ and $7.0 \pm 0.3 \times 10^{26}$ cells, respectively (Flombaum et al. 2013). Although there is no direct evidence in paleontological data, molecular dating methods allowed reconstruction of the earliest events in cyanobacterial evolution. It has been suggested that the first *Synechococcus* cells may have appeared in hot springs before 3 BYA (Dvořák et al. 2014a). While the order contains 70+ genera, the most abundant are *Synechococcus*, *Prochlorococcus*, *Leptolyngbya*, and *Pseudanabaena* (Whitton and Potts 2000). The order Synechococcales is not a morphologically coherent group, containing both unicellular and filamentous cyanobacteria. Since it is the largest order of the non-heterocystous cyanobacteria in terms of number of genera, we cannot cover the whole diversity of this order. Thus, we will focus on the most commonly encountered and abundant taxa.

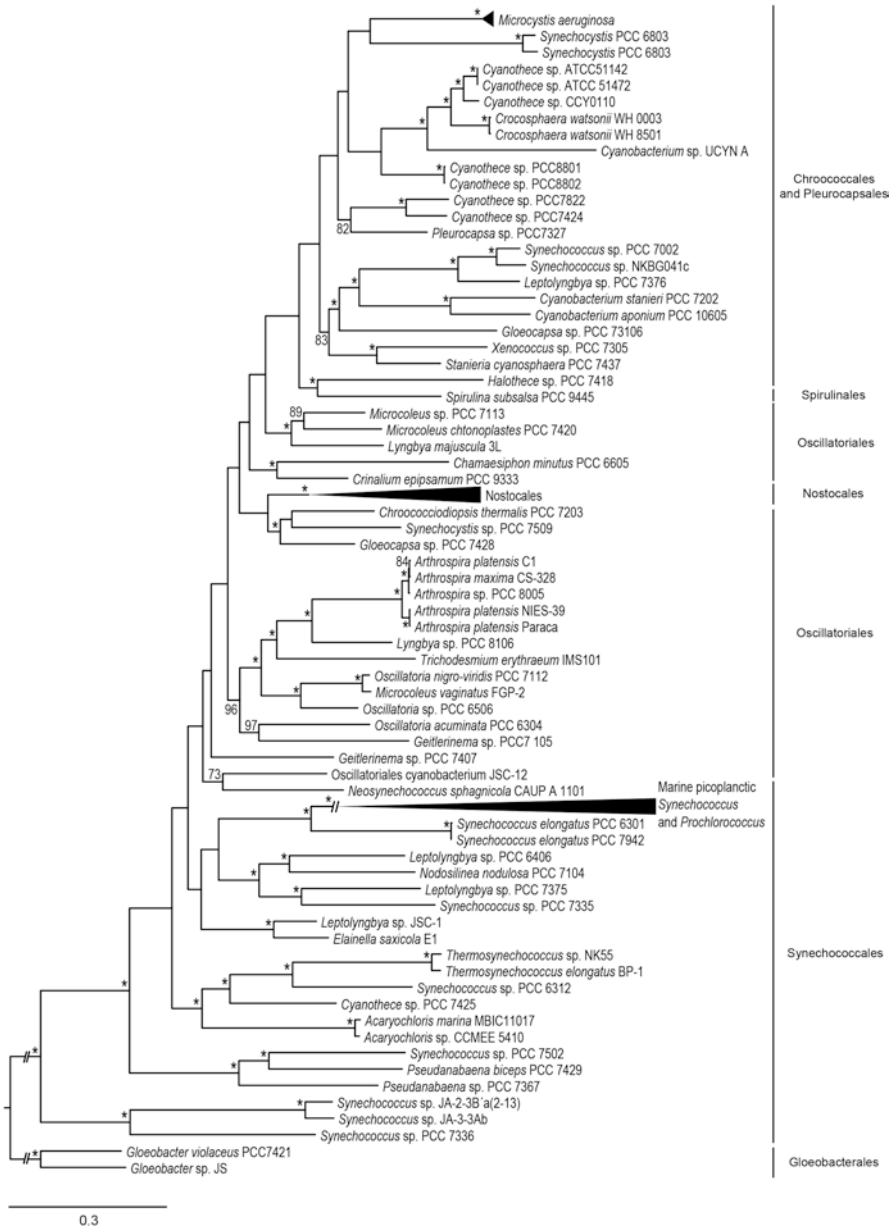

Fig. 2 Phylogenomic reconstruction of cyanobacterial evolution based on 69 concatenated, orthologous protein coding loci. These loci were selected, aligned, and concatenated using Hal (Robbertse et al. 2011). The tree was reconstructed using maximum likelihood optimality criterion and the CAT + LG model in RaxML 8.0.2 (Stamatakis 2006). The topology was tested by 500 rapid bootstrap replicates. Bootstrap support is located at the nodes with an *asterisk* representing 100 % bootstrap support. Orders of cyanobacteria *sensu* Komárek et al. (2014) are labeled

Synechococcus *and* Prochlorococcus

Under the name *Synechococcus* is hidden an example of morphologically similar yet genetically distant lineages. *Synechococcus* is a morphologically simple, yet polyphyletic genus. Described by Nägeli (1849), members are unicellular, small (<4 μm), sometimes forming pseudofilaments, and may possess involuted cells (unusual elongation of cells without cross-wall formation). On the other hand, this lineage exhibits immense genetic variability and extreme polyphyletic origins. For example, Dvořák et al. (2014a) found 12 unrelated lineages with congruent morphology. In subsequent paragraphs, we will focus on some of them according to Fig. 1 in Dvořák et al. (2014a). It should be noted that all of these clades have apparently very distinct evolutionary histories. However, only minor efforts have been made to taxonomically revise this genus, even though the polyphyletic nature of this lineage was amongst the first elucidated with molecular methods (Honda et al. 1999; Robertson et al. 2001). Komárek and Anagnostidis (1998) recognized at least 13 species of *Synechococcus*, which are not linked to type cultures. Moreover, we cannot assign them plausibly to 12 clades from Dvořák et al. (2014a) and to Bergey's Manual (Boone et al. 2001), which recognizes only five, unnamed clusters (see supplementary materials from Dvořák et al. 2014a).

The oldest clade of *Synechococcus* (clade 1) is composed of cyanobacteria inhabiting hot springs, specifically in Yellowstone National Park (Ferris and Ward 1997). This also supports an origin of cyanobacteria in hot springs, currently a prevalent opinion (Butterfield 2015) contrary to a freshwater origin proposed by Blank and Sanchez-Baracaldo (2010). Thermal strains later diverged and are mixed with freshwater strains in clade 6. They are often called the *Thermosynechococcus* (Katoh et al. 2001), but this has not been formally accepted under any code of nomenclature. *Synechococcus* strains dwelling in peat-bogs (*Sphagnum* bogs) are located in clades 8 and 9. *Neosynechococcus sphagnicola* (clade 9) comprises an exception among unnamed or incorrectly named clades, being only recently described (Dvořák et al. 2014b; Fig. 3).

Clade 10 contains the freshwater taxon *Synechococcus elongatus* (PCC 7942, PCC 7943, and PCC 6301), marine and freshwater picoplanktic *Synechococcus*, *candidatus Synechococcus spongiarum*, and *Prochlorococcus* (see details on *Prochlorococcus* below). All these taxa comprise a coherent monophyletic group (Dvořák et al. 2014a), which is, however, highly ecologically and genetically diverse. This group is composed of two clades, which contain either freshwater or marine strains. However, among freshwater strains, there are mixed marine (e.g., WH5701) and thermal (CCAP 1479/1B) strains. It was previously thought that picoplanktic strains of *Synechococcus* were strictly marine, but recent studies (Callieri et al. 2013) showed also their significance and high abundance in a plankton of freshwater lakes. Thus, we may conclude that some strains of *Synechococcus* have high salinity tolerance or were repeatedly introduced to the marine environment. Clade 10 contains *candidatus Synechococcus spongiarum*, which is the most common cyanobacterial symbiont of marine sponges (Erwin and Thacker 2008). It exhibits a very similar

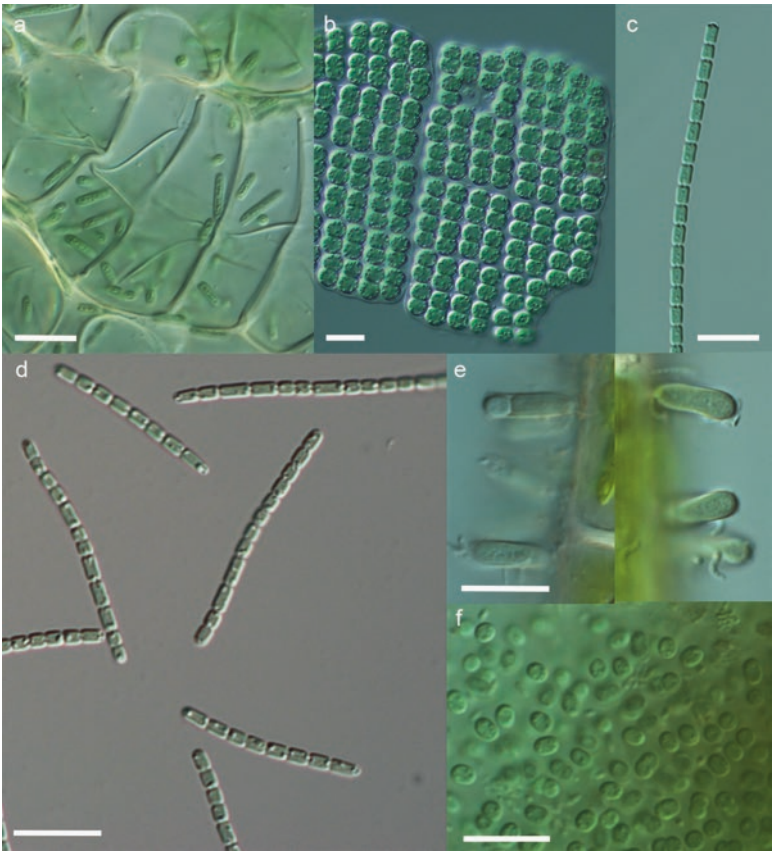

Fig. 3 Microphotographs of Synechococcales. (**a**) *Neosynechococcus sphagnicola*, (**b**) *Merismopedia glauca*, (**c**) *Pseudanabaena galeata*, (**d**) *Pinocchia polymorpha*, (**e**) *Chamaesiphon* sp., (**f**) *Aphanocapsa* sp. Scale bar 10 μm

genome to other *Synechococcus* strains from this clade; however, the genome has been undergoing a streamlining, which is typical for symbiotic bacteria due to more stable environment in the host (Gao et al. 2014).

Prochlorococcus is probably the most abundant oligotrophic cyanobacterium, and exhibits unique physiological features. First of all, it is very small (<2 μm), with simple rod shaped cells and an unusual photosynthetic pigment composition: divinyl-chlorophyll *a* and *b*. Further, it lacks phycobilisomes (specialized cyanobacterial and rhodophyte light harvesting antennae). The genome of *Prochlorococcus* is one of the smallest among cyanobacteria as it has undergone intensive genome reduction since it diverged from *Synechococcus* (Partensky and Garczarek 2010). *Prochlorococcus* is one of the most intensively studied cyanobacteria, and thus its ecological importance and evolutionary impact is extensively reviewed elsewhere: Partensky et al. (1999), Garcıa-Fernandez et al. (2004), and Biller et al. (2015).

Although *Prochlorococcus* seems to be monophyletic, there are four distinct ecotypes. Two of them are more abundant in high light conditions (abbreviated HL). HLll is more abundant in lower latitudes and HLl in higher latitudes. Two other ecotypes are adapted to low light conditions (LL). Ecotype LLl is more abundant in higher latitudes in upper layers of oceans and in lower latitudes in deeper layers around the thermocline. The last ecotype, LLlV, lives only in low latitudes and in the deepest areas of occurrence under the thermocline (Moore et al. 1998; West and Scanlan 1999).

Leptolyngbya

Leptolyngbya is possibly the largest genus of filamentous cyanobacteria in terms of number of species, and is characterized by overall simple morphology: thin filaments (<2 μm) with narrow cells and occasional false branching (reviewed in Komárek and Anagnostidis 2005). *Leptolyngbya* may be found in benthic, aerophytic, subaerophytic, periphytic, and even artificial habitats such as unsanitized urinals (Rulík et al. 2003). Due to their simple morphology, species have been classified based on their ecological preferences. Phylogenetic analyses repeatedly revealed extensive polyphyly within this genus (e.g., Casamatta et al. 2005). Interestingly, some lineages of "*Leptolyngbya*" and "*Synechococcus*" *sensu lato* exhibit similar phylogenetic relationships (e.g., Dvořák et al. 2014a, b). Thus, although they are not coherent in morphology, they often form monophyletic clusters.

The majority of the described species of *Leptolyngbya* have not been sequenced, so molecular revisions are nearly impossible at this stage. Moreover, there are new species with morphology similar to *Leptolyngbya* (based only on morphology), but they are unrelated to the type species. We will discuss a few examples of recently discovered and revised taxa.

The genus *Oculatella* has been proposed by Zammit et al. (2012). Initially isolated from hypogean environments in Malta and Italy, it has a conspicuous apomorphy: a rhodopsin-like reddish inclusion in a terminal cell. Subsequently, Osorio-Santos et al. (2014) proposed another seven species within this monophyletic cluster mostly from desert environments. Some of them lack any unique morphological feature, thus they can be recognized only using sequence data and therefore they are cryptic species.

Ecologically even more diverse are species within the genus *Nodosilinea* (formerly cluster 3 based on Bergey's manual; Boone et al. 2001) which has been established by Perkerson et al. (2011). Initially, this genus contained only four species, isolated from marine and freshwater lakes, but further isolates have been obtained from rocks and desert soils. All strains, though, have a distinctive apomorphy: the production of nodules when grown under low light levels. Interestingly, this appears to have nothing to do with nitrogen fixation (Li and Brand 2007).

Oculatella and *Nodosilinea* are both excellent examples of ecological, biogeographical, and evolutionary differentiation with available molecular data besides the marine picoplanktic *Synechococcus*.

The most comprehensive phylogeny of *Leptolyngbya* has been presented in the paper of Osorio-Santos et al. (2014). It suggests that the monophyly of the genus *Leptolyngbya* will be significantly more disturbed in the future, because there are at least four other species of *Leptolyngbya* outside the *Leptolyngbya sensu stricto* cluster, which contains *L. boryana*, *L. faveolarum*, *L. tenerrima*, and *L. angustata*.

Pseudanabaena

The genus *Pseudanabaena* consists of 36 species, which predominately occur in the plankton and benthos of freshwater or brackish water bodies all around the world. It is filamentous with small cells (mostly <2 μm), distinctive constrictions at cross-walls, rare sheath production, and often with distinctive chromatoplasma (Fig. 3; reviewed in Komárek and Anagnostidis 2005). This chromatoplasm refers to the parietal part of the cell, where thylakoid are concentrated, thus appearing to be darker than the centroplasm, or transparent, central portion of the cell.

Recent investigations have revealed that *Pseudanabaena* is also polyphyletic (Acinas et al. 2009; Dvořák et al. 2015a; Yu et al. 2015). Yu et al. (2015) have attempted to revise the genus by sequencing *P. mucicola*, *P. galeata*, *P. limnetica*, and *P. minima,* showing that they form a monophyletic clade together with *P. catenata*, the type species. Further revisions are forthcoming. For example, Dvořák et al. (2015a) investigated two strains similar to *P. galeata* from a freshwater lake in Vietnam. Although the morphological similarity of these strains to *P. galeata* was remarkable, the strains formed a separate cluster far from *P. galeata.* This represents another evidence of polyphyletic origin of *Pseudanabaena* and new monospecific genus *Pinocchia polymorpha* (Fig. 3) has been described.

The genus *Pseudanabaeana* is also evolutionarily related to the common planktic cyanobacterium *Limnothrix redekei*. While *L. redekei* (a non-toxic, planktic, filamentous cyanobacterium) largely differ in morphology from *P. catenata* and *P. galeata*, they are very closely related in phylogeny of 16S rRNA as shown by, e.g., Suda et al. 2002).

Synechocystis

One of the most popular model organisms for molecular biology is *Synechocystis* sp. strain PCC 6803. Commonly employed by researchers investigating photosynthesis, it was the first sequenced cyanobacterial genome (Kaneko et al. 1995). *Synechocystis* sp. PCC 6803 is also considered as a reference strain for the genus in Bergey's manual (Boone et al. 2001).

Recently, it has been shown that *Synechocystis*, like many other genera, is polyphyletic. Based on phylogenetic position and thylakoid arrangement, the genus *Geminocystis*, containing two species *G. herdmanii* and *G. papuanica*, was split from *Synechocystis* (Korelusová et al. 2009).

Merismopedia

There are 40 species within this genus, but molecular data are available only for three of them. *Merismopedia* is a group of unicellular cyanobacteria, which has a unique colony formation where cells divide in a single plane and create flat colonies (Komárek and Anagnostidis 1998; Fig. 3). *Merismopedia* is most commonly found in periphytic habitats. Palinska et al. (1996) analyzed strains of *M. punctata*, *M. glauca*, *M. elegans* and found that although these strains exhibited a degree of high morphological variability, their near complete 16S rRNA sequence was identical. Thus, it contradicts the vastly more common pattern of the hidden genetic "cryptic diversity" without observable phenotype variability (see an example of *Oculatella* above).

Acaryochloris marina

Acaryochloris marina is a unicellular marine cyanobacterium with simple morphology and has a unique metabolic feature and an unusual photosynthetic pigment: chlorophyll-*d*. First described in 2003 (Miyashita et al. 2003), it has an unexpectedly large genome of 8.3 Mb, which is predominantly typical for morphologically more complex cyanobacteria (Shih et al. 2013). *A. marina* is a monospecific genus, which deserves attention due to its importance as a model organism for understanding photosystem modification and genome expansion in cyanobacteria (Swingley et al. 2008).

Chamaesiphon

This asymmetrically dividing, chroococcalean genus commonly inhabits both submerged and periodically wetted substrates (Fig. 3), in tropical and temperate regions such as on stones, plants, and filamentous algae, especially in running waters. The genus can be divided into three subgenera: *Chamaesiphon sensu stricto*, *Chamaesiphonopsis*, and *Godlewskia* (Sant'Anna et al. 2011), but this is not supported via molecular data. Interestingly, Honda et al. (1999) showed phylogenetic affinity of *Chamaesiphon subglobosus* PCC 7430 to *Leptolyngbya boryanum* PCC 73110 and *Leptolyngbya foveolarum* Komárek 1964/112. Loza et al. (2013) suggested a molecular similarity of *Chamaesiphon subglobosus* PCC 7340 and *Ch. investiens* UAM 386 with genera *Synechococcus* and *Cyanobium*. Komárek et al.

(2014) showed that *Ch. minutus* PCC 6605 forms a separate clade with *Crinalium epipsammum* PCC 9333 within the order Oscillatoriales. Members of subgenus *Godlewskia* probably belong to the order Chroococcales, family Stichosiphonaceae, but it has not been confirmed by molecular methods.

Chroococcales

Members of the order Chroococcales represent unicellular cyanobacteria commonly encountered in aquatic (planktic and periphytic) and terrestrial environments (aerophytic, subaerophytic, soil, epilithic, epiphytic, etc.). Most taxa possess relatively simple morphology, leading to often times indistinct boundaries between species and genera. The order Chroococcales includes eight families, whose members live in single or colonial mode of live, dividing in one or more planes and usually forming irregular type of thylakoid arrangement (Fig. 4; Komárek et al. 2014).

Unicellular cyanobacteria represent an important group in aquatic habitats with respect to their abundance, diversity, and ecological role.

Microcystis

Microcystis is a well-defined genus and possibly the most commonly cited and studied cosmopolitan, bloom-forming, and toxin producing genus (Fig. 4). Planktic *Microcystis* is usually dominated by a few species such as *M. aeruginosa*, *M. wesenbergii*, *M. ichtyoblabe,* often concurrently present with other taxa such as *Woronichinia*, *Snowella,* and *Merismopedia*. The most frequently occurring species (e.g., *M. aeruginosa*, *M. wesenbergii,* or *M. ichtyoblabe*) may be polyphyletic based on 16S rRNA sequence data (Neilan et al. 1997; Komárek and Komárková 2002). Interestingly, *M. aeruginosa*, possibly the most commonly encountered eutrophic cyanobacterium, is quite consistent morphologically across freshwater lakes worldwide. However, it has high 16S-23S ITS diversity and it exhibits geographical barriers on gene flow among populations, with a concurrent lack of phylogeographical pattern (van Gremberghe et al. 2011). Current research reveals specialized clones (chemotypes) of *Microcystis* producing various types of toxic microcystins (Walker et al. 2004). Thus, if we take into account secondary metabolite production as an additional feature, the real biodiversity within *Microcystis* is likely higher than currently suggested.

Aphanothece sensu lato

Members of the genus *Aphanothece* occur worldwide from tropical to polar areas (Gardner 1927; Copeland 1936; Komárek 2003, 2014; Hašler and Poulíčková 2005; Whitton 2005). The genus includes morphospecies with elongated to rod-like cells,

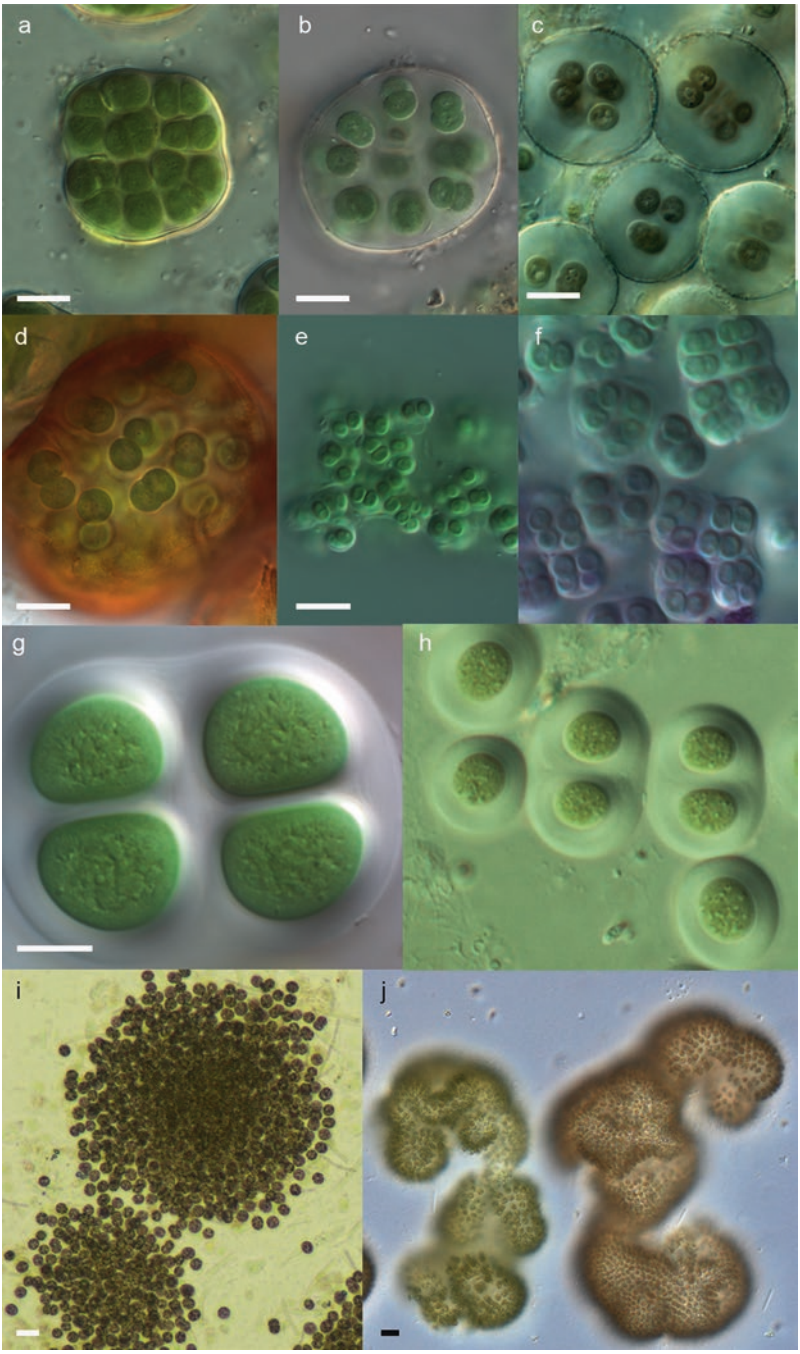

Fig. 4 Microphotographs of Chroococcales. (**a**) *Asterocapsa divina*, (**b**) *Asterocapsa* sp., (**c**) *Asterocapsa* sp., (**d**) *Gloeocapsa novacekii*, (**e**, **f**) *Gloeocapsa* sp., (**g**) *Chroococcus subnudus*, (**h**) *Chroococcus* sp., (**i**) *Microcystis novacekii*, (**j**) *Woronichinia naegeliana.* Scale bar 10 μm

dividing in one plane (perpendicular to main axis) and forming small gelatinous colonies. Although only few records of 16S rRNA exist in GenBank, the genus seems to have a polyphyletic origin. For instance, Komárek et al. (2011) pointed out wide genetic and morphological variability within the genus *Aphanothece*.

Several subgenera (or separate genera if subsequently elevated) of the genus *Aphanothece* can be distinguished: *Aphanothece sensu stricto*, *Anathece*, *Cyanogastrum*, *Halothece-Euhalothece* complex. The new genus *Anathece* represents morphospecies forming oval or rod-like forms, based on the type species *Anathece clathrata* (basionym: *Aphanothece clathrata*). Molecular and morphological features indicate an *Anathece* affiliation with members of the order Synechococcales (Komárek et al. 2011). The remaining species of *Aphanothece* group belong to the order Chroococcales.

Gloeothece

Coccoid cyanobacteria from the genus *Gloeothece* form oval, elongated, or rod-like cells, enveloped with distinct mucilaginous sheaths. *Gloeothece* plays a substantial role within aerophytic or subaerophytic communities or on submerged substrates all over the world (Komárek and Anagnostidis 1998). Their morphology, type of cell division, and molecular features are similar to *Aphanothece*. Sequence similarity of the 16S rRNA from GenBank ranges from 88 to 100 %, clearly falling outside of the traditionally accepted levels for within generic variation. *Gloeothece fuscolutea* was recently designated as a new type of the genus (Mareš et al. 2013) because of similarity between the former type (*Gloeothece linearis*) and *Gloeobacter violaceus*. However, the proposed neotype is still in conflict with Rippka et al. (2001b) who propose *G. membranacea* PCC 6501 as a reference strain and neotype for a botanical nomenclature.

Chroococcus

Chroococcus is commonly encountered on aerophytic, subaerophytic or submerged substrates, where it typically forms small microscopic colonies consisting of hemispherical cells, covered with mucilaginous envelopes, often layered (Fig. 4). Members of this unmistakable genus occur all over the world where they frequently occur in tropical or temperate zones, and less towards the poles (Komárek and Anagnostidis 1998). Molecular analyses show that this genus consists of ca. 60 species in a polyphyletic lineage (e.g., Komárková et al. 2010; Kováčik et al. 2011). Recently, *Chroococcus* was split into two new genera: *Limnococcus* and *Chroococcus sensu stricto*. Species of *Limnococcus* are planktic and differ morphologically and molecularly from the members of *Chroococcus sensu stricto*. Despite a molecular characterization of *Chroococcus sensu stricto,* it is still not clear what the “true”

Chroococcus is, because sequences of the type species, *Ch. rufescens,* are lacking. Further, the phylogenetic placement of some small species is unclear due to molecular similarity with other genera such as *Eucapsis* or *Synechocystis*. For example, Rippka et al. (2001a) note that there is morphological and molecular similarity among several PCC strains of small *Chroococcus* and *Gloeocapsa*. Thus, the true diversity of *Chroococcus* in nature across its range remains unclear and is still based on morphological investigation only.

Gloeocapsa

Several members of the genus *Gloeocapsa* possess morphological similarity with *Chroococcus. Gloeocapsa* represents a relatively heterogeneous group of colonial cyanobacteria forming small or medium sized spherical or oval cells, covered with distinct or indistinct envelopes of various colors (Fig. 4). The majority of species prefer aerophytic or subaerophytic habitats such as moist rocks or bark of trees (Komárek and Anagnostidis 1998). *Gloeocapsa* exhibits a specific life cycle including several distinct morphological stages, the observance of which is essential in the process of species identification. Available sequences of "*Gloeocapsa*" correspond to different genera such as *Chroococcidiopsis*, *Gloeocapsopsis*, *Cyanothece*, *Gloeothece*, but whether or not this is due to convergent evolution of simple morphologies or as a result of misidentifications of strains remains to be seen. For instance, *Gloeocapsa* sp. PCC 7428 (isolated from a moderate hot spring in Sri Lanka) corresponds to the genus *Gloeocapsopsis* (Azua-Bustos et al. 2014). *Gloeocapsa* "*alpicola*" FACHB-400 (=*G. atrata*?) shares 99 % identity with *Gloeothece* sp. PCC 6909 or *Synechocystis* sp. LEGE 06083, and probably does not represent the *Gloeocapsa* at all. Several morphologically distinct lineages within *Gloeocapsa* exist, which can be recognized based on cell size and color of mucilaginous envelopes. Small species (cells <6 μm in diameter) such as *G. atrata*, *G. aeruginosa*, *G. punctata*, *G. compacta* or *G. fusco-lutea* occur frequently among aerophytic habitats, where they form various eco- and morphospecies. Species delimitations without molecular confirmation can be problematic because of the overlapping morphologies. Our knowledge about the diversity of species producing large cells (>6 μm) is incomplete. Current research is based only on floristic data and ecologies of aerophytic/subaerophytic populations.

Peculiar Chroococcales with No Sequence Data

The genera *Cyanophanon*, *Clastidium*, *Stichosiphon* and probably *Chamaecalyx* belong to the currently established family Stichosiphonaceae (Komárek et al. 2014). They represent typical, globally distributed epilithic or epiphytic taxa in streams, rivers, stagnant water bodies or artificial water bodies such as aquaria, channels or basins (Komárek and Anagnostidis 1998). There is currently no molecular data for the family as a whole.

