Palacký University Olomouc

Faculty of Science

Department of Botany

Morphological and genetical variability of cyanobacteria

Ph.D. Thesis

Mgr. Petr Dvořák

Supervisor: Prof. RNDr. Aloisie Poulíčková, CSc.

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Declaration

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

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Mgr. Petr Dvořák

Abstract

Recent discoveries point to the significance of cyanobacteria as primary producers in diverse ecosystems. However, given their enormous diversity, the majority remain uncharacterized. In this thesis, I have investigated the morphological and molecular features of benthic and aerophytic cyanobacteria and explored the global spatial and temporal patterns of their dispersal within free-living cyanobacteria.

The taxonomy of *Nodularia sphaerocarpa*, *N. harveyana*, and *N. moravica* was investigated by a combination of 16S rRNA sequencing, AFLP and ecophysiological experiments using a gradient of salinity. Ecophysiological experiments revealed that *N. sphaerocarpa* together with *N. moravica* are more sensitive to higher salinity while, *N. harveyana* is less sensitive and its morphology (i.e. cell width) is more stable. The status of *N. moravica*, previously only described according to morphology, was confirmed by molecular data. In conclusion, there is convincing evidence for the importance of ecological parameters in the taxonomy of cyanobacteria.

Epipelic cyanobacteria of genera *Microcoleus*, *Phormidium* and *Geitlerinema* sampled from European lakes were investigated. *Microcoleus vaginatus* and *Phormidium autumnale* are a monophyletic complex of species which should be revised as neither belong to the original genus. *M. vaginatus* isolated from desert soil crusts clustered together with epipelic isolates based on 16S rRNA. Only 16S-23S ITS secondary structures could be used to differentiate between such dissimilar habitats. *P. formosum* was confirmed as a genuine species by molecular markers. Moreover, there may be two cryptic species. It had been suggested that genus *Geitlerinema* is polyphyletic. 16S rRNA phylogeny showed three groups spread within cyanobacteria. *G. splendidum, G. carotinusum,* and *G. pseudacutissimum* formed separate clusters but their original assignment to Pseudanabaenaceae was challenged because all studied species were closely related to Phormidiaceae.

The new genus *Johansenia* was derived from the epipelic genus *Komvophron* based on sequences of 16S rRNA and 16S-23S ITS obtained by Single Filament PCR optimized for epipelic cyanobacteria. The phylogeny revealed the position of *Johansenia* within Pseudanabaenaceae. Moreover, the validity of *K. hindakii* was confirmed but it clustered together with members of the Gomontiellaceae. In conclusion, the genus *Komvophoron* is a polyphyletic group which deserves more attention.

Global spatial and temporal dispersal patterns of *M. vaginatus* were characterized by phylogeny of 16S rRNA, 16S-23S ITS and molecular clock based dating. The first evidence for geographical isolation within free-living, non-extremophylic cyanobacteria on a continental level

was found. However, dating analysis revealed that the geographical barriers have not been permanent over time. Dating analysis proves to be a unique way of dating the divergence of cyanobacterial species.

Abstrakt

Sinice jsou významnými primárními producenty v široké škále akvatických i terestrických ekosystémů. Nicméně diverzita sinic je natolik rozsáhlá, že většina z ní zůstává nepopsaná. V této dizertační práci byla zkoumána morfologická a molekulární diverzita bentických a aerofytických sinic. Navíc byly zkoumány globální prostorové a časové změny v rozšíření volně žijících sinic.

Kombinace sekvenovaní 16S rRNA, AFLP analýzy a ekofyziologických experimentů v gradientu salinity byla využita k získání nových poznatků z taxonomie druhů *Nodularia sphaerocarpa, N. harveyana,* a *N. moravica*. *N. sphaerocarpa* a *N. moravica* mají podle ekofyziologických experimentů vyšší senzitivitu ke zvýšené úrovni salinity. Oproti tomu *N. harveyana* je méně citlivá a navíc morfologie (šířka buňky) je v gradientu salinity stabilnější. *N. moravica* byla původně popsána pouze na základě morfologie. Validita tohoto druhu byla potvrzena s použitím molekulárních dat. Ekofyziologické experimenty také poskytly přesvědčivý důkaz o důležitosti ekologických parametrů v taxonomii sinic.

Byly analyzovány epipelické sinice rodů *Microcoleus, Phormidium* a *Geitlerinema* izolované z evropských jezer. *Microcoleus vaginatus* a *Phormidium autumnale* tvoří monofyletický komplex druhů, který by měl být podroben revizi, protože ani jeden z těchto druhů nenáleží do původního rodu. Kmeny *M. vaginatus* izolované z pouštních krust náležely do stejného kladu s epipelickými izoláty na základě analýzy 16S rRNA. Kmeny bylo možné rozlišit pouze na základě sekundárních struktur v 16S-23S ITS. Druh *P. formosum* byl potvrzen s použitím molekulárních dat. Navíc byly v rámci *P. formosum* klastru identifikovány dva pravděpodobně kryptické druhy. Rod *Geitlerinema* byl již v minulosti shledán polyfyletickým. Toto bylo potvrzeno fylogenetickou analýzou 16S rRNA, kde byly nalezeny tři separátní linie v rámci sinic. *G. splendidum, G. carotinusum,* a *G. pseudacutissimum* tvořily oddělené linie, které byly blízce příbuzné k čeledi Phormidiaceae, ačkoliv byly původně řazeny do Pseudanabaenaceaea.

Pomocí sekvencí 16S rRNA a 16S-23S ITS získaných Single Filament PCR optimalizovaných pro epipelické vzorky byl oddělen nový rod *Johansenia* od rodu *Komvophoron*. *Johansenia* náleží na základě fylogenetické analýzy do čeledí Pseudanabaenaceae. Původně morfologický popsaný druh *K. hindakii* byl potvrzen fylogenetickou analýzou 16S rRNA, podle které náležel k členům čeledi Gomontiellaceae. Rod *Komvophoron* je tedy polyfyletická skupina zasluhující větší pozornost.

Fylogenetická analýza a molekulární hodiny založené na 16S rRNA a 16S-23S ITS byly využity na charakterizování globálních prostorových a časových bariér rozšíření *M. vaginatus*.

Tímto byl získán první důkaz pro existenci geografické izolace u volně žijících neextrémofilních sinic. Nicméně geografické bariéry neměly permanentní charakter. Navíc tato studie přináší unikátní vyobrazení datování divergence druhů u sinic.

