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**Towards a modern revision of the cyanobacteria,
a critically important prokaryotic phylum**

Ph.D. Thesis

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ANNOTATION

With an adoption of modern methods of polyphasic approach to the study of cyanobacteria, an increased demand for the revision of the traditional taxonomy has emerged. This thesis is devoted to the systematic revisions of selected terrestrial cyanobacteria at several taxonomic levels. The methodology included thorough morphological characterization of cultured cyanobacterial strains using light and electron microscopy complemented with analyses of the molecular data: DNA sequencing, phylogenetic analyses based on 16S rRNA gene and the adjacent 16S-23S ITS region, and comparison of the predicted secondary structures of this region. Descriptions of new species, genera, families and an in-depth characterization of a previously poorly known family were achieved.

DECLARATION [in Czech]

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V Českých Budějovicích, 9. srpna 2015

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Markéta Bohunická

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LIST OF ARTICLES AND AUTHOR'S CONTRIBUTION

The thesis is based on the following papers (listed chronologically):

Komárek, J., Sant'Anna, C.L., **Bohunická, M.**, Mareš, J., Hentschke, G.S., Rigonato, J. & Fiore, M.F. (2013) Phenotype diversity and phylogeny of selected *Scytonema*-species (Cyanoprokaryota) from SE Brazil. *Fottea* 13(2): 173–200. (IF = 1.93)

Markéta Bohunická performed isolation and cultivation of majority of the cultured strains, analyses of morphology of these strains including photodocumentation, molecular analyses, prepared some of the line drawings, participated in phylogenetic analyses, and preparation of the manuscript.

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Markéta Bohunická cultivated the studied strain, performed the morphological observations, participated in molecular and phylogenetic analyses and preparation of the manuscript.

Johansen, J. R., **Bohunická, M.**, Lukešová, A., Hřčková, K., Vaccarino, M. A. & Chesarino, N. M. (2014) Morphological and molecular characterization within 26 strains of the genus *Cylindrospermum* (Nostocaceae, Cyanobacteria), with description of three new species. *Journal of Phycology* 50: 187–202. (IF = 2.844)

Markéta Bohunická performed purification of the strains, their cultivation and subsequent analyses of morphology including observations of life-cycle, participated in molecular and phylogenetic analyses, prepared all figure plates, and significantly participated in preparation of the manuscript.

Osorio-Santos, K., Pietrasiak, N., **Bohunická, M.**, Miscoe, L. H., Kováčik, L., Martin, M. P. & Johansen, J. R. (2014) Seven new species of *Oculatella* (Pseudanabaenales, Cyanobacteria): taxonomically recognizing cryptic diversification. *European Journal of Phycology* 49(4): 450–470. (IF = 1.912)

Markéta Bohunická isolated and morphologically characterized one of the strains (species), and participated in preparation of the manuscript.

Hauer, T., **Bohunická, M.**, Johansen, J. R., Mareš, J., Berrendero-Gomez, E. (2014) Reassessment of the cyanobacterial family Microchaetaceae and establishment of new families Tolypothrichaceae and Godleyaceae. *Journal of Phycology* 50: 1089–1100. (IF = 2.844)

Markéta Bohunická performed isolation and/or cultivation of strains, analyses of morphology, participated in molecular and phylogenetic analyses, and preparation of the manuscript.

Bohunická, M., Pietrasiak, N., Johansen, J. R., Berrendero-Gómez, E., Hauer, T., Gaysina, L. A. & Lukešová A. (2015) *Roholtiella*, gen. nov. (Nostocales, Cyanobacteria) – a tapering and branching cyanobacteria of the family Nostocaceae. *Phytotaxa* 197(2): 84-103. (IF = 1.318)

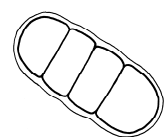
Markéta Bohunická performed isolation and purification of some of the strains, cultivation of all strains, analyses of morphology including life-cycle studies, analysis of ultrastructure of a selected strain, participated in other parts of the study, and was responsible for preparation of the manuscript including the figures and tables.

Bohunická, M., Mareš, J., Lukeš, M., Hrouzek, P., Urajová, P., Šmarda, J., Gaysina, L.A., Strunecký, O. (submitted manuscript) A combined morphological, ultrastructural, molecular, and biochemical study of the peculiar family Gomontiellaceae (Oscillatoriales) reveals a new cylindrospermopsin-producing clade of cyanobacteria.

Markéta Bohunická initiated the study, led the team of authors, performed isolation and/or cultivation of strains, analyses of morphology including photodocumentation and preparation of all figures, participated in analyses of ultrastructure, molecular and phylogenetic analyses, and was responsible for preparation of the manuscript.

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

Why are cyanobacteria so important?

Cyanobacteria are prokaryotic photosynthesizing organisms of crucial ecological and evolutionary importance. Signs of their presence on the Earth surface are as old as 3.5 billion years (Summons et al. 1999, Cavalier-Smith 2006). Since then, they have evolved into a great variety of genetic as well as morphological forms and inhabited nearly all possible photic habitats of our planet. With no doubt, the invention of oxygenic photosynthesis by the first cyanobacteria is the most important bioenergetic process of the planet's surface as during the "Great Oxidation Event" around 2.3 billion years ago, it led to conversion of the early reducing atmosphere to an oxidizing one (e.g. Fischer 1965, Schopf 2014, Schirmer et al. 2015). This change subsequently enabled the evolution and diversification of majority of the life forms known thereafter. Moreover, a subsequent substantial consequence of cyanobacterial activity on Earth is the genesis of all eukaryotic photosynthetic lineages, including the whole kingdom of Plantae and various groups of algae (such as e.g. red and brown algae, dinoflagellates, cryptomonads etc.) This was possible through the process of endosymbiosis in which cyanobacteria were adopted into the cells of primitive eukaryotes and formed their chloroplasts (Mereschkowsky 1905, Martin & Kowallik 1999, Gould et al. 2008). Cyanobacteria were also responsible for the formation of a significant part of the lithosphere through deposition of calcium carbonates in the form of stromatolites (Altermann et al. 2006). Some of the cyanobacteria evolved a mechanism of dinitrogen fixation, which in the most advanced forms occurs in specialized thick-walled cells called heterocytes. A variety of other organisms live in coexistence (most frequently in symbiosis) with cyanobacteria (e.g. lichenized fungi, plants, marine invertebrates), benefiting from this ability that affects the nitrogen cycle and provides a competitive advantage in nitrogen-limited habitats.