The family Gomphospaericeae has also been established only on a basis of morphological features. Members of this lineage typically occur in plankton from tropical to temperate zones, and rarely cold arctic waters. Only one sequence is available: *Gomphosphaeria aponina* SAG 52.96 (freshwater, Austria). The family Entophysalidaceae represents the most morphologically distinctive group of chroococcalean cyanobacteria, but the molecular diversity within the family and species is not known. However, the few deposited sequences of *Chlorogloea* exhibit a high level of heterogeneity. The majority of these species inhabit stony substrates or plants in both aquatic and terrestrial environments, especially in tropical or temperate zones (Komárek and Anagnostidis 1998).

Chroococcidiopsidales

Coccoid cyanobacteria from the genus *Chroococcidiopsis*, formerly placed within the order Chroococcales, now form a separate cluster (Fewer et al. 2002; Azua-Bustos et al. 2014). Interestingly, the genus *Chroococcidiopsis sensu stricto* is more similar in 16S sequence to heterocystous cyanobacteria such as *Fischerella*, *Nostoc*, *Scytonema* than morphologically similar genera from the order Chroococcales or Pleurocapsales. Molecular diversity indicates heterogeneity within the genus *Chroococcidiopsis* (Rippka et al. 2001c; Donner 2013). The reference strain of *Chroococcidiopsis thermalis* PCC 7203 (Rippka et al. 2001b) represents a cluster of freshwater or soil species with cells <5 μm in diameter producing baeocytes <4 μm. Strain PCC 6712 represents freshwater species forming larger cells than members of first cluster. Donner (2013) showed that the genus consists of more lineages than previously found by Rippka et al. (2001c). *Chroococcidiopsis* occurs worldwide, often in extreme environments such as hot and cold deserts, aerophytic, epi/endolithic, on soil or symbiotic in lichens (e.g., Boison et al. 2004; Sompong et al. 2005; Büdel et al. 2009).

Pleurocapsales

Members of the order Pleurocapsales are closely related to the order Chroococcales. They exhibit irregular cell division, specific formations of pseudofilamentous or pseudoparenchymatous thalli, and various types of polarized cells (Fig. 5). Members of the order form a monophyletic group closely related to the Chroococcales (e.g., Komárek et al. 2014). On the other hand, molecular analysis based on genome sequencing placed pleurocapsalean cyanobacteria in the same clade together with members of the order Chroococcales (Shih et al. 2013).

Rippka et al. (2001d) distinguished three clusters based on phenotypic features of PCC strains. *Pleurocapsa minor* strain PCC 7327 seems to be separated from the pleurocapsalean clade (Shih et al. 2013, Fig. 2). It was isolated from a hot spring

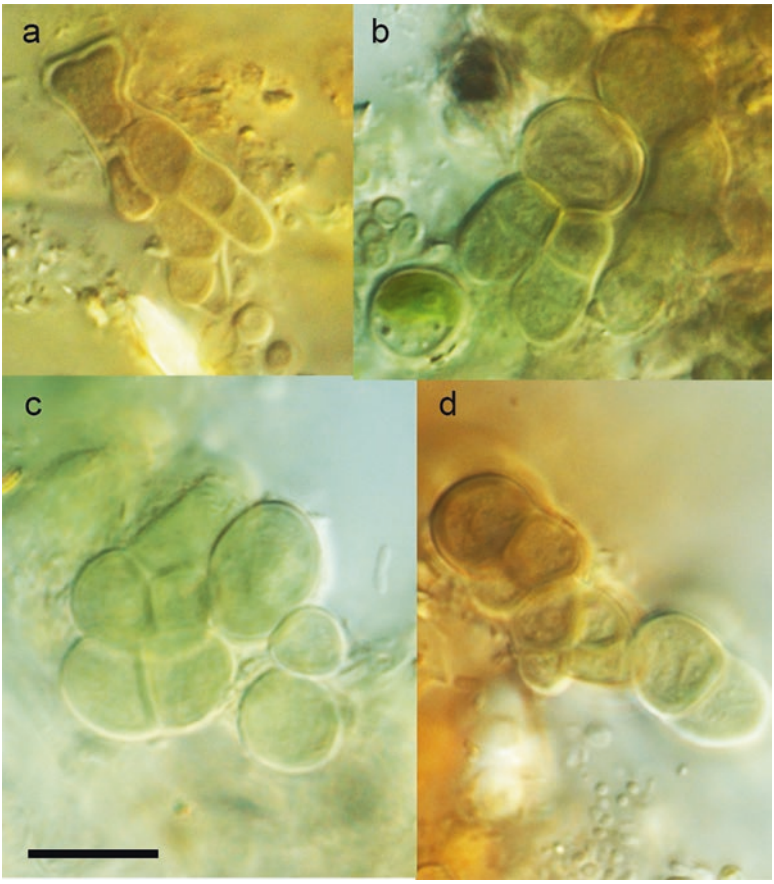

Fig. 5 Microphotographs of Pleurocapsales. (**a**–**d**) *Pleurocapsa minor*. Scale bar 10 μm

and probably represents a different species than designated. The most frequently used strain, PCC 7319, was isolated from a snail shell at intertidal zone in the Northern Mexico. However, the description of *P. minor* originates from streams near Prague (Hansgirg 1890, 1892). Records of *P. minor* from biotopes other than streams in temperate zones should be confirmed by molecular analysis. The genus *Pleurocapsa* includes almost 40 species occurring worldwide (Fig. 5), but only 16S rRNA sequences of *P. minor* and *Pleurocapsa* sp. are available.

The genus *Myxosarcina* is characterized by packet-like colonies and baeocyte production. The type species has not been sequenced and thus we are unable to thoroughly evaluate phylogenetic relationships. However, results from analyses of two complete genomes place *Myxosarcina* in the same clade as *Staineria, Pleurocapsa,* and *Dermatocarpella* (Yu et al. 2015).

Members of the genus *Stanieria* (family Dermocarpellaceae) represent coccoid, baeocyte forming taxa, which inhabit submerged substrates both in fresh and salt

waters. Most of the sequences in GenBank belong to *S. cyanosphaera* and *Stanieria* spp. The phylogeny of Yu et al. (2015) based on 16S rRNA sequence data divides *Stanieria* into two groups including freshwater (e.g., strain PCC 7437) and saltwater species (e.g., strains PCC 7301 and 7302).

Dermocarpella contains six periphytic species with only scarce molecular data available. The most frequent records belong to species originated from stromatolites in Shark Bay (Goh et al. 2009), which may belong to *D. incrassata* (currently designated as *Chamaecalyx incrassatus*) inhabiting snail shells at intertidal zone in Mexico.

Likewise, all available sequences of *Xenococcus* originate from stromatolites at Shark Bay (Goh et al. 2009), geothermal springs in Costa Rica, symbiosis with marine sponges, epiphytic species of seagrasses in East Africa, periphytic in saltwater aquaria, or species inhabiting rock chips (unpublished, only stored in GenBank). Sequences of the type, *X. schousboei*, are lacking. Both molecular and morphological/ecological features of *Xenococcus* indicate a genus with polyphyletic character. Few genera of Pleurocapsales have been sequenced because of their inability to grow under laboratory conditions or rare occurrence. Thus, the real biodiversity within the order must be reexamined using field studies and molecular data.

Oscillatoriales

The Oscillatoriales were introduced in the monograph of Gomont (1892), and contained 15 genera characterized by the type of sheath and trichomes characteristics (Anagnostidis and Komárek 1988; Fig. 6). The order has been significantly expanded since its description and now contains 47 genera (Komárek et al. 2014). The order Oscillatoriales contains filamentous taxa with mostly fasciculated, radial, or irregular thylakoid arrangement (Fig. 6). One coccoid genus (*Cyanothece*; Fig. 6) has been transferred to Oscillatoriales based on thylakoid arrangement (Komárek et al. 2014). As in the other cyanobacteria, the majority of Oscillatoriales is polyphyletic.

Geitlerinema *sensu lato*

Geitlerinema (Fig. 6) consists of the filamentous cyanobacteria with terminal cell prolonged or hooked, parietal thylakoid arrangement, tricheomes <4 μm wide, prominent granules (usually), and the formation of mats on submersed substrates (wood, plants, stones, etc.) or in soils (Komárek and Anagnostidis 2005; Hašler et al. 2012). Originally this taxon was described as a subgenus of genus *Phormidium*, the type species is *Geitlerinema splendidum* (Anagnostidis and Komárek 1988). Hašler et al (2012) suggest *Geitlerinema* is polyphyletic with a subsequent revision by Strunecký et al. (2017) erecting a new genus *Anagnostidinema*, named in honor of the original author of the genus.

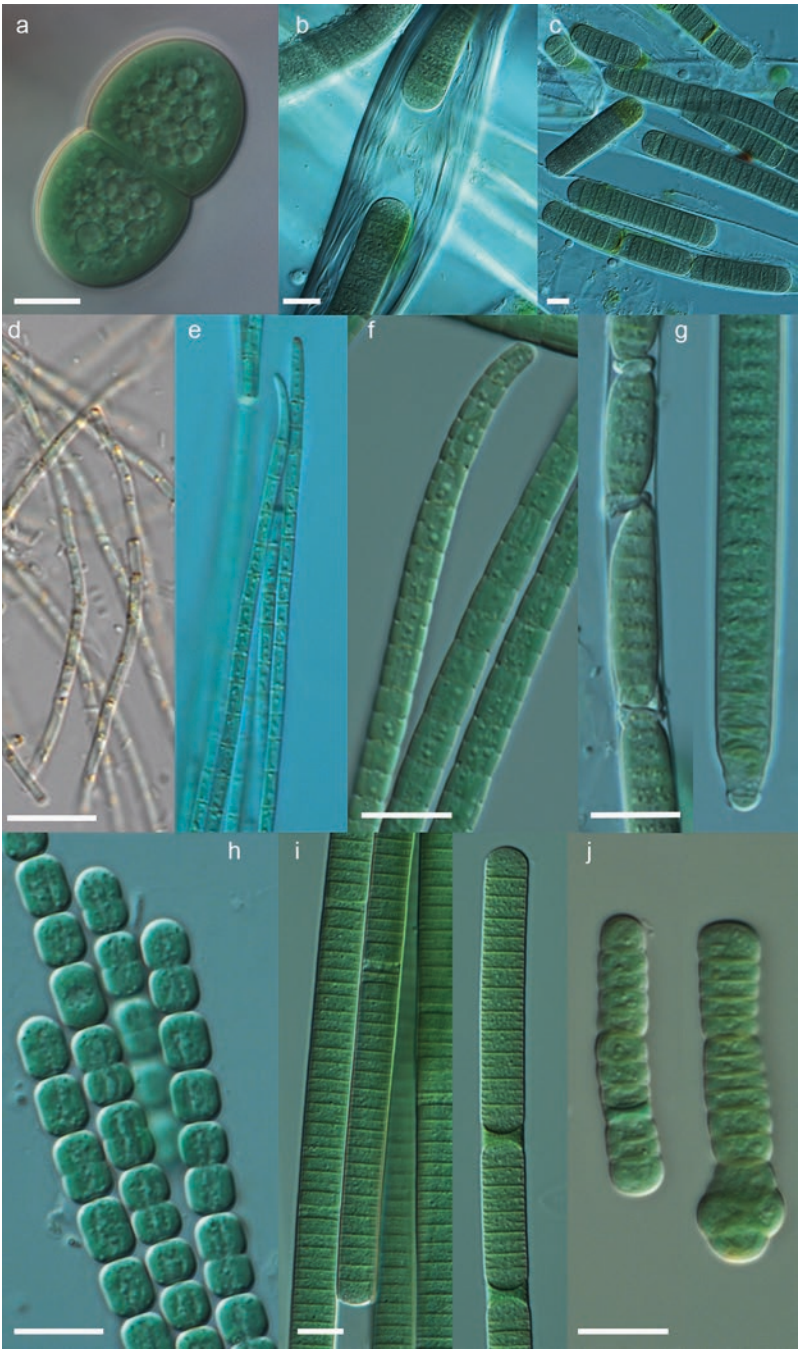

Fig. 6 Microphotographs of Oscillatoriales. (**a**) *Cyanothece aeruginosa*, (**b**, **c**) *Blennothrix* sp., (**d**) *Geitlerinema carotinosum*, (**e**) *Geitlerinema pseudacutissimum*, (**f**) *Kamptonema animale*, (**g**) *Phormidium* sp., (**h**) *Johanseninema constrictum*, (**i**) *Oscillatoria* sp., (**j**) *Crinalium* sp. Scale bar 10 μm

Microcoleus *and* Phormidium

Microcoleus was described by Gomont in 1892, and recent studies suggest that this taxon is polyphyletic. Type species, *M. vaginatus*, is one of the most abundant cyanobacterial species in soil crusts (e.g., Garcia-Pichel et al. 2001). *Microcoleus* forms multiple filaments in one sheath. Trichomes are cylindrical with usually narrowed, straight ends and may be terminated by calyptra. *Microcoleus* is morphologically quite similar to *Phormidium* and their morphological characters can overlap. Traditionally they have been separated on the basis of sheath formation (Komárek and Anagnostidis 2005), though this is not a stable feature (Dvořák et al. 2012; Hašler et al. 2012).

Phormidium sensu lato includes more than 200 species but it is polyphyletic, likely containing several unrecognized genera (Casamatta et al. 2003). Still, this genus is related to *Microcoleus* (Hašler et al. 2012; Strunecký et al. 2013).

Strunecký et al. (2013) revised and transferred *Phormidium autumnale* to *Microcoleus sensu strict*o based on phylogenetic analysis. It should be noted that two species, *M. vaginatus* and *M. atumnalis,* may be recognized only using a stable molecular feature: an 11 base pair insert within the 16S rRNA, which occurs only in *M. vaginatus* (Boyer et al. 2002).

Coleofasciculus originated by a revision of the *Microcoleus chtonoplastes*. It has been erected according to molecular, ecological, and secondary structure data. *Coleofasciculus* is a filamentous cyanobacterium with multiple trichomes covered by unlamellated and colorless sheath. Trichomes are non-tapering, terminal cells are conically rounded, and without calyptra. A typical habitat for *Coleofascicculus* is a littoral of brackish or marine habitats, and never occupies freshwater or terrestrial environments (Siegesmund et al. 2008).

Another genus separated from the genus *Phormidium* is *Wilmottia.* It has been derived from *Phormidium murrayi* (Strunecký et al. 2011), which was originally described from the Antarctica in 1911 as *Leplolyngbya murrayi* (West and West 1911). It is a simple filamentous cyanobacterium with rounded terminal cells, conspicuous granulation, cells <5 μm, and parietal thylakoids (Strunecký et al. 2011).

Larger and more complex species of the genus *Phormidium* have been recently revised as well. For example, Strunecký et al. (2014) erected *Kamptonema* (Fig. 6) by revising *P. animale*, a common freshwater littoral and epipelic cyanobacterium with tapering terminal part of the filament (Hašler et al. 2012). *Oxynema,* derived from the *Phormidium* of the group I *sensu* Komárek and Anagnostidis (2005) and characterized by a pointed terminal cell, was erected by Chatchawan et al. (2012). The genus *Phormidium* as a whole still has unexplored species diversity. For instance, a morphologically indistinguishable, yet genetically distinct, new cyanobacterium *Ammassolinea* (Hašler et al. 2014a) was isolated from the epipelon of the subtropical lakes in Florida.

Planktothrix

This genus was described by Anagnostidis and Komárek (1988) with type species *Planktothrix agardhii*. It consists of filamentous cyanobacteria characterized by the presence of aerotopes, straight filaments with rounded terminal cells, and without sheath (Komárek and Anagnostidis 2005). *Planktothrix* belongs to a major group of water-bloom forming cyanobacteria, which have a cosmopolitan distribution in the freshwater, eutrophic habitats (Komárek and Komárková 2004). Some species produce a variety of toxic, bioactive secondary metabolites, such as *P. agardhii* and *P. rubescens* (Walker et al. 2004). Suda et al. (2002) recognized four species based on a combination of molecular and morphological data: *P. agardhii*, *P. rubescens*, *P. mougeotii*, and *P. pseudoaghardii*. The genus as a whole was revised early, after the introduction of the molecular methods and it seems to be monophyletic.

Komvophoron

Anagnostidis and Komárek (1988) separated the genus *Komvophoron* from *Pseudanabaena* due to spherical or barrel-shaped cells and with different organization. On the basis of the shape of the vegetative and apical cells, this genus has been separated into two subgenera: *Alyssophoron* (type = *A. minutum*) and *Komvophoron* (type = *K. schmidlei*) (Anagnostidis and Komárek 1988; Hašler and Poulíčková 2010). *Komvophoron* mainly inhabits benthic areas, growing on the sand and muddy sediments in freshwater reservoirs (Komárek and Anagnostidis 2005). *Komvophoron* is likely largely overlooked or ignored and has only scant molecular data because benthic cyanobacteria in general are poorly explored (Hašler and Poulíčková 2010; Pulíčková et al. 2014) and *Komvophoron* is resistant to conventional cultivation techniques. However, Hašler and Poulíčková (2010) were able to employ single-filament PCR techniques, obtaining 16S rRNA and 16S-23S ITS sequences of *K. hindakii* and *K. constrictum*. This work showed that *Komvophoron* is polyphyletic, leading to the description of a new genus *Johanseninema* (Fig. 6; Hašler et al 2014b).

Oscillatoria

Oscillatoria is filamentous cyanobacterium with discoid cells (Fig. 6). Trichomes are slightly waved or straight, never branched. Trichomes are >8 μm, usually without sheath. *Oscillatoria, Phormidium,* and *Lyngbya* were distinguished from each other (Geitler 1932) based on sheath properties, but sheath production is not necessarily phylogenetically informative as this character heavily depends on local conditions (Whitton 1992). *Oscillatoria* often creates macroscopic layered, smooth mats. The type species is *Oscillatoria princeps* (Anagnostidis and Komárek 1988).

Although *Oscillatoria* is a very common cyanobacterium, we lack enough molecular data for wholesale revisions at this time. However, we may only infer from our phylogeny (Fig. 2) that *Oscillatoria* is polyphyletic as previously suggested by Ishida et al. (2001).

Lyngbya

This genus was described by Gomont (1892), with the type *L. confervoides*. Marine strains of *Lyngbya* are very important diazotrophic (fix atmospheric nitrogen) organisms and primary producers, but they are also rich in bioactive secondary metabolites, which are mostly toxic (Hoffmann 1994). Water blooms of *Lyngbya* can have adverse effects on coral reefs, especially on coral larvae recruitment (Kuffner and Paul 2004), because *Lyngbya* filaments are not consumed by herbivores and very quickly exploit available surfaces (Paul et al. 2005). On the basis of morphological similarities *Lyngbya, Phormidium*, and *Plectonema* were classified as the "LPP group" (Rippka et al. 1979), subsequently shown to be polyphyletic (Komárek et al. 2014). Although the genus *Lyngbya* is largely coherent in morphological features, it is polyphyletic based on 16S rRNA phylogeny (Engene et al. 2010, 2013). *Lyngbya* may be separated into three distant clades according to ecology: a mixed halophilic/brackish/freshwater lineage, a lineage more closely related to the genus *Oscillatoria* (freshwater), and a marine lineage (Engene et al. 2010). *Moorea* (Engene et al. 2012) and *Okeania* (Engene et al. 2013) were recently erected to include the marine members, which are potent producers of bioactive secondary metabolites. *Moorea* contains two species and *Okeania* five. Finally, *Limnoraphis* has been proposed by Komárek et al. (2013) for some of the freshwater strains, which are responsible for heavy water-blooms in some tropical reservoirs in South America.

Symplocastrum

Gomont (1892) was originally described *Symplocastrum* as a subgenus of the genus *Schizotrix*. Recently elevated to genus level (Anagnostidis 2001; Komárek and Anagnostidis 2005), the type species, *Sy. friesii. Symplocastrum*, is a relatively poorly researched taxon of Oscillatoriaceae (Pietrasiak et al. 2014). Morphologically, this taxon is similar to both *Hydrocoleum* and *Microcoleus*. Only recently sequenced (Pietrasiak et al. 2014), *Symplocastrum* is phylogenetically related to the newly erected genus *Kastovskya* (Mühlsteinová et al. 2014).

Spirulinales

Members of the Spirulinales are solitary or colonial (mats), have trichomes without sheathes, are regularly screw-like coiled, and usually possess an intense motility. Trichomes are not constricted at the cross walls, without branching, and without necridic cells. They reproduce by a disintegration of trichomes or by motile hormogonia (Komárek and Anagnostidis 2005). *Spirulina* is an important organism in biotechnology, as animal fodder, and as a human dietary supplement (Khan et al. 2005). However, this generic name is actually merely conserved; *Spirulina* was revised and the species *S. platensis*, the taxon of most human interest, now belongs to the genus *Arthrospira* (Vonshak 1997), which clusters with the order Oscillatoriales (Komárek et al. 2014). The genus *Halospirulina* has been split from *Spirulina* on the basis of morphology and high halotolerance (salinity between 3 and 13 %+; Nübel et al. 2000).

Nostocales

The Nostocales represent a species rich, diverse lineage of cyanobacteria typified by the ability (obligatory or not) to produce specialized cells (Fig. 7), mainly heterocytes (dedicated to nitrogen fixation) and akinetes (overwintering cells). This class was historically broken into two major groups: those taxa which undergo cell division in multiple planes (the Stigonematales *sensu stricto*, e.g., *Hapalosiphon*, *Mastigocladus*, *Stigonema*) and those taxa that never show reproduction in multiple planes (the Nostocales *sensu stricto*, e.g., *Nostoc*, *Anabaena*, *Calothrix*). These distinctions based solely on morphological features arose from the earliest works of cyanobacterial researchers (e.g., Bornet and Flahault 1886; Geitler 1932; Desikachary 1959) and while useful at a gross level, there were some inherent limitations to these classification schemes. First, some of the features, such as the production of heterocytes, have been shown to be environmentally inducible (in the classic case of the Nostocales) and thus might not be present in all examined specimens, potentially leading to taxonomic confusion. Second, cell division in >1 plane has been shown to be present in several families of the heterocytous clades, making its use in phylogenetic reconstructions questionable.

The relatively recent advent and propagation of modern molecular markers (mainly of the 16S rDNA gene sequence, secondary folding structures of the 16S-23S Internally Transcribed Spacer {ITS} region and comparison of the genes used in nitrogen fixation {e.g., *hetR*, *nifH*}) have allowed more finely nuanced, robust phylogenetic assessments. Rather than relying upon potentially environmentally plastic (e.g., sheath production) or inducible characters (e.g., specialized cells), a wealth of potentially phylogenetically informative characters may be obtained. While many members of this clade are difficult to fully assess due to difficulties in culturing or sequencing, modern approaches are yielding new

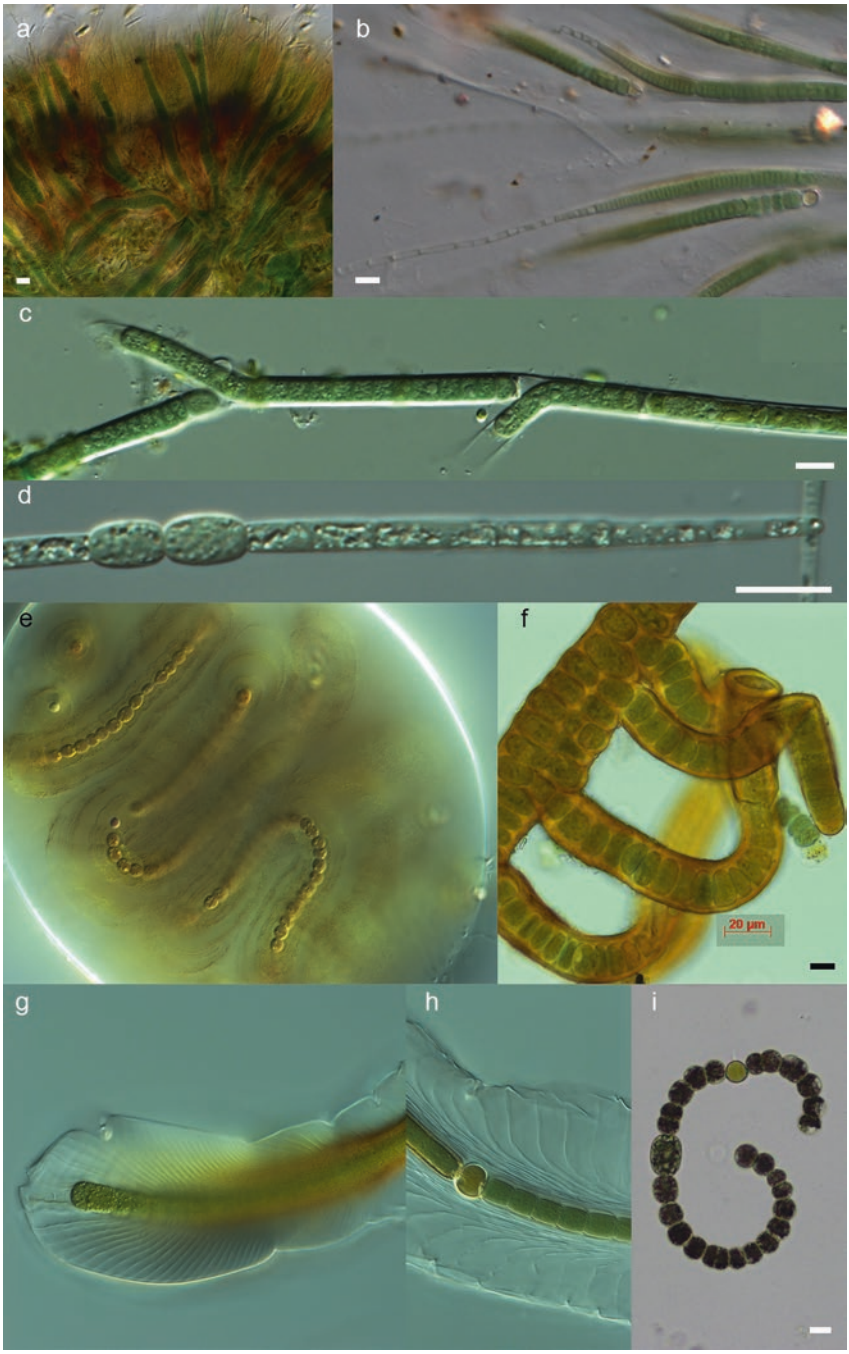

Fig. 7 Microphotographs of Nostocales. (**a**, **b**) *Rivularia* sp., (**c**) *Tolypothrix* sp., (**d**) *Cuspidothrix issatschenkoi*, (**e**) *Nostoc microscopicum*, (**f**) *Stigonema* sp., (**g**, **h**) *Petalonema alatum*, (**i**) *Dolichospermum* sp. Scale bar 10 µm

methods for elucidating phylogenetic relationships (e.g., Mareš et al. 2015). Even though this lineage represents one of the most morphologically character-rich lineages of cyanobacteria, there are most likely not enough unique morphologies to truly differentiate this diverse lineage. Thus, characters such as the structures of the ITS region (e.g., *Roholtiella*, *sensu* Bohunická et al. 2015), physiology (e.g., *Halotia*, *sensu* Genuário et al. 2015), or polyphasic approaches also utilizing ecology (e.g., *Dapisostemon, sensu* Hentschke et al. 2016) are increasingly being employed.

While the Nostocolaes are widely distributed and found in terrestrial, aquatic, subaerial, and symbiotic environments, the exploration of the molecular phylogenies has been rather unevenly applied. As Komárek et al. (2014) point out, 50 new cyanobacterial genera have been erected since 2000, with an additional 16 proposed just at the 2013 IAC meeting. However, many of these new taxa are from the other cyanobacterial lineages. Many Nostocalean taxa are difficult to culture and thus difficult to describe. Also, many lineages possess thick, copious mucilaginous sheaths, necessitating additional steps before successful sequencing (e.g., Mareš et al. 2015). Numerous Nostocalean taxa may be endemic or have restricted ranges (e.g., *Rexia sensu* Casamatta et al. 2006). Thus, the total diversity of this lineage has only been cursorily examined, especially in tropical or seldom sampled habitats (Dvořák et al. 2015b; Hentschke et al. 2016). Lastly, certain lineages, especially the more commonly encountered lentic taxa (e.g., *Anabaena*, *Dolicospermum*, *Cylindrospermopsis*), have received much greater attention than others, and thus have greater phylogenetic resolution. Recent expansion of geographic ranges, such as seen in *Cylindrospermopsis* (Padisák 1997), or the discovery of novel, bio-active secondary metabolites (e.g., *Aetokthonos*, *sensu* Wilde et al. 2014) point to the need for much more comprehensive assessments of the phylogeny of this lineage.

In a recent paper, Komárek et al. (2014) proposed a complete revision of the cyanobacteria as a whole. After examining molecular data sets from all available lineages, the researchers note that the Nostocales is only monophyletic when all taxa capable of producing specialized cells are included, and propose the order Nostocales to encompass these taxa. While all of these taxa have irregular thylakoids, familial level designations are posited based on a number of characters.

The Nostocales represents a diverse lineage in terms of morphology, genetics, and ecological preferences. Many members are associated with aquatic habitats, where they may be planktic (e.g., *Cylindrospermopsis* and *Dolichospermum*), benthic (e.g., *Anabaena*) or associated with the margins of the aquatic and terrestrial landscapes (e.g., *Mastigocladus*). The Nostocales are also commonly encountered as terrestrial microbes, where they have been described from tropical (e.g., *Dapistostemon*) to arctic habitats (e.g., *Nostoc*). Further, they have been described from polar (Vincent 2007), hot, arid (Řeháková et al. 2007), and temperate soils (Lukešová et al. 2009). They are also commonly involved in symbiotic associations (e.g., *Trichormus* and *Azolla*). Many Nostocales are also known to produce a wide array of bio-active compounds, which may be potent neuro-, hepato-, and dermatotoxins (for a review, see Codd et al. 1997).

