

School of Doctoral Studies in Biological Sciences

University of South Bohemia in České Budějovice

Faculty of Science

**Polyphasic approach to the taxonomy of selected
cyanobacteria**

Ph.D. Thesis

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ANNOTATION

The modern taxonomic revision of cyanobacteria is a work in progress, with both theoretical and practical challenges to be addressed. This thesis investigated selected terrestrial cyanobacterial taxa using a polyphasic approach, based on molecular phylogenetic analysis and accompanied by phenotypic characterization and nomenclatural treatment. Diverse methodological approaches were used including optical and transmission electron microscopy, molecular analysis (DNA sequencing) of cyanobacterial strains, single cells and filaments, and phylogenetic analysis of multiple genomic loci. This study provided systematic revisions of individual cyanobacterial genera and the entire phylum, and suggestions for future study projects.

DECLARATION [IN CZECH]

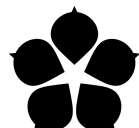
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Jan Mareš

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LIST OF PAPERS AND AUTHOR'S CONTRIBUTIONS

- I. **Mareš J.**, Hrouzek P., Kaňa R., Ventura S., Strunecký O. & Komárek J. (2013). The primitive thylakoid-less cyanobacterium *Gloeobacter* is a common rock-dwelling organism. *PLoS ONE* 8: e66323. (IF=3.53)
Jan Mareš performed isolation and cultivation of strains, analyses of morphology and ultrastructure, molecular analyses, participated in phylogenetic analyses, and was responsible for preparation of the manuscript.
- II. **Mareš J.**, Komárek J., Compère P. & Oren A. (2013). Validation of the generic name *Gloeobacter* Rippka et al. 1974, Cyanophyceae. *Cryptogamie Algologie* 34: 255-262. (IF=0.67)
Jan Mareš conducted the taxonomic investigation and participated in preparation of the manuscript.
- III. **Mareš J.**, Komárek J., Compère P. & Oren A. (2013). (2194) Proposal to conserve the name *Gloeobacter violaceus* against *Aphanothece caldariorum*, *Gloeothece coerulea*, and *Gloeothece linearis* (Cyanophyceae). *Taxon* 62:1055. (IF=3.05)
Jan Mareš conducted the taxonomic and nomenclatural investigation and participated in preparation of the manuscript.
- IV. **Mareš J.**, Hauer T., Komárek J. & Compère P. (2013). (2195) Proposal to conserve the name *Gloeothece* (Cyanophyceae) with a conserved type. *Taxon* 62:1056. (IF=3.05)
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Jan Mareš organized the study, participated in experimental design, isolation of the material, molecular and phylogenetic analyses, and prepared the manuscript for publication.
- VI. Komárek J., Kaštovský J., **Mareš J.** & Johansen J. R. (2014). Taxonomic classification of cyanoprokaryotes (cyanobacterial genera) 2014, using a polyphasic approach. *Preslia* 86:295-335. (IF=2.78)
Jan Mareš performed phylogenetic analyses, created all figures, and participated in writing the manuscript.

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CHAPTER I. GENERAL INTRODUCTION

Cyanobacteria represent the most abundant and most important phylum of photosynthetic prokaryotes on Earth. They inhabit almost all imaginable aquatic and terrestrial habitats, in which they frequently perform crucial ecological services (Whitton 2012). The significance of cyanobacteria in the global biosphere largely follows from their extremely long evolutionary history (Cavalier-Smith 2006). Their continuous presence and biological activity on Earth has probably lasted since approximately 3 billion years BP. In the course of this long period, they have been involved in the origins of the oxygen-rich atmosphere during the „Great Oxidation Event“ (Planavsky et al. 2014, Schopf 2014, Schirrmeister 2013), and in formation of a great portion of biogenous calcium carbonates (stromatolites, travertines) (Altermann et al. 2006, Riding 2000). The extensive evolutionary radiation and ecological success of cyanobacteria has been driven by an impressive set of metabolic skills. These include photosynthesis *via* chlorophyll *a* with both photosystems typical for plants (Bjorn and Govindjee 2009), fixation of the atmospheric nitrogen (Thompson and Zehr 2013, Flores and Herrero 2010, Berman-Frank et al. 2003), and production of a tremendous variety of secondary metabolites (Dittmann et al. 2013, Leflaive and Ten-Hage 2007). Cyanobacteria as free-living microorganisms hold substantial importance in a wide spectrum of aquatic communities such as ocean picoplankton (Flombaum et al. 2013), plankton of lakes and reservoirs (Komárek and Mareš 2012, Komárek 2003, Lyra et al. 2001), and benthos or periphyton of shallow waters and marshlands (Vargas and Novelo 2007, Rejmánková et al. 2004). The planktonic types are frequently capable of forming excessive accumulations of biomass in eutrophic water bodies (O’Neil et al. 2012, Yamamoto et al. 2009, Jozwiak et al. 2008), sometimes containing high levels of powerful toxins (Pearson et al. 2010, Leflaive and Ten-Hage 2007). The occurrence of harmful cyanobacterial blooms has brought serious problems for water management and health protection in heavily populated regions (Koreiviene et al. 2014, Svircev et al. 2014, Funari and Testai 2008). On the other hand, cyanobacteria have been demonstrated to form essential components of sensitive microbial communities such as soil crusts and sub-aerial microbial biofilms (Garcia-Pichel et al. 2013, Gorbushina 2007). Furthermore, they have been found to thrive in extreme environments including hot springs (Kaštovský et al. 2014, Dadheech et al. 2013, Pepe-Ranney et al. 2012), salt marshes (Currin et al. 2011, Řeháková et al. 2009), polar regions (Komárek et al. 2012, Strunecký et al. 2010, Casamatta et al. 2005, Taton et al. 2003) and a variety of deserts (de los Rios et al. 2014, Patzelt et al. 2014, Garcia-Pichel and Wojciechowski 2009). In the role of chloroplast-forming