Cyanobacteria can be found on all continents. They form not only an important part of oceanic and freshwater phytoplankton and benthos being the base of the aquatic food chain (Iturriaga & Mitchell 1986), but they inhabit semiaquatic and terrestrial habitats such as soils (Wu et al. 2003), dry and wet solid surfaces including rocks, the bark of trees and manmade materials (Hauer et al. 2015), and extreme environments such as hot and cold deserts (Bahl et al. 2011), glaciers and other Polar region habitats (Vincent 2002), hypersaline habitats (Kirkwood et al. 2008) or hot springs (Papke et al. 2003); an endolithic way of life is also possible (Wierchos et al. 2006). Cyanobacteria are often pioneer organisms colonizing newly established habitats, enabling gradual succession of other forms of life (Lan et al. 2013). Terrestrial cyanobacteria frequently form microbial communities (biofilms), which are highly important especially in nutrient-poor or/and

environmentally extreme habitats such as desert soils. Facing all the biotic and abiotic stresses, they have evolved a broad range of secondary metabolites (Gademann & Portmann 2008, Rastogi & Sinha 2009, Yadav et al. 2011, Dittmann et al. 2013), which have potential application in medicine and pharmaceuticals (Nunnery et al. 2010, Singh et al. 2011), cosmetics (Yabuta et al. 2014), biofuel production (Machado et al. 2012) as well as in agriculture. In some parts of the world, cyanobacterial populations help in food production (e.g. rice fields – Roger & Ladha 1992) or are directly harvested and processed as a food source (e.g. *dihé* – Abdulqader et al. 2000) or nutritional supplements (e.g. “*Spirulina*” – Belay 2008).

On the other hand, cyanobacteria are capable of forming high population densities in eutrophic waters as well as production of highly toxic compounds. This ability can not only limit various human activities (especially swimming in water reservoirs), but may threaten our food sources or have direct impact to our health (Azevedo et al. 2002), especially when sources of drinking water are affected. With the eutrophication of surface waters, the rise of the world’s human population, and global climate change, the present significance of deleterious impacts of cyanobacteria on our health and prosperity is certain (Carmichael 2008, O’Neil et al. 2012, Paerl & Otten 2013). Thus, efforts to understand as much as possible from aspects of cyanobacterial life, their distribution, ecology, physiology, relationships and last but not least their proper identification and classification is highly desirable.

Changes in approach to the study of cyanobacteria

Cyanobacteria were recognized as a separate group as early as in 1833 (as Myxophyceae, Wallroth 1833) and their deeper systematic examination dates back to the end of the 19th century (Thuret 1875, Bornet & Flahault 1886-1888, Gomont 1892). These first cyanobacterial monographs were later accepted as a starting point in taxonomic referencing. Early phycologists used morphology as observed in bright field light microscopes and followed botanical nomenclatural rules, and cyanobacteria were understood as a distinct group of microscopic algae. In the first quarter of the 20th century, revisions of all taxa known at the time from North and Central America and adjacent regions was published by Tilden (1910, 1917). Similar works in Europe were accomplished by Geitler (1925, 1932, 1942). Both authors characterized all the main groups of cyanobacteria by description of the diacritical features of the species such as type of branching, sheath formation, specialized cell and structure formation (heterocytes, akinetes, hormogonia, hairs), cell shape and dimensions, and included keys to the species. Morphological data were supplemented by the brief description of the habitat of occurrence and often also by line drawings of the organisms. These monographs (especially Geitler’s from 1932 summarizing 1300 species in 145 genera,

20 families and 3 orders) were used as the fundamental basis for identification of cyanobacteria until the end of the 20th century. In the meanwhile, several authors made effort to update the current state of knowledge in the field, but the classical morphology-based system for cyanobacterial classification (sometimes referred to as the “Geitlerian approach”) remained widely accepted (e.g. Elenkin 1936-49, Desikachary 1959, Starmach 1966, Kondrateva 1968, Bourrelly 1970).

Later, a period of reducing the number of recognized cyanobacterial taxa occurred. A thorough revisionary work was completed by Drouet, who proposed that each cyanobacterial species produced a wide range of naturally variable morphologies in response to diverse environmental stimuli (“ecophenes” of the species). Thus he broadened the definition of a few species to encompass the majority of previously separate taxa (Drouet & Daily 1956, Drouet 1968, 1973, 1978, 1981). Drouet’s effort is valued especially for gathering bibliographic information on the sources of all cyanobacterial species described prior to 1960, often including information on type species of genera as well as physical location of type materials for all species. In the third quarter of the 20th century, classical morphological observations of natural cyanobacterial populations were supplemented by examination of cultured strains following bacteriological methods (Waterbury & Stanier 1977, Rippka et al. 1979). This shed light particularly on their phylogenetic relationships (Bonen et al. 1979, Giovannoni et al. 1988), genome size and structure (Herdmann et al. 1979, Doolittle 1979) and cell structure (Golecki 1977, Lang 1977). However, the results revealed incongruence with the traditional morphological approach and led to an adoption of a system of widely defined “form-genera” ranked in the “sections” (Castenholz 2001), again substantially reducing the number of recognized distinct taxonomic units. Under this approach, no new genera and species were recognized and described, obscuring the estimation of the overall cyanobacterial diversity. In consequence, it was inapplicable or at least inconvenient for classical phycological work in water management, floristic or ecological studies. These two taxa-reducing approaches were later rejected by botanically trained phycologists and systematists, who reverted back to a taxonomic paradigm that recognized greater diversity as reported by earlier classical workers.