Type of Cell Division

The majority of cyanobacteria, filamentous, coccoid, or unicellular, undergo binary fission as the main form of reproduction. Some Nostocales exhibit false-branching, where a new filament is not formed as a result of cell division and does not result in the thallus exhibiting division in multiple planes, leading to the appearance of filaments which appear to pass each other (e.g., *Tolypothrix*). However, unique among the Nostocales is a second type of cell division known as true-branching. Generally defined as cell division in which one or more of the cells change the polarity of growth, true-branching allows some cyanobacteria the capability of erect or creeping growth (although this is not a necessary condition for such growth). True branching is differentiated into three main forms: T-, V-, and Y- (for a review, see Golubic et al. 1996). Traditionally considered an important phylogenetic character, and serving to differentiate the Stigonematales, recent works have indicated that this might be a useful feature for familial assignments (Gugger and Hoffman 2004), but not higher levels.

While some Nostocalean taxa exhibit cell division in multiple planes, many taxa reproduce by the liberation of solitary cells generated by division perpendicular to the trichome axis. Other taxa employ hormogonia, which are distinct segments of the trichome, often arising from the formation of adjacent necridial cells or by the result of fragmentation. Those taxa capable of also generating gas vesicles often employ this method to propagate themselves in lentic habitats (e.g., *Dolichospermum*, formerly *Anabaena sensu lato*).

Ultrastructural Features

Recent work has shown this to be a polyphyletic character, but thus far all known Nostocalean taxa have irregularly arranged thylakoids (for a review of thylakoids see Komárek 2013).

Another important cell ultrastructural feature is the presence of gas vesicles, a form of inclusion body which may be filled with atmospheric gases. While they may be induced in some lineages, the presence or absence of gas vesicles is being employed in a phylogenetic sense. For example, gas vesicles were recently employed when the polyphyletic genus *Anabaena* was split into *Anabaena sensu stricto* (containing mainly periphytic species without gas vesicles), *Dolichospermum* (mainly planktic species with gas vesicles), and *Sphaerospermum* (planktic with gas vesicles).

Heterocytes and Akinetes

Perhaps the most distinguishing aspect of the Nostoclaes is their specialized cells, the most common of which are heterocytes and akinetes. The size, shape, and placement of heterocytes are frequently employed in identifications (e.g., *Anabaena*).

Heterocytes may be apoheterocytic (developing from vegetative cells between heterocytes) or paraheterocytic (developing from vegetative cells outside of heterocytes). Heterocytes may form at the ends of trichomes (terminal or basal), within a trichome (intercalary), at a right angle from a trichome (lateral), or within a multiseriate trichome (lateral). The formation of heterocytes along the trichome may also be solitary, in pairs, or as several in a row. Further, the actual shape and size of heterocytes differs by taxa. Although the formation and frequency of heterocytes may be environmentally inducible, the size and position in trichomes appears to be genetically controlled.

Akinetes are thick-walled resting cells, often environmentally inducible, typically used to survive adverse environments. These may also be used in phylogenetic reconstructions. For example, *Gloeotrichia* is separated from other members of the Rivulariaceae by the obligatory presence of akinetes (it should be noted that Komárek et al. 2014 separated this into a new family, the Gloeotrichiaceae). Akinetes, like heterocytes, have been used in phylogenetic assessments as the size, shape, and placement of akinetes seem genetically fixed (but the number produced seems environmentally influenced).

Modern Systematic Scheme

The clade containing the Nostocales/Stigonematales has long been recognized as taxonomically challenging. While monophyletic on the grossest level (e.g., the clade containing taxa with the capability of forming specialized cells), relationships within this clade have proven to be confusing, leading to calls from numerous researchers to resolve some of the phylogenetic uncertainties (Fig. 8; e.g. Kaštovský and Johansen 2008; Lukešová et al. 2008; Hauer et al. 2014; Komárek 2015, etc.). Numerous changes have been proposed and as newly discovered taxa are isolated, sequenced, and examined, our understanding of the relationships within this lineage is ever changing. A recent paper from Komárek et al. (2014) has proposed a taxonomic scheme integrating molecular, cellular, ecological, and morphological data in a total evidence (polyphasic) approach. This scheme involved the examination of the largest data set of cyanobacteria published thus far and represents an excellent, testable hypothesis of evolutionary relationships going forward.

Familial Designations

Aphanizomenonaceae

Characterized by unbranched, isopolar or subsymmetric filaments with akinetes and typically aerotopes (e.g., *Aphanizomenon*), this is a widely distributed, commonly encountered lineage, especially in eutrophic systems. A rather confusing clade in

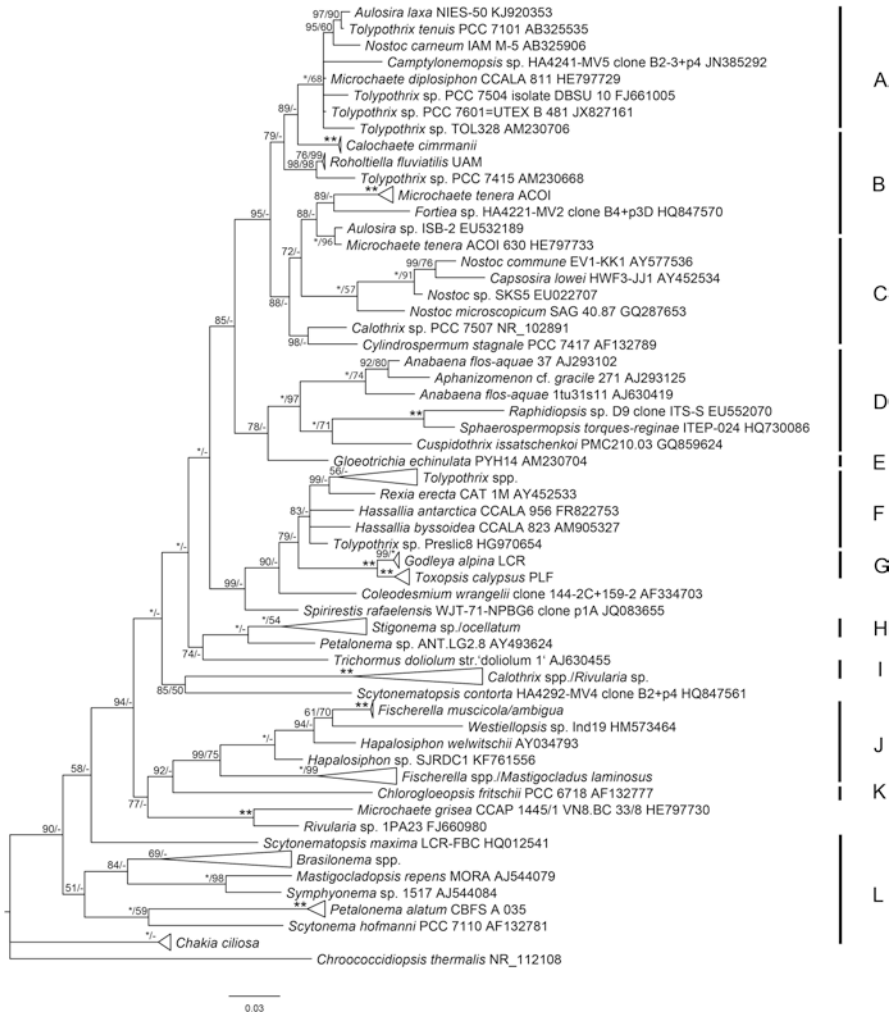

Fig. 8 Phylogenetic reconstruction of the main Nostocalean lineages based on 16S rRNA gene sequence data. The tree was inferred in MrBayes 3.2.1 (Ronquist and Huelsenbeck 2003) using the F81+G+I model. Two separate runs, each with 4 chains (1 cold and 3 heated) were run for 4,115,000 generations and sampled every 1000th generation. The bootstrap analysis was performed in RaxML 8.0.2 (Stamatakis 2006) with 1000 bootstrap replicates and GTR+GAMMA model. Posterior probabilities and bootstrap supports are at the nodes and *asterisk* represents 100 % posterior probability and bootstrap supports. A—Tolypotrichaceae, B—Fortieaceae, C—Nostocaceae, D—Aphanizomenonaceae, E—Gloeotrichiaceae, F—Tolypotrichaceae, G—Godleyaceae, H—Stigonemataceae, I—Rivulariaceae, J—Hapalosiphonaceae, K—Chlorogloeopsidaceae, L—Scytonemataceae and Symphyonemataceae

terms of morphological assessments, this includes the common planktic genera *Aphanizomenon*, *Cylindrospermopsis*, *Dolichospermum,* and *Raphidiopsis*. Many of these genera have long been considered polyphyletic, but recent investigations of morphology, coupled with 16S gene sequence data have allowed researchers to create monophyletic lineages for several genera. For example, Wacklin et al. (2009) were able to find a stable morphological character, the presence of aerotopes, which separated the planktic forms of "*Anabaena*" into the monophyletic genus *Dolichospermum*. However, it should be noted that other relationships within the Aphanizomenonaceae itself remain less clear, but monophyletic groupings are being elucidated and described, especially for clades of human interest (e.g., Werner et al. 2012).

Dapisostemonaceae

This family was recently described from examination of aerophytic taxa from Brazil. Not included in the Komárek et al. (2014) paper, Hentschke et al. (2016) separated this family from the Tolypothricaceae based on 16S rDNA sequence data. Characterized by heteropolar filaments, intercalary, bipolar heterocytes, lack of akinetes and single false branching (e.g., *Dapisostemon*). This mono-generic family probably represents a larger lineage of taxa that have not yet been identified or sequenced. The original materials were collected from seldom sampled tropical, aerophytic habitats and thus sister taxa may still need to be described, leading to uncertain familial assignments. For example, Hentschke et al. (2016) note that a new, closely related genus *Streptostemon*, putatively placed in the Scytonemataceae or Tolypothrichaceae, may actually be elevated to the first member of a heretofore undescribed family.

Scytonemataceae

A species-rich lineage characterized by isopolar filaments and false branching (e.g., *Brasilonema*, Fiore et al. 2007). There exists a wide range of morphological and ecological variability within this lineage, and numerous genera have been poorly or not at all characterized molecularly (e.g., *Chakia* and *Petalonema*). Some generic designations appear to be well supported (e.g., *Brasilonema*), while others are poorly resolved. Note: Komárek et al. (2014) caution that the relationship of this family to the Symphyonemataceae needs to be further elucidated.

Symphyonemataceae

A small family contains at least two genera (*Mastigocladopsis* and *Symphyonema*), characterized by both isopolar filaments and Y-type true branching (but see note above). Lamprinou et al. (2011) erected two new genera of cave dwelling cyanobacteria (*Iphinoe* and *Loriellopsis*) that may or may not belong to this family; current phylogenies lack resolution to clearly place them.

Rivulariaceae

A well-characterized, widely distributed, commonly encountered lineage whose taxa possess tapering, heteropolar filaments with facultative false branching, and the presence of a long, thin hair-like projection (e.g., *Calothrix*). The current disposition of some of the most known genera (e.g., *Calothrix*) from this lineage is in a state of flux. The original type of *Calothrix* was isolated from marine habitats, but has not been sequenced. Berrendero et al. (2016) have set about to resolve these issues by erecting new genera and attempting to create smaller, monophyletic units within this lineage, but do not propose any wholesale alterations to the family.

Chlorogloeopsidaceae

A poorly understood lineage, characterized by isopolar filaments or cell aggregates, the capacity for true-branching remains unclear (e.g., *Chlorogloeopsis*). Rarely described from nature, very little is known of the ecology of this potentially monotypic family (*Chlorogloeopsis fritschii* is the only taxon thus described). The majority of the work on this family has come from isolated strains in culture (and mainly of strains from PCC), so phenotypic plasticity remains a question.

Hapalosiphonaceae

A morphologically character-rich, yet difficult to properly identify lineage, characterized by isopolar filaments with T-type true branching, filaments and branches which may be uni- or multiseriate. A difficult clade to sequence due to recalcitrant mucilaginous sheaths, certain genera appear polyphyletic (e.g., *Fischerella*, a separate family in some phylogenies) while others appear monophyletic (e.g., *Westiellopsis*). However, assessments of this lineage are hampered by misidentifications of deposited cultures and sequences in GenBank. Komárek et al. (2014) suggest that this family is in need of revision with more sister taxa and strains in culture collections sequenced.

Stigonemataceae

A rarely reported (except for *Stigonema*), morphologically complex clade possessing filaments and branches that may be uni- or multiseriate with T-type true branching (e.g., *Stigonema*). Difficult to work with since strains do not survive well in culture, this lineage is challenging to investigate. Strains exhibit a wide array of morphological variation, further adding to taxonomic confusion.

Godleyaceae

A recently erected family containing two genera (*Toxopsis* and *Godleya*), characterized by both iso- and heteropolar tapering filaments and false branching (e.g., *Toxopsis*). This lineage may be sister to the Tolypothrichaceae (Lamprinou et al. 2012). Thus far, all known isolates have been from subaerial or terrestrial habitats.

Tolypothrichaceae

Recently revised in order to create monophyletic lineages from former Microchaetacean taxa (Hauer et al. 2014), this family is now monophyletic and characterized by non-tapering trichomes with heteropolar filaments and frequent false branching (e.g., *Tolypothrix*). Members of the Tolypothrichaceae are typically found in terrestrial or freshwater habitats (never marine) and are commonly encountered (Hauer et al. 2014).

Capsosiraceae

A poorly understood lineage, characterized by polar growth of colonies, which may be filamentous or aggregates, and possessing the ability for true-branching. Caution is warranted with this lineage, as the only sequenced member thus far, *Capsosira lowei*, may actually represent a taxon from a phylogenetically related lineage (Casamatta et al. 2006). Further collection and sequencing is warranted.

Gloeotrichiaceae

Previously assigned to the Rivulariaceae, this morphologically distinct lineage possesses tapering trichomes with heteropolar filaments, and spherical colonies that form akinetes (e.g., *Gloeotrichia*). This is a phylogenetically unsettled lineage, and future revisions might employ akinete characteristics (certain taxa exhibit akinetes in pairs, others as solitary cells) and the presence or absence of gas vesicles. Thus far only a single taxon has been sequenced (*G. echinulata*), so additional data will help resolve final phylogenetic placements.

Nostocaceae

Perhaps the most well known of the noctocalean lineages, typified by iso- or heteropolar filaments with facultative false-branching and akinete production (e.g., *Anabaena*). Species rich and ecologically permissive, these taxa can be found

worldwide. Well studied, it is also clear that many genera are both morphologically character-poor and polyphyletic. While some members are difficult to identify, total evidence approaches employing characters such as ecology, ITS structures or slight morphological differences are increasingly being employed to separate monophyletic clusters (e.g., *Mojavia* isolated from desert soils or *Halotia* from Antarctica soils). This is also an interesting clade as many members have definable, diagnosable development sequences (e.g., *Nostoc*).

The Dark Matter of Cyanobacterial Diversity

Cyanobacteria represent 23.4 % of known prokaryotes and are among the most morphologically distinct prokaryotes. Nabout et al. (2013) note 2698 described species, with ca. 15 new species per year. Many cyanobacterial species remain to be described, with some models predicting at least 6280 species (Nabout et al. 2013). Some cyanobacterial groups are more studied than others (number of papers in ISI Web of Knowledge database; Thomson Reuters, New York; accessed 19.1.2016; search based on order name in title: Synechococcales 3 papers, Chroococcales 99, Pleurocapsales 7, Oscillatoriales 99, Nostocales 126 and Stigonematales 36 papers published), so additional work is needed. In addition, there exist gaps in our knowledge of cyanobacterial diversity from certain geographic regions (e.g., tropics) and specific habitats (e.g., benthic or aerophytic habitats).

There is a long-standing joke that the distribution of microalgae depends on the distribution of phycologists. For example, the vast majority of cyanobacteria have been described from Europe, the center of floristic research over the last three centuries. For example, Hauer et al. (2015) have gathered floristic records of terrestrial cyanobacteria dwelling on rocks and found 401 taxa recorded from Europe, 155 from North America, 175 from South America, 72 from Africa, 280 taxa from Asia, 86 from Australia and Oceania, and 27 taxa from Antarctica and Arctic regions. These habitats host approximately 30 % of the entire described cyanobacterial diversity (Nabout et al. 2013). Likewise, all studies of hypogeal cyanobacteria are from the Mediterranean area (Hauer et al. 2015). Reliable comparisons of diversity among geographical regions are not available, due to the lack of studies from different areas of the world. One of the questions that can now be addressed is the geographic distribution of the cyanobacteria and existence of endemic taxa (Taton et al. 2003; Finlay 2002; Dvořák et al. 2012). For example, a recent molecular study (Taton et al. 2003) provided evidence that cyanobacterial diversity and endemism in Antarctica is greater than assumptions of diversity/endemism based on microscopic analysis. Nadeau et al. (2001) suggest a bipolar distribution for several oscillatorians taxa, in congruence with results of some other authors (Jungblut et al. 2010; Comte et al. 2007). Globally dispersed microorganisms have been reported from geothermal environments (Papke et al. 2003; Ward et al. 2008). On the other hand, some genera (e.g., *Rexia*) have been postulated as being endemic to very specific, limited geographic distributions (Casamatta et al. 2005).

Tropical regions represent large geographic areas with a variety of habitats. Coupled with high humidity and low seasonality, this may enable many species to coexistence (Mittelbach et al. 2007). The estimated proportion of possibly undescribed microalgae was about 60 % (Neustupa and Škaloud 2008). Indeed, the exploration of little-known habitats in tropical regions has led to discoveries of new taxa (Sant'Anna et al. 2011; Dadheech et al. 2014; Hašler et al. 2014a; Dvořák et al. 2015b). Neustupa and Škaloud (2010) concluded that tropical, corticolous habitats harbor higher diversity than corresponding temperate habitats. Their results indicate that the microhabitat conditions, in the case of terrestrial phototrophs, typically humidity and light, may play a crucial role in determining algal and cyanobacterial diversity.

Although freshwater habitats in general have received more attention than terrestrial ones, the studies focused on microalgal assemblages are not equally distributed. The ratio of papers on planktic, epiphytic, and epipelic microalgal assemblages was 62:32:4 as of January 2013 (Pulíčková et al. 2014). Since 2007, when the epipelon was proposed as a major unexplored freshwater cyanobacterial habitat (Poulíčková et al. 2008), several new cyanobacterial taxa were distinguished using a polyphasic approach (Hašler et al. 2012, 2014a, b).

Additional Information

For further information and copious illustrations, the authors suggest the works of Komárek and Anagnostidis (1998, 2005) for Synechococcales, Chroococcales, Oscillatoriales, Spirulinales and Chroococcidiopsidales, Komárek (2013) and Komárek and Johansen 2015 for Nostocales. The most comprehensive web sources are CyanoDB (http://www.cyanodb.cz/) and AlgaeBase (http://www.algaebase.org/).

Conclusions and Future Prospects

The diversity of cyanobacteria is immense. While we have already discovered thousands of species, it seems to be but a glimpse of the real biodiversity, illustrated by the growing number of the new taxa erected every year, especially from tropical habitats. Further, only a minority of described species has been sequenced. However, phylogenetic reconstructions based on obtained sequences mostly exhibited entangled relationships with polyphyletic genera. This problem is amplified by the fact that there is debate about species concepts and the inability to cultivate a majority of cyanobacteria.

Nevertheless, we remain optimistic, because recent advances in genomics, metagenomics, single cell genomics, and related fields promise development of tools which may allow us to tackle the problems outlined above. We expect that the number of newly described taxa will only increase in the future. With a growing body of whole genome data, we will be able to recognize more nuanced differences among lineages, thus precisely resolving species relationships.

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PAPER I.

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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 $\mu S.cm^{-1}$, 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 $\mu mol.m^{-2}.s^{-1}$, 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 $ng.\mu l^{-1}$). 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:

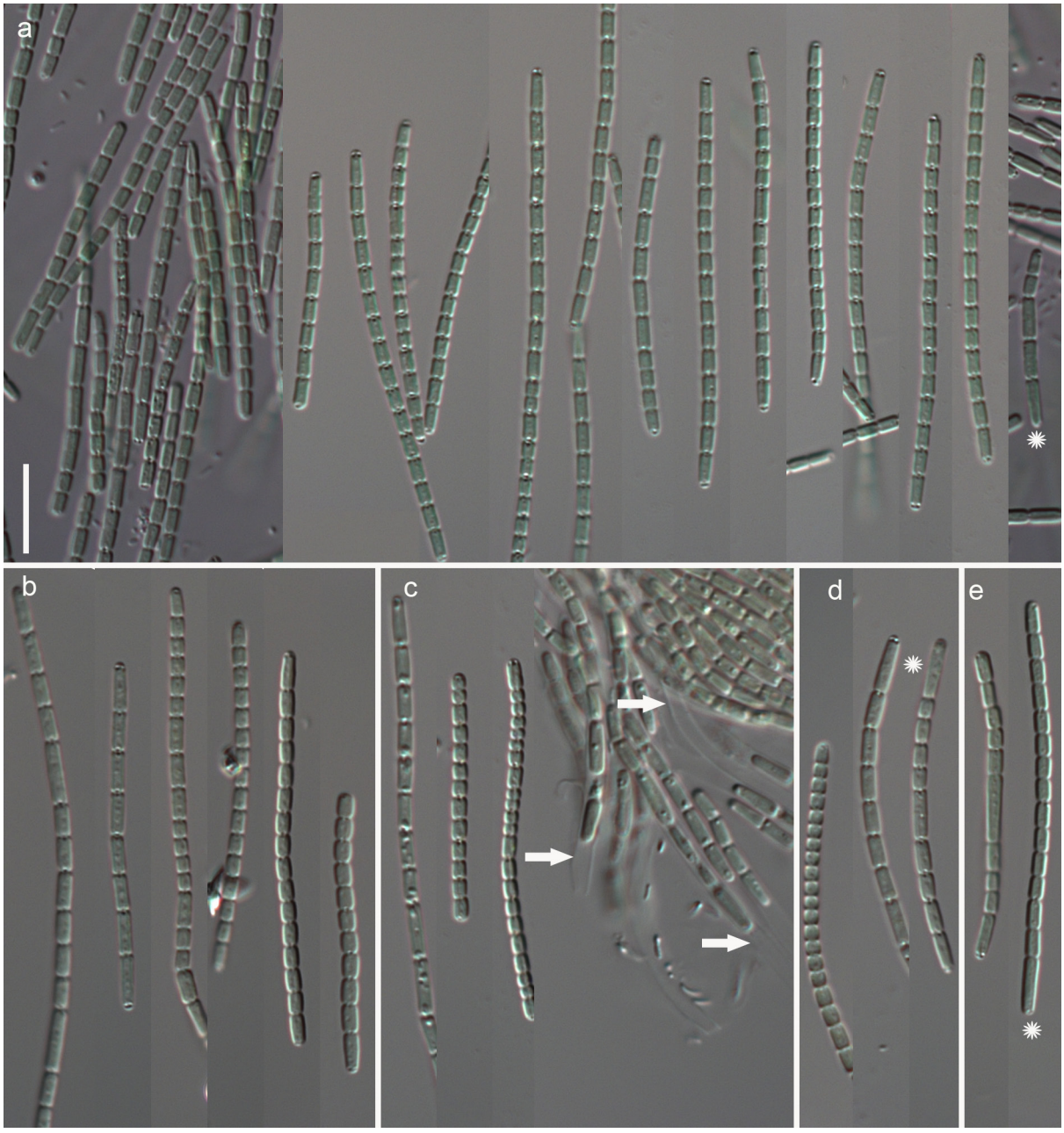

Fig. 1. *Pinocchia polymorpha* sp. nov.: (a) strain E 5 in full Z medium and 26 °C; (b) the same in medium without nitrogen; (c) the same in medium without phosphorus; (d) the same strain in medium withouth both N and P; (e) the same strain in full Z medium and 16 °C. Scale bar 10 µm, asterix show elongated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

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.

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

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 *Pseu-*

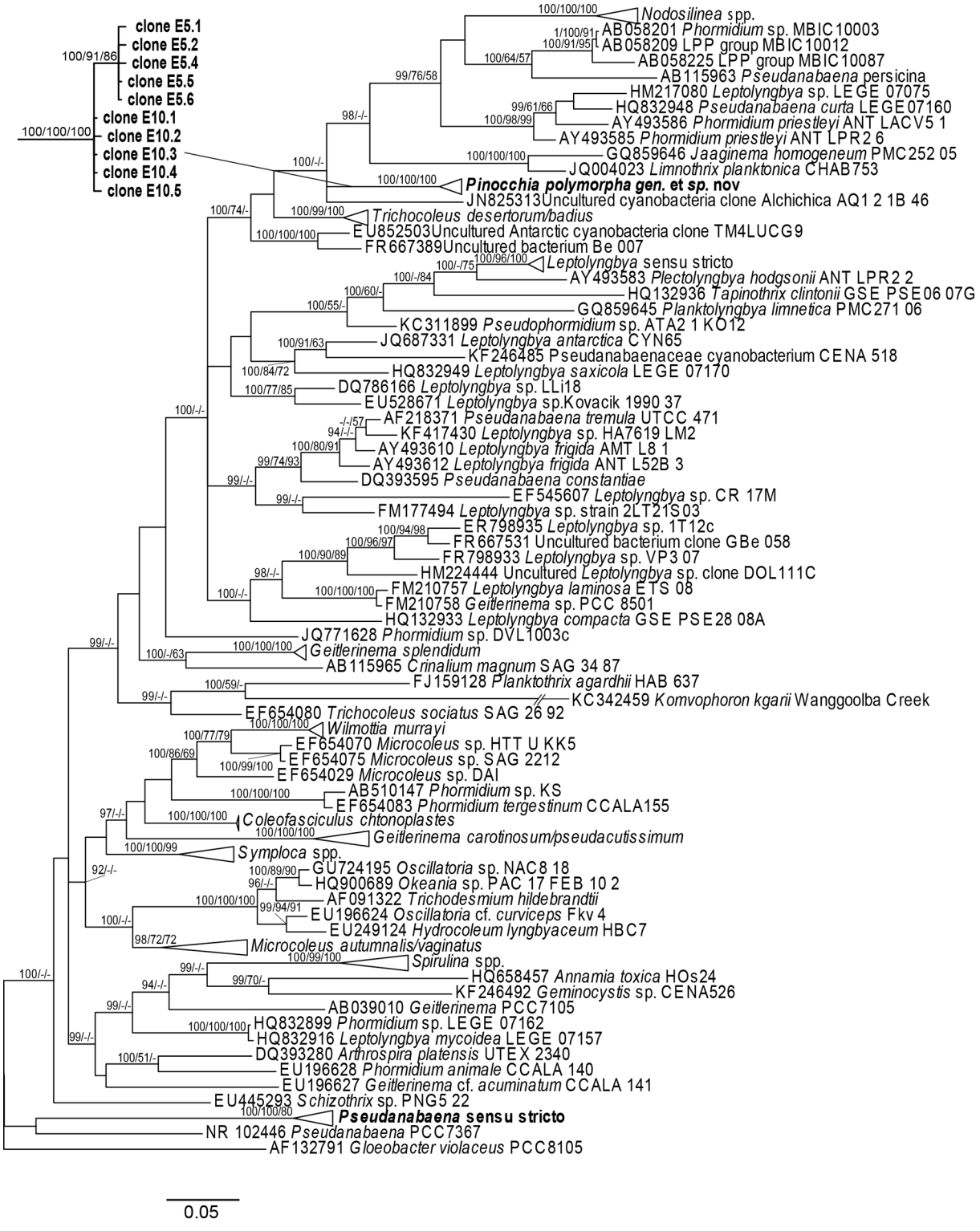

Fig. 2. A phylogenetic reconstruction based on 16S rRNA using Bayesian inference. Studied strains and *Pseudanabaena sensu stricto* are in bold. Supports at the nodes (Bayesian inference/maximum likelihood/maximum parsimony) represent only bootstrap values >50 and posterior probabilities >90. A collapsed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

danabaena 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).