intracellular endosymbionts, cyanobacteria initiated the evolution of all plants and photosynthetic algae (de Alda et al. 2014, McFadden 2014, Blank 2013). They have further taken part in consortia with a number of plants, lichenized fungi and oceanic reef invertebrates (Usher et al. 2007).

Given the ubiquitous distribution and tremendous importance of cyanobacteria in the biosphere, creation of a convenient cyanobacterial taxonomic system has been desirable. In the course of 19th and early 20th century, cyanobacteria were considered to be an integral part of the existing Linnean botanical system as a peculiar group of simple algae. Indeed, their considerable morphological diversity and photosynthetic mode of life has primarily drawn the attention of phycologists rather than classical microbiologists. Following several pioneering works (Gomont 1892, Bornet and Flahault 1888, Thuret 1875), a comprehensive taxonomic system was conceived by Geitler in the first half of 20th century (Geitler 1925, 1932, 1942). In his elaborate monograph (Geitler 1932), Geitler provided a clear morphological definition of all contemporary cyanobacterial taxa (Cyanophyceae) based on cell division mode, presence of filaments and their structure, branching patterns, presence of mucilaginous formations, etc. This system, with minor modifications (Elenkin 1936-49, Starmach 1966, and others), has then served phycologists and hydrobiologists for several decades. Although being a „mere taxonomic handbook“, it has helped (if not entirely enabled) establishing cyanobacterial research as a modern discipline.

Despite their alga-like nature, cyanobacteria have long been recognized as prokaryotes. In the second half of 20th century, introduction of modern techniques of study such as electron microscopy, biochemical and molecular analyses raised broader interest in investigation of their bacterial properties (Wilmotte et al. 1992, Rippka et al. 1979, Bonen and Doolittle 1978, Waterbury and Stanier 1977). Concurrently, efforts have been made to improve the existing system to better reflect the (actual or supposed) morphological plasticity, ecological distribution and evolutionary relationships within cyanobacteria. Still within the boundaries of botanical taxonomy, Francis Drouet and several other authors suggested a revision of the system in order to contain a significantly reduced number of widely defined taxa (Drouet 1968, 1973, 1978, 1981, Bourelly 1970). Although this view was later proven to be rather inadequate, Drouet's thorough revisionary work helped in establishing and validating many existing species (Drouet and Daily 1956). Meanwhile, a growing group of interested bacteriologists have started investigating cyanobacteria using pure laboratory strains (Rippka et al. 1979). The cultivation-based approach enabled a radical breakthrough in understanding the cyanobacterial cell structure and phylogenetic relationships. Recognizing the major

discrepancies of their findings with the traditional botanical system, bacteriologists decided to create a provisional system of cyanobacterial „form-genera“, „families“, and „sections“ (Castenholz 2001). While being very useful for laboratory work with clonal cyanobacterial strains, this system has no standing in either botanical or bacteriological nomenclature. Moreover, its application in cultivation-independent floristic surveys, ecological studies, and water quality monitoring is quite problematic.