However, the distribution of cyanobacterial diversity was still being discussed. Crucial role was played by adoption of an ecological theory known as the Bass-Becking Hypothesis (Baas-Becking 1934). The key synopsis of this hypothesis is that "everything is everywhere, *but* the environment selects". A fundamental tenet of this hypothesis is that microbes are so abundant in terms of population size, and so small in terms of physical size, that there are no real barriers to their dispersal (de Wit & Bouvier 2006). The taxonomic implication that follows is that there are no biogeographic patterns to be detected in microbes, and endemic species consequently do not exist. This theory still

enjoys support among many microbiologists. Following this idea and using the above described morphological approach, many ecologists and taxonomists tended to force all new cyanobacterial discoveries from various habitats and geographical localities into previously described, mainly European species. Despite the fact that cyanobacteria are the most complex prokaryotes, they are to some extent limited in their morphological variability, especially at the lower taxonomic levels. Therefore such determination is possible, although in contradiction with species definition recognized by modern taxonomists, because it does not necessarily lead to recognition of taxa reflective of evolutionary history. Similar morphotypes can occur due to plesiomorphy and homoplasy, and consequently morphologically comparable populations may not share common ancestry.

To revise these old mistakes of cyanobacterial taxonomy and to avoid making new ones, a polyphasic approach for species recognition, including morphology, ecology, physiology, and molecular biology has been proposed (Hoffmann 1988, Komárek 2003). This new formal system combined bacteriological (culture-based) as well as botanical approaches. It helped researchers to re-evaluate their previous opinions about species distribution (Komárek 1985, 2003) and to realize that many morphologically indistinguishable strains that do not share a common evolutionary history are incorrectly placed in polyphyletic morphospecies. It was first used in the systematic reevaluation of the larger taxonomic units within cyanobacteria (Anagnostidis & Komárek 1985, Komárek & Anagnostidis 1986, Anagnostidis & Komárek 1988, Komárek & Anagnostidis 1989, Anagnostidis & Komárek 1990). Following this effort, monographs on Chococcales, Oscillatoriales and heterocytous genera were published within the series of Süßwasserflora von Mitteleuropa (Komárek & Anagnostidis 1999, 2005, Komárek 2013). These fundamental key books summarize the current knowledge on the cyanobacterial taxa from temperate zones and provide an overview of the taxa from other climatic zones, fully accepting their potential endemism. The classical morphological, ecological and distribution data are supplemented with molecular evidence, where available.

The polyphasic approach to the cyanobacterial classification gradually accepted by the majority of the phycologists of the end of 20th and the beginning of the 21st century is in agreement with the species concept (a theoretical logical basis of a species nature) proposed for cyanobacteria by Johansen & Casamatta (2005). They recommended adoption of a concept by Mishler & Theriot (2000), which they refer to as the “Monophyletic Species Concept” to distinguish it from a number of other phylogenetic species concepts. The ranking criterion for species that Johansen & Casamatta (2005) recommended is the existence of any autapomorphic feature in any character set that can lead to reliable recognition of the species lineage in future collections. This can

incorporate a variety of different criteria, such as molecular sequence, biochemistry, morphology, ultrastructure, biogeography, and ecophysiology and thus it forms a good theoretical background for the application of the polyphasic approach.

Despite the intensive effort of current phycologists, only small portion of cyanobacterial diversity has been analyzed using the methods of molecular biology so far, and the results are disproportionately distributed among certain cyanobacterial groups (strongly favoring bloom-forming taxa). The most commonly examined molecular marker of prokaryotic organisms including cyanobacteria is the 16S rRNA gene of the ribosomal operon (also called SSU). This so-called house-keeping gene is highly conserved in some regions and variable in others, and thus phylogenies based on this gene allow convenient differentiation at all taxonomic levels, especially above species (Nübel et al. 1997). The 16S rRNA marker is a cornerstone of the molecular part of the polyphasic evaluation, as convenient genetic comparison of a newly obtained 16S rDNA sequence is possible through steadily growing sequence databases (e.g. NCBI). It is also often used for classical bacteriological similarity comparisons and evaluation of the degree of relatedness of the tested strains. It has been proposed that members of a prokaryotic species should be at least 97% similar (Stackebrandt & Goebel 2004), which was later adjusted to 98.7% (Stackebrandt & Ebers 2006), the genera cutoff was set at <95% (Stackebrandt & Goebel 2004). However, these criteria are continuously being disputed by the cyanobacterial taxonomists, especially for the heterocytous taxa, in which the similarities are often much higher at both levels (Lyra et al. 2001, Flechtner et al. 2002, Rajaniemi et al. 2005, Řeháková et al. 2007, Kaštovský & Johansen 2008, Lukešová et al. 2009, Vaccarino & Johansen 2012, Řeháková et al. 2014).

Recently, it has been demonstrated that using the internal transcribed spacer (ITS), the region following the 16S rDNA and preceding the 23S rDNA, has a potential to separate the individual species of a genus. The ITS alone or together with the 16S rDNA, can be used to construct a phylogeny of closely related taxa (e.g. Mühlsteinová 2014). As the ITS is a non-protein-coding part of the DNA that must be removed from the rRNA during post-transcriptional processing, it is particularly important to assemble the alignment reflecting the more conserved parts of the secondary structure, which possibly have structural function during the processing. Often, these conserved parts are being identified and their secondary structures (typically hairpin helices) are being predicted. Comparison of similarity of these structures in related taxa provides an accessory piece of evidence of the degree of their evolutionary divergence (e.g. Vaccarino & Johansen 2011, Kaštovský et al. 2014, Genuário et al. 2015).

To refine phylogenetic resolution, other protein-coding genes (e.g. genes involved in photosynthesis, RNA transcription, or oxidative phosphorylation) are being sequenced and included in some cyanobacterial multi-locus phylogenies (Seo & Yokota 2003,

Malone et al. 2015, Řeháková et al. 2014, Mareš et al. 2013, Rajaniemi et al. 2005, Tanabe et al. 2007, Mazard et al. 2012). This effort especially improves bootstrap support at the backbone of the phylogenies, which is often missing when only 16S rRNA gene sequence is used. It seems that multi-locus analyses will be an inevitable component of future revisionary work in cyanobacterial taxonomy. Besides, with the recent advances in the whole genome sequencing, such phylogenies can be constructed using multiple selected genes from the available genomic data (Shih et al. 2013). This was critical in the re-evaluation of higher level taxonomy proposed by Komárek et al. (2014), in which 31 conserved protein sequences were used to construct a phylogenetic tree of all (available) cyanobacterial genera and to show their relationships with increased accuracy. Taxonomic conclusions of this important study, including order and family assignments of all presently described cyanobacterial genera (and covering some that will be described in near future) are followed in the ensuing subchapter.