Contents

1. Introduction

Cyanobacteria (Cyanophyta, Cyanophyceae, Cyanoprokaryota, Blue-Green Algae) represent a specific group of Gram-negative bacteria which evolved very early in the earth's history. It is morphologically, physiologically, and genetically a diverse group of organisms inhabiting almost all aquatic and terrestrial environments (Castenholz 2001). The most courageous estimates of the age of the oldest cyanobacteria fossils are 3.3 – 3.5 billion years. These fossils were excavated in the Apex Cherts of the Warrawoona Group in Western Australia (Schopf & Packer 1987), but the authenticity of the fossil record has been re-examined several times. In fact, the microfossils may be secondary artifacts within graphite rock (e.g. Brasier et al. 2002). Tomitani et al. (2006) synthesized molecular, physiological, paleontological, and geochemical data and proposed origin of heterocysts and akinetes between 2.45 to 2.1 billions years. Undoubtedly, cyanobacteria are the oldest known autotrophic organisms inhabiting the Earth.

Owing to the unique features of their primary metabolism, particularly oxygenic photosynthesis and the ability to actively fix atmospheric nitrogen, cyanobacteria have substantially transformed the global ecosystem during evolution (Kopp et al. 2005). The great oxidation event happened between 2.45 and 2.22 billion years ago (Bekker et al. 2004). An increase in the oxygen concentration resulted in evolution of the oxygen based life we know today.

Apart from important primary metabolism, cyanobacteria produce a large variety of functional secondary metabolites. These compounds are predominantly known for their toxicity to humans. Cyanotoxins are mainly produced by cyanobacteria which form water-bloom due to eutrophication of freshwater and saline aquatic environments (Carmichael 1992). They show very variable biological activity – e.g. anticancer, antibacterial, and antiviral and hence their potential use is as pharmaceuticals which target diverse diseases (Singh et al. 2011).

1.1. Taxonomy of cyanobacteria: an overview

Cyanobacterial diversity was firstly explored during the 19th century when cyanobacteria were recognized as a separate group of organisms. The classification was based entirely on morphology of isolated strains and specimens. The most important morphological traits were cell dimension, cell/filament morphology, type of cell division and presence of sheath/envelope (e.g. Bornet & Flahault 1886-1888, Gomont 1892, Geitler 1932). This trend continued to the second half of the 20th century. From that time, molecular markers (especially 16S rRNA) have revolutionized cyanobacterial systematics. However, it should be emphasized that the importance

of morphological features is eminent. For this reason, a combination of morphological, ecological and molecular data was used and this led to the concept of "polyphasic approach" which is today the most respected route to the practical determination and description of cyanobacterial taxa (e.g. Johansen & Casamatta 2005, Siegesmund et al. 2008, Komárek 2010). The existence of most of the traditional genera revised by Geitler (1932) was confirmed by molecular studies. At the same time, the majority of cyanobacteria particularly at a species level are uncharacterized. This is underlined by the number of newly described species (e.g. Komárek 2010). Traditionally, cyanobacterial taxa were described according to the International Code of Botanical Nomenclature due to their original classification as plants. Later, application of the International Code of Bacteriological Nomenclature was applied which stressed cyanobacterial evolutionary relations to bacteria. However, a simple transfer of taxa from botanical to bacteriological code is problematic as cyanobacteria possess unique evolutionary, ecological, and physiological features (Komárek 2010, Komárek 2011). No consensus of nomenclature has been universally accepted even after the attempts of the IAC (The International Association of Cyanophyte/Cyanobacteria Research) Symposia (Komárek 2011).

A basic taxonomical unit in the systematics of cyanobacteria is species but there are numerous species concepts that attempt to define a species both theoretically and practically (Johansen & Casamatta 2005). Nonetheless, the evolution of cyanobacteria is shaped from different driving forces than eukaryotes and cyanobacteria are incapable of sexual reproduction. Thus the Biological Species Concept *sensu* Mayr (1969), which is generally used for sexually reproducing macroorganisms, cannot be applied. Johansen & Casamatta (2005) recommended use of the Monophyletic Species Concept *sensu* Mishler & Theriot (2000), formerly Autapomorhpic Species Concept. This concept defines a species as the smallest monophyletic group which can be delimited by recognizable autapomorphies. The authors also suggested following practical criteria for defining cyanobacterial species. The crucial point is finding evidence of separation from present species which may be accomplished by (1) characterizing morphological differences, (2) genetic distance in 16S rRNA sequence, (3) differences in 16S-23S ITS secondary structures, (4) biochemical dissimilarity (composition of secondary metabolites), and (5) ecophysiological characteristics predominantly defined by biotope of studied strain.

In the light of recent research, the real biodiversity is underestimated using the criterion of morphological variability. There are numerous examples of species entities which are morphologically indistinguishable but do not share a common evolutionary history, i.e. their molecular phylogeny is more diverse than morphological. This discrepancy resulted in the concept of "cryptic species" complexes which often occur in cyanobacteria (e.g. Boyer et al.

2002, Casamatta et al. 2003, Siegesmund et al. 2008).

The definition of a cyanobacterial species is still blurred. However more confusing is delimiting higher taxonomical units. The often accepted system described in Bergey's Manual of Systematic Bacteriology (Castenholz 2001) separates cyanobacteria into 5 groups based on thallus characteristics. The first two groups possess coccal thallus; the third group is filamentous, non-heterocytous; the fourth group is filamentous with heterocysts; and the fifth group is filamentous with heterocysts and true branching. Such distinctive morphological features, may give the impression of stability during evolution. However, these groups apart from the fourth and fifth have a polyphyletic origin. It follows that multicellularity and also unicellularity have evolved several times during evolution (Shirrmeister et al. 2011).

1.2. Molecular markers used in taxonomy of cyanobacteria

Analysis of DNA (protein) sequences and other molecular markers have become key methods for understanding the evolution of organisms. A similar trend evolved in the molecular systematics and population genetics of cyanobacteria (e.g. Giovanonni et al. 1988, Boyer et al. 2001, Castenholz 2001, Komárek 2010). The most widely used gene is 16S rRNA (SSU) which codes small ribosomal subunits. The significance and expansion of 16S rRNA is shown in the statistics for number of 16S rRNA sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/) and processed by RDP (Ribosomal Database Project; http://rdp.cme.msu.edu/). The RDP database provides cohesive and regularly updated ribosomal related data, mainly 16S rRNA sequences of bacteria and archea (Cole et al. 2009). Using the RDP tool Browser, there are 57 040 16S rRNA sequences assigned to cyanobacteria from a total number of 2 639 157 sequences (accessed on 8th December 2012). If rough comparison with other sequence markers is made, we find that they are used to less extent. For instance 13 096 sequences of 16S-23S ITS (Internal Transcribed Spacer), are deposited, or 1 149 sequences (NCBI database accessed on 8th December 2012) coding rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit).