Since approximately the 1980s, another way of understanding the cyanobacterial diversity has arisen, attempting to unify the traditional knowledge with the results of modern studies. The „Modern approach“ series by Komárek and Anagnostidis (Anagnostidis and Komárek 1990, Komárek and Anagnostidis 1989, Anagnostidis and Komárek 1988, Komárek and Anagnostidis 1986, Anagnostidis and Komárek 1985) have pledged for an as-natural-as-possible taxonomy of cyanobacteria, adjusted from the old botanical system, however newly based on a combination of all useful morphological, ultrastructural, biochemical, ecological and molecular data. Such an approach, including molecular phylogenies available to date, was implemented in the new comprehensive „Süßwasserflora von Mitteleuropa“ book series (Komárek and Anagnostidis 1999, 2005; Komárek 2013). This monumental monograph series has, after many decades, been the first to effectively replace the system proposed by Geitler (1932). After years of modern taxonomic study inspired by the ideas of Komárek and Anagnostidis, and publication of several important works (Komárek and Kaštovský 2003, Hoffmann et al. 2005), the results eventually led to re-defining of the concept of cyanobacterial species and genera. Johansen and Casamatta (2005) proposed to adopt a phylogenetic species concept (Mishler and Theriot 2000, Wheeler and Platnick 2000) adjusted for cyanobacteria as asexual organisms, in which the species are „the smallest monophyletic groups worthy of taxonomic recognition“. In this view, genera are simply defined as monophyletic clusters of species. Based on these modern concepts, together with the requirement for utilization of all available data, a so-called polyphasic approach achieved a dominant position in recent cyanobacterial taxonomy (Komárek 2010). In the framework of this taxonomic concept, a system of monophyletic taxa at all levels, including species, genera, families and orders, is inferred from an evolutionary reconstruction (phylogenetic tree) based on molecular sequence data. Ideally, all these taxa are then supported using stable apomorphies (morphology, thylakoid arrangement, biochemical and physiological traits) that allow reliable identification of the species in both natural samples and culture isolates. The polyphasic approach to cyanobacterial study has experienced a true boom in the last decade, resulting in the successful revision of numerous traditional taxa and description of many new

ones, mostly on the level of genera and species (from the latest studies, e.g. Dadheech et al. 2014, Johansen et al. 2014, Kaštovský et al. 2014, Mühlsteinová et al. 2014 a, b, Rodarte et al. 2014, Strunecký et al. 2014, Lien et al. 2013, Zapomělová et al. 2012). Nevertheless, the data collected throughout the years have also demonstrated serious problems and shortcomings in the polyphasic approach, which need to be addressed by the community of cyanobacterial taxonomists.

Despite the considerable amount of work accomplished in recent years, a large proportion of traditional genera and species still await modern revision. Furthermore, the state of higher-level cyanobacterial taxonomy is rather unsatisfying in the long term. The progress in reconstruction of the phylum-wide evolutionary relationships has been hindered for several independent reasons. First, although the situation is slowly improving, the research has been still biased in favour of aquatic taxa important for water management, typically water-bloom forming and toxic cyanobacteria (Engene et al. 2013, Zapomělová et al. 2011, Moustaka-Gouni et al. 2010, Laamanen et al. 2001). Second, until recently, a major part of taxonomic studies have been restricted to the temperate climatic region, mostly Europe and North America. Consequently, inappropriate European determination literature has been applied in tropical countries and other less studied areas. Recent reviews (Hauer et al. 2015, Nabout et al. 2013) clearly demonstrated this disproportion in cyanobacterial diversity investigation. In the last few years, it has become evident that relatively understudied terrestrial habitats host a tremendous diversity of little known cyanobacterial morphotypes and genotypes (Ferreira et al. 2013, Hrouzek et al. 2013, Mühlsteinová et al. 2013, Mühlsteinová and Hauer 2013, Komárek et al. 2013, Branco et al. 2009). One can claim, that almost every taxonomist's sampling trip to a tropical forest or wetland, polar region, desert of any kind, or another peculiar habitat has resulted in the description of new species and genera. Ongoing efforts are needed in terrestrial and non-temperate biotopes before we can reasonably estimate cyanobacterial diversity, and construct a taxonomic system that would cover most of the currently undocumented diversity.

Such efforts are partly stymied by the failure to isolate cyanobacteria into pure laboratory cultures. Analysis of cultured cyanobacteria, especially DNA sequencing in strains with clearly defined morphologies, is an essential part of a typical polyphasic study. However, many of the most interesting morphotypes, often terrestrial species with complicated thallus structure and prominent mucilaginous envelopes (e.g. *Stigonema*, *Petalonema*, *Gloeocapsa*, *Entophysalis*, *Porphyrosiphon*, *Schizothrix*), resist all attempts at isolation and growth under artificial conditions. This may be due to special growth requirements as well as inevitable

contaminant microorganisms, frequently attached to the gelatinous sheaths. Common techniques of culture-independent DNA analysis, such as the denaturing gradient gel electrophoresis (Boutte et al. 2006) or high-throughput sequencing of 16S rRNA amplicons (Kleinteich et al. 2014, Lindemann et al. 2013) in environmental samples have brought only limited insight into the diversity of the cultivation-resistant cyanobacteria. These approaches largely rely on DNA extraction yields, which may be problematic in species with massive mucilaginous envelopes (Billi et al. 1998). Even more importantly, there is no way of linking the obtained sequence data directly to the morphotypes observed in the microscope. One of the methods, less frequently used in cyanobacteria but promising for taxonomic work with uncultured material, is sequencing of single colonies, filaments, and cells. In several genera such as *Microcystis*, *Prochlorococcus*, *Woronichinia*, *Microcoleus*, and *Lyngbya*, minuscule amounts of material directly inspected in optical microscope were successfully sequenced (Kashtan et al. 2014, Lara et al. 2013, Engene et al. 2010, 2011, Janse et al. 2004, Boyer et al. 2002). Application of such an approach to a wider variety of taxa, including morphologically derived terrestrial types, would have a potential to substantially improve the standing cyanobacterial taxonomy.