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

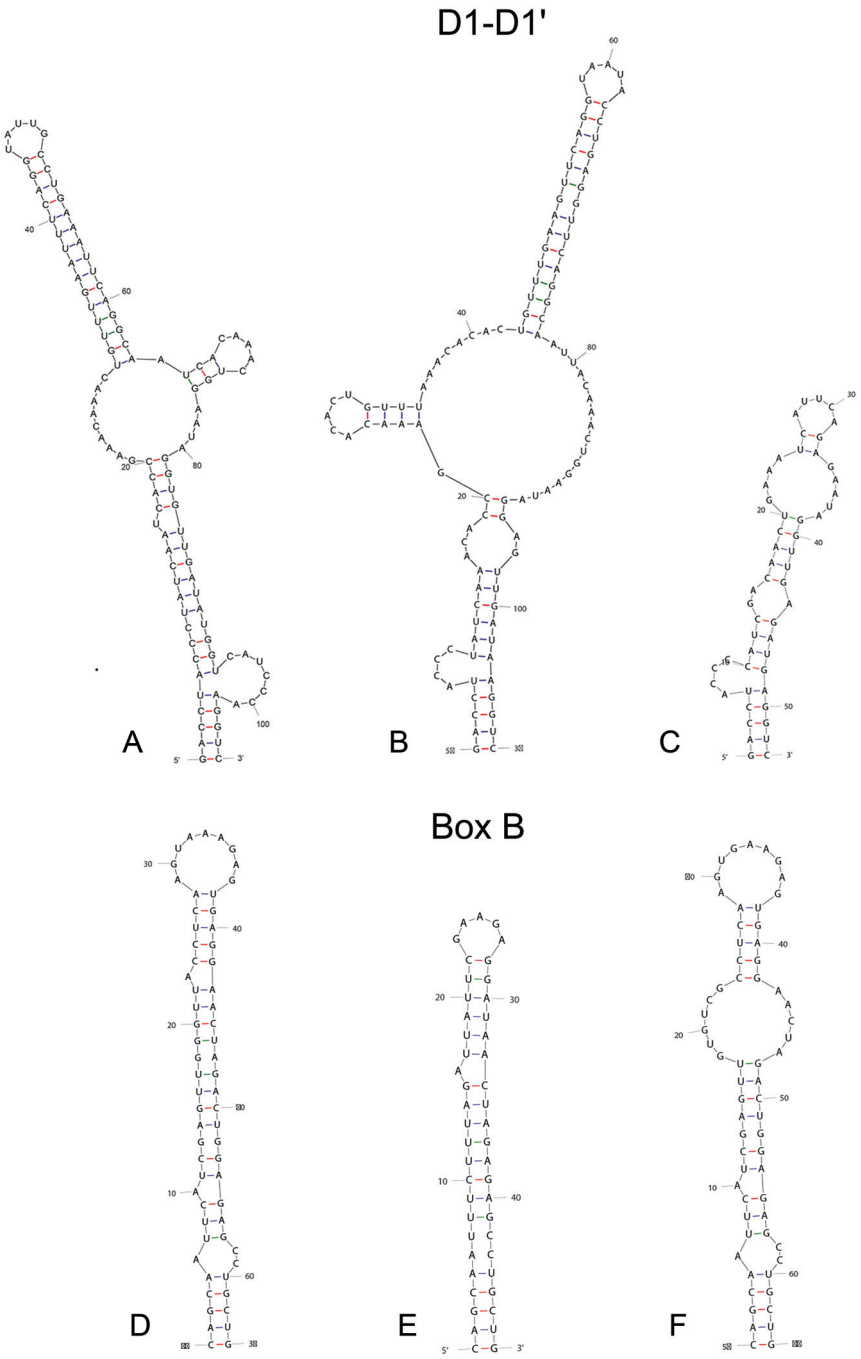

Fig. 3. Estimated 16S–23S ITS secondary structures D1–D1' and Box–B helices of *Pinocchia*: (A, D) for clones E5.1, E5.2, E5.4, E5.5, E5.6; (B, E) for clones E10.1, E10.3; (C, F) for clones E10.2, E10.4, E10.5

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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Revealing hidden diversity among tropical cyanobacteria: the new genus *Onodrimia* (Synechococcales, Cyanobacteria) described using the polyphasic approach

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Abstract

Leptolyngbya represents a group of common mat forming cyanobacteria with very simple trichome morphology and a polyphyletic evolutionary origin. In this paper, we used a polyphasic approach to describe a new genus morphologically similar to *Leptolyngbya*. Three strains of Leptolyngbyaceae cyanobacteria were isolated from submersed bark of tree branches which fell into the Hot-water spring from a rainforest in West Java. A phylogeny of the 16S rRNA gene indicated that these strains fell into a well-supported clade separate from *Leptolyngbya* sensu stricto. Although our strains possessed only minor morphological differences from other similar Leptolyngbyaceae species, these new taxa may be differentiated based on a peculiar form of reproduction, where hormogonia and hormocytes form tree-like tuft structures. Thus, based on a phylogenetic position, morphological, and ecological evidence, we propose a new genus, *Onodrimia*.

Key words: *Leptolyngbya,* new genus, phylogeny, tropical cyanobacteria, 16S rRNA, 16S-23S ITS

Introduction

Cyanobacteria belong to the most widespread, morphologically distinct, abundant known bacteria (Whitton 1992), and played an important role in the evolution of the Earth's biosphere as primary producers of oxygen (Mur *et al.* 1999). Moreover, cyanobacteria colonized almost all biotopes on our planet, including oceans, rivers, lakes, tree bark, soil, as well as extreme habitats such as deserts, hot springs, glaciers and polar regions (Bellinger & Siegge 2010, Whitton & Potts 2000, Stal 2002, Stibal *et al*. 2006). They are also the ancestors of plant and algal plastids (Reyes-Prieto *et al.* 2007).

The taxonomy of cyanobacteria has a very long and complicated history. Species were initially recognized using phenotypic criteria such as cell morphology, sheath characteristics or cell ultrastructure. Recently, researchers have started to define species based on the polyphasic approach, which utilizes data from morphological, molecular, ecological, ultrastructure data, ITS secondary structures, etc. (Johansen & Casamatta 2005). However, reconstruction of evolutionary relationships as evidenced from genome studies within cyanobacteria has shown that evolutionary patterns do not necessarily correspond with morphological characters. Furthermore, researchers have noted the presence of cryptic taxa which cannot be identified on the basis of morphological features alone, but also require molecular markers, for example 16S rRNA (Dadheech *et al.* 2014, Řeháková *et al.* 2014, Komárek *et al.* 2014, Dvořák *et al.* 2015a, Dvořák *et al.* 2015b). At the species level, morphology alone seems to often be insufficient to differentiate taxa, especially those which possess little morphological variability (e.g. *Leptolyngbya* Anagnostidis & Komárek (1988: 390) and *Oculatella* Zammit, Billi et Albertano (2012: 352) in Osorio-Santos *et al.* 2014). Furthermore, a majority of morphologically coherent genera appear to be polyphyletic based on the phylogenetic reconstructions, which have been called "cryptogenera" (Komárek 2010, Komárek *et al.* 2014). This may be a consequence of frequent evolutionary convergence among many lineages of cyanobacteria (Dvořák *et al.* 2014a).

Leptolyngbya was described in 1988 by Anagnostidis & Komárek and initially placed in the Pseudanabaenaceae prior to transfer to the Leptolyngbyaceae (Komárek *et al.* 2014). *Leptolyngbya* and other Leptolyngbyaceae species are common, mat-forming cyanobacteria with the following features: filamentous, unbranched (rarely pseudobranched) taxa with thin filaments ca. 3.5 μm width, and surrounded by sheath (Anagnostidis & Komárek 1988, Komárek &

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Anagnostidis 2005). There are currently >100 taxa circumscribed in this character poor genus (Johansen *et al.* 2011). Under the bacteriological code, *Leptolyngbya* fell within the "LPP group B" cluster (Rippka *et al.* 1979). The initial erection of *Leptolyngbya* encompassed taxa originally assigned to different oscillatoriallacean genera such as *Lyngbya* Agardh ex Gomont (1893: 118)*, Plectonema* Thuret ex Gomont (1893: 96) and *Phormidium* Kützing ex Gomont (1893: 156) (Komárek & Anagnostidis 1988). All of these genera are characterized by straight trichomes, cylindrical or isodiametric cells, variable cell constrictions, reproduction by trichome breakage, and facultative sheath production and motility (Rippka *et al.* 1979). *Leptolyngbya* may be found in numerous habitats, but most often occurs in mats (growing on different substrates) or as aerophytes.

Based on phylogenetic reconstruction of the 16S rRNA gene, *Leptolyngbya* is polyphyletic (Perkerson *et al.* 2011, Cassamatta *et al.* 2005, Komárek & Anagnostidis 2005). Identifications are notoriously difficult due to few significant morphological characteristics (Alberto & Kovácik 1994, Casamatta *et al*. 2005). Based on a combined polyphasic approach, *Leptolyngbya* has recently been partially revised by the erection and splitting of new genera such as *Oculatella* Zammit, Billi & Albertano (2012: 352), *Nodosilinea* Perkerson & Casamatta in Perkerson *et al*. (2011: 1405), *Stenomitos* Miscoe & Johansen in Miscoe *et al.* (2016: 83), *Kovacikia* Miscoe & Johansen in Miscoe *et al.* (2016: 85), and *Haloleptolyngbya* Dadheech, Mahmoud, Kotut & Krienitz (2012: 273).

In this paper, we use morphological, molecular, and ecological data to erect another new cyanobacterial genus in the Leptoylngbyaceae from a tropical area.

Materials and methods

Strain isolation:—Strains were isolated from submersed bark of tree branches which fell into the hot water spring in the rainforest, near Tamanjayae, Ujung Kulon NP, West Java (GPS: 6° 47'0.81''S', 105°30'9.14''E, 49 m a.s.l.) on 25th February 2012. Three cultures (E27, E28 and E30) 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:—Morphological characters of the strains were analyzed using a Zeiss AxioImager light microscope (objectives EC Plan-Neofluar 40x/ 1.3 N.A., oil immersion, DIC; Plan-Apochromat 100x/ 1.4 N.A., oil immersion, DIC) with a high resolution camera (AxioCam HRc 13MPx). During morphological evaluation of strains, the following characters were assessed: cell shape, terminal cells, cell dimensions, reproduction, sheaths, and granulation of cells.

PCR amplification and sequencing:—Genomic DNA was isolated from ca. 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).

PCR amplification of the partial 16S rRNA and the whole 16S-23S ITS was obtained using 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 commercially sequenced. Sequences were stored in GenBank (accession numbers: MG000960, MG000961, MG000962).

Phylogenetic analyses:—The most similar sequences of 16S rRNA were obtained 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 *Leptolyngbya* were added. Multiple sequence alignment was performed in Mafft (Katoh *et al.* 2002). The tree was rooted to the outgroup *Gloeobacter violaceus* Rippka, Waterbury & Cohen-Bazire (1974: 436). 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: model GTR+G+I. 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 100,000,000 generations. The sampling frequency was each 10,000th generation. 25% trees were discarded as burn-in. Maximum likelihood analysis was performed in RaxML 8.0.2 (Stamatakis 2006) with a GTR+G model. Maximum parsimony analyses were performed in MEGA version 6.0. (Tamura *et al.* 2013) 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

Class **Cyanophyceae**
Order **Synechococcales**
Family **Leptolyngbyaceae**

Onodrimia Jahodářová, Dvořák & Hašler, *gen. nov.*

Thallus usually macroscopic, in mats, occasionally creeping. Filaments straight to bent, occasionally coiled or entangled together, frequently false branched. Sheath colorless, roundly closed at the ends or opened after hormogonia release, exceeding trichome or with trichome protruding from sheath. Trichomes narrowed at the ends, immotile. Cells usually rectangular, isodiametric to longer then wide, with visible parietal chromatoplasm and inner pale centroplasm. Apical cells rounded or conical, without calyptra. Reproduction into short hormogonia or hormocytes by help of necridic cells. Both hormogonia and hormocytes frequently form groups of tree-like tufts and attach via sheath to other filaments.

Etymology:—The branching filaments of *Onodrimia* resembles tree branches. The genus epithet is derived from name Onodrim, which is an Elvish name for the giant tree-like beings, also known as Ents, who appeared in the unforgettable epic story of The Lord of the Rings written by John R. R. Tolkien.

Type species:—*Onodrimia javanensis* Jahodářová, Dvořák & Hašler.

Onodrimia javanensis Jahodářová, Dvořák *et* Hašler, *sp. nov.* (Figs. 1–3)

Thallus usually macroscopic, in mats, occasionally creeping, bright green to grey-green. Filaments straight to bent, occasionally coiled or entangled together, frequently false branched. Sheath facultative, thin, colorless, distinct, roundly closed at the ends or opened after hormogonia release, exceeding trichome or with trichome protruding from sheath. Trichomes, bright green, grey-green to yellowish, slightly constricted at cross-walls, narrowed at the ends, immotile, sometimes in tight loops. Cells usually rectangular, isodiametric to longer then wide, 1.39–2.55 µm (average 1.9 µm) wide, 1.5–8.35 µm (average 3.6 µm) long, with visible parietal chromatoplasm and inner pale centroplasm, with distinct storage granules. Apical cells usually longer than wide, rounded or conical, without calyptra, sometime pale with orange granules (probably dying cells). Reproduction into short hormogonia or hormocytes by help of necridic cells. Both hormogonia and hormocytes frequently form groups or tree-like tufts and attach via sheath to other filaments.

Type:—INDONESIA. Java, near the Tamanjaya village, Ujung Kulon NP, 6° 47’0.81’’S’, 105°30’9.14’’E, 49 m a.s.l., submersed bark of tree branches which fell into the hot water spring in rainforest, *coll.* L. Majeský, 25 February 2012 (holotype: OLM! Botany 24: Lichens and others No. 9224 dried sample is deposited in Regional Museum in Olomouc, Czech Republic. Reference strain: UPOC E28/2016, deposited at the culture collection of Department of Botany, Palacký University Olomouc, Czech Republic).

Habitat:—Submersed bark of tree branches which fell into the hot water spring in rainforest.

Etomology:—The species name was established based on the first place of discovery of *Onodrimia.*

Phylogeny:—All 16S rRNA sequences of *Onodrimia* were 100% identical. Bayesian inference based on a partial sequence 16S rRNA phylogeny revealed a monophyly of *Onodrimia* lineage among other cyanobacterial genera – most related were *Phormidesmis* Turicchia, Ventura, Komárková & Komárek (2009: 179), *Stenomitos*, *Neosynechococcus* Dvořák, Hindák, Hašler & Hindáková in Dvořák *et al*. (2014b: 26), *Pantanalinema* Vaz *et al.* (2015: 301) and some unspecified Leptolyngbyaceae species. Other compact and highly supported genera were *Nodosilinea, Spirulina* Turpin ex Gomont (1893: 249)*, Symploca* Kützing ex Gomont (1893: 104)*, Oculatella, Coleofasciculus* Siegesmund, Johansen & Friedl in Siegesmund *et al*. (2008: 1575)*, Wilmottia* Strunecký, Elster et Komárek (2011: 62) (Fig. 4). *Onodrimia´s* cluster was strongly supported by both posterior probability and bootstrap values. Uncultured bacterium TG-102 (JQ769612.1) forms a sister lineage to *Onodrimia,* but the support is not significant. In our phylogenetic tree, *Leptolyngbya* appeared in 10 separate lineages. Thus, we show that polyphyly of *Leptolyngbya* is larger than previously shown (see Fig 2).

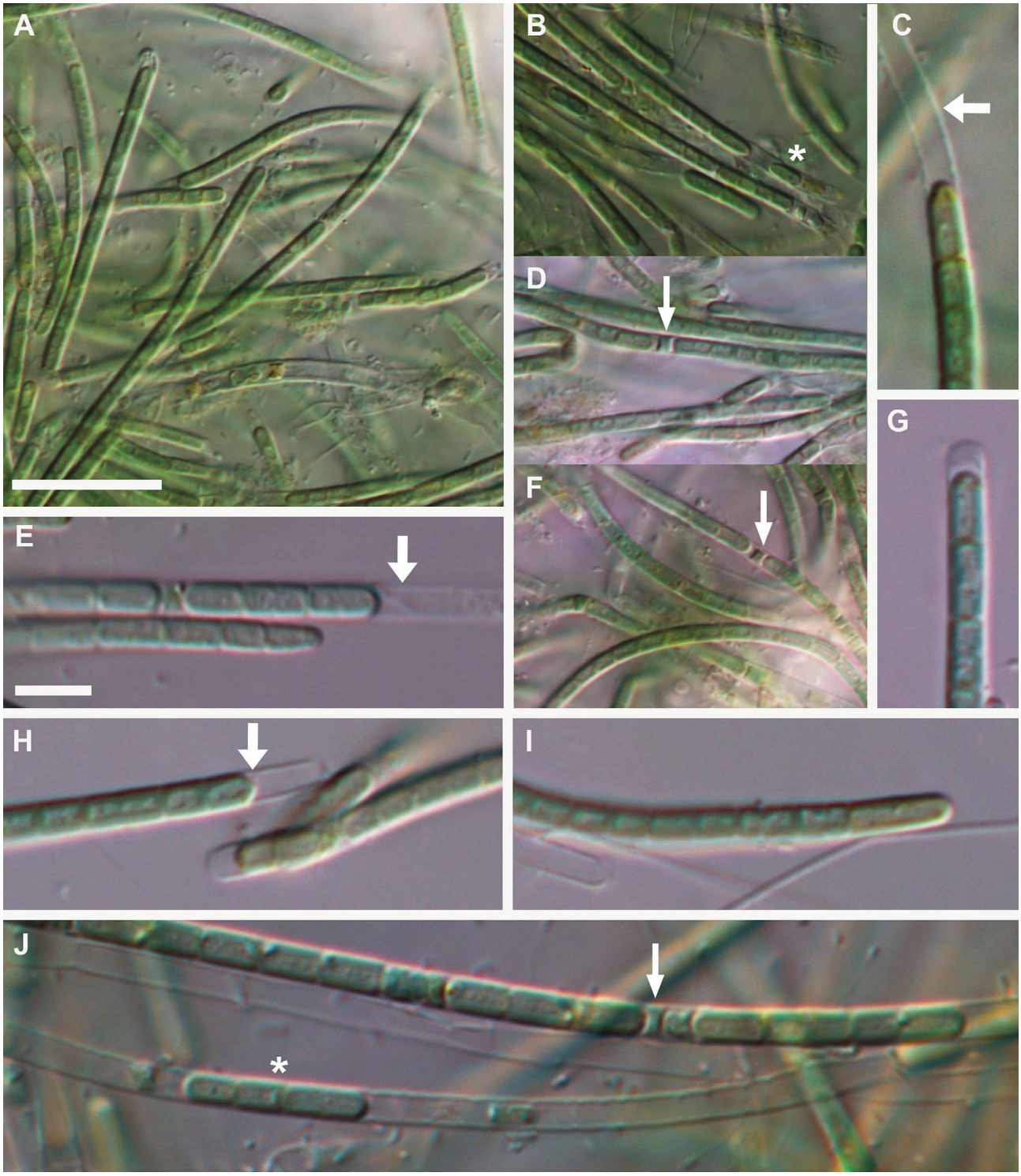

FIGURE 1. Morphological variability of *Onodrimia javanensis* sp. nov. (A–B) arrangement of trichomes in colony and old sheaths. (D, F, J) trichomes with necridic cells (narrow arrow). (C, E, H) trichomes with exceeding sheath (bold arrow). (I, G) appearance of apical cells. (B, J) formation of hormogonia (asterisk). Scale bar 10 μm.

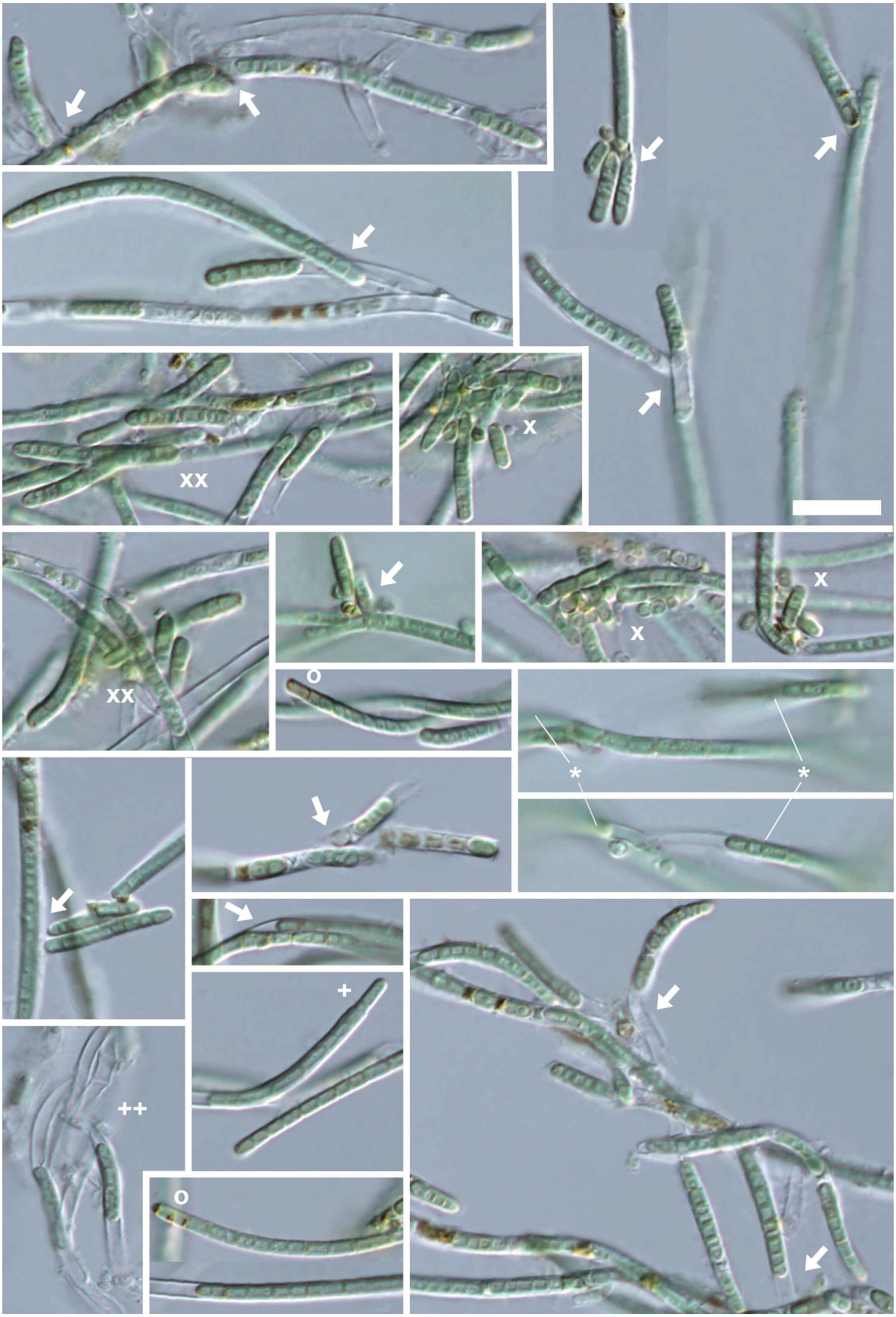

FIGURE 2. Variability in reproduction of *Onodrimia javanensis* sp. nov. Arrow=pseudobranching, asterisk=attachment of sheath to trichome, cross=clusters of hormogonia and hormocytes, double cross=growing trichomes from hormogonia and hormocytes, circle=pale apical cell with orange granules (probably dying cell), plus=trichome protruding from sheath, double plus=empty sheaths suggesting branching of trichomes. Scale bar 10 μm.

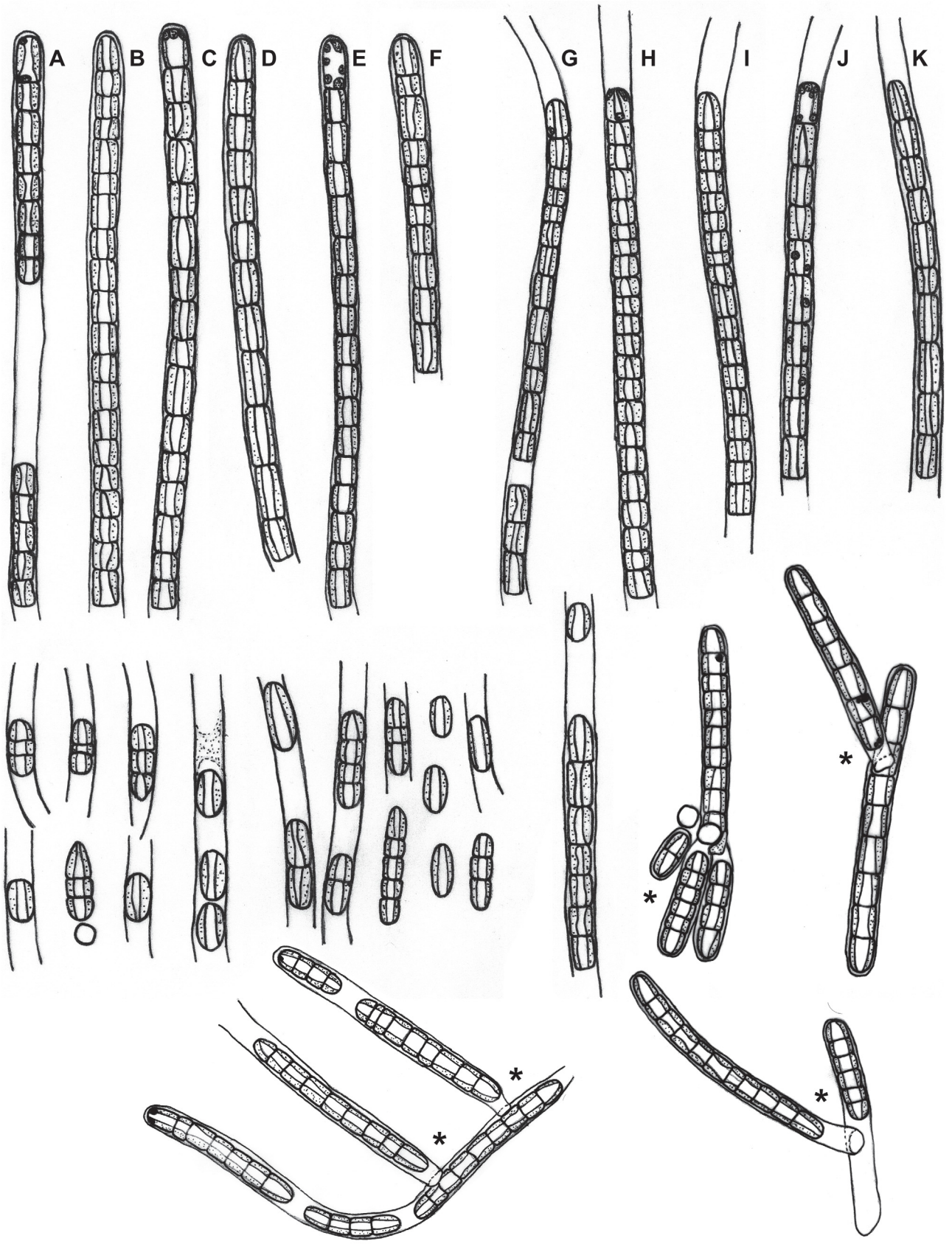

FIGURE 3. Iconotype of *Onodrimia javanensis*. A–F filaments with close sheath. G–K filaments with open sheath. In the lower part are drawn hormogonia, hormocytes and tree-like tufted hormogonial production (asterisk).

FIGURE 4. A phylogenetic reconstruction of 146 taxa based on 16S rRNA using Bayesian inference. Studied strains *Onodrimia javanensis* and *Leptolyngbya* sensu stricto are in bold. Supports at the nodes (Bayesian inference/maximum likelihood/maximum parsimony) represent only posterior probabilities >90 and bootstrap values >50, symbol of asterisk on the nodes represent 100 posterior probabilities and bootstrap values. Lines on site of the tree represent separate lineages of *Leptonygbya* sensu stricto and other Leptolyngbyaceae genera including *Onodrimia*.

The closest relative to *Onodrimia* based on a BLAST search (28th of July 2015) was an uncultured bacterium TG-102 (JQ769612.1; with sequence similarity 96.2%) (Table S1), which was found in biological soil crust of a copper mine tailings wasteland.

Secondary structure of 16S-23S ITS:—All three strains (E27, E28, E30) possessed a single, identical operon containing genes for $tRNA^{Ile}$ and $tRNA^{Ala}$ (490 nt total). The D1-D1' helix (105 nt) and Box-B (46 nt) was the same between all strains (Fig. 5). The base of the stem always consisted of 5 bp clamp followed by a 3' asymmetric loop of 9 nt. The middle part contains three loops. The first loop consisted of 6 nt, with the second largest loop (14 nt) and the third loop the smallest (5 nt). The terminal loop of the D1-D1´ consisted of 10 nt. The Box-B helix was

conserved in all strains. Many cyanobacteria have a V2 helix between the two tRNA genes but all of our strains lack this structure, having only 9 nt between the tRNA genes. We compared D1-D1' helix and Box-B of *Onodrimia* with secondary structures of *Nodosilinea nodulosa* (Z.Li & J.Brand) Perkerson & Casamatta in Perkerson *et al.* (2011: 1405) UTEX 2910 (KF307598), *Oculatella subterranean* Zammit, Billi & Albertano (2012: 352), *Leptolyngbya boryana* (Gomont) Anagnostidis & Komárek (1988: 391) UTEX B 485 (EF429291), *Leptolyngbya appalachiana* Johansen & Olsen in Johansen *et al.* (2008: 24) GSM-SFF-MF60 (EF429286), *Phormidesmis* sp. WJT36-NPBG20 (KJ939034), *Phormidesmis* sp. WJT67-NPBG4A (KJ939043), *Stenomitos rutilans* Miscoe & Johansen in Miscoe *et al.* (2016: 85) HA7619-LM2 (KF417430) and *Neosynechococcus sphagnicola* CAUP A 1101 (KJ469130). We found only little resemblance in a shape of base composition except the fact that *L. appalachiana* also showed an exceptionally long D1-D1' helix similar to *Onodrimia* (*Onodrimia* 105 nt and *L. appalachiana* 115 nt), but they differed in overall shape. Moreover, *L. appalachiana* exhibited also an exceptionally long Box-B (74 nt), while *Onodrimia* had only 46 nt.

Discussion

Sundaland, with Java Island, is one of the 25 places with the highest biodiversity of animals and plants, places widely known as biodiversity hot spots, mostly located in the tropics (Myers *et al.* 2000). The diversity of tropical cyanobacteria seems to be much richer than previously expected (Fiore *et al.* 2007). In the last few years, a number of new tropical cyanobacterial taxa have been proposed such as the heterocytous *Dapisostemon* Hentschke, Sant'Anna & Johansen in Hentschke *et al.* (2016: 136), *Streptostemon* Sant' Anna *et al.* (2010: 220), and *Brasilonema* Fiore *et al.* (2007: 794) from Brazil. Oscillatorialean cyanobacteria are also a potent source of newly described taxa, such as *Ammassolinea* Hašler, Dvořák, Poulíčková & Casamatta in Hašler *et al.* (2014) and *Jacksonvillea* Hašler *et al.* (2017: 289) from Florida. Interestingly, the morphologically simplest cyanobacteria (order Synechococcales) also offer unexpectedly high diversity, which is exemplified by newly erected genera such as *Pantanalinema* and *Alkalinema* Vaz *et al.* (2015: 302) from tropical Brazilian alkaline lake, *Kovacikia* and *Stenomitos* from Hawaiian caves, *Elainella* Jahodářová, Dvořák & Hašler in Jahodářová *et al.* (2017) and *Pinocchia* Dvořák, Jahodářová & Hašler in Dvořák *et al.* (2015: 115) from Vietnam. The taxonomic darkness is slowly subsiding from the tropical areas and revealing countless new taxa, but we are still far from understanding tropical cyanobacterial diversity.