The 16S rRNA gene is reliable, generally accepted, and possesses a vast number of sequence for comparison, but several difficulties arose in interpreting the phylogeny. Some doubts regarding using 16S rRNA were raised very early after the expansion of molecular methods in the taxonomy of bacteria. Fox et al. (1992) compared the similarity of some bacterial genomes based on DNA-DNA hybridization to the similarity of 16S rRNA sequences from the same strains. They showed that similarity between genomes does not always correlate with similarity between 16S rRNA sequences. One extreme example may be illustrated: *Serpula hyodysenteriae*

B78 compared to *Serpula innocens* 4171 displayed 40% similarity in DNA-DNA hybridization and 99.1% similarity between 16S rRNA. It should be mentioned that the limit for separation of species by DNA-DNA hybridization is 70% and by 16S rRNA 97.5%. More exactly expressed, when the 16S rRNA sequence similarity is smaller than 97.5%, the similarity by DNA-DNA hybridization is smaller than 70% (Stackerbrandt & Goebel 1994). In conclusion, these exact limits should be considered more arbitrarily as otherwise species would be defined as a phenetic construct which is not an appropriate species definition (Johansen & Casamatta 2005).

Another useful marker in taxonomy of cyanobacteria is 16S-23S ITS region. It is an internally transcribed spacer between genes for small (16S) and large (23S) ribosomal subunit. Its applicability in taxonomy and population genetics was tested in Boyer et al. (2001). 16S-23S ITS region is worth applying under the species level in population genetics because it possesses sufficient variability. This has the advantage over use of the 16S rRNA which seems to have more suitable resolution for genus level or above. Sequences of 16S-23S ITS region are used for reconstruction of phylogenetic trees or for comparison of RNA secondary structures among studied strains (Boyer et al. 2001, Boyer et al. 2002, Perkerson et al. 2010, Perkerson et al. 2011, Siegesmund et al. 2008).

A combination of several molecular markers should be used for adequate phylogenetic resolution. MLST (Multilocus Sequence Typing) was developed for typifying pathogenic bacterial strains (Maiden et al. 1998). Generally, MLST requires use of several housekeeping genes (ribosomal operon, circadian genes, and cytochrome b6) together. This approach was also found suitable for cyanobacteria. An investigation of population structure and recombination of *Microcoleus* (*Coleofasciculus*) *chthonoplastes* based on MLST is described in Lodders et al. (2005). Similarly, Acinas et al. (2009) studied Spanish and Baltic populations of *Pseudanabaena* strains and confirmed a multilocus approach as suitable for cyanobacteria. They also used genes connected to photosynthesis specific for cyanobacteria, e.g. phycocyanin operon.

1.3. Some problematic generic/species complexes in this study

1.3.1. Benthic *Nodularia*

The genus *Nodularia* Mertens ex Bornet & Flahault is composed of heterocystous cyanobacteria which mainly inhabit saline benthic and planktonic habitats (e.g. Laamanen et al. 2001, Lyra et al. 2005). The most widely studied species is planktonic bloom-forming *N. spumigena* which cause heavy toxic blooms in the Baltic Sea (e.g. Bolch et al. 1999). Benthic species are largely overlooked (Lyra et al. 2005). Geitler (1932) recognized only two species of *Nodularia* based on filament width – *N. harveyana* was composed of species with narrower

filaments (<8 μm) and *N. spumigena* had filaments wider than 8 μm. Similarly, Nordin & Stein (1980) also identified only these two species. They explained wide variability in morphology of *N. harveyana* in their experiments as the effect of salinity and pH. Subsequently, genus *Nodularia* was divided into two groups based on the presence or absence of gas vacuoles – a group of planktonic species with gas vacuoles and a group of benthic species without gas vacuoles (Komárek et al. 1993, Hindák et al. 2003). They recognized altogether 5 benthic species including a new species *N. moravica* (Hindák et al. 2003) which was described using the morphological approach. Laamanen et al. (2001) and Lyra et al. (2005) revised the genus *Nodularia* using different molecular markers but they studied only *N. spumigena*, *N. sphaerocarpa*, and *N. harveyana*. They confirmed separation of planktonic *N. spumigena* from benthic species by examining strain toxicity (nodularin production) which showed that the benthic strains are unable to produce toxins. They also hypothesized that there may be two cryptic lineages within *N. harveyana*. However, they did not find any ecological features which could rigorously separate *N. harveyana* and *N. sphaerocarpa*.

1.3.2. Genera *Phormidium* and *Microcoleus*

Phormidium Kützing ex Gomont is filamentous, non-heterocytous cyanobacterium which represents a morphologically very diverse genus composed of more than 100 species. Considering morphological features of the terminal part of the filament, *Phormidium* is divided into 8 groups (Komárek & Anagnostidis 2005). Since there is very extensive morphological variability within the genus, it is not surprising that it has been found polyphyletic and composed of several separate species using sequences of 16S rRNA and 16S-23S ITS (Marquardt & Palinska 2007, Palinska & Marquardt 2008, Siegesmund et al. 2008). Recently, genus *Phormidium* has been partly revised mostly by establishing new genera from previously identified polyphyletic lineages.

Turicchia et al. (2009) identified the genus *Phormidesmis* (including species *P. molle*) which was previously assigned to *Phormidium molle*. 16S rRNA based phylogeny and thylakoid arrangement showed close relationship to the family Pseudanabaenaceae. Shortly afterward, Komárek et al. (2009) confirmed the existence of the genus *Phormidesmis* and described another species – *P. priestleyi*.

The Antarctic cyanobacterium *Phormidium murrayi* forms separate lineage and it was described as new genus *Wilmottia murrayi* related to the family Pseudanabaenaceae (Strunecký et al. 2011).

Genus *Microcoleus* Desmazieres ex Gomont was partly revised by establishing a new

genus *Colefasciculus* with one species *C. chthonoplastes* (Siegesmund et al. 2008) which frequently occurs in the litoral zone of oceans and seas worldwide. Same authors also showed that the genus *Microcoleus* is polyphyletic. *M. vaginatus* in particular, based on 16S rRNA phylogeny, belongs to the family Oscillatoriaceae. *M. vaginatus* also share the same evolutionary lineage with *Phormidium autumnale* which probably places them in a different new genera out of both *Phormidium* and *Microcoleus*. *M. vaginatus* and *P. autumanale* share similar morphological characteristics. Thus they are almost undistinguishable except that *M. vaginatus* exhibits multiple filaments in a common sheath. Nevertheless, this feature disappears during culturing or it is not often present even in natural samples. The only absolute identifying character is 11-bp insert in 16S rRNA of *M. vaginatus* (Garcia-Pichel et al. 2001, Boyer et al. 2002).