Another set of problems originates in the type of molecular data used in cyanobacterial systematics, and taxonomic concepts, which are based on interpretation of these data. The genomic locus most frequently analysed in cyanobacteria, as well as in other groups of prokaryotes, is the small subunit ribosomal RNA (16S rRNA) gene. In bacterial taxonomy, 97% and 95% thresholds of 16S rRNA gene sequence similarity between strains, as an approximation of DNA-DNA hybridization capability (Stackebrandt and Goebel 1994), are sometimes used for definition of separate species and genera, although the „gold standard“ threshold has been disputed even by the author of the original study (Stackebrandt and Ebers 2006, Gevers et al. 2005). On the contrary, in the cyanobacterial polyphasic approach, this criterion has been mostly rejected or used only as a supplementary piece of information as it does not correspond with the observed diversity. Especially in heterocytous cyanobacteria (Nostocales) the 16S rRNA similarity between genera frequently exceeds 95% (Řeháková et al. 2014 b). Moreover, DNA similarity is a continuous quantitative variable, in principle allowing only phenetic classification, which is in direct contradiction to the accepted phylogenetic species concept (Johansen and Casamatta 2005). In spite of that, the ribosomal RNA operon has remained essential for the definition of cyanobacterial species and genera. According to the most recent view (Osorio-Santos et al. 2014, Řeháková et al. 2014 a, Johansen et al. 2014), the 16S rRNA gene together with the adjacent internal transcribed

spacer (ITS) region, provide sufficient information for reliable separation of cyanobacterial phylogenetic species (worthy of recognition using phenotypic characters). The analyses recommended by these authors include both phylogenetic reconstructions and comparisons of the predicted rRNA secondary structure. Osorio-Santos et al. (2014) suggested in their in-detail study of *Oculatella* that also morphologically cryptic species may be diagnosed and described. Therein, clusters of related cryptic species are considered to be evolutionary lineages (monophyletic clades), which can be clearly distinguished based on the predicted ITS secondary structures with help of p-distances of ITS sequences, however exhibiting only minor, and sometimes overlapping phenotypic and/or ecological differences. The usefulness of such an approach has to be tested in future studies. Cryptic species can also be found in more distant phylogenetic lineages with convergent or plesiomorphic characters, especially in cyanobacteria with simple morphologies (Dvořák et al. 2014). It is important to note that the 16S rRNA gene alone (without ITS) frequently fails to reflect the species diversity, at least as currently understood. In a recent study by Řeháková et al. (2014 b), a tree of *Nodularia* based on the 16S rRNA and RuBisCO large subunit (*rbcLX*) genes contained morphospecies and ecotypes intermixed in a completely erratic way. In contrast to species, genera are usually well recovered in 16S rRNA trees, perhaps with exception of those that only recently diverged, such as *Nodularia/Anabaenopsis/Cyanospira* (Řeháková et al. 2014 b).

Although the 16S+ITS rRNA is a powerful tool for phylogenetic reconstruction in cyanobacteria, one has to keep in mind that it has little to do with the phenotypic traits. Many of the genes influencing the commonly used morphological characters (branching, mucilage, presence and position of heterocytes and akinetes) and ecophysiological adaptations are likely located in genomic islands (Coleman et al. 2006). The genomic islands, in part acquired by horizontal gene transfer, are areas of the genome that differ also among closely related strains, and are subject to increased rate of recombination. The rapid evolution in these genomic regions is partly driven by environmental factors (niche differentiation), and it is only vaguely related to the neutral evolution of conserved housekeeping loci. Thus the cyanobacterial morphology is not always congruent with the phylogenetic position in rRNA-based trees, and the taxonomic value of certain phenotypic traits may vary in different lineages. A recent example was given by Johansen et al. 2014 who reported two morphologically divergent genera *Cylindrospermum* and *Cronbergia* to form an inseparable monophyletic cluster. Whereas the ribosomal RNA operon has proven to be useful for the definition of cyanobacterial species and genera, it mostly fails to support higher phylogenetic clusters in large data sets (Schirrmeyer et al. 2011). Furthermore, a substantial portion of genes, rarely

including also rRNA genes and other conserved loci, are horizontally transferred among (cyano)bacteria, creating complex evolutionary networks (Calteau et al. 2014, Dagan et al. 2013, Green 2005). For these reasons, phylogenetic studies based on multi-locus sequence typing or genomic data mining are necessary for unravelling the relationships among cyanobacterial genera, families and orders. Several such studies have already been published (Seo and Yokota 2003), the most recent one using all available whole-genome sequencing data (Shih et al. 2013). Unfortunately, these results have not been interpreted with sufficient taxonomic insight and genuine interest to help establishing a robust updated system of cyanobacteria. For example, Shih et al. (2013) have stuck to the dated nomenclature-independent bacteriological system of sections and form-genera (Castenholz 2001). A polyphasic analysis of higher cyanobacterial taxa based on robust multi-locus phylogenies is clearly missing.