We report a new, periphytic, filamentous cyanobacterium from Java. The newly erected genus *Onodrimia* fits within the circumscription of *Leptolyngbya* based on the description of Komárek & Anagnostidis (2005). However, as previously shown by Perkerson *et al.* (2011), Osorio-Santos *et al.* (2014), and many others, our phylogenetic reconstruction using 16S rRNA revealed that *Leptolyngbya* is polyphyletic, although the polyphyly is more extensive than previously reported with possibly up to 10 lineages (Fig. 4) and *Onodrimia* represents one of these lineages. *Onodrimia* is clearly delimited from other Leptolyngbyaceae taxa and comprises a highly supported clade, sharing only 96.2% sequence similarity with Uncultured bacterium clone TG-102 (JQ769612) and 94% sequence similarity with Uncultured bacterium clone TG-104 (JQ769614). Both Uncultured bacteria are basal lineages to *Onodrimia* and were collected from biological soil crust of copper mine tailings wastelands in China. Since the sequences of TG-102 and TG-104 come from environmental sampling, we are not able to compare their morphology with our strains. Moreover, *Onodrimia* does not cluster with *Leptolyngbya* sensu stricto represented by type species *L. boryana*.

Onodrimia can be distinguished from *Leptolyngbya* based on the morphological feature of tree-like hormogonial tufts (see Table S2). Moreover, *Onodrimia* may be distinguished from other Leptolynbyaceae based on its unusual habitat. It was isolated from submersed bark of tree branches which fell into the hot water spring in the Java rainforest. *Leptolyngbya corticola* Johansen *et al.* (2011: 291) is the only species occupying a similar habitat (tree bark), but it was isolated from temperate forest (Johansen *et al.* 2011).

The 16S rRNA phylogenetic tree showed that *Onodrimia* formed a cluster with *Phormidesmis*, *Stenomitos, Pantanalinema* and *Neosynechococcus*. However, all those genera are morphologically distinct from *Onodrimia.* Cells of *Phormidesmis* are isodiametric, *Stenomitos* has thinner trichomes (less than 2.5 μm), *Pantanalinema* has isodiametric or wider than long cells and *Neosynechococcus* is a unicellular cyanobacterium.

Komárek *et al.* (2014) have proposed a new taxonomic classification system for cyanobacteria. Based on this proposed system, *Onodrimia* would be classified in the order Synechococcales, family Leptolyngbyaceae based on 16S rRNA phylogeny (Fig. 4). Other Leptolyngbyaceae taxa are located at the terminal part of the phylogenetic tree. Most of these taxa should be classified to the order Synechococcales, family Leptolyngbyaceae or family Pseudanabaenaceae.

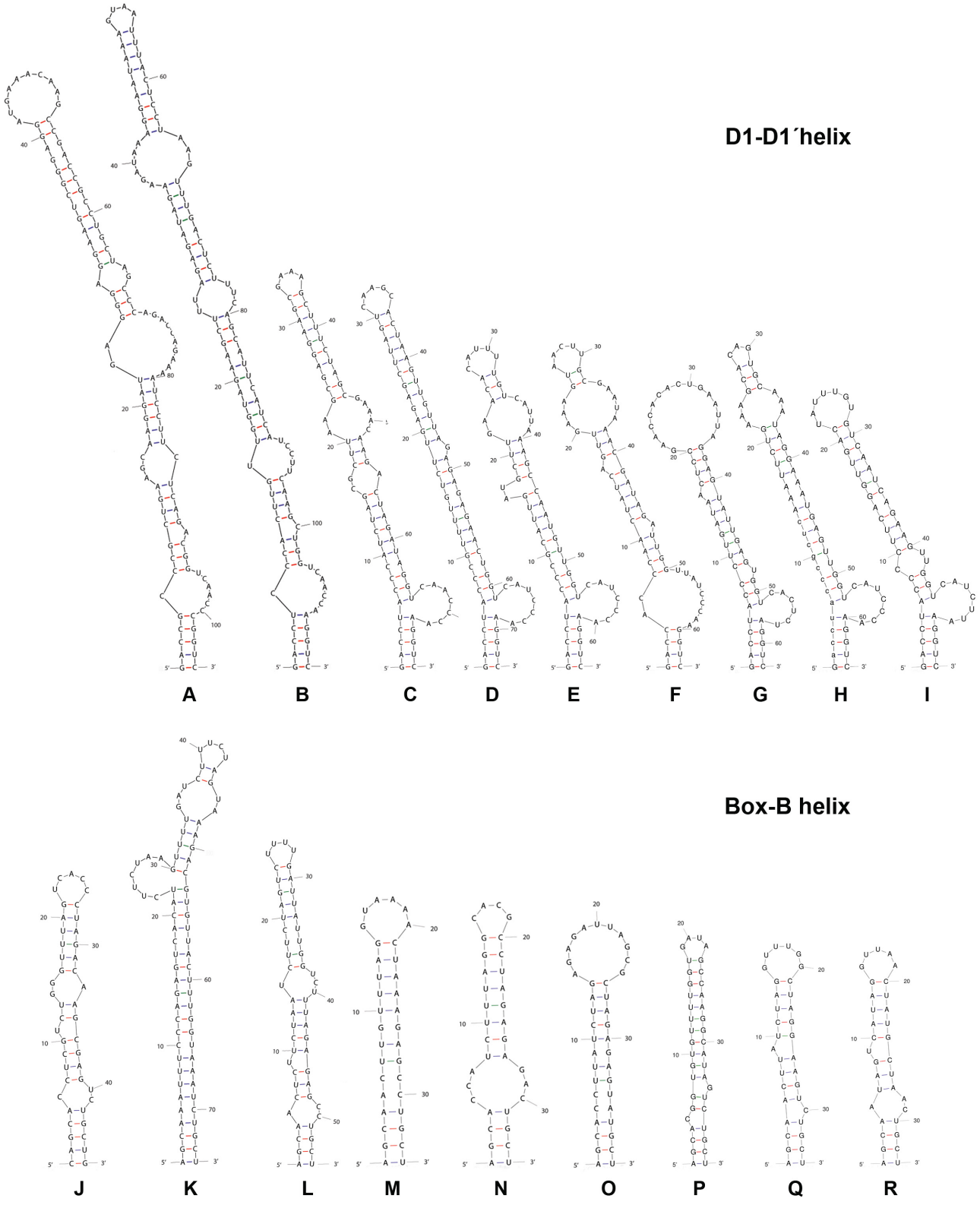

FIGURE 5. Estimated 16S-23S ITS secondary structures D1-D1´and Box-B helices of (A, J) *Onodrimia javanensis* E27, E28, E30, (B, K) *Leptolyngbya appalachiana* GSM-SFF-MF60 (EF429286), (C, L) *Phormidesmis* sp. WJT36-NPBG20 (KJ939034), (D, M) *Phormidesmis* sp. WJT67-NPBG4A (KJ939043), (E, N) *Stenomitos rutilans* HA7619-LM2 (KF417430), (F, O) *Neosynechococcus sphagnicola* sy1 (KJ469130), (G, P) *Nodosilinea nodulosa* UTEX 2910 (KF307598), (H, Q) *Oculatella subterranea*, (I, R) *Leptolyngbya boryana* UTEX B 485 (EF429291).

The secondary structures of the ITS region between 16S-23S rRNA provide effective tools for recognition of taxa at the species level (e.g., Siegesmund *et al.* 2008; Hašler *et al*. 2014). The ITS region contains several semi–conservative motifs (D1-D1′ helix, V2, Box-B, V3), which are critical to post-transcriptional processing and thus constrained by natural selection (Johansen *et al.* 2011). Secondary structures have been used as an autapomorphy in the description of novel taxa such as *Coleofasciculus*, *L. appalachiana* and *L. badia* Johansen & Lowe in Johansen *et al.* (2008: 26). Also, it has been used as supporting evidence in the description of *Rexia erecta* Casamatta, Gomez & Johansen (2006: 23), *Pinocchia polymorpha* Dvořák, Jahodářová & Hašler in Dvořák *et al.* (2015: 114), etc. We found no ITS variability among *Onodrimia* strains (Fig. 5). The D1-D1′ helix and Box-B were identical among all strains of *Onodrimia*. When we compared ITS secondary structures of *Onodrimia* with other members of Leptolygbyaceae, we found that the only similar structures appeared in *Leptolyngbya appalachiana*, which also possesses extremely long D1-D1′ helices, separating them from all other Leptolyngbyaceae. However, *L. appalachiana* is not related to *Onodrimia* based on 16S rRNA phylogeny and the long helix is not a synapomorphy of these two genera, because they have evolved independently.

Although our strains possessed only minor morphological differences from other filamentous species within the Leptolyngbyaceae, they could be distinguished from all of these taxa by morphological autapomorphies (tree-like tufted hormogonial production), molecular autapomorphies (distinctive secondary structures of the conserved domains of the ITS regions), phylogenetic position, and low sequence identity values with all other species in the order Synechococcales. Based on all of this evidence and tropical occurrence, we conclude our strains belong to a new species in a new genus, and name them *Onodrimia javanensis*.

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PAPER III.

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Elainella gen. nov.: a new tropical cyanobacterium characterized using a complex genomic approach

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ABSTRACT

Cyanobacteria represent an ancient, monophyletic lineage of bacteria with the ability to undertake oxygenic photosynthesis. Although they possess a relatively high degree of morphological variability compared with other prokaryotes and there is a wealth of molecular data, there are still significant gaps in our knowledge of cyanobacterial diversity, especially in tropical areas. Here, we present a novel, filamentous, tropical cyanobacterium, which could be classified as *Pseudophormidium* based on morphological criteria. A total evidence investigation employing ecological, morphological and genomic data, indicated that our strains form a new and ancient evolutionary lineage among cyanobacteria unrelated to *Pseudophormidium*. Based on this polyphasic assessment, our strains represent a novel, monospecific genus: *Elainella*. This new genus represents an example of phenotypic convergence, which seems to be a prevalent macroevolutionary pattern in cyanobacteria, a likely cause of the frequently cited polyphyly within a majority of genera.

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KEYWORDS 16S rRNA; genome sequencing; new genus; phylogenomics; *Pseudophormidium*; tropical cyanobacteria

Introduction

Cyanobacteria are probably the most ancient lineage of oxygenic primary producers, with fossil records dated 3.5–3.8 BYA (Schopf, 2002; Sleep, 2010). Cyanobacteria are ubiquitous across a myriad of habitats (e.g. fresh water, marine, terrestrial, subaerial, etc.) and often occur in extreme biotopes such as hot springs, polar regions, or soil crusts (Whitton & Potts, 2002). They are also ecologically and evolutionarily important, for example, marine cyanobacteria account for 25% of all primary productivity (Stal, 2002; Whitton & Potts, 2002; Bellinger & Sigee, 2010; Flombaum *et al.*, 2013). Most explorations of cyanobacterial diversity have focused on temperate zones (Dvořák *et al.*, 2015*a*) with forays into polar regions (Taton *et al.*, 2003; Casamatta *et al.*, 2005), but there is increasing evidence that biodiversity in tropical and subtropical regions has been underestimated (Fiore *et al.*, 2007; Bohunická *et al.*, 2011; Perkerson *et al.*, 2011; Hašler *et al.*, 2014; Dvořák *et al.*, 2015*a*; Genuário *et al.*, 2015; Vaz *et al.*, 2015; Hentschke *et al.*, 2016).

Traditionally, cyanobacteria were identified using only morphological features. However, environmental factors and culture conditions are known to affect morphological characters, such as the presence of heterocytes or sheaths (Casamatta *et al.*, 2003; Hašler *et al.*, 2011, 2012; Dvořák *et al.*, 2015*a*; Hentschke & SanťAnna, 2015). For example, Berrendero *et al.* (2011) showed that morphological characters of *Calothrix* changed in culture, causing them to resemble *Tolypothrix*. To circumvent issues arising from phenotypic plasticity, evolutionary relationships among the cyanobacteria are currently being inferred using the 16S rRNA gene (Giovannoni *et al.*, 1988) since it is highly conserved among all prokaryotes (Coenye & Vandamme, 2003). However, 16S rRNA phylogenies alone lack sufficient resolution to reliably recognize many species or ecotypes, which is particularly problematic for parasitic bacteria. Thus, Maiden *et al.* (1998) developed multilocus sequence typing (MLST), which allows for a more sensitive approach for species identification, because it uses up to 10 different markers of protein coding sequences. However, more recently, it has been shown that MLST might also be insensitive to some minute differences among ecotypes, which may be considered as species. For instance, ecotypes within *Bacillus subtilis* may be recognized only based on whole genome data (Kopac *et al.*, 2014). Moreover, the whole genome sequence may provide a statistical species definition such as average nucleotide identity (ANI; Richter & Rosselló-Móra, 2009).

Research into the taxonomy and biodiversity of cyanobacteria is further complicated by entangled evolutionary relationships, which is most strikingly illustrated by the polyphyly of phenotypically

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coherent genera (sometimes called 'cryptogenera'; Komárek *et al.*, 2014; Dvořák *et al.*, 2015*b*). For instance, the globally distributed cyanobacterium *Synechococcus* contains at least 12 polyphyletic lineages (Dvořák *et al.*, 2014), each of which may be considered a separate genus. This phenomenon seems to be common among cyanobacteria. Komárek *et al.* (2014) suggest that the majority of cyanobacterial genera are polyphyletic. The reasons for this rampant polyphyly are numerous, including convergent evolution, lack of distinguishable morphological characters and the antiquity of this lineage. Recently, the evolutionary patterns responsible for this frequent polyphyly have been combined within the 'model of serial convergence' proposed by Dvořák *et al.* (2014), which suggests that frequent gene exchange within local gene pools (*sensu* Polz *et al.*, 2013) via horizontal (lateral) gene transfer and homologous recombination are the crucial factors responsible for convergence.

Polyphyly can also be observed in the genus *Pseudophormidium* (Taton *et al.* 2006; Alwathnani & Johansen 2011; Osorio-Santos *et al.* 2014). The genus was originally one of the sections of the genus *Plectonema* with one species, *Plectonema phormidioides*. This section was elevated to the genus level *Pseudophormidium* (Anagnostidis & Komárek, 1988). Komárek & Anagnostidis (2005) characterized *Pseudophormidium* as follows: filamentous, falsely branched cyanobacterium mostly with sheaths and with relatively wide trichomes (up to 10 μm), mostly distinctly constricted at cross-walls; apical cells usually rounded and reproduction by hormogonia. *Pseudophormidium* differs from the genus *Phormidium* by the presence of false branching trichomes (Anagnostidis & Komárek, 1988). *Pseudophormidium* contains mostly periphytic species, growing on stones or other substrate in unpolluted streams and on soils (Anagnostidis & Komárek, 1988; Anagnostidis, 2001).

In this paper, we present a new enigmatic genus of *Pseudophormidium*-like cyanobacterium isolated from the plankton of an ephemeral waterbody in the forest of the National Park Cat Tien and from granite and sand in a waterfall in Pongour, Vietnam. We provide ecological, morphological and genomic data allowing a complex evaluation of evolutionary history and taxonomy of this new cyanobacterium.

Materials and methods

Strain isolation

Algal samples were collected from plankton of ephemeral waterbodies in the forest of the National Park Cat Tien, Đồng Nai province, Vietnam on 22 and 24 November 2010 (GPS: 11 26.752 N, 107 23.184 E; GPS: 11 25.712 N, 107 25.716 E) and from granite and sand from Pongour waterfall 875 m above sea level, Lâm Đồng province, Vietnam on 29 October 2010 (GPS: 11 41.302 N, 108 15.877 E) (Table 1). Strains were isolated from fresh samples using standard isolation techniques. Three study cultures (E1, E8 and E11) were cultivated in 90 mm Petri dishes under the following conditions: temperature 26±1°C, illumination 20 μmol photons m^{-2} s^{-1}, light regime 12h light:12h dark, and liquid Zehnder medium (Z medium; Staub, 1961).

Table 1. Environmental parameters of localities where *Elainella saxicola* occurred.

	E. saxicola E1	*E. saxicola* E8	*E. saxicola* E11
Locality	Ephemeral waterbody in the forest, Cat Tien National Park	Ephemeral waterbody in the forest, Cat Tien National Park	Waterfall Pongour, 875 m.a.s.l.
State/Province	Vietnam/Đồng Nai	Vietnam/Đồng Nai	Vietnam/Đồng Nai
Date	24.11.2010	22.11.2010	29.10.2010
GPS N	11 26.752 N	11 25.712 N	11 41.302 N
GPS E	107 23.184 E	107 25.716 E	108 15.877 E
Conductivity (μS cm^{-1})	21	101	62
pH	6.06	5.84	7.10
Temperature (°C)	26.7	NA	22.9
Transparency (m)	0.25	NA	NA
Habitat	Plankton	Plankton	Epilithon, epipsammon

Morphological assessment

Strain morphology was studied 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). Cultured samples were used for strain morphology and evaluated using the following characters: cell shape, terminal cells, cell dimensions (100 cells), reproduction, sheaths, branching and granulation of cells.

PCR amplification and sequencing

Genomic DNA was extracted from *c.* 50 mg of fresh biomass using an UltraClean Microbial DNA Isolation Kit (MOBIO, Carlsbad, California, USA) following the manufacturer's manual. DNA quality and consistence was checked on GelRed (Biotinum Inc., Hayward, California, USA) stained 1.5% agarose gel. DNA was quantified using the NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, Delaware, USA). A partial 16S rRNA sequence and the whole 16S–23S ITS sequence were obtained using PCR amplification with primers: forward P2 (5′-

GGGGAATTTTCCGCAATGGG-3′), and reverse P1 (5′- CTCTGTGTGCCTAGGTATCC-3′) previously described in Boyer *et al.* (2001). 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 given in Dvořák *et al.* (2012). PCR products were cloned using StrataClone PCR Cloning kit (Agilent Technologies, Stratagene Product Division, La Jolla, California, USA) following the manufacturer's manual with modifications described in Dvořák *et al.* (2012).

De novo *genome sequencing*

Since the 16S rRNA and 16S–23S ITS sequences for all investigated strains were identical, we only sequenced one strain, E1, for the *de novo* genome as the representative for the new genus. Genomic DNA was extracted from 50 mg of wet biomass using UltraClean Microbial DNA Isolation Kit (MOBIO, Carlsbad, California, USA). An ethidium bromide stained 1.5% agarose gel and NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, Delaware, USA) were used to evaluate the quality and concentration of DNA, respectively.

The library for sequencing was prepared by TruSeq Nano DNA kit (Illumina Inc, San Diego, California, USA) from 200 ng DNA according to manufacturer's instructions, but DNA was digested by Bioruptor Plus (Diagenode, Liege, Belgium) and size selection was modified to achieve insert size of *c*. 1000 bp. The insert size of libraries was defined by Agilent High Sensitivity DNA Kit (Agilent Technologies, Inc.) and the concentration of library was assessed by KAPA Library Quantification Kit for Illumina (Kapa Biosystems, Woburn, Massachusetts, USA). MiSeq Reagent Kit v3 (Illumina Inc, San Diego, California, USA) was used for sequencing to produce pair end reads with length of 300 bp.

A total of 2 880 274 pair ended reads with a mean length of 226 bp were assembled *de novo* using the MIRA 4 assembler (Chevreux *et al.*, 1999; parameters: -job = denovo,genome,accurate, -NW: cmrnl = no, autopairing). We used the following procedure for the removal of contaminant contigs. We ran BLASTN against complete bacterial genomes with default parameters with all contigs. We regarded contigs showing a match to a sequenced bacterial genome rather than cyanobacteria as contaminants (1274 contigs). The *de novo* assembled genome resulted in 284 contigs (>500 bp) with an N50 73 085 bp, and a theoretical coverage of 38× based on the estimation of a length of 8.7 megabases. Rapid Annotation using the Subsystems Technology (RAST) pipeline (Aziz *et al.*, 2008) was used for annotating with default options except enabled fix frameshift; tRNA was predicted using tRNAscan-SE 1.21 (parameters: cove searching, covariance model—bacterial; Lowe & Eddy, 1997). CRISPRs (clustered regularly interspaced short palindromic repeats) and CRISPR spacers were identified using CRISPRfinder (Grissa *et al.*, 2007). Putative secondary metabolite gene clusters and molecule structures were predicted by antiSMASH 3.0 (Weber *et al.*, 2015). Average nucleotide identity (ANI) was determined by Jspecies (Richter & Rosselló-Móro, 2009). A visual representation of the BLAST searched genome similarities of *Elainella* with *Leptolyngbya* sp. JSC-1 was performed in BRIG (Alikhan *et al.*, 2011). The genome has been deposited in GenBank (http://www.ncbi.nlm.nih.gov/; BioProject: PRJNA313515, accession number: LUGL00000000).

Phylogenetic and phylogenomic analyses

The most similar sequences of 16S rRNA were obtained 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 *Pseudophormidium* and other cyanobacteria were added. Multiple sequence alignment was performed in MEGA 6 (Tamura *et al.*, 2013) using Muscle algorithm (Edgar, 2004) (see Supplementary dataset S1). The tree was rooted to the outgroup *Gloeobacter violaceus*. The most appropriate model for maximum likelihood analyses was determined in jModelTest 0.1.1 (Posada, 2008) based on both the Bayesian and the Akaike Information Criterion as follows: GTR+I+G. A 50% majority consensus tree was constructed in MrBayes 3.2.1 (Ronquist & Huelsenbeck, 2003). Four separate runs were performed, each with three heated and one cold chain for 100 000 000 generations. The sampling frequency was each 10 000th generation. 25% trees were discarded as burn-in. Maximum likelihood analysis was performed in RaxML 8.0.2 (Stamatakis, 2006) with a GTR+G 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.

A total of 129 available and annotated genomes (both draft and complete) of cyanobacteria were acquired from the ftp server of GenBank (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/), database version 27 January 2015. Other genomes of cyanobacteria from GenBank were added to cover the broad evolutionary array of this group, representing most major niches/habitats. Genomes of *Leptolyngbya boryana* PCC 6306, *Geitlerinema* sp. PCC 7105, *Spirulina subsalsa* PCC 9445 and *Nodosilinea nodulosa* PCC 7104 were

re-annotated using RAST due to lack of annotation in the GenBank database (Supplementary table S1).

The super alignment of 69 protein sequences (see Supplementary dataset S2) for a subsequent phylogenomic reconstruction of a cyanobacterial species tree was obtained using phylogenomic Perl pipeline Hal (Robbertse *et al.*, 2001) with options described in Dvořák *et al.* (2014).

The phylogenomic reconstruction based on a resulting super alignment with a total of 15 141 amino acids was performed in RAxML 8.1.15 (Stamatakis, 2006) using CAT+LG model. The most suitable substitution matrix was identified in PROTTEST 3.3 (Abascal *et al.*, 2005). *Gloeobacter violaceus* and *Gloeobacter* sp. were used as outgroup taxa. Tree topology was tested using rapid bootstrapping with 500 bootstrap replicates with the same model settings.

The dating of the phylogenomic reconstruction has been performed using calibration points and settings of penalized likelihood (Sanderson, 2002), as previously used by Dvořák *et al.* (2014) in r8s (Sanderson, 2012) with the following exception: the root of the tree was calibrated with the first likely evidence of cyanobacteria photosynthesis 3.2 BYA (Satkovski *et al.*, 2015).

Results

Elainella saxicola E. Jahodářová, P. Dvořák & P. Hašler, gen. et sp. nov. (Figs 1–21)

Holotype: Holotype OLM! Botany 24: Lichens and others No. 9222 dried sample is deposited in Regional Museum in Olomouc, Czech Republic.

Type strain: UPOC E1/2017, deposited at the culture collection of Department of Botany, Palacký University Olomouc, Czech Republic.

Type locality: Ephemeral waterbody in the forest, Cat Tien National Park, province Đồng Nai, Vietnam, coll. E. S. Gusev, 24 November 2010.

Etymology: The genus epithet is derived from Elaine, The Lady of the Lake, who lived in a castle that was beneath a lake surrounding island of Avalon. Elaine gave Arthur the magic sword Excalibur. The species name refers to the fact that this genus was found on a stone, Latin – *saxum*.

Description

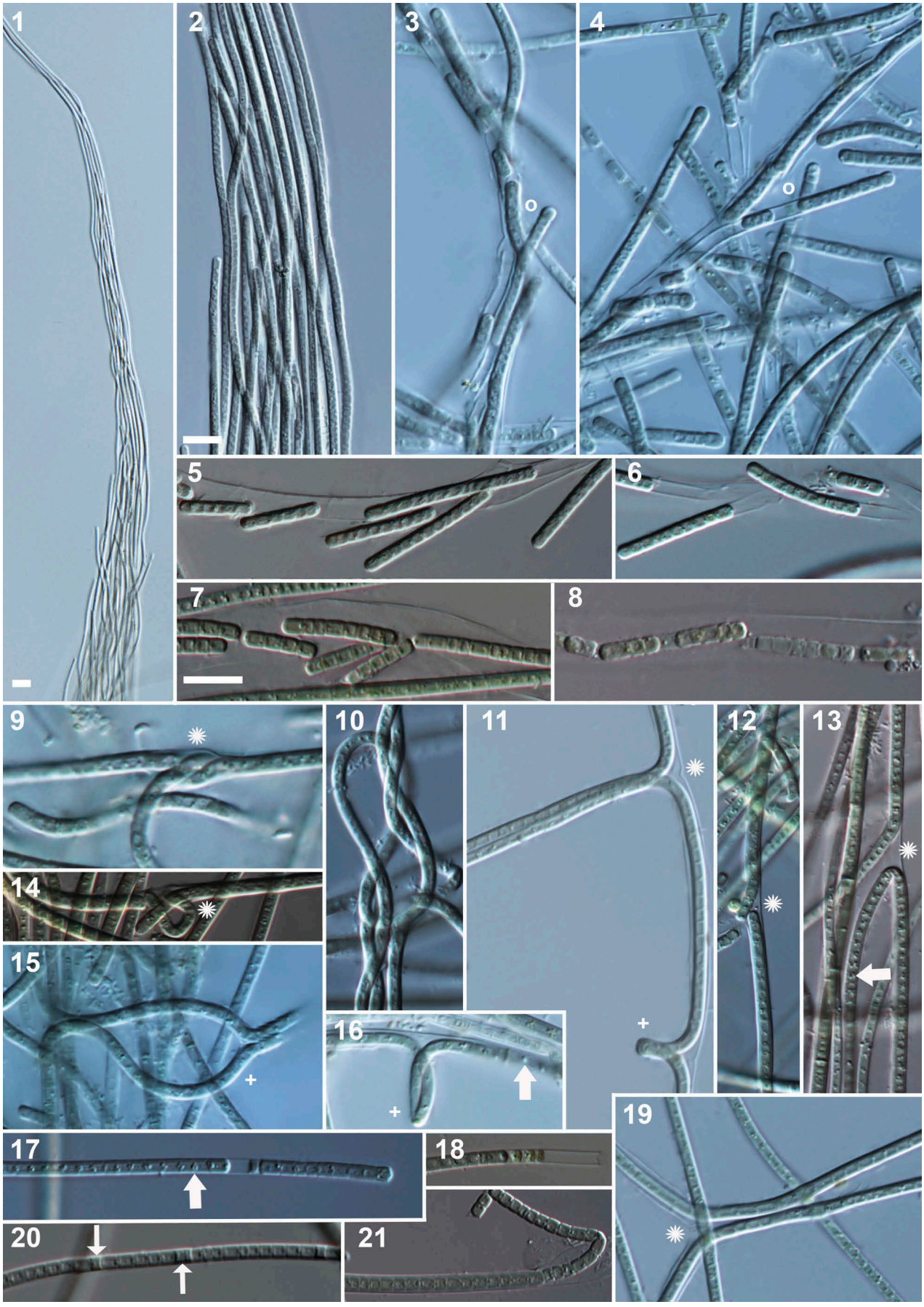
Colonies macroscopic, dark green, in fascicles or tufts. Filaments yellow-green, green, grey-green, straight, curved, undulate, often with loops. Sheath colourless, thin and distinct, variable in length. Sheath exceeds trichome or trichome protruding from sheath. Trichomes cylindrical, not attenuated at the end, slightly or not constricted at cross-walls, immotile. False branching present, usually both of chiasmatic type formed after breakage of trichome loop formation, and Y type formed during simultaneous growth of hormogonia. Cells isodiametric or longer than wide 1.73–2.63 μm (average 2.27 μm) wide and 1.34–3.76 μm long (average 2.3 μm). Cell content divided into visible peripheral chromatoplasm and central pale nucleoplasm often with granules, without aerotopes. Apical cell rounded, without calyptra. Reproduction by necridic cells, via trichome breakage and subsequent disintegration, releasing hormogonia.

The genome

The total length of the draft, near-complete genome was 8 702 141 bp with 47.6% G+C content. It is one of the largest genomes among the non-heterocytous cyanobacteria sequenced so far. RAST genome annotation revealed a total of 8472 coding sequences (CDSs) and 102 RNAs. All common tRNAs were present as in other cyanobacterial genomes. 51.3% of CDSs were annotated based on known proteins with biological function and 48.7% were identified as hypothetical proteins. The genome contains genes for nitrogen fixation (*nifB, nifS, nifU, nifH, nifD* and *nifK*), similar in composition to some other non-heterocytous, N-fixing cyanobacteria such as *Cyanothece* sp. PCC 7425. Furthermore, the genome of *Elainella* contains 24 confirmed CRISPRs with 223 CRISPR spacers. On the other hand, it lacks genes, which has been demonstrated by Gan *et al.* (2014) crucial for Far-Red Light Photoacclimation (FaRLip), e.g. *ApcA2* and *ApcB2*. A comparison of the basic genome properties with the closest cyanobacterium with a genome sequence, *Leptolyngbya* sp. JSC-1, can be found in the supplementary information, including ANI and 16S rRNA (Supplementary tables S2, S3, Supplementary fig. S1). AntiSMASH revealed 10 putative biosynthetic gene clusters in total and only two of them had significant similarity to known gene clusters searched by ClusterBlast implemented in antiSMASH – puwainaphycins and cryptophycin biosynthetic gene cluster (see details in Supplementary fig. S2).