Objectives and content of the thesis

With the growing volume of sequence data available and the subsequent uses of these data in phylogenies, the necessity of deeper reevaluation of many traditionally defined – but now recognized as polyphyletic – taxonomic units has become evident. This effort, although far from completion, already resulted in creation of several new families (Komárek et al. 2014). However, to meaningfully revise higher taxonomic units, we need to start with well-established basic building blocks of the whole system. These building blocks are individual species that should be properly identified, characterized and reliably assigned into monophyletic genera. **Table 1** summarizes 45 new genera published since 2010. It clearly demonstrates that recent intense employment of molecular markers in cyanobacterial taxonomy has led to recognition of previously neglected diversity.

In this thesis I aimed to contribute my part to the collective attempt of cyanobacterial taxonomic revision. I used methods of polyphasic approach to redefine cyanobacterial taxonomic units on the level of species, genera, and families. The main group of interest were terrestrial soil and rock inhabiting taxa of cyanobacteria. The thesis is composed of three main chapters, corresponding to the taxonomic level revised.

Chapter I addresses the revisionary work at the species and subspecies level. In **Paper I**, samples of the genus *Scytonema* (Nostocales, Scytonemataceae) from the biodiversity hotspot of Mata Atlântica, Brazil were examined using classical morphological observations and complemented with 16S rRNA gene analysis for some of the isolated strains. Two new species, *Scytonema chora* and *S. papillicapitatum* were described. **Paper II** is a strain-based study of the genus *Cylindrospermum* (Nostocales, Nostocaceae), in which a total of 26 strains were examined, three new species described

(*C. badium*, *C. moravicum*, *C. pellucidum*) and *Cronbergia siamenis* recombined to *Cylindrospermum siamense*. The 16S rDNA phylogeny was supplemented with a stand-alone ITS phylogeny for each of the ITS operons (including the one with both tRNA^{Ile} and tRNA^{Ala} genes and the one missing both tRNAs). The ITS secondary structures were compared and discussed (D1-D1', V2 and Box-B helices). **Paper III** introduces seven new species of a recently described genus *Oculatella* (Synechococcales, Leptolyngbyaceae) using morphological features, ITS structures, 16S and 16S-23S ITS rDNA phylogenies and comparison of similarities of these regions. Three of the species isolated from desert soils had overlapping morphologies and were defined using solely molecular evidence. These three species are therefore cryptic and their valid description marks an important shift in the traditional perception of recognizing cyanobacterial species as the “*smallest monophyletic groups deemed worthy of formal recognition*” (Mishler & Theriot 2000).

Chapter II covers revisions at the generic level. **Paper IV** describes *Calochaete* (Nostocales, Fortieaceae), a new genus of tapered and false branched heterocytous cyanobacteria from a soil crust collected at 3600 m a.s.l. in Costa Rica. Its morphology was documented and the phylogenetic placement of the new finding based on 16S rDNA phylogeny provided. Heterogeneity of the traditional family Microchaetaceae arising from the results obtained was discussed. **Paper V** deals with another newly described genus, *Roholtiella* (Nostocales, Fortieaceae). The study examined 16 strains isolated from soil habitats that possessed morphological features of three different families: Rivulariaceae (tapering trichomes), Tolypotrichaceae (single false branching) and Nostocaceae (arthrospores). Phylogenetic analyses revealed their assessment to Nostocaceae (later reclassified to Fortieaceae by Komárek et al. 2014) in which they formed a strongly supported monophyletic clade. Four new species were described, two of which were cryptic, with indistinguishable morphotypes, but recognizable using 16S-23S ITS region (especially Box-B and V3 helices).

Chapter III includes two revisions at the family level. **Paper VI** presents the first part of a work on reassessment of the polyphyletic cyanobacterial family Microchaetaceae. Morphology, ecology, biogeography, and phylogeny of 16S rRNA gene were used to study strains assigned in culture collections to the nominate genus *Microchaete*. Although morphologically in compliance with the genus definition, it was found that these strains are not related to the traditional members of Microchaetaceae such as *Tolypothrix* or *Hassallia*, but rather belong to the unrelated families Nostocaceae and Rivulariaceae. To reflect the revealed phylogenetic relationships, two new families, Tolypotrichaceae and Godleyaceae within the order Nostocales were established. The study also described three new species of *Fortiea* (*F. contorta*, *F. laiensis*, *F. coimbrae*). **Paper VII** is a multidisciplinary study of a little known cyanobacterial family

Gomontiellaceae (Oscillatoriales). Five strains in three genera (*Hormoscilla*, *Crinalium*, and *Starria*) were characterized in detail using a combination of morphological, ultrastructural, molecular, and biochemical analysis, information on morphology of the type genus and species of the family, *Gomontiella subtubulosa* was also included. The family was found to be monophyletic and the unusual feature of cellulose incorporated into the cell walls was verified for all tested strains. Unique morphological and ultrastructural characteristics were additionally documented. Moreover, production of hepatotoxin cylindrospermopsin was detected in the genus *Hormoscilla*, which represents the first report of the presence of this toxin in soil cyanobacteria.

Table 1. Descriptions of new cyanobacterial genera using modern approach to the cyanobacterial taxonomy (2010 – July 2015). Order and family designation based on Komárek et al. 2014. Genus *Nisada* was described only based on thorough morphological observations, molecular data were not collected. Papers presented in this thesis are marked by an asterisk.