As demonstrated in the previous text, the current cyanobacterial taxonomy is facing the challenge of integrating the extensive data on cyanobacterial „botanical“ biodiversity with the requirements of modern microbiology, and the ever-growing database of molecular sequences. In all groups of organisms, including cyanobacteria, a viable taxonomic hypothesis has to be reflected in a respective system of valid taxonomic names, a nomenclature code. Unfortunately, the taxonomic dualism in cyanobacterial research has also given rise to nomenclatural struggle between the two available systems – the International Code of Nomenclature for Algae, Fungi and Plants (ICN, Mc Neill et al. 2012) and the Bacteriological Code (Lapage et al. 1992). Formally, cyanobacteria have been part of algae and have been described as such under the provisions of the botanical code, which has remained a common practice until now. Logically, the prokaryote nature of cyanobacteria, and the progress in their cultivation and modern study have caused some researchers to advocate their inclusion under the Bacteriological Code. Repeated proposals and efforts were made to enable the formal description of cyanobacterial genera and species under these rules (Oren 2011, 2004, Stanier et al. 1978). Moreover, a rule was incorporated in the ICN, which ensures the validity of cyanobacterial species described under the Bacteriological Code also in the botanical system. Surprisingly, the community of bacteriologists has been extremely reluctant to accept the proposals for designation of cyanobacterial taxa (although otherwise fulfilling the respective requirements), and only extremely few such descriptions were achieved. As a result, one of the leading advocates of inclusion of cyanobacteria in the Bacteriological Code has recently suggested they should be further handled exclusively under the provisions of the ICN (Oren and Garrity 2014). Other taxonomists have proposed creating a special system suitable for

cyanobacterial taxonomy (Komárek 2011) or anticipated unification of all nomenclature codes under a universal „Biocode“ (Palinska and Surosz 2014). For now, the only usable and practical code for the taxonomy of cyanobacteria is the ICN. If a system of cyanobacteria consisting of valid taxa is to be achieved using the ICN, it must be based on clearly defined type species (generitypes). In order to define viable higher taxa, the generitypes need to be included in multilocus (or genome-wide) phylogenies and characterized by the methods of polyphasic approach. Creation of such a robust phylogenetic backbone composed of clearly defined and valid taxa is an essential prerequisite for a comprehensive taxonomic revision of cyanobacteria, which is missing at the moment.

In this thesis, I aimed to perform a careful polyphasic analysis of several chosen terrestrial cyanobacterial genera whose taxonomy has long remained questionable. Such kind of taxonomic revision frequently requires nomenclatural amendment to achieve valid taxa. Thus, the disentanglement and validation of the chosen problematic cyanobacterial genera presented another challenge for my work. The next aim was to test and exploit molecular methods less common in cyanobacterial taxonomy, such as sequencing of single cells in cultivation-resistant cyanobacteria, and construction of multilocus phylogenies. The last goal of the thesis was to summarize the existing knowledge collected in polyphasic studies worldwide, contributing to the presentation of the most current state of cyanobacterial classification.

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CHAPTER II presents an exemplary polyphasic study resolving the taxonomic identity of an intriguing epilithic terrestrial cyanobacterial genus, *Gloeobacter*, which forms the basal lineage in all cyanobacteria with great importance in evolutionary and experimental research.

PAPER I

The primitive thylakoid-less cyanobacterium *Gloeobacter* is a common rock-dwelling organism

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PloS ONE (2013) 8:e66323

Abstract

Cyanobacteria are an ancient group of photosynthetic prokaryotes, which are significant in biogeochemical cycles. The most primitive among living cyanobacteria, *Gloeobacter violaceus*, shows a unique ancestral cell organization with a complete absence of inner membranes (thylakoids) and an uncommon structure of the photosynthetic apparatus. Numerous phylogenetic papers proved its basal position among all of the organisms and organelles capable of plant-like photosynthesis (i.e., cyanobacteria, chloroplasts of algae and plants). Hence, *G. violaceus* has become one of the key species in evolutionary study of photosynthetic life. It also numbers among the most widely used organisms in experimental photosynthesis research. Except for a few related culture isolates, there has been little data on the actual biology of *Gloeobacter*, being relegated to an “evolutionary curiosity” with an enigmatic identity. Here we show that members of the genus *Gloeobacter* probably are common rock-dwelling cyanobacteria. On the basis of morphological, ultrastructural, pigment, and phylogenetic comparisons of available *Gloeobacter* strains, as well as on the basis of three new independent isolates and historical type specimen, we have produced

strong evidence as to the close relationship of *Gloeobacter* to a long known rock-dwelling cyanobacterial morphospecies *Aphanothece caldariorum*. Our results bring new clues to solving the 40 year old puzzle of the true biological identity of *Gloeobacter violaceus*, a model organism with a high value in several biological disciplines. A probable broader distribution of *Gloeobacter* in common wet-rock habitats worldwide is suggested by our data, and its ecological meaning is discussed taking into consideration the background of cyanobacterial evolution. We provide observations of previously unknown genetic variability and phenotypic plasticity, which we expect to be utilized by experimental and evolutionary researchers worldwide.