1.3.3. Genus *Geitlerinema*

Geitlerinema (Anagnostidis et Komárek) Anagnostidis was established by a separation of some species of *Phormidium* and *Oscillatoria* and assigned to the family Pseudanabaenaceae (Anagnostidis 1989). It is non-heterocystous, filamentous cyanobacterium with peripheral arrangement of thylakoids, with thin (<4 μm) and motile filaments (Komárek & Anagnostidis 2005). *G. splendidum* and *G. amphibium* often occur in the epipelic assemblages of lakes (Hašler et al. 2008). However, the taxonomy of *Geitlerinema* has not yet been properly revised based on molecular data. Perkerson et al. (2010) studied 5 strains of *Geitlerinema* sp. and recognized 4 different polyphyletic lineages. Only one of them actually belonged to the former genus *Geitlerinema sensu* Anagnostidis (1989). Thus revision of the genera looks inevitable.

1.3.4. Genus *Komvophoron*

Komvophoron (Skuja) Anagnostidis et Komárek is a genus of filamentous cyanobacteria which are characteristic for muddy or sandy sediments or less often for thermal springs (Komárek & Anagnostidis 2005). *Komvophoron* was established by Anagnostidis & Komárek (1988) and it was placed in the family Borziaceae. It is an overlooked genus. I found only 8 papers in the Web of Knowledge (5th December 2012) under the term "*Komvophoron*". The reason for the paucity of publications is probably the rarity of *Komvophoron* and impossibility of establishing a culture (Hašler & Poulíčková 2010). These authors have done the most extant revision of the *Komvophoron*. They described a new species *K. hindakii* which is morphologically similar to the type species *K. schmidlei*. This work also summarizes rare floristic data on the occurrence of *Komvophoron* in the Czech Republic. The genus has been overlooked because of its occurrence in epipelon (Hašler et al. 2008).

1.4. Ecology of benthic and aerophytic cyanobacteria

Cyanobacteria have acquired remarkable adaptations to the most diverse aquatic and terrestrial environments during evolution. They inhabit all latitudes from tropical to polar and all altitudes from high mountains to lowlands. They survive the most severe conditions of deserts, hot springs, and hypersaline ecosystems. Moreover, cyanobacteria are partner in symbiosis with other organisms (Whitton & Potts 2000).

1.4.1. Microbial mats: a general view

A microbial mat is a community composed of a large variety of prokaryotic and eukaryotic microorganisms which often create a laminated, coherent, "mat" like structure growing on different substrates (aquatic and terrestrial). For instance, microbial mats may be found in intertidal zones, hot springs, freshwater sediments, and soil crusts. Moreover, as mats are also termed benthic microbial communities although their structure is less coherent (e.g. epipelon). The crucial group, which often creates a microbial mat, is the filamentous cyanobacteria. A typical laminated structure is composed of several layers. A surface layer is usually mucilaginous rich in scytonemin which has a protective function (against UV radiation). Right under the surface layer are cyanobacteria which may be accompanied by diatoms. Finally, under the cyanobacterial layer, there are several bacterial layers composed of purple sulphur and green sulphur bacteria (Stal 2000). The deepest layer is composed of sulphide-reducing bacteria. However, these bacteria may be present throughout the whole mat (Visscher et al. 1992).

1.4.2. Cyanobacterial mats of saline environments

Cyanobacteria often dominate microbial mats in environments with higher salinities. The most hostile saline habitats, where cyanobacteria occur, are hypersaline lagoons, solar lakes and hypersaline sulphur springs. However, even there they possess significant cyanobacterial biomass (Javor 1989).

Halophylic communities are often dominated by filamentous *Microcoleus* (*Coleofasciculus*) *chthonoplastes* and coccoid cyanobacterium *Aphanothece halophytica*. *C. chthonoplastes* has a very large range of halotolerance. It occurs in low salinity brakish water (Baltic Sea; e.g. Lodders et al. 2005), but also in salterns where the salinity may exceed 30% (Zavarzin et al. 1993). The existence of several cryptic species is presumed because of such a high halotolerance (Siegesmund et al. 2008). *C. chthonoplastes* is physiologically well-adapted

for hypersaline conditions.

Brakish environments, like river estuaries, are inhabited by different species of genus *Nodularia*. Either planktic (*N. spumigena*) or benthic (e.g. *N. sphaerocarpa*, *N. harveyana*) may be found. Benthic species are characterized by the absence of gas vacuoles. They are able to glide and they are non-toxic (Komárek et al. 1993, Laamanen et al. 2001, Lyra et al. 2005). Moreover, benthic *Nodularia* species are able to grow in freshwater conditions, i.e. they are not obligate halophylic cyanobacteria (Nordin & Stein 1980).

1.4.3. Epipelic communities

An assemblage of microorganisms associated with fine muddy or organic sediment is called an epipelon. Epipelic communities are usually present in litoral zones of lakes (Poulíčková et al 2008). Muddy sediments are efficiently sampled using the method of Round (1953) who suggested glass tubes for sucking in sediment by negative pressure. Autotrophic organisms of epipelon are dominated by cyanobacteria and diatoms. Although the epipelon is investigated less intensively than plankton, some studies have revealed their key importance for lake environments. For example, Wetzel (2001) reported that epipelic communities of some Alaskan ponds have 6-10 times higher primary production than phytoplankton.

Cyanobacteria may represent 20-80% of autotrophic epipelic communities. Hašler et al. (2008) analyzed in detail 45 sediments from ponds across the whole trophic gradient. They identified 39 species of cyanobacteria in the epipelic assemblage. The majority of identified species were characteristic just for epipelon.

Špačková et al. (2009) investigated the seasonal succession of epipelic algae in the mesotrophic pond Bezednik (Czech Republic). They suggested that the composition of different algal groups varies during the year. Diatoms predominated in spring and autumn. On the other hand, cyanobacteria and euglenophytes dominated during the summer. Thus it actually partly resembles the dynamics of a lake phytoplankton.

Due to a specific condition within lake sediments, some unusual taxa of cyanobacteria occur in epipelons. For instance, the genus *Komvophoron* is almost exclusively found in epipelon. Moreover, a new species *K. hindakii* was described in Czech and British ponds (Hašler et al. 2008, Hašler & Poulíčková 2010).

1.4.4. Soil communities

Cyanobacteria are important primary producers within surface soil and the layers beneath it (Whitton 2000). A species composition of cyanobacterial soil community is influenced by light,

moisture, pH, mineral nutrients, and combined nitrogen (Granhall 1975). A soil community is mostly composed of filamentous heterocystous or non-heterocystous cyanobacteria which are frequently motile. Taxonomically, a community is composed usually of e.g. *Microcoleus vaginatus*, *Nostoc*, *Phormidium*, *Calothrix*, *Leptolyngbya* (Whitton 2000, Garcia-Pichel et al. 2001, Boyer et al. 2002, Komárek & Anagnostidis 2005). *Microcoleus vaginatus* is distributed world-wide and it inhabits the soil crust of deserts (Boyer et al. 2002) to high moutain soil in the Himalaya (Řeháková et al. 2011). It builds large biomass on soil surface or it is a part of microbial crust (Boyer et al. 2002, Řeháková et al. 2011).