Phylogeny

16S rRNA phylogeny, based on a Bayesian inference, revealed that strains of *Elainella* form a monophyletic lineage among other filamentous cyanobacteria such as *Pseudanabaena*, *Nodosilinea, Trichocoleus, Oculatella, Spirulina* and *Symploca* (Fig. 22). The most closely related cyanobacterium to *Elainella* is *Leptolyngbya* sp. D1C10 (KJ654308) with 100% node support.



Figs 1–21. Microphotographs of *Elainella saxicola* E1 type strain. (Figs 1, 2) Colony in tufts. (Figs 3, 4) Y-like false branching. (Figs 5–7) Hormogonia. (Figs 3, 4, 9–14, 19) False branching. (Figs 15, 16) Loops. (Figs 17, 18, 20) Straight trichomes. (Figs 8, 21) Disintegrated trichomes. Scale = 10 μm; asterisk = divided trichomes (false branching); broad arrow = granules; narrow arrow = necridic cells; plus = loops; circle = Y-like false branching.

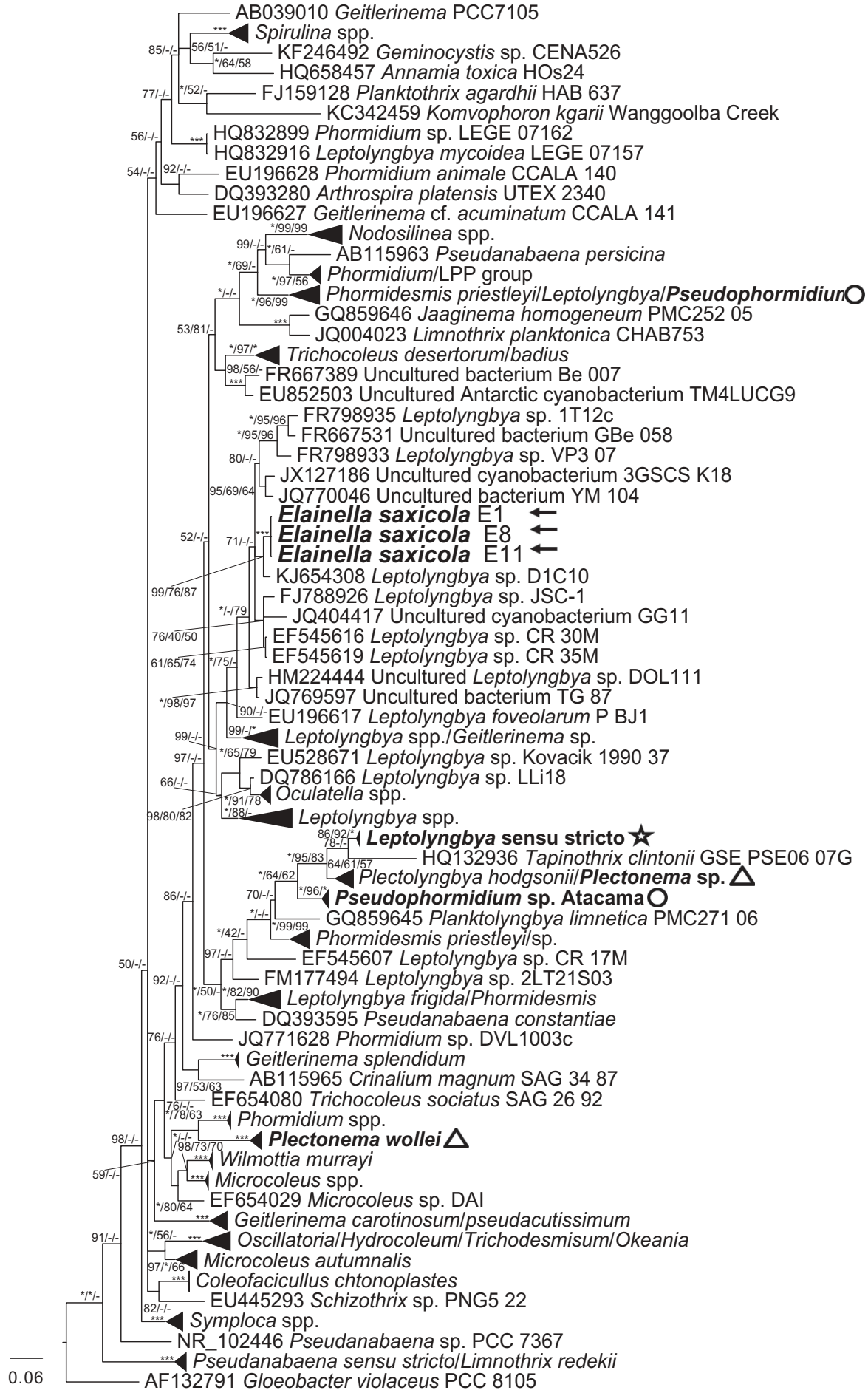


Fig. 22. A phylogenetic reconstruction of 133 taxa based on 16S rRNA using Bayesian inference. *Leptolyngbya sensu stricto* is marked with a star, *Pseudophormidium* strains with open circles, *Plectonema* strains with open triangles and strains of *Elainella saxicola* with arrows. Supports at the nodes (Bayesian inference/maximum likelihood/maximum parsimony) represent only bootstrap values and posterior probabilities >50.

The closest sister clades to *Elainella* included uncultured cyanobacteria from disparate habitats and parts of the world. The first clade included *Leptolyngbya* sp. 1T12c (FR798935; fountain in Italy), the uncultured cyanobacterial clone 3GSCS K18 (JX127186; endolithon, Germany), and the uncultured bacterial clone YM 104 (JQ770046; epilithon, Spain). The second clade contained the extremophile *Leptolyngbya* sp. JSC-1 (FJ788926; hot spring in Great Yellowstone area), the uncultured cyanobacterial clone GG11 (JQ404417; Forbidden City marble sculpture, China), *Leptolyngbya* sp. CR 30M (EF545616; hot spring of the Miravelles volcano, Costa Rica), and the uncultured *Leptolyngbya* sp. clone DOL111 (HM224444; dolomite rock, China). However, the relationship of these cyanobacteria to *Elainella* is unclear since they are connected by nodes with low node support.

Phylogenomic dating

We evaluated the evolutionary history of *Elainella* in the context of all cyanobacteria using a dated phylogeny 69 gene super-alignment (a set of orthologous genes used for phylogenetic reconstruction is available in Supplementary table S4). *Elainella* clustered with *Leptolyngbya* sp. JSC-1 and these taxa are likely to have diverged 2.24–2.47 BYA, thus, before the Great Oxygenation Event (GOE; Kopp *et al.* 2005; Fig. 23). They were connected by a node with 100% bootstrap support (split 0.66–0.73 BYA), which contradicted 16S rRNA analysis, where their clustering had only low node support (Fig. 22).

Discussion

The type species of the genus *Pseudophormidium* (*P. phormidioides*) has not yet been sequenced so we were unable to add it to the phylogenetic analysis. Although *Elainella* is similar to *Pseudophormidium*, it may be morphologically and ecologically distinguished from the type species of *Pseudophormidium* (see details in Table 2). *Pseudophormidium* is a relatively species-poor genus with very unclear phylogeny, and likely to be in need of considerable revision (Komárek *et al.*, 2014). Based on morphological characters, *Elainella* is similar to *Plectonema* species described by Gardner (1927) from Puerto Rico. These species were later combined or designated as unclear species of the genera *Pseudophormidium* and *Leptolyngbya* (Komárek & Anagnostidis 1988, 2005). The unclear taxonomic status of Gardner's species requires their future revision. It should be noted that the descriptions of *Plectonema* and *Pseudophormidium* largely overlap (e.g. thallus formation, filaments branching and hormogonia formation) (Komárek & Anagnostidis, 2005). However, *Plectonema* can be distinguished from *Pseudophormidium* by trichome width. *Pseudophormidium* typically possesses narrower trichomes (<10 μm) than *Plectonema* (8–25, up to 72 μm). Delimitation of both genera is well supported by 16S rRNA gene analysis (see Fig. 22 in this study). Although Gardner (1927) described four *Plectonema* species sharing morphological similarity with *Elainella saxicola* we cannot verify the taxonomic status of Gardner's species because of the lack of 16S rRNA and ITS sequences for them. *Elainella saxicola* frequently forms fasciculate colonies and inhabits ephemeral freshwater bodies, in contrast to Gardner's metaphytic species inhabiting soils and walls. On the other hand, our analyses of the 16S rRNA gene support the validity of *Elainella* and indicate that *Leptolyngbya, Plectonema* and *Pseudophormidium* represent a non-coherent group of polyphyletic taxa which must be completely revised.

We found other morphologically similar species to *Elainella*, but they differed in key morphological or ecological features. For example, *Ps. battersii* has slightly attenuated trichomes towards the ends, blackish trichomes and it occurs in littoral marine zones (Komárek & Anagnostidis, 2005). *Ps. hollerbachianum* can be differentiated from *Elainella* by cell dimensions and constriction at the cross-walls. Ecologically, *Ps. pauciramosum* is similar but inhabits salt waters (saline lakes). *Pl. andinum* belongs to an unrevised species and it differs by constriction at the cross-walls (see details in Table 2). It should be noted that living strains and molecular data are largely lacking for these species. Current progress in molecular techniques reveals a high level of intrageneric and intraspecific variability which exceeds their morphological variability. Thus, new species and genera have been recently established due to a high support for molecular difference despite morphological similarity amongst known taxa (Hašler *et al.*, 2014; Osorio-Santos *et al.*, 2014; Dvořák *et al.*, 2015*a*).

Elainella was found to form a highly supported clade distinct from *Pseudophormidium* and *Plectonema*. *Leptolyngbya* sp. D1C10 (KJ654308) formed the closest sister lineage with 98% sequence similarity. Furthermore, the clade containing both *Elainella* and *Leptolyngbya* sp. D1C10 is distant from *Leplolyngbya sensu stricto*, and based on the position of *Leptolyngbya* sp. D1C10 in the phylogeny, this leads us to hypothesize that *Leptolyngbya* sp. D1C10 is probably a species belonging to the genus *Elainella*. However, additional information about this strain (except for the fact that it has been found in India in a saline ecosystem) cannot be obtained, and therefore we cannot proceed with the revision of its taxonomic status.

Phylogenetic reconstruction further suggested that *Pseudophormidium* had a polyphyletic origin, which

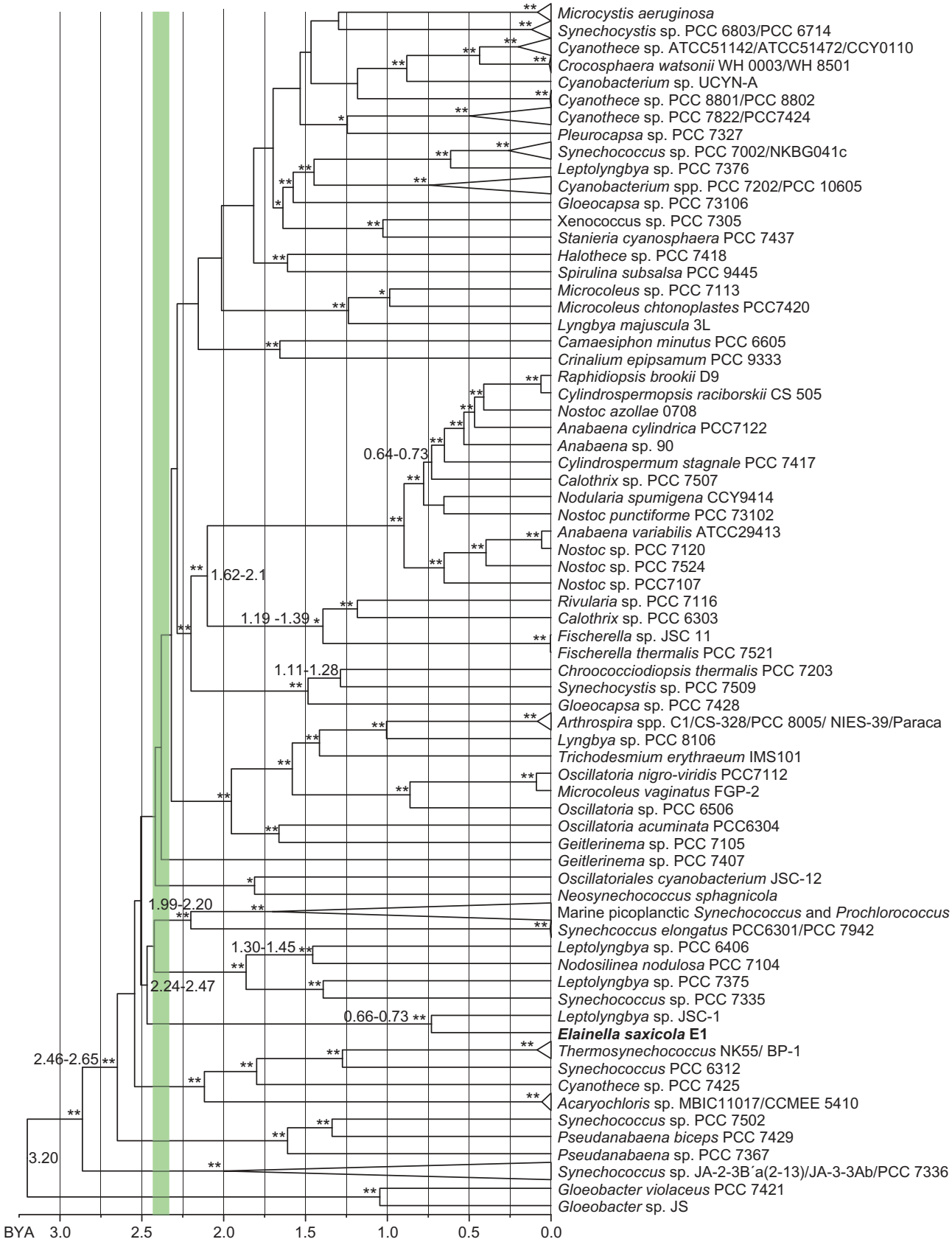


Fig. 23. A chronogram based on super alignment of 69 orthologous gene families of cyanobacterial genomes constructed using maximum-likelihood criterion. Both time estimates are combined in this figure. Bootstrap supports ≥70 are represented by asterisk and ≥90 by two asterisks together with the age at the nodes. Studied strain is in bold. BYA = billion years ago. Great Oxidation Event (GOE) is represented by the shaded stripe.

Table 2. Morphological comparison of *Elainella saxicola* with other closely related *Pseudophormidium, Plectonema* and *Leptolyngbya* species.

	Filaments	False branching	Presence of sheath	Cell dimensions (μm)	Trichome constrictions	Apical cell shape	Necridic cells	Hormogonia	Pigmentation	Presence of granules	Occurrence
***E. saxicola* E1**	Straight, bent, undulate, often with loops	+ (rarely)	+	L: 2.55±1.21 W: 2.18±0.45	-(or slightly)	Rounded	+	+	Yellow-green, green, grey-green	+	Plankton of ephemeral waterbody in the forest, on granite and sand in waterfall
Pl. andinum	Filaments long	+ (scarcely)	+ (sometimes lacking)	W: 2.4±0.4	-	Rounded	NA	NA	Bright blue-green	-	Freshwater, aerophytic, among mosses on rock
Pl. spirale	Coiled; attached at one end; variable width at different periods of growth and in different parts of the filaments, young tapering gradually towards the apices, later bulging in the middle and toward the base on account of the coiling and contortion of the trichome, increasing the length of diameter 2-3 times	-	+ (very thin)	W: 2.9±0.9	+	NA	NA	+	Blue-green, later dirty green to brownish	NA	Described from cultures (soils?), growing on an old pump
***Leptolyngbya* sp. JSC-1**	Entangled; organized in astral colonies in viscous or liquid media	NA	+	Short cells L: 2.25±0.41; W: 2.16±0.13 Narrow cells L: 2.89±0.27; W: 1.62±0.17	-	Rounded	+	+	Can change from green to reddish	+	Hot spring in Great Yellowstone area
***L. tenuissima* (*Pl. tenuissimum*)**	Individual filaments among other algae, 1.4–1.6 μm wide	+ (very sparse)	+ (very thin)	NA	+ slightly	Rounded	NA	NA	Blue-green	NA	On walls
***L. muralis* (*Pl. murale*)**	Contorted and geniculate, 3.6–4 μm wide	+	+	W: 1.65 ±0.15	+ slightly	NA	NA	NA	Blue-green	NA	On walls
***Ps. flexosum* (*Pl. flexosum*)**	Individual filaments among other algae, 5.8–6.5 μm wide	+	+ (very thin)	NA	-	Rounded Blunt-conical	NA	NA	Blue-green	NA	On soils
Ps. hollerbachianum	Trichomes variously curved, densely entangled	+ (richly)	+ (thin)	L: 1.25±0.25 W: 2.05±0.65	+	Rounded	NA	+	Pale to bright blue-green	-	Subaerophytic, edaphic
Ps. battersii	Long, flexuous, slightly attenuated towards ends	+ (richly and repeatedly)	+ (thick in main filaments)	W: 2.75±0.75	+	Rounded	NA	+	Blackish	-	Marine, epilithic in littoral water level zone
Ps. pauciramosum	Long, entangled, forming small clusters or insufficiently clearly expressed tufts, attached to substratum	+ (rarely)	+ (thin)	L: 1.75±0.55 W: 3.5±0.7	-(or slightly constricted)	Rounded	NA	NA	Grey to greenish	+	In saltwater (saline lake), epipelic on *Enteromorpha*
Ps. phormidioides	Thallus thin, membranaceous, somewhat lumbricous, expanded, with densely coiled filaments, rarely solitary filaments. Filaments 6–9 (10) μm wide	+ (richly)	+ (thin)	W: 6.3±0.7	+	Rounded	NA	+	Blue-green, greyish blue-green, olive-green or dirty brownish-violet	-	Freshwater, stones in mountain clear streams

is in agreement with Osorio-Santos *et al.* (2014). Comparison of *Pseudophormidium* sequences from the Atacama Desert (Osorio-Santos *et al.*, 2014) to those of *Elainella* revealed that they were completely unrelated. As further support for the polyphyletic origin, the habitat of *Pseudophormidium* species from the Atacama Desert differed from those habitats colonized by the type species *P. phormidioides* and by *Elainella*. *Pseudophormidium* sp. (HQ832909) falls into the clade containing *Nodosilinea* strains and is morphologically similar to them. Moreover, in the description of *Pseudophormidium* sp. (HQ832909) there is no mention of false branching, which is a typical feature of *Pseudophormidium*. Thus, it is probable that *Pseudophormidium* sp. (HQ832909) should be transferred to *Nodosilinea*, suggesting that the polyphyly within *Pseudophormidium* is more extensive than anticipated.

We were able to compare morphological features of these taxa based on data from Brown *et al.* (2010). We found that *Elainella* possessed pseudobranching and *Leptolyngbya* sp. JSC-1 exhibited two types of cells: short and narrow (they differ in length/width ratio; see Table 2 and Brown *et al.*, 2010 for details). Thus, together with a significant difference in ANI, 16S rRNA (Supplementary table S3), different genome size and composition (Supplementary fig. S1 and Supplementary table S2), and different autecology (Table 2 and Supplementary table S1), we can distinguish between these two strains. Since *Leptolyngbya* JSC-1 is unrelated to *Leptolyngbya sensu stricto*, as evidenced by the 16S rRNA phylogeny, it is unlikely to belong to the genus *Leptolyngbya* and its taxonomic status should be revised in the future. Taken together, based on the extensive evidence rising from presented data, we may safely assume that *Elainella* represents a new tropical monospecific genus of cyanobacteria.

Based on a new taxonomic classification system for cyanobacteria proposed by Komárek *et al.* (2014), *Elainella* would be classified in the order Synechococcales, family Leptolyngbyaceae based on 16S rRNA phylogeny and phylogenomic inference.

Leptolyngbya JSC-1 is the first cyanobacterium in which FaRLiP has been observed. FaRLiP provides an advantage in low light conditions in environments such as soils, mats and stromatolites (Gan *et al.*, 2014). *Elainella* does not possess such an advantage, because it does not contain FaRLiP, thus, it is tempting to hypothesize that it is better adapted to habitats with higher light intensity or less adapted to lower light intensities. This hypothesis could be tested experimentally in the future.

The genome of *Elainella* contains genes with possible nitrogen fixation function similar to other N-fixing cyanobacteria (reviewed in Bothe *et al.*, 2010) and it is likely that *Elainella* can fix atmospheric nitrogen. However, the activity of the nitrogenase enzyme should be evaluated to confirm its biological function.

Cyanobacteria produce a myriad of secondary metabolites that may have toxic properties, which can have a significant impact on both the environment and human health (reviewed in Leflaive & Ten-Hage, 2007). *In silico* analysis of the *Elainella* genome revealed putative biosynthesis pathways for 10 different secondary metabolites, two of which were assigned to puwainaphycins and cryptophycin. The rest are likely to be unknown groups of secondary metabolites. Some puwainaphycins have general cytotoxic activity (Hrouzek *et al.*, 2012), while cryptophycin exhibits high cytotoxicity against multi-drug resistant cancer cells (see cf. Weiss *et al.*, 2013). Although we have not confirmed biological activity of compounds produced by *Elainella*, we suggest that it is a potentially attractive organism to assess for bioactive compounds.

The phylogenomic tree topology, as well as dating analysis (Fig. 23) is in concordance with previous reconstructions (e.g. Komárek *et al.*, 2014; Shirrmeister *et al.*, 2015). *Elainella* seems to belong among lineages which diverged very early in the evolution of cyanobacteria before GOE. However, there is a weak bootstrap support at this split, thus this assumption has to be tested further.

Elainella represents a new evolutionary lineage among cyanobacteria and the first *Pseudophormidium*-like cyanobacterium with whole genome data. This also adds to our knowledge of tropical rock-dwelling microbial biodiversity, which is still rather limited. Furthermore, *Elainella* provides more evidence that phenotype convergence is a prevailing pattern in macroevolution in cyanobacteria. This fact complicates ongoing transitions from classical and molecular-based taxonomy and provides a cautionary warning to ecological or taxonomic studies that rely on phenotypic data alone.

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Disclosure statements

No potential conflict of interest was reported by the authors.

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Supplementary information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at https://doi.org/10.1080/09670262.2017.1362591

Supplementary table S1. Taxa list with GenBank accession numbers used in phylogenomic analysis.

Supplementary table S2. Genome properties of *Elainella saxicola* E1 and *Leptolyngbya* sp. JSC-1.

Supplementary table S3. 16S rRNA similarity of related taxa to *Elainella saxicola* E1.

Supplementary table S4. A list of the orthologues used for the phylogenomic reconstruction.

Supplementary fig. S1. A circular visualization of the comparison of *Elainella saxicola* genome with *Leptolyngbya* sp. JSC-1. The inner circle represents GC skew and the outer BLAST based similarity of protein coding regions.

Supplementary fig S2. Putative gene cluster and chemical structure of secondary metabolites.

Supplementary dataset S1.

Supplementary dataset S2.

Author contributions

E. Jahodářová identified the new cyanobacterium, performed PCR, Sanger sequencing, 16S rRNA phylogeny, prepared figures, and wrote the manuscript; P. Dvořák designed experiments, analysed genome data, performed phylogenomic dating and wrote the manuscript; P. Hašler performed morphological evaluation and took microphotographs; K. Holušová performed genome sequencing; A. Poulíčková coordinated work, took microphotographs and wrote the manuscript. All authors reviewed and edited the final manuscript. E. Jahodářová and P. Dvořák contributed equally to this manuscript.

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PAPER IV.

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Difference without distinction? Gaps in cyanobacterial systematics; when more is just too much

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Abstract: Cyanobacteria are amongst the most abundant, ubiquitous, ecologically and evolutionarily significant microbes on Earth. Unique among the Bacteria in their capacity to be identified using morphology, understanding the evolutionary relationships and describing the diversity of this lineage is both important and challenging. The advent of modern sequencing technology has proven a boon to those studying cyanobacterial systematics as it has provided copious amounts of sequence data (mainly of the 16S rRNA gene sequence). However, this influx of data has also led to taxonomic confusion and recognition of polyphyly in many genera. Thus, the purpose of this paper is to describe this apparent paradox of increasing data yet poor phylogenetic resolutions by employing the Poisson Tree Process (PTP) algorithm and to propose some ameliorative efforts.

Key words: convergence, cyanobacteria, DNA database, metagenomics, phylogeny, species concept, taxonomy

Introduction

The cyanobacteria represent a large and diverse phylum of photo–oxygenic bacteria. They exist in aquatic, terrestrial and subaerial environments, inhabiting a wide variety of environments spanning the poles to the equator, from freshwaters to marine habitats. Being so widely distributed, they provide significant inputs to both the global oxygen and nitrogen cycles (Whitton & Potts 2000). Although cyanobacteria are an evolutionary and ecologically important group of organisms, significant gaps exist regarding their taxonomy and systematics. These problems are exacerbated due to difficulty of culturing, low sampling efforts outside the temperate zone, and problematic species concepts (for reviews see Castenholz 1992; Komárek 2003; Johansen & Casamatta 2005; Komárek et al. 2014; Dvořák et al. 2015a, b).

As a whole, the cyanobacteria exhibit an extensive amount of phenotypic variability. Their classification was initially based on features such as morphology of filaments, cells, sheaths, types of branching, cell differentiation, reproduction, etc., while employing botanical nomenclature due to their similarity to eukaryotic algae (Komárek & Anagnostidis 1998, 2005; Komárek 2013). Unfortunately, some of these characters (e.g., sheaths) have been shown to be phenotypically plastic, and thus their use in phylogenetic reconstructions remains open to debate (e.g., Casamatta & Vis 2003). Over the last two decades, molecular markers, most notably the 16S rRNA gene sequence, have shown that some morphological features do not necessarily correspond to phylogenetic reconstructions elucidated by molecular methods. More recently, it has been suggested that many of the most common, traditional genera of cyanobacteria are in fact polyphyletic (reviewed in Komárek et al. 2014). For example, Robertson et al. (2001) found five polyphyletic lineages in the morphologically simple, coccoid genus *Synechococcus*. A more recent analysis by Dvořák et al. (2014a) found 12 lineages in this same genus and proposed that cyanobacteria undergo serially convergent events due to genome dynamics through horizontal gene transfer and homologous recombination. These and similar findings provide support for splitting polyphyletic genera into smaller, monophyletic lineages and subsequently describing new taxa. However, some cyanobacterial lineages are morphologically indistinguishable due to convergent evolution and their lack of identifiable character poor morphologies (Dvořák et al. 2014a; Komárek et al. 2014).

Since the majority of prokaryotes cannot be cultured using standard laboratory techniques (e.g. Amann et al. 1995), a complete description of their biodiversity

seems to be an impossible endeavor. Fortuitously, recent advances in metagenomics (genetic material recovered directly from uncultured organisms from environmental samples) have offered a direct approach to circumvent this limitation. However, this approach is used infrequently in taxonomy due to logistical constraints. The concept has recently been proposed under the International Code for Nomenclature of Prokaryotes (ICNP, http://icsp.org/) by expansion of the *Candidatus* species concept (Konstantinidis & Rosselló–Móra 2015). As a result, this approach allows for naming of taxa that cannot be grown and maintained in culture.

Conversely, the International Code for Algae, Fungi and Plants (ICN, http://www.iapt–taxon.org/nomen/main.php), which is the primary code employed with cyanobacterial taxa, makes provision for the description of new taxa based on type material stored as dried biomass. Thus, new approaches to elucidating cyanobacterial diversity such as the use of single filament PCR may be applied, although results employing this approach are still scarce in the literature. For example, Mareš et al. (2015) performed a revision of selected Stigonematales cyanobacteria and Hašler et al. (2014a) revised the culture–resistant genus *Komvophoron* utilizing this approach. In any case, it should be noted that the biodiversity of prokaryotes is so vast (with potentially many millions of species) that description of taxa using metagenomics tools would help to elucidate the uncultured (and undescribed) majority of prokaryotes (Konstantinidis & Rosselló–Móra 2015). Cyanobacterial systematic endeavors are further complicated by the fact that they have traditionally been named under the ICN, but may also be validly described under the ICNP.

Nabout et al. (2013) noted that the total number of described cyanobacterial species is 2698, as obtained from the continuously updated CyanoDB database (database version 2013, Komárek & Hauer 2011). The authors estimated that an expected number of species of cyanobacteria is 6280 (with a confidence interval of 4402 to 8159), the upper limit of which is close to an earlier estimation of 8000 species by Guiry (2012). It should be noted that these quantifications are mostly based on traditional (and often flawed due to convergent evolution) morphological approaches using a phenetic species concept under the ICN. The introduction of molecular markers for unraveling the systematics of cyanobacteria has allowed researchers to use species concepts derived from evolutionary principles, such as the phylogenetic or monophyletic species concepts, which are increasingly being employed by cyanobacterial taxonomists (reviewed in Johansen & Casamatta 2005).

Currently, there is no definitive indication of how many species of cyanobacteria may exist, how many have been described using phylogenetic based species concepts, or how many species may be anticipated among sequences gathered from metagenomics data without culturing. Thus, the purpose of this paper is to use the available sequences of cyanobacteria to elucidate some of the phylogenetic relationships. Further, we seek to quantify an overlap between described species and available DNA sequence–based species (e.g., taxa with names and 16S sequence data) and we will use these results to evaluate the reliability of database DNA sequence data for taxonomic and metagenomics purposes.