Následující pasáž o rozsahu 11 stran obsahuje skutečnosti chráněné autorskými právy a je obsažena pouze v archivovaném originálu dizertační práce uloženém na Přírodovědecké fakultě Jihočeské univerzity v Českých Budějovicích. Publikace vyšla tiskem v časopise PloS ONE.

CHAPTER III carries out the validation of the generic name *Gloeobacter* under the current rules of botanical nomenclature and proposes for conservation of the generic names *Gloeobacter* and *Gleothece*, which have suffered from serious confusion.

PAPER II

Validation of the generic name *Gloeobacter* Rippka et al. 1974, Cyanophyceae

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Cryptogamie Algologie (2013) 34:255–262

Abstract

The genus name *Gloeobacter* with the single (= type) species *Gloeobacter violaceus* (Cyanophyta, Cyanoprokaryota, Cyanobacteria) was described by Rippka, Waterbury et Cohen-Bazire (Arch. Microbiol. 100: 419-436, 1974). However, this is not a validly published name and so it currently has no standing under the botanical International Code of Nomenclature (ICN, Mc Neil et al. 2012) or the International Code of Nomenclature of Prokaryotes (ICNB/ICNP, Lapage et al. 1992). The lack of valid publication of the genus name causes many problems in the taxonomy of this phylogenetically and experimentally important cyanophyte/cyanobacterium. The lack of thylakoids, a feature unique among all known cyanobacteria, as well as the phylogenetic position of the representative of this genus, warrant valid publication of this generic name. The type strain was deposited in the collection PCC in Paris under the number PCC 7421 and later introduced into numerous other strain collections; however, the dried specimens were not yet conserved. The type strain is cited as holotype in Castenholz (Bergey's Manual, 2001). We here propose validation of the names *Gloeobacter* Rippka et al. 1974, gen. nov (type: *Gloeobacter violaceus*) and *Gloeobacter violaceus* Rippka et al. 1974, sp. nov., utilising the description by Rippka et al. reproduced here, and supported by the exsiccate BRNM No. HY 2366 (under the rules of the botanical

Code of Nomenclature - ICN), using exsiccate from the type strain 7421 (PCC 7421(T) = ATCC 29082(T)).

Keywords

Cyanophyceae, Cyanobacteria, *Gloeobacter*, taxonomy, validation

*Následující pasáž o rozsahu 8 stran obsahuje skutečnosti chráněné autorskými právy a je obsažena pouze v archivovaném originálu dizertační práce uloženém na Přírodovědecké fakultě Jihočeské univerzity v Českých Budějovicích. Publikace vyšla tiskem v časopise *Cryptogamie Algologie*.*

PAPER III

(2194) Proposal to conserve the name *Gloeobacter violaceus* against *Aphanothece caldariorum*, *Gloeothece coerulea*, and *Gloeothece linearis* (Cyanophyceae)

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PAPER IV

(2194) Proposal to conserve the name *Gloeobacter violaceus* against *Aphanothece caldariorum*, *Gloeothece coerulea*, and *Gloeothece linearis* (Cyanophyceae)

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Taxon (2013) 62:1056

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CHAPTER IV introduces a methodology for single-cell and single-filament sequencing of cultivation-resistant terrestrial cyanobacteria, providing new insights into the phylogeny of two taxonomically interesting heterocytous genera, *Stigonema* and *Petalonema*.

PAPER V.

Phylogenetic analysis of cultivation-resistant terrestrial cyanobacteria with massive sheaths (*Stigonema* spp. and *Petalonema alatum*, Nostocales, Cyanobacteria) using single-cell and filament sequencing of environmental samples.

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Abstract

Molecular assessment of a large portion of traditional cyanobacterial taxa has been hindered by the failure to isolate and grow them in culture. In this study, we developed an optimized protocol for single cell/filament isolation and 16S rRNA gene sequencing of terrestrial cyanobacteria with large mucilaginous sheaths, and applied it to determine the phylogenetic position of typical members of the genera *Petalonema* and *Stigonema*. A methodology based on a glass-capillary isolation technique and a semi-nested PCR protocol enabled reliable sequencing of the 16S rRNA gene from all samples analyzed. Ten samples covering seven species of *Stigonema* from Europe, North and Central America, and Hawaii, and the type species of *Petalonema* from Slovakia were sequenced. Contrary to some previous studies, which proposed a relationship with heteropolar nostocalean cyanobacteria, *Petalonema* appeared to belong to the family Scytonemataceae. Analysis of *Stigonema* specimens recovered a unique coherent phylogenetic cluster, substantially broadening our knowledge of

the molecular diversity within this genus. Neither the uni- to biseriate species nor the multiseriate species formed monophyletic subclusters within the genus. Typical multiseriate species of *Stigonema* clustered in a phylogenetic branch derived from uni- to biseriate *S. ocellatum* Thuret ex Bornet & Flahault in our analysis, suggesting that species with more complex thalli may have evolved from the more simple ones. We propose the technique tested in this study as a promising tool for a future revision of the molecular taxonomy in cyanobacteria.