1.5. Biogeography of cyanobacteria

Microorganisms have small dimensions and enormous dispersal abilities which differentiate them from macroorganisms. For this reason, the potential structuring of geographical barriers and distributional patterns differs significantly (Martiny et al. 2002). A study of microbial biogeography can provide important information on global epidemiologies in humans, animals and plants and, identification of areas where beneficial bacteria prosper in the environment etc. Moreover, the study of biogeography would enhance knowledge of global patterns of the diversity and evolution of bacteria (Ramette & Tiedje 2006).

The first widely accepted description of the distribution of microorganisms was published by Baas Becking (1934). He summarized microbial distributional patterns (biogeography) under the following famous tenet which may be translated from Dutch as follows: "Everything is everywhere, but, the environment selects". He considered all microbial species as ubiquitous. Their distribution is only restricted by local environmental factors. Finlay (2002) claimed ubiquity for all organisms smaller than 1 mm. For these reasons, allopatry cannot play any important role in speciation. However, his observations were based only on morphological identification of species.

Recent molecular evidence has provided new insights into the study of biogeographical patterning in cyanobacteria. Some studies confirmed Baas Becking's principle and some showed that biogeography may exist on a species level (see review of Ramette & Tiedje 2006). Jungblut et al. (2010) studied the differences between arctic and antarctic cyanobacterial communities based on analysis of 16S rRNA. Polar regions represent ideal areas for studying the distribution of microorganisms as they are geographically remote, sharing vast dispersal barriers, and very similar climate characteristics (Staley & Gosink 1999). Jungblut et al found that there are many almost identical (99.9% similarity in 16S rRNA) phylotypes in both polar regions. Moreover, they disproved the endemicity of some of the antarctic species proposed by Taton et al. (2006),

e.g. *Phormidium priestleyi* and *Leptolyngbya frigida*.

Microcystis aeruginosa are bloom-forming, toxin producing cyanobacteria that are distributed world-wide (Huisman et al. 2005). Van Gremberghe et al. (2011) analyzed the global distributional patterns of this species using the 16S-23S ITS marker which has high resolution at a species and population level (Boyer et al. 2001). Parsimony network revealed no particular dispersal pattern. For this reason, the authors assume that gene flow between populations is common and local events of bottleneck and selective sweep drive speciation. Very similar results were reported by Garcia-Pichel et al. (1996 and 2001) who selected *Microcoleus chthonoplastes* (formerly *Coleofasciculus chthonoplastes*, see Siegesmund et al. 2008) and *M. vaginatus* as models to study dispersal patterns. They also found no biogeographical pattern within these species. However, their analyses were based only on DGGE (Denaturing Gradient Gel Electrophoresis) and sequences of 16S rRNA which may not have sufficient resolution under the species level.

On the other hand, some distributional patterns were identified in thermophylic cyanobacteria. Papke et al. (2003) described biogeographical patterns in cyanobacterium *Synechococcus* spp. on a continental scale. The strains were isolated from 48 hot springs and showed correspondence between phylogeny of 16S rRNA and geographical origin. This study also provided evidence for the existence of allopatric speciation within cyanobacteria. Resembling pattern revealed investigation of stigonemetalean cyanobacterium *Mastigocladus laminosus* (Miller et al. 2007).

2. Aims

The principal goal of this thesis was to investigate filamentous cyanobacteria from different types of benthic and aerophytic microbial mats based on morphological and genetic variability (polyphasic approach). Second, using appropriate molecular markers to challenge recent taxonomical findings, leading to taxonomical revision. Third, this thesis also focused on finding spatial and temporal patterns in global cyanobacterial distribution. Particular aims are listed in points below:

- investigate taxonomical relationships within some benthic representatives of the genus *Nodularia* using the polyphasic approach
- explore molecular and morphological diversity of some epipelic cyanobacteria
- revise the genus *Komvophoron* using molecular markers and the Single Filament PCR approach
- challenge acceptance of the ubiquity within non-extremophylic cyanobacteria below the species level and reconstruct the temporal dimensions of the cyanobacterial evolution using molecular clocks.

3. Conclusions and future prospects

In this thesis, I have explored the morphologic and genetic variability of cyanobacteria inhabiting benthic and aerophytic habitats, and revised the taxonomy using the polyphasic approach. Further, I have found evidence contradicting the assumption of universal ubiquity within prokaryotes and dated evolution of some cyanobacterial species using molecular clocks.

3.1. Polyphasic characterization of the benthic *Nodularia*

Benthic representatives of the genus *Nodularia* typically occur in brakish environments like the Baltic Sea or river estuaries (e.g. Laamanen et al. 2001, Lyra et al. 2005), and also in different freshwater habitats with low salinity (Komárek et al. 1993, Hindák et al. 2003). Paper I is focused on a polyphasic characterization of *N. sphaerocarpa* isolated from an epipelic sample in Olomouc, *N. moravica* (type strain provided by Prof. F. Hindák), and another *N. sphaerocarpa* and *N. harveyana* obtained from culture collections. Halotolerance and changes in morphology were assessed in a gradient of salinity. AFPL (Amplified Fragment Length Polymorphism) and sequence of 16S rRNA were analyzed. *N. harveyana* exhibited higher tolerance to salinity in comparison to *N. sphaerocarpa* and *N. Moravica* which appears to be better physiologically adapted to freshwater environments. Moreover, *N. harveyana* was morphologically more stable (i.e. vegetative cell, heterocyst, and akinete width) across salinity gradients than *N. sphaerocarpa*. The importance of salinity to the taxonomy of *Nodularia* was suggested by Nordin & Stein (1980). However, these authors recognized only two species – *N. spumigena* and *N. harveyana*. Paper I showed that there are more benthic species of *Nodularia* with different species specific ecological demands. These species were confirmed by AFPL and 16S rRNA analyses. *N. moravica* was revised and the validity of species was confirmed. In conclusion, salinity has a strong physiological effect and is an important factor which should be considered in taxonomy. In addition, molecular markers are congruent with ecophysiology of strains. *N. sphaerocarpa*, *N. harveyana* and *N. moravica* were separated based on different halotolerace, morphology changes in salinity gradients and molecular markers. It was also shown that AFPL analysis offered advantages as a molecular marker of use in the taxonomy of cyanobacteria.

3.2. Morphological and molecular diversity of some common epipelic cyanobacteria

Genera *Phormidium*, *Microcoleus* and *Geitlerinema* frequently occur in the epipelon (Hašler et al. 2008). All of these genera were found to be composed of a few polyphyletic or cryptic lineages (Boyer et al. 2002, Siegesmund et al. 2008, Perkerson et al. 2010). I investigated

following species: *P. autumnale*, *P. formosum*, *M. vaginatus*, *G. splendidum*, *G. carotinosum*, and *G. pseudacutissimum* (Paper II).