	Genus	Type species	Order	Family	Reference
1	<i>Aerosakkonema</i>	<i>funiforme</i>	Oscillatoriales	Oscillatoriaceae	Thu et al. 2012
2	<i>Aetokthonos</i>	<i>hydrillicola</i>	Nostocales	Hapalosiphonaceae	Wilde et al. 2014
3	<i>Alkalinema</i>	<i>pantanalense</i>	Synechococcales	Leptolyngbyaceae	Vaz et al. 2015
4	<i>Ammassolinea</i>	<i>attenuata</i>	Oscillatoriales	Microcoleaceae (?)	Hašler et al. 2014c
5	<i>Anathece</i>	<i>clathrata</i>	Synechococcales	Synechococcaceae	Komárek et al. 2011
6	<i>Annamia</i>	<i>toxica</i>	Oscillatoriales	Microcoleaceae	Nguyen et al. 2013
7	<i>Caldora</i>	<i>penicillata</i>	Oscillatoriales	Microcoleaceae (?)	Engene et al. 2015
8	<i>Calochaete</i>	<i>cimrmanii</i>	Nostocales	Fortieaceae	Hauer et al. 2013*
9	<i>Cephalothrix</i>	<i>komarekiana</i>	Oscillatoriales	Oscillatoriaceae	Malone et al. 2015
10	<i>Chakia</i>	<i>ciliosa</i>	Nostocales	Scytonemataceae	Komárková et al. 2013
11	<i>Chalicogloea</i>	<i>cavernicola</i>	Chroococcales	Chroococcaceae	Roldán et al. 2013
12	<i>Chryso sporum</i>	<i>ovalisporum</i>	Nostocales	Aphanizomenonaceae	Zapomělová et al. 2012
13	<i>Cronbergia</i>	<i>siamensis</i>	Nostocales	Nostocaceae	Komárek et al. 2010
14	<i>Cyanocohniella</i>	<i>callida</i>	Nostocales	Nostocaceae	Kaštovský et al. 2014
15	<i>Dactylothamnos</i>	<i>antarcticus</i>	Nostocales	Tolypotrichaceae	Komárek et al. 2015
16	<i>Desertifilum</i>	<i>tharensense</i>	Oscillatoriales	Coleofasciculaceae	Dadheech et al. 2012a
17	<i>Desmonostoc</i>	<i>muscorum</i>	Nostocales	Nostocaceae	Hrouzek et al. 2013
18	<i>Godleya</i>	<i>alpina</i>	Nostocales	Godleyaceae	Novis & Visnovsky 2011
19	<i>Haloleptolyngbya</i>	<i>alcalis</i>	Oscillatoriales	Leptolyngbyaceae	Dadheech et al. 2012b
20	<i>Halotia</i>	<i>branconii</i>	Nostocales	Nostocaceae	Genuário et al. 2015b
21	<i>Iphinoe</i>	<i>spelaeobios</i>	Nostocales	Symphyonemataceae	Lamprinou et al. 2011
22	<i>Johanseninema</i>	<i>constrictum</i>	Oscillatoriales	Microcoleaceae	Hašler et al. 2014a, b
23	<i>Kamptonema</i>	<i>animale</i>	Oscillatoriales	Microcoleaceae	Strunický et al. 2014
24	<i>Kastovskya</i>	<i>adunca</i>	Oscillatoriales	Coleofasciculaceae	Mühlsteinová et al. 2014
25	<i>Limnoraphis</i>	<i>hieronymusi</i>	Oscillatoriales	Oscillatoriaceae	Komárek et al. 2013
26	<i>Limnococcus</i>	<i>limneticus</i>	Synechococcales	Merismopediaceae	Komárková et al. 2010
27	<i>Loriellopsis</i>	<i>cavernicola</i>	Nostocales	Symphyonemataceae	Lamprinou et al. 2011
28	<i>Microseira</i>	<i>wollei</i>	Oscillatoriales	Oscillatoriaceae	McGregor & Sendall 2015
29	<i>Moorea</i>	<i>producing</i>	Oscillatoriales	Oscillatoriaceae	Engene et al. 2012
30	<i>Neosynechococcus</i>	<i>sphagnicola</i>	Synechococcales	Leptolyngbyaceae	Dvořák et al. 2014
31	<i>Nisada</i>	<i>stipitata</i>	(molecular data not available)		Gold-Morgan et al. 2015
32	<i>Nodosilinea</i>	<i>nodulosa</i>	Synechococcales	Leptolyngbyaceae	Perkenson et al. 2011
33	<i>Oculatella</i>	<i>subterranea</i>	Synechococcales	Leptolyngbyaceae	Zammit et al. 2012

34	<i>Okeania</i>	<i>hirsuta</i>	Oscillatoriales	Oscillatoriaceae	Engene et al. 2013
35	<i>Ophiothrix</i>	<i>epidendron</i>	Nostocales	Scytonemataceae	Sant'Anna et al. 2010
36	<i>Oxynema</i>	<i>thaianum</i>	Oscillatoriales	Microcoleaceae	Chatchawan et al. 2012
37	<i>Pantanalinema</i>	<i>rosanae</i>	Synechococcales	Leptolyngbyaceae	Vaz et al. 2015
38	<i>Pinocchia</i>	<i>polymorpha</i>	Synechococcales	Leptolyngbyaceae (?)	Dvořák et al. 2015
39	<i>Plectolyngbya</i>	<i>hodgsonii</i>	Synechococcales	Leptolyngbyaceae	Taton et al. 2011
40	<i>Roholtiella</i>	<i>edaphica</i>	Nostocales	Fortieaceae	Bohunická et al. 2015*
41	<i>Roseofilum</i>	<i>reptotaenium</i>	Oscillatoriales	Coleofasciculaceae	Casamatta et al. 2012
42	<i>Sphaerospermopsis</i>	<i>reniformis</i>	Nostocales	Aphanizomenonaceae	Zapomělová et al. 2009, 2010
43	<i>Streptostemon</i>	<i>capitatus</i>	Nostocales	Tolypotrichaceae	Sant'Anna et al. 2010
44	<i>Toxopsis</i>	<i>calypsus</i>	Nostocales	Godleyaceae	Lamprinou et al. 2012
45	<i>Wilmottia</i>	<i>murrayi</i>	Oscillatoriales	Coleofasciculaceae	Strunecký et al. 2011

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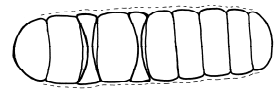
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CHAPTER I.

Revision at the species and sub-species level

PAPER 1.