Keywords

16S rRNA gene, cyanobacteria, morphology, *Petalonema*, phylogeny, single cell, single filament, *Stigonema*, taxonomy

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CHAPTER V summarizes the state of art in cyanobacterial taxonomy based on both historical and modern studies, and uses a phylogeny-based polyphasic approach to present a new revised and updated classification of the whole cyanobacterial phylum.

PAPER VI.

Taxonomic classification of cyanoprokaryotes (cyanobacterial genera) 2014, using a polyphasic approach

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Abstract

The whole classification of cyanobacteria (species, genera, families, orders) has undergone extensive restructuring and revision in recent years with the advent of phylogenetic analyses based on molecular sequence data. Several recent revisionary and monographic works initiated a revision and it is anticipated there will be further changes in the future. However, with the completion of the monographic series on the Cyanobacteria in Süßwasserflora von Mitteleuropa, and the recent flurry of taxonomic papers describing new genera, it seems expedient that a summary of the modern taxonomic system for cyanobacteria should be published. In this review, we present the status of all currently used families of cyanobacteria, review the results of molecular taxonomic studies, descriptions and characteristics of new orders and new families and the elevation of a few subfamilies to family level. All recently defined cyanobacterial genera (some still invalid) are listed in the family to which they are likely to belong and an indication is given of their taxonomic validity and level of polyphasic characterization of each genus.

Keywords

concept of genera, cyanobacteria, molecular methods, taxonomic classification, polyphasic approach

Následující pasáž o rozsahu 41 stran obsahuje skutečnosti chráněné autorskými právy a je obsažena pouze v archivovaném originálu dizertační práce uloženém na Přírodovědecké fakultě Jihočeské univerzity v Českých Budějovicích. Publikace vyšla tiskem v časopise Preslia.

CHAPTER VI. SUMMARY OF THE RESULTS

In general, this thesis aimed at improving the current state of cyanobacterial taxonomy by revising selected taxa and introducing some modern methodical approaches. In spite of recent advancements, the revisionary work in cyanobacterial taxonomy is largely in the making. One of the most important tasks in this long-term endeavour is the revision of traditional cyanobacterial genera by means of the polyphasic approach. A large proportion of the extant genera still have not been inspected using modern methods. The examination of other genera has already been initiated, however, it frequently resulted in recognition of their polyphyly or unclear intergeneric boundaries. The taxonomic circumstances are generally worse in certain groups of terrestrial cyanobacteria, which have been less studied and less represented in culture collections. Therefore, the first aim of this thesis was to contribute to the revision of selected terrestrial cyanobacterial genera. In **paper I**, the only known representative of the most basal cyanobacterial lineage, a rock-dwelling coccoid genus *Gloeobacter*, which lacks thylakoids, was examined. Although it is a popular model organism, especially in photosynthesis research, this genus has never been isolated from nature again after its description in 1974. In our study, we isolated several new strains and investigated them in detail using a whole spectrum of polyphasic analyses. These included a phylogeny based on two housekeeping loci, proof of missing thylakoids using transmission electron microscopy, careful morphological analysis, and determination of the pigment composition in batch cultures. Such a powerful combination of different approaches is not very common, and can serve as a model study for further revision of cyanobacterial genera. Moreover, we determined the most probable relationship of *Gloeobacter* to several unclear terrestrial members of two other coccoid genera, *Aphanothece* and *Gleothece*. Along with morphological studies, the relationship of *Gloeobacter* and *Aphanothece caldariorum* was unravelled using molecular analysis of a well-preserved herbarium specimen more than 100 years old. This accomplishment encourages further attempts to analyse historical specimens of cyanobacteria. Nevertheless, the intra-generic variability (number of species) in *Gloeobacter* and its relationship to several more taxa remain to be addressed in future studies.