P. autumnale and *M. vaginatus* are a complex morphologically almost undistinguishable species. This complex has been studied (Boyer et al. 2002, Siegesmund et al. 2008), but the strains were isolated mostly from deserts of aerophytic habitats where these species usually occur (Komárek & Anagnostidis 2005). Paper II presents the morphological and molecular characteristics of epipelic representatives of this complex. *P. autumnale* and *M. vaginatus* differed only slightly in filament width and *M. vaginatus* usually have prominent intracellular granules (Paper II). *M. vaginatus* should however possess fasciculated filaments in a commons sheath, but this feature disappears in culture and it is often not even present in natural populations (e.g. Boyer et al., Paper II). The only reliable distinguishing character is 11-bp insert within 16S rRNA of *M. vaginatus* proposed by Garcia-Picher et al. (2001). The reliability of the 11-bp insert as *M. vaginatus* apomorphy has been confirmed several times (Boyer et al. 2002, Siegesmund et al. 2008, Paper II, Paper IV). Phylogenetic analysis of 16S rRNA constructed using Bayesian inference confirmed the monophyletic origin of *M. vaginatus* and *P. autumnale* complex. Moreover, close relation to the family Oscillatoriaceae has been shown (Paper II, Paper IV), while *P. autumnale* should have originally belonged to the family Phormidiaceae (Komárek & Anagnostidis 2005). This conclusion is similar to phylogenetic analysis performed in Siegesmund et al. (2008). Epipelic strains of *M. vaginatus* had very close positions in the phylogenetic tree to the aerophytic strains isolated from desert crusts. A significant difference was found only based on analysis of secondary structures within 16S-23S ITS. This fact once again confirmed the advantages of the 16S-23S ITS marker in the taxonomy of cyanobacteria and the importance of combining different molecular markers. Moreover, such remarkable ecological and geographical variability indicates the existence of some cryptic species inside this lineage (Paper II, Paper IV).

P. formosum occurs very frequently in epipelic community (Hašler et al. 2008), but it has not been studied using molecular markers so far. Altogether 5 strains were isolated and analyzed in Paper II. The morphology was in agreement with the latest description in Komárek & Anagnostidis (2005). 16S rRNA phylogeny revealed the common evolutionary origin of all strains. Moreover, they were separated into two groups. The first contains strains isolated in Bohemia and second in Moravia. This fact was also confirmed by analysis of 16S-23S ITS. However, the Moravian lineage also contained *P. animale* SAG 1459/6 isolated in the United Kingdom. We suggest reidentification of this strain. In conclusion, *P. formosum* is a valid species whose existence was confirmed by polyphasic approach, but it is probably composed of two cryptic species (Paper II).

Different representatives of the genus *Geitlerinema* were investigated in Paper II. Although it is a widely distributed cyanobacterium, there is no comprehensive study of its molecular variability (Meyers et al. 2007, Bittencourt-Oliveira et al. 2009, Perkerson et al. 2010). 16S rRNA based phylogeny (Paper II) revealed the polyphyletic origin of the *Geitlerinema*. This was pointed out by Perkerson et al. (2010). One lineage characterized by ultrastructure (Anagnostidis 1989), clustered with family Pseudanabaenaceae. The other two lineages were more closely related to the family Phormidaceae and were investigated in Paper II Therefore the evolutionary origin of this genus is polyphyletic. The first, Phormidiaceae related lineage was *G. splendidum* which had a distinctive monophyletic lineage in 16S rRNA phylogeny. Moreover, the morphology, especially the shape of the terminal cell and 16S-23S ITS structures differed significantly from other *Geitlerinema* species. The second Phormidiaceae related lineage was composed of *G. carotinosum* and *G. pseudacutissimum*. These species are very similar, thin filamentous, with prominent caroteniod granules. Only *G. pseudacutissimum* possesses fasciculated, *Microcoleus*-like thallus. 16S rRNA and 16S-23S ITS structures reliably distinguished these species and confirmed the original description of the species of Geitler (1956). Interestingly, *G. carotinosum* has been until now only found at the type locality and in surroundings though *G. pseudacutissimum* is more widely distributed (Italy, Czech Republic).
The 16S rRNA phylogeny showed *W. murrayi* lineage clustering with the *Microcoleus steenstrupii* and *Colefasciculus chthonoplastes*. Although some authors assign their position to Pseudanabaenaceae (Strunecký et al. 2011), our results show that these genera clearly belong to the family Phormidiaceae. I think, that the difference is due to insufficient taxa sampling for phylogenetic analysis and samples containing only *Geitlerinema* representatives from only one existing lineage (Paper II).

3.3. Molecular diversity of *Komvophron* based on Single Filament PCR

Besides the genera discussed above, epipelic communities are characterized by different species of *Komvophoron* which are insufficiently characterized by molecular markers mainly because *Komvophoron* strains do not grow in cultures (Hašler et al. 2008, Hašler & Poulíčková). Turicchia et al. (2009) described two new species, *K. apiculatum* and *K. rostratum* from alkaline marches of northern Belize. We have doubts about these species. Unfortunately, they cannot be re-investigated, because their sequences are not deposited in the GenBank and cultures are not available either (J. Komárek, pers. com.). Moreover, their phylogenetic position and affiliation to the genus *Komvophoron* is based on a false interpretation of GenBank sequence. Under accession number AF355398 is deposited *Leptolyngbya schmidlei* (Johansen, pers.com.) not *K.*

Schmidlei. There are only three, short sequences for *Komvophoron* in GenBank (searched on the 13th December 2012). Paper III pioneers the taxonomy of *Komvophoron* using Single Filament PCR which was specially modified for epipelic samples. The Single Filament PCR was used because *Komvophoron* species have not been successfully cultivated (Hašler & Poulíčková 2010). 16S rRNA and 16S-23S ITS sequences of *K. hindakii* and *K. constrictum* were obtained and the morphological variability of natural populations was characterized. A synthesis of morphological features with phylogeny of the 16S rRNA sequences led to a partial revision of the genus *Komvophoron* as it currently remains. Sequences were separated into two lineages. *K. hindakii* formed monophyletic lineage which clustered with *Hormoscilla pringsheimii* and with *Crinalium* spp. Thus the validity of species description was confirmed. This also means that *K. hindakii* belongs more likely to the family Gomontomelliaceae, than to Borziaceae Anagnostidis & Komárek (1988).

The second lineage was formed by two *K. constrictum* morphotypes which are closely related to the family Pseudanabaenaceae. This led to description of a new genus *Johansenia* with two species – *J. constricta* and *J. pseudoconstricta*. The molecular data are congruent with morphology in this case. In conclusion, the evolutionary history of *Komvophoron* is polyphyletic.