Phenotype diversity and phylogeny of selected *Scytonema*-species (Cyanoprokaryota) from SE Brazil

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Abstract

Members of the genus *Scytonema* belong to prominent components of microflora of tropical and subtropical soils, but their diversity and taxonomic classification is still little known. Molecular analyses of isolated strains, but also the morphological and ecological examination of natural populations are important for the starting revisions. Here we present phenotype characteristics of fourteen morphotypes of the cyanobacterial genus *Scytonema* from the SE Brazil, mostly from the state São Paulo and from the Atlantic Rainforest (ecosystem of “Mata Atlântica”). The populations of studied *Scytonema* species are ecologically significant, as they hold the dominant position in the microvegetation communities of lateritic and forest soils and of stony substrates (less frequently also aquatic forms). Species commonly distributed throughout the tropical regions (such as *S. guyanense*, *S. javanicum*, *S. stuposum*), as well as the types with restricted geographic distribution in S America are included in the study. Two new species from a little known subgenus *Myochrotes*, in which the cultivation is difficult, are described: *S. papillicapitatum* Sant'Anna et Komárek and *S. chorae* Sant'Anna et Komárek. Our analyses support the principle of congruency of morphological characters with phylogenetic position of the studied species.

Key words: Cyanobacteria, *Scytonema*, taxonomy, new species, polyphasic approach, ecology, Brazil (SP)

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PAPER II.

Morphological and molecular characterization within 26 strains of the genus *Cylindrospermum* (Nostocaceae, Cyanobacteria), with descriptions of three new species

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Abstract

Twenty-six strains morphologically identified as *Cylindrospermum* as well as the closely related taxon *Cronbergia siamensis* were examined microscopically as well as phylogenetically using sequence data for the 16S rRNA gene and the 16S-23S internal transcribed spacer (ITS) region. Phylogenetic analysis of the 16S rRNA revealed three distinct clades. The clade we designate as *Cylindrospermum sensu stricto* contained all five of the foundational species, *C. maius*, *C. stagnale*, *C. licheniforme*, *C. muscicola*, and *C. catenatum*. In addition to these taxa, three species new to science in this clade were described: *C. badium*, *C. moravicum*, and *C. pellucidum*. Our evidence indicated that *Cronbergia* is a later synonym of *Cylindrospermum*. The phylogenetic position of *Cylindrospermum* within the Nostocaceae was not clearly resolved in our analyses. *Cylindrospermum* is unusual among cyanobacterial genera in that the morphological diversity appears to be more evident than sequence divergence. Taxa were clearly separable using morphology, but had very high percent similarity among ribosomal sequences. Given the high diversity we noted in this study, we conclude that there is likely much more diversity remaining to be described in this genus.

Keywords: 16S rRNA gene; 16S-23S ITS; *Cylindrospermum*; Nostocaceae; polyphasic approach

Abbreviations: ITS, internal transcribed spacer

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PAPER III.

Seven new species of *Oculatella* (Pseudanabaenales, Cyanobacteria): taxonomically recognizing cryptic diversification

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Abstract

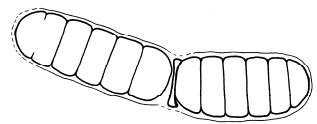
A total of 27 strains of *Oculatella* were isolated, characterized and sequenced, and analysed phylogenetically with an additional environmental clone from the Atacama Desert and 10 strains isolated and sequenced by others. The strains were clearly separated based upon phylogenetic analyses conducted with a concatenated alignment of the 16S rRNA and 16S-23S ITS region of the ribosomal operons in the genus *Oculatella*. Differences in secondary structure of the conserved domains of the ITS region, as well as comparative analysis of P-distance among ITS regions, served to separate the strains into distinct taxonomic units. Seven new species of *Oculatella* were described, including four from arid to semi-arid soils (*O. atacamensis*, *O. mojaviensis*, *O. coburnii*, *O. neakamentiensis*) and three from more mesic habitats, including a temperate lake (*O. hafneriensis*), a desert waterfall (*O. cataractarum*) and a Hawaiian sea cave (*O. kauaiensis*). The soil forms show statistically significant morphological differences, but the ranges overlap to a degree that they are not diagnosable by morphology, and these four cryptic species are characterized here using molecular characters. The more mesic species, including the type species from Mediterranean hypogea, *O. subterranea*, are all morphologically distinct from each other and from all four soil taxa. This report is the first to use solely molecular criteria to distinguish cryptic species of cyanobacteria.

Key words: 16S rRNA gene sequence, 16S-23S ITS, Atacama Desert, Colorado Plateau, cryptic species, Cyanobacteria, Europe, Hawaii, Mojave Desert, *Oculatella*, Pseudanabaenaceae, rRNA secondary structure

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CHAPTER II.

Revision at the genus level

PAPER IV.

***Calochaete* gen. nov. (Cyanobacteria, Nostocales), a new cyanobacterial type from “páramo” zone in Costa Rica**

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Abstract

A new tapered and false branched morphotype of filamentous heterocytous cyanobacterium was isolated from soil material collected on a massif of Chirripó Mountain, Costa Rica. The strain was analyzed morphologically and a sequence of its 16S rRNA gene was compared with available 16S rDNA sequences of organisms with similar morphology, especially those with heteropolar tapering filaments. Phylogenetic analyses revealed that the strain was significantly different from Rivulariaceae, but was closely related to several strains designated as *Tolypothrix*. However, according to the original descriptions in the literature, members of the genus *Tolypothrix* possess only very slightly tapering filaments. With regard to all these differences, we decided to describe a new genus—*Calochaete* gen. nov. with type species *C. cimrmanii*.

Key words: 16S-23S ITS, 16S rRNA gene, Central America, Cyanoprokaryota, Microchaetaceae, morphology, new genus, taxonomy

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PAPER V.

***Roholtiella*, gen. nov. (Nostocales, Cyanobacteria)—a tapering and branching cyanobacteria of the family Nostocaceae**

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Abstract

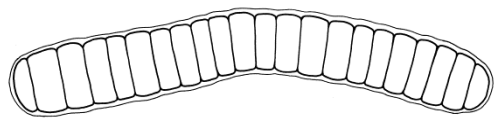
A total of 16 strains phylogenetically placed within the Nostocaceae were found to possess morphological features of the Rivulariaceae and Tolypothrichaceae (tapering trichomes and single false branching, respectively) in addition to their typical Nostocacean features (production of arthrospores in series). These strains formed a strongly supported clade separate from other strains that are phylogenetically and morphologically close. We describe four new species within the genus *Roholtiella* gen. nov. The four species include three distinguishable morphotypes. *Roholtiella mojaviensis* and *R. edaphica* are morphologically distinct from each other and from the other two species, *R. fluviatilis* and *R. bashkiriorum*. *Roholtiella fluviatilis* and *R. bashkiriorum* are cryptic species with respect to each other. All four species are easily distinguished based on the sequence of the 16S-23S ITS regions, in particular the flanking regions to the conserved Box-B and V3 helices. The species are further established by the elevated p-distance between species that is much reduced among strains within the same species. *Calochaete cimrmanii*, a recently described tapering species from tropical biomes, is the most likely sister taxon to *Roholtiella*.