Another scope of the thesis was to strengthen the outputs of taxonomic revisions using nomenclatural amendments. The cyanobacterial nomenclature teems with complicated issues such as the presence of invalid taxa, unclear or missing holotypes, lost herbarium specimens, and vague descriptions of species. These problems are rarely solved by polyphasic studies alone, and the majority of researchers are not familiar with or not willing to deal with the

complexity of nomenclatural rules. In **papers II-IV**, directly related to **paper I**, we attempted to solve complicated relationships of the name *Gloeobacter* and the names of several old species of *Aphanothece* and *Gloeothece*. *Gloeobacter* itself first had to be validated (**paper II**) because its initial description lacked designation of a holotype and a Latin diagnosis (mandatory at that time). Unfortunately, the classical type species of *Gloeothece*, *G. linearis*, which is a species unrelated to all *Gloeothece* in the modern sense, was found among the few taxa most probably synonymous with *Gloeobacter* (**paper III**). This would make the name *Gloeobacter* invalid again, and leave majority of the genus *Gloeothece* without a name. To solve this inconvenient situation, proposals for conservation of both genera (*Gloeobacter* and *Gloeothece*) were submitted (**papers III and IV**). These contributions demonstrated that the nomenclatural treatment of cyanobacterial taxa, although complicated, can be accomplished, and brings the possibility of achieving a much clearer and more comprehensible taxonomic system.

A further aim of the thesis was to broaden the scope and quality of taxonomic revisions in cyanobacteria using modern molecular approaches. Currently, the vast majority of polyphasic studies have been restricted to strains of relatively easily cultivable cyanobacteria. The assessment of a large number of interesting morphospecies that resist laboratory cultivation has been blocked. This is mainly due to the impossibility of analysing clearly defined morphotypes from mixed assemblages by molecular methods. The study presented in **paper V** brought one possible solution to this inconvenience. Employing isolation of single cyanobacterial cells or trichomes under an optical microscope, we retained the opportunity to check the morphology of an individual separated from an environmental sample before its molecular processing. The method was successfully applied to samples of terrestrial cyanobacteria with massive sheaths, which are otherwise resistant to both cultivation and DNA extraction. The study further contributed to the general scope of this thesis with partial revision of the genera *Stigonema* and *Petalonema*. However, the variability inside these two classical genera is considerable, and more studies are required to cover the whole range of their morphological, ecological, and molecular diversity. One of the most valuable results of **paper V** is confirmation of the possibility to apply single-cell sequencing protocols to desiccated herbarium specimens of terrestrial cyanobacteria. Some of the samples analysed were 5-7 years old and yielded very good results. This is again encouraging for future molecular studies using herbarium specimens. Such potential samples include not only historical specimens, but, in particular, field collections from uneasily accessible sites and places with limited laboratory facilities. On the other hand, one of the limitations of the

molecular analysis presented in **paper V** is its restriction to the 16S rRNA gene. Phylogenies inferred solely from this gene often suffer from insufficient informative power at both subgeneric and suprageneric (family, order) levels. Hopefully, future studies will overcome this limitation, e.g. by amplifying whole genomes from single cells and conducting larger-scale sequencing of the genome amplicons.

The final aim of this thesis was to summarize the recent revisions in cyanobacterial taxonomy. In the last few years, the number of polyphasic studies has rapidly increased, mainly as a result of wide application of molecular techniques. Consequently, the classification of cyanobacteria has been in an urgent need for a phylum-wide revision that would reflect the current state of art in this field. **Paper VI** presents an attempt to construct an updated classification of cyanobacterial genera, families, and orders. In this study, we made efforts to provide a substantial improvement in implementing the phylogenetic concept of cyanobacterial taxa and a critical review of their plausibility. A robust phylogenetic reconstruction of 146 available cyanobacterial genomes covering all known major lineages was inferred from 31 conserved proteins. Broad phylogenetic studies of this kind, based on whole genome data, have been rather scarce, and none of them has drawn explicit taxonomic consequences. The resulting phylogenetic tree in **paper VI** enabled us to recognize the extensive polyphyly in some groups, and to start the necessary revisions by designating several entirely new orders and families and new combinations. Furthermore, we presented a complete list of cyanobacterial genera with an indication of their taxonomic clarity. Individual taxa were further commented on in the main text, which referred to almost all available recent literature on cyanobacterial taxonomy. It is important to note that this study presents only the first step in a long way towards a more natural system of cyanobacteria, corresponding to their evolutionary relationships. We hope that sufficient data will be soon obtained to allow splitting of the obviously polyphyletic orders Synechococcales and Oscillatoriales and designation of several smaller but well-defined taxonomic units.

In summary, this thesis has explored several well-established and several less common ways of investigation in cyanobacterial systematics. The results obtained during this study confirm that the polyphasic approach to cyanobacterial taxonomy is the most effective methodological approach for exploring diversity in this group of prokaryotes. However, this approach is in need of constant re-evaluation and updating. Use of other data in addition to morphology and 16S rRNA gene sequences (e.g. biochemical analysis) may substantially improve the understanding of cyanobacterial diversity. It is advisable to use multilocus or whole genome molecular data to infer reliable phylogenies, especially at higher taxonomic

levels. The relatively small proportion of cultivable cyanobacteria creates an urgent need for development and application of cultivation-independent techniques such as single-cell molecular analysis. Last but not least, taxonomic revisions should be accompanied by nomenclatural treatments.