Material and Methods

We acquired a comprehensive dataset of 16S rRNA sequences of cyanobacteria from GenBank (database version 21st January 2015) using search query (((900:2500[Sequence Length]) AND cyanobacteria [organism]) AND 16S) to ensure we gathered sufficiently long sequences for analyses. Stored GenBank sequences were obtained mostly using Sanger dideoxy sequencing and 454 pyrosequencing. Multiple sequence alignment (MSA; Dataset S1 Supporting Information) was performed using MAFFT (Katoh et al. 2002) with automatic diagnostics of alignment parameters. Large gaps and uninformative regions were eliminated from the alignment using Gblocks (Castresana 2000) with the following settings: Maximum Number Of Contiguous Nonconserved Positions set to 50, Minimum Length Of A Block 2, and Allowed Gap Positions: half. Identical sequences were removed from alignment and their list has been stored for further analyses (Tab. S1 in the Supporting Information). Gblocks was employed in order to restrict sequence length and remove as much uninformative sequence as possible to shorten computing time. We chose our cutoff b.p. criteria for two reasons. First, many researchers only amplify a portion of the 16S rRNA gene and thus complete genes are not typically available. Second, we could not employ shorter reads (<899 b.p.) for MSA because these sequence fragments may be from the beginning or end of the 16S rRNA gene and might not overlap. The upper limit was set due to genome assemblies, which are very large and would make MSA impossible. The ITS region was not employed due to variability within and among strains, which would lead to erroneous MSA.

A phylogenetic reconstruction was performed using maximum likelihood criterion in RaxML 8.0.0 (Stamatakis 2014) under substitution model GTR+GAMMA (Dataset S2 in the Supporting Information). A phylogenetic reconstruction using neighbor joining optimality criterion was performed in MEGA 6 (Tamura et al. 2013) using Kimura 2–parameter model (Dataset S3 in the Supporting Information). Species were delimited in the Python programmed package PTP (Poisson Tree Process; Zhang et al., 2013), which uses the phylogenetic species concept *sensu* Eldredge & Cracraft (1980) and most recently modified by Nixon & Wheeler (1990). PTP uses substitution per site difference (not a genetic distance with a particular cutoff) for species identification. It assumes that there is a significantly (statistically speaking) higher evolutionary distance (measured in substitution per site) among species than within species. Since it uses substitution per site, it does not require an ultrametric tree. Thus, PTP also reflects different evolutionary rates within different species.

PTP produces a list of identified species or operational taxonomic units (OTUs). For consistency and simplicity, we will use the term "PTP–defined species" throughout the text. Our analysis employed units that include at least one cultured strain and PTP–defined species composed of sequences from uncultured cyanobacteria. We employed and assessed taxa with cultured strains if they were identified as an existing and validly

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described species under the ICN or the ICNP since species of cyanobacteria are considered under both codes. However, it should be noted that cyanobacterial species described under ICNP are valid under ICN, but ICNP does not accept species under ICN (Oren 2011). Also, species described under ICNP are much less frequent than under ICN. PTP outperforms other automatic delimitation algorithms such as GMYC (Fujisawa & Barraclough 2013; Pons et al. 2006), because it does not require an often error–prone ultrametric tree and it has significantly faster performance on large datasets (Zhang et al. 2013).

We used the following procedure to identify which sequences employed in the GenBank dataset corresponded to definitely named and reliably identified PTP–defined species (e.g., units consisting of both cultured and uncultured taxa which are definitively identified to species by a taxonomic expert; including species described based on single cell/filament PCR techniques). We also considered all identical sequences, which were subsequently assigned to PTP–defined species (Tab. S1 in the Supporting Information). First, all PTP–defined species with sequences deposited solely as sequences from "uncultured cyanobacteria" were removed from the list of definitively named and reliably identified species (excluding species described based on single cell/filament PCR techniques). Second, PTP–defined species containing only sequences without species epithets or with unsure species epithets were removed from the list of reliably identified species (GenBank accession numbers and available literature were checked to ensure missing epithets). Finally, we did an extensive GenBank and literature search to confirm whether a particular PTP–defined species contains any described species valid under either the ICN or the ICNP (including synonyms). Species with a *Candidatus* status under the ICPN were not employed in this analysis. If the PTP–defined species contained at least one definitively named and reliably identified species, we employed it in further analyses. The phylogeny reconstructed for this paper has been used as a template for taxonomic decisions. Multiple sequence alignment, template trees, and a list of identified cultured or uncultured species are available in Supporting Information.

Results and Discussion

How many "unculturable" cyanobacterial taxa actually exist?

The final alignment contained 10037 sequences with 4983 non–identical sequences. Employing the PTP species delimitation articulated earlier, we recovered 2741 PTP–defined species of cyanobacteria (Fig. 1, Table S2 in the Supporting Information). It has been suggested that only ca. 1% of prokaryotes may be cultured (Amann et al. 1995), but this figure is postulated mainly with heterotrophic bacteria in mind. This is in sharp contrast to our findings that only 51% of PTP–defined species were from uncultured environmental samples. However, this number may be biased since only sequences longer than 900 b.p. were used, while many metagenomics analyses employ far shorter sequences, often on the order of 300–500 b.p. Nevertheless, we excluded these shorter fragments since they would not be appropriate to utilize in our multiple sequence alignment.

This finding may imply either a high success rate of culturing the vast majority of cyanobacteria or that habitats with high uncultured cyanobacterial diversity are lacking in metagenomic data. For instance, fine, freshwater sediments (epipelon) are known for very low sampling efforts (Poulíčková et al. 2014), even though they are inhabited by a complex and diverse community of cyanobacteria and other algae (Mann et al. 2008; Poulíčková et al. 2008). Moreover, the sampling efforts of cyanobacteriologists are traditionally concentrated in temperate zones, although tropical latitudes may serve as hot spots of biodiversity (see Hohner–Divine et al. 2004 for review). Describing the taxonomy of tropical cyanobacteria is an active research endeavor, especially in aerophytic habitats (e.g. Fiore et al. 2007; Neustupa & Škaloud 2008). However, a dearth of tropical papers is evident when compared to temperate zones, as seen in the number of Web of Knowledge indexed papers (Dvořák et al. 2015a). Together with the rapid pace of new species descriptions in recent years (Komárek et al. 2014), we conclude that most of the cyanobacteria biodiversity remains undescribed and that a sizable portion of uncultured biodiversity may remain unnamed.

On the incompatibility of molecular and traditional cyanobacterial systematics

We found that only 571 PTP–defined species (20.9%) may be assigned to definitively named and reliably identified species under either the ICN or ICNP (Fig. 1, see Supporting Information for details). Furthermore, the 571 PTP–defined species represents 12.7–21.2% of the total described species under the ICN included in either AlgaeBase (Guiry & Guiry 2015; 4484) or the CyanoDB (information from Nabout et al. 2013; 2698 species), respectively. It should be noted that species under the ICN and ICNP are described using various species concepts, mostly phenetic with some form of a phylogenetic species concept, but a majority of recently published descriptions and revisions do not cite any particular species concept (for a review, see Johansen & Casamatta 2005). Species identified using the PTP are purely phylogenetic species as advocated by Nixon & Wheeler (1990). Therefore, the total numbers of species may differ based on the species concepts employed. Since modern molecular methods are able to provide greater taxonomic resolution due to a bounty of additional data (e.g. Johansen & Casamatta 2005; Erwin & Thacker 2008), we consider the discrepancy between the number of phylogenetic species and number of described species to reflect that the total diversity is underestimated.

The vast increase in described cyanobacterial diversity over the last 20 years is a direct result of molecular methods, which has in turn significantly influenced the taxonomic reasoning of cyanobacteriologists. Both 16S rRNA gene sequence data and metagenomic or community level assessments appear to have taken different directions. 16S rRNA sequences are deposited in DNA databases at near exponential levels yet are themselves flooded by ambiguously identified sequences resulting from previously, unequivocally identified sequences.

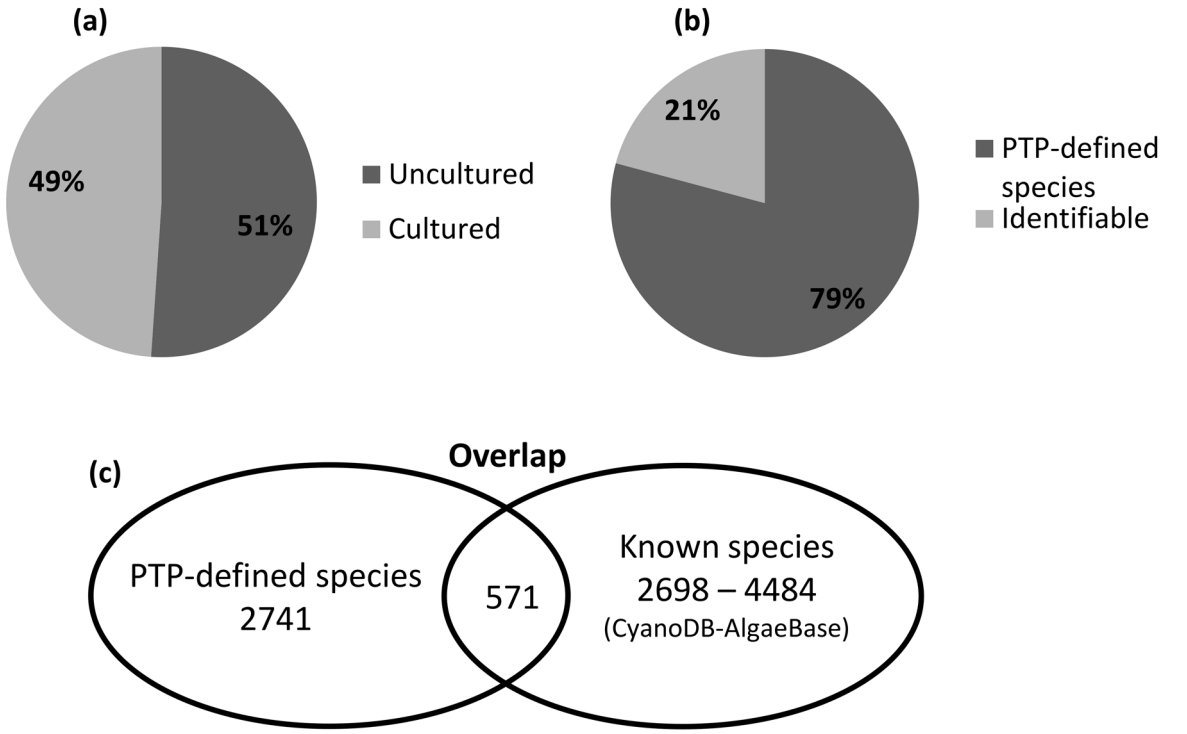

Fig. 1. Graphical representation of PTP analysis results. a) The ratio of uncultured and cultured PTP–defined species. b) The portion of PTP–defined species, which may be assigned to existing described species based on CyanoDB. c) A Venn diagram representing an overlap of PTP–defined species and species present in databases CyanoDB and AlgaeBase.

Thus, the difference between both systems is expanding daily due to erroneously identified sequences and species.

One possible reason for such a striking difference may rise from the entangled evolutionary relationships among cyanobacteria. As previously noted, a majority of described genera are currently considered polyphyletic (Komárek et al. 2014; Fig. S1 in the Supporting Information). The ultimate goal in reconstructing evolutionary relationships is that separate lineages should be classified and named based on rules of cladistics and phylogeny. Our results show that not much effort has been given to resolve this conundrum until now. The most striking example is the genus *Synechococcus*, which contains 12 polyphyletic lineages (Dvořák et al. 2014a), of which only one has been properly revised under the ICN (Dvořák et al. 2014b) and none under the ICNP. For example, the marine picoplanktic *Synechococcus* are one of the most abundant organisms on earth (Flombaum et al. 2013), but this lineage lacks proper taxonomic treatment. There are multiple species within this group, as previously recognized by Robertson et al. (2001) and our analysis in this paper. Thus, we may anticipate further disagreement of molecular data with traditional systems. Similarly, extensive evidence of polyphyly has been found in other taxa–rich genera such as *Leptolyngbya* (Osorio–Santos et al. 2014; Jahodářová et al. 2017), *Phormidium* (Hašler et al. 2012), or *Cylindrospermum* (Johansen et al. 2014).

Another source of taxonomic inconsistency may emerge from practical use of different taxonomic classifications by researchers. First, there is no consensus among researchers whether to use the ICN or ICNP. While the ICN accepts all names generated under the ICNP, the reciprocal is not so. Recently, however, Pinevich (2015) proposed changes to some principals of the ICNP, which would allow acceptance of valid species published under the ICN to also be valid under the ICNP. Second, the traditional ICN has many versions of the nomenclatural schemes, for example those proposed by Geitler (1932), Desikachary (1959), or Komárek et al. (2014). Unfortunately, there is typically no corresponding change to the appropriate cyanobacterial databases, leading to taxonomic confusion. Third, and perhaps most importantly, carefully articulated taxonomic revisions are undertaken and published slowly, while ambiguously identified sequences are still accumulating very rapidly in GenBank. Coupled with the enigmatic evolutionary histories of most cyanobacteria, this can lead to much confusion and uncertainty. One possible remedy would be to establish an approved repository for all cyanobacterial taxonomic and systematic endeavors, easily accessible to all interested researchers.

Another potential source of inconsistency may be variability in the ease of DNA recovery resulting in over–representation of some groups and under–representation of others. While some taxa with thin or lacking sheathes are relatively easy to amplify, other lineages, especially those with firm, copious sheaths (e.g., *Nostoc*, *Petalonema*, *Gloeocapsa*) might be recalcitrant to DNA analyses (Mareš et al. 2015).

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A capability of PTP to recognize cyanobacterial species

Automatic species delimitation approaches are often at odds with manually curated delimitations. This paper represents a novel approach to apply automatic delimitation of species to cyanobacteria, barring barcoding efforts which have met with limited success (Eckert et al. 2015). Thus, we endeavor to evaluate the performance of PTP on several well–defined and revised cyanobacterial genera.

For example, PTP recognized 6 of 7 PTP–defined species within *Oculatella* (except *O. mojaviensis*; Osorio–Santos et al. 2014), while *O. subterranea* was divided into three PTP–defined species. In the genus *Nodosilinea*, only one species (*Nodosilinea* sp. NB1a–A5) has not been recognized by PTP. On the other hand, new sequences have appeared in GenBank since Perkerson et al. (2001) established *Nodosilinea*; we found three PTP–defined species of *Nodosilinea* sp. CENA 183, CENA 144, and CENA 137. Less persuasive results were shown by analysis of *Cylindrospermum*, which belongs to the heterocystous cyanobacteria. Recently revised by Johansen et al. (2014), PTP recognized *C. badium*, *C. moravicum*, and *C. marchicum*. However, *C. catenatum*, *C. pellucidum*, *C. licheniforme*, and *C. muscicola* were collapsed into one PTP–defined species and the same appeared also in cases of *C. allatosporum* and *C. maius*. Thus, we may conclude that PTP species delimitation provides a hint for species identification and enumeration, but results should be cautiously interpreted because there are certainly gaps in this method. In any case, the purpose of this paper is to show how significant the gaps are between molecular and phenotype based species delimitations. It seems that PTP adequately serves this purpose.

Conclusions: limitations of available DNA sequence data in systematics of cyanobacteria.

Cyanobacteria are certainly not unique, but they are challenging in the employment of molecular data for identification and taxonomy. The ambiguity of cyanobacterial systematics creates a potential pitfall for metagenomic research and DNA barcoding. Based on our results, reliable species identifications based solely on sequence data from DNA databases may be unlikely. Moreover, recent work has indicated that different species within a genus cannot be differentiated solely by 16S rRNA sequences alone. Thus, additional data, such as provided by the ITS region *rbc*L gene sequences, are increasingly being employed (e.g. Osorio–Santos et al. 2014). While traditional employment of the 16S rRNA gene might be sufficient for generic level assignments, we see many misidentified strains due to the polyphyletic nature of many currently circumscribed genera (Komárek et al. 2014). Thus, it is likely that cyanobacteria with entirely different evolutionary histories are conflagrated, as evidenced with *Synechococcus*. On another front, practical identifications of cyanobacteria (for applied phycology, ecology, and genetics) are still obtained mainly using morphological observations which may be increasingly problematic as more polyphyletic lineages are elucidated and rarely revised. Further, there is a lack of morphological apomorphies for some newly described lineages (Komárek et al. 2014; Dvořák et al. 2015b). The potential use of cyanobacteria for environmental bioassessment and biomonitoring efforts is largely limited by available sequence data (for those employing genetic markers) and proper species descriptions using phenotypic data (e.g. morphology, Manoylov 2014). For example, *Phormidium retzii*, considered one of the most commonly encountered lotic taxa in North America, has been shown to be a collection of cryptic taxa, and thus, further taxonomic revisions are warranted (Casamatta et al. 2003).

Taken together, we have shown that genetic and taxonomic databases (e.g. AlgaeBase, CyanoDB, GenBank, etc.) may not clearly articulate the diversity of cyanobacteria. Many species are ambiguously identified or come from uncultured specimens with a concurrent low certainty of proper identification. To avoid further confusion, we propose the following recommendations: Curation of taxonomic revisions and descriptions should be more widely linked with DNA databases and authors should be more actively involved with the curation of databases. For example, authors should update their sequence identifiers even after publication. Furthermore, cyanobacterial taxonomists using the ICN have an opportunity to make descriptions of new taxa without cultures. If they took advantage of this opportunity, it would allow for faster and more effective taxonomic revisions and naming of new taxa from unculturable species (e.g., *Johanseninema*; Hašler et al. 2014a, b). Taxonomists using ICNP may use the *Candidatus* concept to provide a putative name to uncultured taxa. However, *Candidatus* names are not valid under ICN or ICNP. Therefore, two names for the same species may be proposed, one for each code and eventually both validated, leading to heterotypic synonyms.

Revisions of polyphyletic genera are essential for proper identification of cyanobacteria. Without this effort, we would not be able to recognize a majority of species within polyphyletic clusters (e.g., *Leptolyngbya*, *Phormidium*, and *Synechococcus*).

Species identifications based on metagenomic data should be assessed more carefully. Sequence data should be named only based on properly described or bar–coded species. DNA database accessions should contain a statement containing a level of confidence and elucidation of the method of identification.

All revisions should be performed only with robust taxon sampling with numerous, abundant, phylogenetically relevant outgroups and sister taxa. For example, if only "*Leptolyngbya*" sequences are employed in an analysis this might mask polyphyletic relationships as one might recover a single, monophyletic clade. This might be illustrated with *Leptolyngbya nodulosa* which was originally described and phylogenetically analyzed with rather depauperate sister and outgroup taxa (all that

was available at the time) before subsequently being transferred to a new genus. Revisions using only phenotypic data are discouraged, due to serial convergence of morphotypes (Dvořák et al. 2014a, 2015b). It should be noted that cyanobacteria have a relatively rich herbaria presence in museums, and thus an investigation of type material is often possible and recommended (Komárek et al. 2014; Palinska & Surosz 2014). If type species are unavailable, they may be retypified based on recent samples from type locality or close to type locality.

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Supplementary material

the following supplementary material is available for this article:

Table S1. A list of identical sequences removed from multiple sequence alignment.
Table S2. All cultured cyanobacterial species analyzed in this paper. XLS table with all cultivable cyanobacterial species used for this paper. Each line represents one PTP–defined species. Identifiable (green; see methods section for definition) and unidentifiable (red) species are labeled.
Dataset S1. Multiple sequence alignment of cyanobacteria. A PHYLIP formatted 16S rRNA
multiple sequence alignment of cyanobacteria with removed duplicated sequences.
Dataset S2. Phylogenetic tree of cyanobacterial 16S rRNA in a Newick format reconstructed in
RaxML based on Dataset S1.
Dataset S3. Phylogenetic tree of cyanobacterial 16S rRNA in a Nexus format reconstructed in
MEGA (neighbor joining optimality criterion) based on Dataset S1.
Fig. S1. Phylogenetic relationships among cyanobacteria based on 16S rRNA. Parallel lines represent clades, which are on in a real scale. They were shortened due to their excessive length. Each collapsed clades is annotated by one strain name from each genus in particular clade.

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

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SUMMARY OF DOCTORAL THESIS

CRYPTIC DIVERSITY OF CYANOBACTERIA

SUMMARY OF DOCTORAL THESIS

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The review on the doctoral thesis has been written by the Department of Botany, Faculty of Science, Palacký University. The summary of doctoral thesis was sent out on …….

The oral defence of the thesis takes place in front of the Commission for Ph.D. thesis for Study Subject Botany in the conference room at the Department of Botany, Faculty of Science, Palacký University, Olomouc-Holice, Šlechtitelů 27, on …….

The Ph.D. Thesis is available in the Library of Biological Department of Faculty of Science at Palacký University, Olomouc-Holice, Šlechtitelů 27.

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CONTENTS

1. GENERAL INTRODUCTION

Cyanobacteria (Cyanoprokaryota, Cyanophyceae, Blue-green algae) are an ancient group of autotrophic prokaryotes with the ability to produce oxygen by photosynthesis. They colonize a myriad of ecosystems (e.g. marine, freshwater, terrestrial, etc.), they occur in extreme biotopes such as deserts, polar regions or hot springs. The origin of cyanobacteria is dated to a period of Archean 2.7–3.5 (3.8) billion years ago (BYA) (Sleep 2010; Blank & Sanchez-Baracaldo 2010; Schopf 2001). The oldest cyanobacterial fossil record was found in Apex Cherts (Western Australia), from 3.3–3.5 BYA (Schopf & Packer 1987). As primary producers, cyanobacteria have enormous influence on the global ecosystem (marine cyanobacteria produce 25% of total oxygen) and they can also affect the evolution of other organisms (Flombaum et al. 2013).

The traditional phenotypic classification was based on a filament or cell morphology, cell dimensions, presence of sheath or envelope, type of cell division, color, and type of branching (e.g. Bornet & Flahuatl 1887; Gomont 1892; Geitler 1932; Komárek & Anagnostidis 1998; Komárek & Anagnostidis 2005; Komárek 2013). The late 20th century was a revolutionary time for cyanobacterial taxonomy. A revision of cyanobacterial system based on ecology, ultrastructural properties, molecular markers (especially 16S rRNA) and also morphological data led to establishment of a new framework for cyanobacterial classification called “polyphasic approach”. It has become a respected method of cyanobacterial taxonomy and determination widely used by phycologists (Johansen & Casamatta 2005; Siegesmund et al. 2008; Komárek 2010; Hašler et al. 2014b; Dvořák 2017; Dvořák et al. 2017a; Jahodářová et al. 2018 etc.).

However, reconstruction of evolutionary history does not necessarily correspond with morphological pattern, as demonstrated by phylogenetic reconstructions. This led to a proposal of cryptic taxa which are phenotypically indistinguishable. They could be only identified by molecular markers from one gene or whole genome (16S rRNA, ITS region etc.) (Dadheech et al. 2014; Komárek et al. 2014; Dvořák et al. 2015a; Jahodářová et al. 2018).

Furthermore, unrelated taxa share similar morphological characters and therefore create polyphyletic clusters caused by morphological convergence. A possible explanation comes from unclear evolutionary relationships of cyanobacteria (cryptic taxa, horizontal gene transfer, and homologous recombination). Dvořák et al. (2014) suggest that

horizontal (lateral) gene transfer (HGT) and homologous recombination (HR) are the essential evolutionary factors which frequently exchange genes within local gene pools (Polz et al. 2013). In fact, as an example of convergent evolution, new genera were derived from the genus *Leptolyngbya*: *Onodrimia* (Jahodářová et al. 2017), *Nodisilinea* (Perkerson et al. 2011), *Oculatella* (Zammit et al. 2012) and *Stenomitos* (Miscoe et al. 2016). Komárek et al. (2014) suggest that the majority of genera described using morphological criteria are polyphyletic.

Recent research has shown that 16S rRNA is not sufficient for recognition of different species among the species clusters within defined genus. Additional data should be used for detailed identification, for instance, ITS region or another gene sequence (Osario-Santos et al. 2014). On the other hand, in practical "daily" identification for applied phycology, ecology and genetics, morphological observations are still easiest to use for cyanobacterial determination. But with an increasing number of newly described taxa, moreover, without any morphological apomorphy, the situation will be more complicated

In the last decade, a new trend of sequencing and analyzing of whole bacterial genomes started to be more popular and more affordable. Some researchers argue that inconspicuous evolution events driven by environmental diversification are capturable only by whole genome sequencing approach (Kopac et al. 2014; Olsen et al. 2015). Bacterial DNA is organized mostly to a single circular chromosome, which can have many copies (polyploidy). There are three types of gene groups in the bacterial genome based on their distribution among prokaryotes (Koonin & Wolf 2008). First and the smallest group contains conservative set of housekeeping genes which are core part of a genome and thus present in all prokaryotes. The second group (shell genes) is moderately common. Finally, so called “cloud” genes are the least common. A number of protein-coding genes correlates with genome size in prokaryotes (0.8–1.2 gene by 1 kb). Bacterial genome size fluctuates mostly from 2 to 5 Mb (Koonin & Wolf 2008).

Horizontal gene transfer and homologous recombination are important forces in cyanobacterial speciation. Bacteria lack sexual reproduction, but HGT and HR are the way how to gain new genes with variable functions. HGT does not take place randomly in a genome, but is concentrated in heterogeneous genome regions, called genomic islands (Dobrindt et al. 2004). There are three ways how could bacteria incorporate a foreign fragment of DNA to its cell: natural transformation, conjugation, and transduction. But only transformation and conjugation have been observed in cyanobacteria. There are two ways how to identify HGT, using parametric and

phylogenetic methods. Parametric methods investigate G+C content variation (McLean et al. 1998), nucleotide composition, oligonucleotide frequencies (Lawrence & Ochman 1998) or structural characters of genomes (Worning et al. 2000). Explicit phylogenetic methods utilize conflict tree topology in phylogenetic reconstruction for detection of HGT. Implicit phylogenetic methods using sequence similarities and evolutionary distances (Haggerty et al. 2009; Ravenhall et al. 2015).

2. AIMS

The principal goal of this thesis was to investigate new filamentous cyanobacteria isolated from different habitats using polyphasic approach with focus on cryptic lineages.

2.1. SPECIFIC AIMS

- To identify and describe new lineages from families Leptolygbyaceae and Pseudanabaenaceae.
- To date evolution of a new genus *Elainella* using whole genome data.
- To evaluate a reliability of sequence data of cyanobacteria in GenBank database for taxonomy and metagenomics.

3. MATERIAL AND METHODS

3.1. TAXONOMICAL PART

Strain isolation

Cyanobacterial samples were collected from plankton and periphyton of a lake Hồ Đâu Co in Vietnam (strains E5, E10) and from plankton of ephemeral waterbodies in the forest of the National Park Cat Tien in Vietnam (strains E1, E8, E11), and from submersed bark of tree branches, near Tamanjayae, Ujung Kulon NP, West Java (strains E27, E28, E30). Cultures were maintained in 90 mm Petri dishes under the laboratory conditions as follows: temperature 26±1 °C, illumination 20 μmol photons m^{-2} s^{-1}, light regime 12h light:12h dark, and liquid Zehnder medium (Z medium) (Staub 1961).

Morphological assessment

Strain morphology was studied using light microscope Zeiss AsioImager (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 D512 12MPx). Cultures were used for studying and evaluation of morphology (see Dvořák et al. 2015a; Jahodářová et al. 2017; Jahodářová et al. 2018).

Nutrient and temperature experiment with Pinocchia culture

To find morphological apomorphy, for direct recognition of *Pinocchia* from *Pseudanabaena*, I used modified Z medium (Zehnder) without nitrogen, medium without phosphorus and medium without both of elements. I cultured strains in two different temperatures, at 16 °C and 26 °C. The difference was found among all cultures without nitrogen, phosphorus or both elements maintained at 26 °C.

PCR amplification and sequencing

Genomic DNA was extracted from ca 50 mg of fresh biomass using an UltraClean Microbial DNA Isolation Kit (MOBIO, Carlsbad, California, USA). DNA quality and consistence was checked on GelRed (Biotinum Inc., Hayward, California, USA) stained 1.5% agarose gel. DNA was quantified using the NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, Delaware, USA). A partial 16S rRNA sequence and the whole

16S–23S rRNA ITS sequence were obtained using PCR amplification. PCR products were cloned using StrataClone PCR Cloning kit (Agilent Technologies, Stratagene Product Division, La Jolla, California, USA).

Phylogenetic analyses

The most similar sequences of 16S rRNA were retrieved from the NCBI database and identified using nucleotide BLAST. Multiple sequence alignment was performed in MEGA 6 (Tamura et al. 2013) using Muscle algorithm (Edgar 2004) or in Mafft with E-INS-I algorithm (Katoh et al. 2002). The phylogenetic tree was rooted using *Gloeobacter violaceus* as the outgroup. The most appropriate model for Bayesian inference was determined by jModelTest 0.1.1 (Posada 2008) based on both the Bayesian and the Akaike Information Criterion. Bayesian inference majority consensus tree was constructed in MrBayes 3.2.3 (Ronquist & Huelsenbeck 2003). Maximum likelihood analysis was performed in RaxML 8.0.2 (Stamatakis 2006). Maximum parsimony analyses were performed in MEGA version 6.0. (Tamura et al. 2013) or in PAUP*4.0b10 (Swofford 2002).

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).

3.2. GENOMIC PART

In the case of *Elainella saxicolla* I used phylogenomics assessment.

De novo genome sequencing

Genomic DNA was extracted from wet biomass using UltraClean Microbial DNA Isolation Kit (MOBIO, Carlsbad, CA, USA). DNA quality and consistence was checked on GelRed (Biotinum Inc., Hayward, California, USA) strained 1.5% agarose gel. The quantification of DNA was performed by NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

The library for sequencing was prepared by TruSeq Nano DNA kit (Illumina Inc, San Diego, CA, USA) using 200 ng DNA according to manufacturer's instruction, but DNA was digested by Bioruptor Plus (Diagenode, Liege, Belgium) and size selection was modified to achieve insert size about 1,000 bp. The insert size of libraries was defined by Agilent High Sensitivity DNA Kit (Agilent Technologies, Inc.) and concentration of

library was assessed by KAPA Library Quantification Kit for Illumina (Kapa Biosystems, Woburn, MA, USA). MiSeq Reagent Kit v3 (Illumina Inc, San Diego, CA, USA).

A total of 2,880,274 pair ended reads with an average length of 226 bp were assembled *de novo* using the MIRA 4 assembler. We used a following procedure of contaminant contigs removal. We ran BLASTN with all contigs against complete bacterial genomes.