Key words: 16S rRNA gene, 16S-23S ITS, cryptic species, morphology, new genus, Nostocophycideae, polyphasic approach, taxonomy

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CHAPTER III.

Revision at the family level

PAPER VI.

Reassessment of the cyanobacterial family Microchaetaceae and establishment of new families Tolypothrichaceae and Godleyaceae

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Abstract

The family Microchaetaceae is a large group of heterocytous cyanobacteria, whose members bear typical morphological features of uniseriate heteropolar filaments never terminated by thin hairs and with simple false branching. However, phylogenetic analyses of the gene for 16S rRNA showed that members of this traditionally morphologically delimited family form several distant groups and therefore the current concept is hereafter indefensible. In this study, we provide reassessment of the status of the family Microchaetaceae based on morphology, ecology, biogeography, and phylogeny of 16S rRNA gene. Thorough examination of strains of the nominate genus *Microchaete* revealed their affiliation to two groups, Nostocaceae and Rivulariaceae, and their distant position to other traditional members of Microchaetaceae such as *Tolypothrix*, *Hassallia*, and *Coleodesmium*. To reflect the phylogenetic relationships and to accommodate members of the traditional family Microchaetaceae that are clearly not related to any of the *Microchaete* representatives, we propose establishment of two new families, Tolypothrichaceae and Godleyaceae. Based on both molecular and morphological evidence, we also provide a description of three new species of the genus *Fortiea*.

Keywords: 16S rRNA gene; Cyanobacteria; Godleyaceae; Microchaetaceae; Tolypothrichaceae; *Tolypothrix*; *Fortiea*; new species

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PAPER VII.

A combined morphological, ultrastructural, molecular, and biochemical study of the peculiar family Gomontiellaceae (Oscillatoriales) reveals a new cylindrospermopsin-producing clade of cyanobacteria

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Article submitted to Journal of Phycology

Abstract

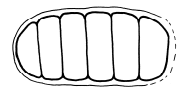
Members of the morphologically unusual cyanobacterial family Gomontiellaceae were studied using a polyphasic approach. Cultured strains of *Hormoscilla pringsheimii*, *Starria zimbabweensis*, *Crinalium magnum*, and *Crinalium epipsammum* were thoroughly examined, and the type specimen of the whole family, *Gomontiella subtubulosa*, was investigated. The results of morphological observations using both light microscopy and transmission electron microscopy were consistent with previous reports and provided evidence for the unique morphological and ultrastructural traits of this family. Analysis of the 16S rRNA gene confirmed the monophyletic origin of non-marine representatives of genera traditionally classified into this family. The family was phylogenetically placed among other groups of filamentous cyanobacterial taxa. The presence of cellulose in the cell wall was analyzed and confirmed in all cultured Gomontiellaceae members using Fourier transform infrared (FTIR) spectroscopy and fluorescence microscopy. Evaluation of toxins produced by the studied strains revealed the hepatotoxin cylindrospermopsin (CYN) in available members of the genus *Hormoscilla*. Production of this compound in both *Hormoscilla* strains was detected

using HPLC-HRMS and confirmed by positive PCR amplification of the *cyrJ* gene from the CYN biosynthetic cluster. To our knowledge, this is the first report of CYN production by soil cyanobacteria, establishing a previously unreported CYN-producing lineage. The present study indicates that cyanobacteria of the family Gomontiellaceae form a separate but coherent cluster defined by numerous intriguing morphological, ultrastructural, and biochemical features, and exhibiting a toxic potential worthy of further investigation.

Key index words: 16S rRNA; cylindrospermopsin; *cyrJ*; cellulose; *Crinalium*; *Cyanothece*; *Hormoscilla*; *Gomontiella*; *Starria*; toxins

Abbreviations: CCALA, Culture Collection of Autotrophic Organisms, Třeboň, Czech Republic; CYN, cyanotoxin cylindrospermopsin; EPS, extracellular polysaccharides; FTIR, Fourier transform infrared; HRMS, high resolution mass spectrometry; SAG, Sammlung von Algenkulturen, Goettingen, Germany.

Následující pasáž o rozsahu 25 stran obsahuje skutečnosti chráněné autorskými právy a je obsažena pouze v archivovaném originálu dizertační práce uloženém na Přírodovědecké fakultě Jihočeské univerzity v Českých Budějovicích.



SUMMARY OF THE RESULTS

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The main objective of this thesis was to contribute to better knowledge of cyanobacterial diversity in general. It aimed at the improvement of the current state of cyanobacterial taxonomy by careful polyphasic revisions at different taxonomic levels. Studied were selected interesting groups of mostly terrestrial cyanobacteria from specific habitats such as soils of hot deserts, steppes or high mountains and wet walls of various climatic regions.

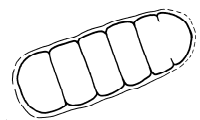
Terrestrial cyanobacteria often live in communities of microbial biofilms or soil crusts. These communities are usually composed of a unique mix of microorganisms (bacteria, microscopic fungi, algae and cyanobacteria), frequently functionally linked. Thus, isolation of a cyanobacterium and its purification to a strain is a lengthy process that requires great patience and precision. Despite this difficulty, work with unicyanobacterial strains remains the basis for their polyphasic characterization. For each of the papers presented in this thesis, novel cyanobacterial strains were isolated from natural samples. First of all, these strains were examined in a light microscope. Morphological characteristics were observed and recorded through the whole life cycle of the particular cyanobacterium. In general, this includes observations of a fresh culture (approx. up to 1 month old), aging culture (several observations within following 4 to 12 months depending on the strain) and dying culture. It is especially important for more complicated cyanobacteria, such as heterocytous types. Their life cycle starts by elongation and growth of filaments from hormogonia/arthrospores/akinetes, is followed by maturing of the filaments, formation of typical morphological features (e.g. heterocytes, branching, tapering, sheath etc.), releasing of new hormogonia, and terminates by formation and liberation of resting cells (e.g. arthrospores, akinetes). Such a complex sequence of the life cycle stages can hardly ever be observed from a natural population collected at one time. Detailed documentation including photographs and descriptions of the life cycles of the studied cyanobacteria was provided in **Paper I, III, IV, V and VI**. Original line drawings of the taxa under examination can be found in **Paper I, II and III**.