3.4. A phylogeography of the *Microcoleus vaginatus*

Since Baas Becking's (1934) famous microbial evergreen was published, molecular analyses of globally distributed microorganisms have provided a plethora of evidence proving and disproving the hypothesis. However, evidence confirming the assumption of ubiquity even in cyanobacteria prevails. The most recent analysis of the global population of a freshwater cyanobacterium *Microcystis aeruginosa* revealed random distribution of globally distributed ITS genotypes (van Gremberghe et al. 2011). Paper IV contributes to this discussion with an analysis of the spatial and temporal distributional patterns of the globally distributed cyanobacterium *Microcoleus vaginatus*.

The studied strains were isolated from three continents (Europe, North America and Asia). Sequences of 16S rRNA and 16S-23S ITS were analyzed using different phylogenetic approaches. Phylogenetic tree, network and PCoA (Principal Coordinate Analysis) performed with 16S-23S ITS sequences revealed that strains originating from Europe were geographically isolated from strains isolated in North America and Asia. In the other words, phylogenetic clustering was congruent with the geographical origins of isolated strains. The correlation between genetic and geographic distance was evaluated by the Mantel test and it was found to be

highly significant.

The distribution of organisms is changing not only in space but also over time. Nevertheless, only a few attempts to date cyanobacterial evolution have been made. Since paleontological reconstruction bacterial evolution is very scarce, calibrating points for molecular clocks are lacking. Bacterial molecular clocks may be calibrated by host fossil record, but it is not appropriate for free-living organisms. Moreover, molecular clocks can be calibrated based on association with ecological events or inferred from eukaryotes (Ochman et al. 1999). None of these methods is entirely appropriate for cyanobacteria. For this reason, a novel calibration from fossil DNA was developed for this purpose for dating the dispersal of *M. vaginatus* (for more details see Material and Methods, Paper IV). 16S rRNA based Bayesian chronogram revealed that dispersal barriers among populations have not been permanent, i.e. gene flow among geographical distant populations may have been in existence from time to time. This fact is also stressed by the very long duration of species evolution in cyanobacteria. For instance, *M. vaginatus* diverged from other cyanobacteria around 39.5 million years ago and *Coleofasciculus chthonoplastes* 65.42 million years ago. Using molecular clocks thus provided unique evidence for the long existence of the cyanobacterial species. In conclusion, reconstruction of *M. vaginatus* evolutionary history revealed that geographical barriers on a continental level may have played an important role in the evolution of the cyanobacteria. In fact, this is the first evidence for the specific distributional pattern resembling geographical isolation in non-extremophylic cyanobacteria. For this reason, allopatric speciation is probably an important factor in speciation within cyanobacteria. However, dispersal barriers have not been persistent over long time periods.

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5. List of papers included in the thesis

I. Hašler, P., Dvořák, P., Ondřej, V., Kitner, M., Hloušková, P. & Poulíčková, A. (2011): The importance of the polyphasic approach in a comparative study of *Nodularia* Mertens ex Bornet et Flahault (Nostocales, Cyanobacteria). Preslia, 83: 167–182.

II. Hašler, P., Dvořák, P., Johansen, J.R., Kitner, M., Ondřej, V. & Poulíčková, A. (2012): Morphological and molecular study of epipelic filamentous genera *Phormidium*, *Microcoleus* and *Geitlerinema* (Oscillatoriales, Cyanophyta/Cyanobacteria). Fottea, 12: 341–358.

III. Hašler, P., Dvořák, P. & Poulíčková, A. (2012): *Johansenia*, a new genus among filamentous epipelic cyanobacteria. Preslia (submitted).

IV. Dvořák, P., Hašler, P. & Poulíčková, A. (2012): Phylogeography of the *Microcoleus vaginatus* (cyanobacteria) from three continents – a spatial and temporal characterization. PLoS ONE 7(6): e40153. DOI:10.1371/journal.pone.0040153.

Paper I

The importance of the polyphasic approach in a comparative study of *Nodularia* (*Nostocales*, *Cyanobacteria*)

Význam komplexního přístupu při srovnávacím studiu sinic rodu *Nodularia* (*Nostocales*, *Cyanobacteria*)

Petr H a š l e r, Petr D v o ř á k, Vladan O n d ř e j, Miloslav K i t n e r, Petra H l o u š k o v á & Aloisie P o u l í č k o v á

Department of Botany, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic, email: petr.hasler@upol.cz

Hašler P., Dvořák P., Ondřej V., Kitner M., Hloušková P. & Poulíčková A. (2011): The importance of the polyphasic approach in a comparative study of *Nodularia* (*Nostocales*, *Cyanobacteria*). – Preslia 83: 167–182.

This paper focuses on the morphology, taxonomy and ecology of the widespread cyanobacteria of the genus *Nodularia* Mertens ex Bornet & Flahault. In this study the benthic strain of *N. sphaerocarpa,* isolated from a sand-pit near Olomouc (Czech Republic), is compared with brackish and seawater strains. Changes in morphology and growth parameters (biomass and chlorophyll *a*) recorded in varying salinity gradients were studied and a 16S rRNA sequencing and AFLP analysis conducted. Morphological and ecophysiological characteristics found were in congruence with molecular data. Three major subgroups of the benthic *Nodularia* (*N. sphaerocarpa*, *N. moravica* and *N. harveyana*) were found using the polyphasic approach. The results of both the molecular and morphological study clearly separated *N. moravica* and *N. sphaerocarpa*, as freshwater species prefering a low salinity and the *N. harveyana* strains originating from a marine environment preferring a high salinity.

K e y w o r d s: AFLP, cyanobacteria, ecology, morphology, *Nodularia*, salinity, 16S rRNA

Introduction

The genus *Nodularia* Mertens ex Bornet & Flahault is a widespread group of ecologically and morphologically complicated species, which usually occur in brackish coastal waters and freshwater alkaline water bodies. This genus rarely occurs in the Czech Republic being represented exclusively by bentic species (Kaštovský et al. 2010). Previous identification was primarily based on the width of the filament. Based on Geitler's species concept (Geitler 1932), two main groups of species are recognized; the *N. harveyana* group (filaments narrower than 8 μm) and the *N. spumigena* group (filaments wider than 8 μm). Before the 1980s a great number of species, forms and varieties were described. After this date several taxonomic revisions were made and many taxa were combined (e.g. Nordin & Stein 1980, Komárek et al. 1993). The number of species varies depending on the authors. Komárek et al. (1993) and Hindák et al. (2003) classified *Nodularia* species, using the presence of gas vesicles as the main diacritical feature, with filament width secondary. Following this treatment *Nodularia* species were divided into a benthic group without gas vesicles (*N. harveyana*, *N. moravica*, *N. sphaerocarpa*, *N. turicensis*, *N. willei*) and a planktonic water bloom forming group with gas vesicles (*N. baltica*, *N. crassa*, *N. litorea*, *N. spumigena*). However, the occurrence of gas vesicles is not a stable feature and gas vesicles can disappear under unfavourable conditions (Poulíčková et al. 2004).