The de novo assembled genome resulted in 284 contigs (>500 bp) with an N50 73 085 bp, and a theoretical coverage of 38× based on the estimation of a length of 8.7 megabases.

Rapid Annotation using the Subsystems Technology (RAST) pipeline (Aziz et al. 2008) was used for annotating; tRNA was predicted using tRNAscan-SE 1.21 (Lowe & Eddy 1997). CRISPRs (clustered regularly interspaced short palindromic repeats) and CRISPR spacers were identified using CRISPRfinder (Grissa et al. 2007). Putative secondary metabolite gene clusters and molecule structures were predicted by antiSMASH 3.0 (Weber et al. 2015). Average nucleotide identity (ANI) was determined by Jspecies (Richter & Rosselló-Móro 2009). A visual representation of the BLAST searched genome similarities of *Elainella* with *Leptolyngbya* sp. JSC-1 was performed in BRIG (Alikhan et al. 2011).

A total of 129 available and annotated genomes of cyanobacteria were acquired from the ftp server of GenBank. Other genomes of cyanobacteria from GenBank were added to cover the broad evolutionary array of this group, representing most major niches/ habitats. Genomes of *Leptolyngbya boryana* PCC 6306, *Geitlerinema* sp. PCC 7105, *Spirulina subsalsa* PCC 9445 and *Nodosilinea nodulosa* PCC 7104 were re-annotated using RAST due to lack of annotation in the GenBank database.

The super alignment of 69 protein sequences for a subsequent phylogenomic reconstruction of a cyanobacterial species tree was obtained using phylogenomic Perl pipeline Hal (Robbertse et al. 2001) with options described in Dvořák et al. (2014). The phylogenomic reconstruction based on a resulting super alignment with a total of 15 141 amino acids was performed in RAxML 8.1.15 (Stamatakis 2006). The most suitable substitution matrix was identified in ProtTest 3.3 (Abascal et al. 2005). *Gloeobacter violaceus* and *Gloeobacter* sp. were used as outgroup taxa. The dating of the phylogenomic reconstruction has been performed using calibration points and settings of penalized likelihood (Sanderson 2002), as previously used by Dvořák et al. (2014) in r8s (Sanderson 2012).

3.3. GAPS IN CYANOBACTERIAL TAXONOMY

I obtained a comprehensive dataset of 16S rRNA sequences of cyanobacteria from GenBank using search query to make sure I congregate sufficiently long sequences for analyses. Multiple sequence alignment was created using MAFFT (Katoh et al. 2002). Identical sequences were removed from alignment. I also could not employ shorter reads (<899 bp) for MSA because these fragments might not overlap. The ITS regions were included.

A phylogenetic reconstruction was performed using maximum likelihood criterion in RaxML 8.0.0. (Stamatakis 2014). A phylogenetic reconstruction using neighbor joining optimality criterion was performed in MEGA 6 (Tamura et al. 2013). Species were delimited in the Python program package PTP (Poisson Tree Process) (Zhang et al. 2013).

A list of phylogenetically identified species (created by PTP) so called operational taxonomic units (OTUs). Analyses exploit and evaluate taxa with cultured strains if they were identified as an existing and validly described species under ICN or the ICNP.

I used the mentioned procedure to identify which sequences employed in the GenBank dataset corresponded to definitely named and reliably identified PTP-defined species. I also considered all identical sequences, which were subsequently assigned to PTP-defined species. All sequences of “uncultured cyanobacteria” and sequences without epithet or with unsure species epithet were removed from the list of definitively named and reliably identified species. I also did an extensive search in GenBank and literature to confirm whether a particular PTP-defined species contains described species valid under either the ICN or the ICNP. The PTP-defined species containing at least one definitively named and reliably species were included in the procedure, whereas the species with *Candidatus* status were excluded from this analysis.

4. PUBLICATIONS INCLUDED IN THE THESIS

4.1. BOOK CHAPTER

DIVERSITY OF THE CYANOBACTERIA

Petr Dvořák, Dale A. Casamatta, Petr Hašler, Eva Jahodářová, Alyson R. Norwich, Aloisie Poulíčková

Abstract

The cyanobacteria are an ancient lineage of photo-oxygenic bacteria. Globally responsible for much of the primary productivity and nitrogen fixation, they are also evolutionarily significant as the photosynthetic members of serial endosymbiotic events leading to the establishment of chloroplasts. Traditionally classified based on morphological characters, recent research revealed an abundance of cryptic diversity evidenced by molecular analyses, most notably the 16S rDNA gene sequence. Explorations of seldom sampled habitats, such as tropics environments, aerophytic habitats, soil crusts, etc., have also revealed a tremendous new diversity of taxa. This increase in the alpha-level diversity, coupled with new molecular techniques, has greatly altered our perceptions of the evolutionary relationships within this clade. Many of the traditional genera have proven to be polyphyletic, but revisions are underway.

Keywords: Cyanobacteria • Phylogeny • Taxonomy • Biodiversity • Morphology

4.2. INDIVIDUAL PAPERS

PAPER I.

A NEW TROPICAL CYANOBACTERIUM *PINOCCHIA POLYMORPHA* GEN. ET SP. NOV. DERIVED FROM THE GENUS *PSEUDANABAENA*

Petr Dvořák, Eva Jahodářová, Petr Hašler, Evgeniy Gusev, Aloisie Poulíčková

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.*

Keywords: 16S rRNA • 16S–23S ITS • cryptogenus • new species

PAPER II.

REVEALING HIDDEN DIVERSITY AMONG TROPICAL CYANOBACTERIA: THE NEW GENUS *ONODRIMIA* (SYNECHOCOCCALES, CYANOBACTERIA) DESCRIBED USING THE POLYPHASIC APPROACH

Eva Jahodářová, Petr Dvořák, Petr Hašler, Aloisie Poulíčková

Abstract

Leptolyngbya represents a group of common mat forming cyanobacteria with very simple trichome morphology and a polyphyletic evolutionary origin. In this paper, we used a polyphasic approach to describe a new genus morphologically similar to *Leptolyngbya*. Three strains of Leptolyngbyaceae cyanobacteria were isolated from submersed bark of tree branches which fell into the Hot-water spring from a rainforest in West Java. A phylogeny of the 16S rRNA gene indicated that these strains fell into a well-supported clade separate from *Leptolyngbya sensu stricto*. Although our strains possessed only minor morphological differences from other similar Leptolyngbyaceae species, these new taxa may be differentiated based on a peculiar form of reproduction, where hormogonia and hormocytes form tree-like tuft structures. Thus, based on a phylogenetic position, morphological, and ecological evidence, we propose a new genus, *Onodrimia*.

Keywords: *Leptolyngbya* • new genus • phylogeny • tropical cyanobacteria • 16S rRNA • 16S–23S ITS • Algae

PAPER III.

ELAINELLA GEN. NOV.: A NEW TROPICAL CYANOBACTERIUM CHARACTERIZED USING A COMPLEX GENOMIC APPROACH

Eva Jahodářová, Petr Dvořák, Petr Hašler, Kateřina Holušová, Aloisie Poulíčková

Abstract

Cyanobacteria represent an ancient, monophyletic lineage of bacteria with the ability to undertake oxygenic photosynthesis. Although they possess a relatively high degree of morphological variability compared with other prokaryotes and there is a wealth of molecular data, there are still significant gaps in our knowledge of cyanobacterial diversity, especially in tropical areas. Here, we present a novel, filamentous, tropical cyanobacterium, which could be classified as *Pseudophormidium* based on morphological criteria. A total evidence investigation employing ecological, morphological and genomic data, indicated that our strains form a new and ancient evolutionary lineage among cyanobacteria unrelated to *Pseudophormidium*. Based on this polyphasic assessment, our strains represent a novel, monospecific genus: *Elainella*. This new genus represents an example of phenotypic convergence, which seems to be a prevalent macroevolutionary pattern in cyanobacteria, a likely cause of the frequently cited polyphyly within a majority of genera.

Keyword: 16S rRNA • genome sequencing • new genus • phylogenomics • *Pseudophormidium* • tropical cyanobacteria

PAPER IV.

DIFFERENCE WITHOUT DISTINCTION? GAPS IN CYANOBACTERIAL SYSTEMATICS; WHEN MORE IS JUST TOO MUCH

Petr Dvořák, Eva Jahodářová, Dale A. Casamatta, Petr Hašler, Aloisie Poulíčková

Abstract

Cyanobacteria are amongst the most abundant, ubiquitous, ecologically and evolutionarily significant microbes on Earth. Unique among the Bacteria in their capacity to be identified using morphology, understanding the evolutionary relationships and describing the diversity of this lineage is both important and challenging. The advent of modern sequencing technology has proven a boon to those studying cyanobacterial systematics as it has provided copious amounts of sequence data (mainly of the 16S rRNA gene sequence). However, this influx of data has also led to taxonomic confusion and recognition of polyphyly in many genera. Thus, the purpose of this paper is to describe this apparent paradox of increasing data yet poor phylogenetic resolutions by employing the Poisson Tree Process (PTP) algorithm and to propose some ameliorative efforts.

Keywords: convergence • cyanobacteria • DNA database • metagenomics • phylogeny • species concept • taxonomy

5. CONCLUSIONS

5.1. BOOK CHAPTER

5.1.1. Diversity of the Cyanobacteria

This chapter is focused on evolutionary history, speciation and taxonomy of cyanobacteria. The introduction describes the origin of cyanobacteria, their impact on the global ecosystem, other organisms or humans. It contains information about morphology, different views on cyanobacterial classification and cyanobacterial phylogeny. Subsequent part is focused on a cyanobacterial speciation and on problems of modern taxonomy as well. The largest part of the chapter discusses the species taxonomy and it is divided according to the latest cyanobacterial system proposed by Komárek et al. (2014) (Synechoccoccales, Chroococcales, Chroococcidiopsidales, Pleurocapsales, Oscillatoriales, Spirulinales, Nostocales). Each section describes the most important members of the individual order. The whole chapter is enriched by phylogenetic and phylogenomic trees, drawings, and photographs.

5.2. INDIVIDUAL PAPERS

5.2.1. Polyphasic characterization of *Pinocchia* gen. and sp. nov.

Benthic, planktic and mataphytic representatives of the genus *Pseudanabaena* occur in the sea, freshwater and also terrestrial habitats (Komárek & Anagnostidis 2005). This genus was found to be composed of polyphyletic or cryptic lineages (Komárek et al. 2014). Paper I is focused on a polyphasic characterization of new *Pseudanabaena*-like cyanobacterium named *Pinocchia polymorpha*. *Pinocchia* was isolated from plankton and periphyton of the lake Hồ Đâu Co, province Đồng Nai, Vietnam. Morphology of the trichomes was an important factor which was considered. *Pinocchia* was morphologically very similar to *Pseudanabaena*, particularly to *Ilyonema galeata* or *P. catenata* (species with polar gas vesicles and specific shape of a terminal cell are ranked to subgenus *Ilyonema* in Komárek & Anagnostidis 2005). *Pinocchia* differs from *I. galeata* by high variability of cell length (this is characteristic for *P. catenata*). Furthermore, *Pinocchia* possess prolongated, pointed and sometimes conical apical cell which is unusual in *I. galeata* and *P. catenata*. Phylogenetic analysis of 16S rRNA revealed the real position of

Pinocchia as a significantly supported monophyletic clade. Furthermore, *Pseudanabaena sensu stricto* was very distant from *Pinocchia*. I found high variability of ITS region in particular strains even in clones. I discover three types of D1-D1´ helix and B-boxes. It might suggest potential existence of two cryptic species in *Pinocchia* genus. I applied nutrient (different concentration of nitrogen and phosphorus) and temperature (cultivation in 16 °C and 26 °C) experiment to find morphological apomorphy. It has been shown many times that nitrogen, phosphorus or it rations affect the intensity of cell division (Poulíčková et al. 2001; Hašler et al. 2003; Hašler & Poulíčková 2010). Obviously, a higher temperature (26 °C) was more convenient for *Pinocchia* growing, probably due to the isolation from the tropical lake. Taken together, *Pinocchia* represents cryptic lineage derivate from polyphyletic *Pseudanabaena*. Its monophyly confirms the tree topology, secondary structures of ITS region and also tropic origin.

5.2.2. Polyphasic characterization of *Onodrimia* gen. and sp. nov.

Polyphyletic genus *Leptolyngbya* contains more than 100 species (Komárek & Anagnostidis 2005; Perkerson et al. 2011; Osario-Santos et al. 2014). All *Leptolyngbya*-like species have thin filaments and simple morphology (Komárek & Anagnostidis 2005). Paper II describes new taxon *Onodrimia javanensis* derived from *Leplogynbya*. Strains were isolated from the submersing bark of tree branches which fell into hot water spring in the rainforest, near Tamanjayae, Ujung Kulon NP, West Java. *Onodrimia* possesses morphological autapomorphy and differs from other Leptolyngbyaceae genera. The peculiar form of reproduction represents hormocytes and hormogonia stuck by sheath on mother trichome and create tree-like tuft structures. Moreover, the phylogeny of 16S rRNA gene confirmed the strong polyphyletic origin of *Leptolyngbya* (*Leptolyngbya* created 10 separate lineages, more than previously reported). *Onodrimia* is clearly separated from other Leptolyngbyace genera and encompass clade with significantly support. Moreover, *Onodrimia* shares 96.2% sequence similarity with Uncultured bacterium TG-102 (JQ769612) and 94% sequence similarity with Uncultured bacterium TG-104 (JQ769614). Above that, *Leptolyngbya sensu stricto* creates a clade on the base of the tree and is not closely related to *Onodrimia*, which clustered with *Phormidesmis*, *Stenomitos*, *Pantanalinema*, and *Neosynechococcus*. On the other hand, all these taxa are morphologically distant from *Onodrimia*. *Leptolyngbya corticola* is the only species characterized by similar ecology (tree bark) but was found in temperature forest (Johansen et al. 2011). The D1-D1´ helix and B-box were identical among all studied

strains, as all *Onodrimia* strains have no variability in ITS structure. I compared ITS secondary structures of *Onodrimia* to other Leptolyngbyace members. *L. appalachiana* keeps extremely long D1-D1′ helix as *Onodrimia*. *Onodrimia javanensis* possess only minor morphological difference from other taxa in Leptolyngbyaceae family, but it could be distinguished by morphological autapomorphy (tree-like hormogonial tufts), phylogenetic position in the tree, secondary structures, low sequence similarity and unique ecology.

5.2.3. The complex genomic approach in a description of *Elainella*

This paper aimed at a description of new genus and species *Elainella saxicola*. *Elainella* is filamentous cyanobacterium, with simple morphology. Strains were collected from plankton of ephemeral waterbody in the forest of the National Park Cat Tien, province Đồng Nai, Vietnam. In Paper III, I used polyphasic approach for the new taxa description. Polyphyly can also be observed in *Pseudophormidum* (Taton et al. 2006; Alwathnani & Johansen 2011; Osario-Santos et al. 2014) which is morphologically very similar to *Elainella*. All selected taxa from *Plectonema* and *Leptolyngbya* have overlapping descriptions with *Elainella* (especially Gardner′s taxa from Puerto Rico, later classify to unclear species of *Pseudophormidium* and *Leptolyngbya*) (Komárek & Anagnostidis 2005; Anagnostidis & Komárek 1988). *Pseudophormidium* and *Plectonema* overlap each other in some morphological characters (for instance thallus formation, branching of filaments and formation of hormogonia) (Komárek & Anagnostidis 2005). Their only distinguishing feature is the width of the trichome. *Plectonema* possesses wide trichomes (8–25, up to 72 μm), on the other hand, *Pseudophormidium* creates narrowed trichomes (less than 10 μm). Bayesian phylogeny of 16S rRNA gene exposed monophyletic origin of *Elainella saxicola* despite of other filamentous lineages as *Pseudanabaena*, *Trichocoleus*, *Nodosilinea*, *Oculatella*, *Symploca*, and *Spirulina*. The most phylogenetically related cyanobacterium to *E. saxicola* is *Leptolyngbya* sp. D1C10 (KJ654308). *Leptolyngbya* sp. D1C10 is probably a species of *Elainella*. Unfortunately, additional information about this strain cannot be acquired. Therefore, I was not able to revise these taxa. I could not add the type species of *Pseudophormidium* (*P. phormidioides*) and also Gartner′s taxa to the phylogeny, because it has not been sequenced yet. *Elainella* was found to form a highly supported clade distant from *Leptolyngbya sensu stricto*, *Pseudophormidium*, and *Plectonema* in 16S rRNA phylogeny.

The total length of the *Elainella* draft genome was 8 702 141 bp, G+C content was 47.6%. RAST annotation detects a total of 8472 coding sequences and 102 RNAs. Based on known proteins with biological function, 51.3% of coding sequences were annotated; 48% of them were identified as potential proteins. Genome of *Elainella* contains genes for fixation of atmospheric nitrogen (*nif*B, *nif*S, *nif*U, *nif*H, *nif*D, *nif*K). The gene composition is similar as in other non-heterocytous, nitrogen-fixing cyanobacteria, for instance, *Cyanothece* sp. PCC 7425 (Bothe et al. 2010). However, the real activity of the nitrogenase should be experimentally tested. Moreover, the genome contains 24 confirmed CRISPRs with 223 CRISPR spacers. Cyanobacteria are able to produce an inexhaustible quantity of secondary metabolites, very often in high concentrations. Some of these substances have a negative impact on human health (cyanotoxins), they can also affect the function of an ecosystem (Leflaive & Ten-Hage 2007). Ten potential biosynthetic gene clusters were detected *in silico* analysis using AntiSMASH, but only two of them are assigned to already known gene: puwainaphycins and cryptophycin. Both puwainaphycin and cryptophycin have exhibit cytotoxic activity (Hrouzek et al. 2012; Weiss et al. 2013). Overleaf, the genome of *Elainella* lacks crucial genes for Far-Red Light Photoacclimation or abbreviated FaRLip (e.g. ApcA2, ApcB2) (Gan et al. 2014). These genes were found in phylogenetically closes cyanobacterium *Leptolyngbya* sp. JSC-1. *Leptoloyngbya* sp. JSC-1 is the first discovered cyanobacterium with this mechanism. FaRLip ensures an advantage in low light conditions in an environment such as mast, soil or stromatolites (Gan et al. 2014). Hypothetically, *Elainella* should be better adapted to the higher light intensity. I attempted to reveal the origin of *Elainella* using dated 69-gene phylogeny to put it in the context of all cyanobacteria. *Elainella* is sister to *Leptolyngbya* sp. JSC-1, these taxa are likely to diverge 2.24–2.47 BYA, before the Great Oxygenation Event (Kopp et al. 2005). In this phylogenomic analysis, they created a clade with 100% support, but in 16S rRNA phylogeny their cluster scored only low node support. I was able to compare morphological characters of both taxa using data from Brown et al. (2010). *Leptolyngbya* sp. JSC-1 creates two types of cells (short and narrow) and *Elainella* possess pseudobranching. Moreover, *Leptolyngbya* sp. JSC-1 is unrelated to *Leptolyngbya sensu stricto* in 16S rRNA phylogeny, its taxonomic status should be revised in the future. Based on Komárek et al. (2014) *Elainella* would be classified to order *Synechococcales*, family Leptolyngbyaceae (pursuant on the phylogeny of 16S rRNA gene). Significant differences in ANI, 16S rRNA, different genome sizes and

different autecology (*Leptolyngbya* sp. JSC-1 was found in hot spring in Great Yellowstone area) give us enough evidence to distinguish these two taxa.
Elainella represents evolutionary new lineages among cyanobacteria and the first *Pseudophormidium*-like cyanobacterium with whole genome data.

5.2.4. Difference without distinction? Gaps in cyanobacterial systematics

The most widely used marker in cyanobacterial phylogeny is 16S rRNA gene. But it is generally known that some morphological features do not necessarily correspond with molecular phylogenetic reconstructions. Nowadays, the majority of cyanobacterial genera are polyphyletic (Komárek et al. 2014). The mentioned problem makes cyanobacterial systematic very unclear. It is obvious to ask questions: how many cyanobacterial species do exist, how many species may be expected among sequences collected from metagenomics data without culturing or how many taxa have been described using phylogenetic species complex (Dvořák et al. 2018). In Paper IV I tried to find answers to these questions and elucidate the real situation as well as I tried to quantify an overlap between described species and available 16S rRNA sequences from the GenBank database.
The final alignment of the 16S rRNA gene comprised 10037 sequences (but only 4983 sequences were unique). From this amount, PTP delimited only 2741 PTP-defined cyanobacterial species. It is interesting, that 51% of PTP-defined species were assigned to uncultured environmental cultures. Though, scientists estimated that only about 1% of prokaryotes are maintained in cultures. These extreme differences between number of sequences in database and number of delimit species are probably caused by the length of sequences used in the analysis, only sequences longer than 899 bp were used (metagenomics analysis exploit sequences under 300–500 bp). I excluded shorter fragments than 900 bp from the alignment. There are two ways to explain this situation, the cultivation of most cyanobacteria is very unsuccessful or that habitats with a high amount of uncultured cyanobacteria are lacking in metagenomics data. Cyanobacterial and algal members of planktic habitats are prominently studied, on the other hand, freshwater sediments are neglected (Hašler et al. 2008, Poulíčková et al. 2014). Despite that fact Mann et al. (2008) and Poulíčková et al. (2014) thought that epipelon communities are very complex and diverse. For example, Hašler & Poulíčková (2010) described a new species of epipelic *Komvophoron* or Hašler et al. (2014a) determinated new genus *Johanseninema* from epipelon. Moreover, there are multiple areas that are

unexplored, especially tropical areas or polar regions, in sharp contrast with temperate zones where the vast majority of phycologists do their research (Dvořák et al. 2017b).

I discover that only 571 PTP-defined species (20.9%) may be assigned to a certain name and reliably identified species under ICN or ICNP. The number of PTP-defined species represent only 12.7–21.2% of all describes species under ICN in either CyanoDB or AlgeaBase databases (Guiry & Guiry 2015, 4484 species; Nabout et al. 2013, 2698 species). PTP-defined species are delimited only phylogenetically (Nixon & Wheeler 1990). New taxa under ICN and ICNP are described using different species concept. Hence, the total number of species may be varied depending on the applied species concept.

In conclusion, I have shown that taxonomic and genetic databases may not to reflect accurately the real diversity of cyanobacteria. There are several ideas on how to improve this situation: 1. Phycologists should do the revisions of polyphyletic taxa, newly described genera should always be characterized by autapomorphy. 2. Metagenomic data should be assessed more carefully. 3. Revisions should be undertaken only with robust and proper taxon sampling.

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7. LIST OF AUTHOR'S PUBLICATIONS

BOOK CHAPTER

DVOŘÁK P, CASAMATTA DA, HAŠLER P, **JAHODÁŘOVÁ E**, NORWICH AR, POULÍČKOVÁ A (2017) Diversity of the cyanobacteria. In: Modern Topics in the Phototrophic Prokaryotes: Environmental and Applied Aspects. (Ed. by Hallenbeck PC), Springer International Publishing, Switzerland. pp 3–46.

PAPER I.

DVOŘÁK P, **JAHODÁŘOVÁ E**, HAŠLER P, POULÍČKOVÁ A (2015) A new tropical cyanobacterium *Pinocchia polymorpha* gen. et sp. nov. derivated from the genus *Pseudanabaena*. Fottea 15: 113–120.

PAPER II.

JAHODÁŘOVÁ E, DVOŘÁK P, HAŠLER P, POULÍČKOVÁ A (2017) Revealing hidden diversity among tropical cyanobacteria: the new genus *Onodrimia* (Synechococcales, Cyanobacteria) described using the polyphasic approach. Phytotaxa 326: 25–40.

PAPER III.

JAHODÁŘOVÁ E, DVOŘÁK P, HAŠLER P, HOLUŠOVÁ K, POULÍČKOVÁ A (2018) *Elainella* gen. nov.: a new tropical cyanobacterium characterized using a complex genomic approach. European Journal of Phycology 53: 39–51.

PAPER IV.

DVOŘÁK P, **JAHODÁŘOVÁ E**, CASAMATTA D, HAŠLER P, POULÍČKOVÁ A (2018) Difference without destinction? Gaps in cyanobacterial taxonomy: where more is just too much. Fottea 18: 130–136.

PAPER V.

HAŠLER P, PENTECOST A, **JAHODÁŘOVÁ E**, DVOŘÁK P, POULÍČKOVÁ A (2018) Taxonomic revision of *Ulva montana* (Lighfoot 1777) and description of a new genus of *Lightfootiella* (Cyanophyceae, Chroococcaceae). Phytotaxa 362: 173–186.

PAPER VI.

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8. PRESENTATIONS AT MEETINGS

I. JAHODÁŘOVÁ E, DVOŘÁK P, HAŠLER P, POULÍČKOVÁ A (2017) Metamorphosis in cyanobacteria: how to change a genus in several days. 11th International Phycological Congress, Szczecin, Poland (poster).

II. JAHODÁŘOVÁ E, DVOŘÁK P, HAŠLER P, CASAMATTA DA, GUSSEV E, POULÍČKOVÁ A (2016) Confusion by cryptic genera: when the same is not the same. Young Systematists' Forum, London, United Kingdom (poster).

III. JAHODÁŘOVÁ E, DVOŘÁK P, HAŠLER P, CASAMATTA DA, HOLUŠOVÁ K, GUSEV E, POULÍČKOVÁ A (2016) When the same is not the same: unveiling a hidden cyanobacterial diversity using polyphasic approach. 57th Meeting of the Czech Phycological Society, Prague, Czech Republic (poster).

9. PARTICIPATION ON PROJECTS

I. Czech Science Foundation 17-13254S (co-worker; 2017–2018, Generating the species-towards a better understanding of speciation mechanisms in eukaryotic microorganisms).

II. Internal grant agency of Palacký University PrF-2015-001, PrF-2016-001, PrF-2017-001 and PrF-2018-001.

10. SOUHRN (SUMMARY, IN CZECH)

Sinice (Cyanobacteria) se na Zemi poprvé objevily asi před 3,5 miliardami let. Patří mezi hlavní organismy ovlivňující světové biogeochemické cykly a jsou prapředky chloroplastů dnešních vyšších rostlin. Sinice jsou schopny přežít v nejrůznějších prostředích. Díky jejich obrovské rozmanitosti zůstává velká většina taxonů systematicky nezařazena. V této práci jsem studovala morfologické i molekulární znaky tří nových rodů sinic z tropických oblastí (*Pinocchia*, *Onodrimia* a *Elainella*), také jsem se zaměřila na mezery v systematice sinic. Spolupracovala jsem na knižní kapitole pojednávající o diverzitě cyanobakterií.

Recentní studie odhalily, že rody jako je *Leptolyngbya* nebo *Pseudanabaena* jsou výrazně polyfyletické. Oba druhově bohaté rody se značí jednoduchou morfologií, vytvářejí tenká vlákna a obývají jak vodní, tak i terestrické habitaty. Nicméně, na základě polyfyletického původu byla *Leptolyngbya* rozdělena do několika nových linií, jako je *Oculatella* nebo *Nodosilinea*. *Pseudanabaena* na svou revizi stále čeká. Podobně jsem i já popsala dva neznámé rody odvozené z rodů *Leptolyngbya* a *Pseudanabaena*. *Pinocchia* a *Onodrimia* jsou jednoduché vláknité cyanobakterie tropického původu. Fylogeneze založená na genu 16S rRNA jednoznačně odlišila monofyletické linie rodu *Pinocchia* a *Onodrimia* od *Pseudanabaena* a *Leptolyngbya sensu stricto*. Snažila jsem se najít morfologickou apomorfii, která by striktně definovala nově popsané rody. V případě rodu *Pinocchia* se jedná pravděpodobně o kryptický rod, avšak některé z trichomů měly výrazně prodlouženou, špičatou, někdy kuželovitou apikální buňku. Pro ověření, zda se skutečně jedná o apomorfii, jsem použila nutriční experiment. Nebyl však nalezen signifikantní rozdíl, který by odlišoval kontrolní a experimentální kulturu. Na druhou stranu, *Onodrimia* je charakteristická zvláštním typem reprodukce (produkuje hormogonia v takzvaných stromečkovitých formacích). Tento charakteristický znak lez považovat za apomorfii.

Další nově popsaná tropická sinice *Elainella* je velice podobná sinici rodu *Pseudophormidium*. *Pseudophormidium* je málo prostudovaný taxon s nejasnou morfologií. Pro definici tohoto nového rodu jsem použila jak fylogenezi na základě 16S rRNA genu a morfologické informace, tak i celo-genomový přístup. Celková délka genomu byla 8 702 141 párů bází. V genomu rodu *Elainella* jsem identifikovala geny sloužící k fixaci vzdušného dusíku a deset potencionálně biosyntetických genových klastrů, pouze dva z nich zřejmě produkující cytotoxické metabolity. Z datované

fylogeneze na základě 69 ortologních genů jsme se pokusila odhalit vznik rodu *Elainella* a kmene *Leptolyngbya* sp. JSC-1. Tento klád se pravděpodobně diversifikoval asi před 2,24–2,47 miliardami let.

Gen kódující malou podjednotku ribozomu (16S rRNA gen) je hojně užívaný marker pro fylogenezi bakterií. V databázích můžeme najít tisíce sekvencí tohoto genu. Na druhou stranu, je obecně známo, že morfologická charakteristika neodpovídá molekulární fylogenezi. Proto jsem chtěla tento zjevný paradox, neustále se zvětšujícího množství dat a nesprávného fylogenetického určení, prostudovat pomocí algoritmu PTP (Poisson Tree Process). Alignovala jsem 10 037 sekvencí genu 16S rRNA a pomocí PTP algoritmu jsem delimitovala počet druhů sinic v databázi GenBank. PTP identifikovalo pouze 2741 PTP-definovaných druhů, avšak 51 % PTP-druhů bylo nekultivovatelných. Také jsem se zaměřila na faktory, které by mohly tuto nelehkou situaci taxonomie sinic zlepšit (např. nově popsané taxony by měly být vždy charakterizovány autapomorfií, metagenomická data by měla být vyhodnocována pečlivěji, revize by měly být prováděny pouze s robustním a správným výběrem taxonů).