Morphological characterization of the strains was followed by sequencing of 16S rRNA gene. The acquired sequences provided information on the closest relatives available from a sequence database and were used for construction of phylogenetic trees (complemented with already published related sequences and sequences of other taxonomic groups). The results revealed the phylogenetic relationships of the tested material and were used as a basis for the taxonomic decisions. In some of the papers included in this thesis, also 16S-23S ITS region was sequenced and used to improve the accuracy of the phylogenetic placement. In **Paper IV**, a separate supplementary phylogenies were constructed from this region, whereas in **Paper III** and **Paper V**, 16S

rRNA was concatenated with the 16S-23S ITS to create a phylogeny of closely related taxa. Percent similarity of the 16S rRNA was computed for the majority of the new species and genera descriptions (**Paper III, IV, V, VI**). Comparison of the predicted secondary structures of the 16S-23S ITS was used as a supplementary criterion for description of new taxa in **Paper I, III, IV and V**. It was especially useful in recognition of cryptic species of *Oculatella* (**Paper III**) and *Roholtiella* (**Paper V**).

Less commonly used methods of cyanobacterial taxa characterization were performed in a part of the included studies. Analysis of ultrastructure using transmission electron microscope, revealing e.g. arrangement of thylakoids within the cells or a nature of the cell wall was accomplished in **Paper V and VII**. **Paper VII** also contains analysis of toxic compounds by HPLC-HRMS with positive result for cylindrospermopsin in the genus *Hormoscilla*, which was then confirmed by positive PCR amplification of the *cyrJ* gene. The presence of cellulose in all tested members of the family Gomontiellaceae was detected using Fourier transform infrared (FTIR). Visualization of the cellulose by Caucofluor white fluorescence dye staining revealed its localization in the cell walls (**Paper VII**).

Altogether, this thesis encompasses characterization of one previously poorly known and almost forgotten cyanobacterial family, description of two new genera and two new families belonging to a chaotic part of the order Nostocales, and description of 20 new species within six cyanobacterial genera. All of these taxonomic decisions were carefully discussed and suggestions for the continuation of the revisionary process were provided. Although this contribution is just a drop in the ocean of yet unresolved part of the cyanobacterial taxonomy, I believe the main aim of the thesis was achieved and the results of the presented papers set a good standard for future modern taxonomic revisions of such an important prokaryotic group.



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Education

- 2002-2006 **Bachelor study in biology (Bc.)**
Faculty of Biology, University of South Bohemia in České Budějovice,
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Bc. Thesis: *Aerophytic vegetation of the wet walls in CHKO Broumovsko, Czech Republic. [in Czech]*
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MSc. Thesis: *Cyanobacteria of wet walls and seeps in Grand Staircase-Escalante National Monument, Utah, USA.*
- 2011 **Rigorous exam (RNDr.)**
Faculty of Science, University of South Bohemia in České Budějovice,
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Faculty of Science, University of South Bohemia in České Budějovice,
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PhD. Thesis: *Towards a modern revision of the cyanobacteria, a critically important prokaryotic phylum.*

Scientific employment

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Conferences

- 2007 **NEAS** - 46th annual Northeast Algal Symposium, Narragansett, Rhode Island, USA
Poster: *Cyanobacteria of wet walls and seeps in Grand Staircase-Escalante National Monument, Utah, USA.*
- 2007 **IAC** - 17th Symposium of the International Association for Cyanophyte Research, Mérida, Yucatán, Mexico
Poster: *Cyanobacteria of wet walls and seeps in Grand Staircase-Escalante National Monument, Utah, USA.*
- 2010 **IAC** - 18th Symposium of the International Association for Cyanophyte Research, České Budějovice, Czech Republic
Poster: *Tapinothrix clintonii sp. nov. (Pseudanabaenaceae, Cyanobacteria), a new species at the nexus of five genera.*
- 2011 **CEDM** - 5th Central European Diatom Meeting, Szczecin, Poland
Poster: *Preliminary study of the diatom flora of the top of Mt. Roraima, Venezuela.*
- 2012 **Mikroskopie 2012**, Bratislava, Slovensko
Invited talk: *Algae and cyanobacteria - Who they are and how can electron microscopy help us in modern phycology.*
- 2012 IV International Conference «**Advances in modern phycology**», Kyiv, Ukraine
Talk: *Comparison of the molecular and morphological diversity within the family Scytonemataceae (Nostocales, Cyanobacteria).*
- 2012 **ISPP** - 14th International Symposium on Phototrophic Prokaryotes, Porto, Portugal
Poster: *Comparison of the molecular and morphological diversity within the family Scytonemataceae (Nostocales, Cyanobacteria).*
- 2013 **IAC** - 19th Symposium of the International Association for Cyanophyte Research, Cleveland, Ohio, USA
Talk: *Revision of a recently described pantropical genus Brasilonema Fiore et al. (Nostocales, Cyanobacteria);*
Poster: *Hormoscilla pringsheimii (Gomontiellaceae, Cyanobacteria), a characterization of an unusual taxon based on morphology, ultrastructure, biochemical properties and molecular data.*

International cooperation

- 2006, 2009, 2012 a month long fellowship at John Carroll University in Prof. J. R. Johansen's laboratory
- 2006-2008 Teaching Assistant at John Carroll University: Microbiology – laboratory, Principles of biology 1 – laboratory
- 2007-2008 Sigma Xi, The Scientific Research Society (associate member)

Publications

- Gomez, E. B., Johansen, J. R., Kaštovský, J., **Bohunická, M.** & Čapková, K. (submitted) *Macrochaete gen. nov.* (Cyanobacteria): the first step in solving the profligately polyphyletic genus *Calothrix*. *Journal of Phycology*.
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