The taxonomically important character should be the ability to form gas vesicles and not their actual presence (Komárek et al. 1993).

Current identification of *Nodularia* species is based on the morphology of vegetative cells, heterocytes, akinetes, ecology and molecular biology (Laamanen et al. 2001, Lyra et al. 2005). Cell size can be variable and can overlap among different species (for detail see Komárek et al. 1993, their Fig. 2), however, in combination with the other features all *Nodularia* species are identifiable. The taxonomic position of *N. sphaerocarpa* is still unclear. Numerous authors consider this species to be a variety of *N. harveyana* (e.g. Geitler 1932, Elenkin 1938, Starmach 1966, Kondrateva 1968, Bourrelly 1970, Nordin & Stein 1980), whereas others classify it as a separate species (Komárek et al. 1993, Hindák et al. 2003).

The aims of this study are: (i) to evaluate the relationship of morphology, ecophysiology and molecular variability in the taxonomic classification within the genus *Nodularia* and (ii) to evaluate the taxonomic position of *N. moravica* and *N. sphaerocarpa*.

Material and methods

A benthic population of *N. sphaerocarpa* was obtained from a eutrophic sandpit near Olomouc (Czech Republic; 49°34'3.775"N; 17°14'58.131"E) in 2007 (pH 8.03, conductivity 1040 µS/cm, salinity 0.7‰) using the sampling methods published in Špačková et al. (2009). Samples of bottom sandy sediments were incubated under standard laboratory conditions (temperature t = 15±1 °C, illumination 20 µmol·m^{-2}·s^{-1}, photoperiod L/D 16/8 hrs) and studied using a Zeiss AxioImager light microscope (AxioCam HRc 13MPx, objectives Planapochromat 100/1.4, Oil, DIC, EC Plan-Nefluar 40/1.3, Oil, DIC). A strain of *N. sphaerocarpa* was isolated in liquid Bristol-Bold (BB) medium (Bold 1949). Several strains including those of *N. harveyana* (strain CCAP 1452/1, origin: marine; SAG 44.85, origin: salt marsh), *N. moravica* (strain Hindák 2000/15; Institute of Botany, Slovak Academy of Sciences, origin: freshwater, benthic), *N. sphaerocarpa* (strain SAG 50.79, origin: thermal water) and *Nodularia* sp. (strain CCAP 1452/6, origin: marine) were used for comparing with the above strain of *N. sphaerocarpa* (strain Dvořák 2009, origin: freshwater, benthic; Table 1).

Experimental growth in salinity gradient

The liquid medium BB was adjusted with sodium chloride to a final salinity gradient as follows: 10, 20, 30, 40, 50, 60 and 70‰. Serological plates (12 wells) were filled with 3.5 ml of culture media and 100 µl of *Nodularia* inoculum (3000 cells per ml) added to each well, with three replicates of each salinity. The plates were kept at the same culture conditions as incubated natural material of *N. sphaerocarpa*. Cultures were maintained for 30 days and regularly checked using an inverted microscope, the Zeiss Axiovert 40C. During the following 30 days, the number of vegetative cells, heterocytes, akinetes and vegetative cells between heterocytes were counted in a Bürker chamber. Morphological parameters (cell length and width) were measured for one hundred filaments per sample. The growth of cultures was evaluated using the chlorophyll-a concentration method of Vernon (1960).

Table 1. – List and characterization of the *Nodularia* strains used in this study.

Strain name and designation	Locality/Habitat	Reference	GeneBank access no.
N. harveyana, CCAP 1452/1	no data/marine	this study	HQ394172
N. harveyana, SAG 44.85	Lincolnshire, near Gibraltar/salt marsh	this study	HQ394175
N. moravica, Hindák 2000/15	Podivín, Czech Republic/freshwater, benthic, sand-pit lake	this study	HQ394173
N. sphaerocarpa, Dvořák 2009	Olomouc, Czech Republic/freshwater, benthic,	this study	HQ394177
N. sphaerocarpa, SAG 50.79	Dax, France/thermal water	this study	HQ394174
Nodularia sp., CCAP 1452/6	Dunstaffnage Bay, Oban, UK /marine, intertidal sediment	this study	HQ394176
N. harveyana, BECID29	Gulf of Finland, Baltic Sea/littoral zone, rock surface	Lyra et al. 2005	AJ781146
N. harveyana, Hübel 1983/300	Hiddensee, Baltic Sea/benthic microbial mat	Lyra et al. 2005	AJ781142
N. harveyana, Bo53	Boiensdorf/shallow coastal water	Lyra et al. 2005	AJ781143
N. harveyana, BECID27	Gulf of Finland, Baltic Sea /littoral zone, plant surface	Lyra et al. 2005	AJ781145
N. sphaerocarpa, BECID35	Gulf of Bothnia, Baltic Sea/littoral zone, mat-like colony	Lyra et al. 2005	AJ781149
N. sphaerocarpa, BECID36	Gulf of Finland, Baltic Sea/littoral pool, rock surface	Lyra et al. 2005	AJ781147
N. sphaerocarpa, Fä19	Fährdorf, Isle of Poel/shallow coastal water	Lyra et al. 2005	AJ781144
N. sphaerocarpa, Hübel 296	no data	Lyra et al. 2005	AJ781141
N. sphaerocarpa, PCC 73104	Spotted lake, Canada/alkaline soil	Lyra et al. 2005	AJ781139
N. sphaerocarpa, Up16a	Gulf of Finland, Baltic Sea/plankton	Lyra et al. 2005	AJ781140
N. sphaerocarpa, UTEX B 2092	Osoyoos, Canada/alkaline soil	Lyra et al. 2005	AJ781151
N. spumigena, AV1	Gulf of Finland, Baltic Sea/plankton	Lyra et al. 2005	AJ781136
N. spumigena, F81	Gulf of Finland, Baltic Sea/plankton	Lyra et al. 2005	AJ781137
N. spumigena, PCC 9350	Gulf of Finland, Baltic Sea/plankton	Lyra et al. 2005	AJ781131
N. spumigena, UTEX B 2093	San de Fuca, Whidbey Island, WA, USA/pond	Lyra et al. 2005	AJ781148

Statistical analyses

Morphological variability was analysed using one-way ANOVA (NCSS software; Hintze 2006). Polynomial correlation was made on significance level of $\alpha = 0.05$. Hierarchical clustering was based on Ward's minimum variance method (NCSS software).

DNA extraction, amplification and sequencing

DNA extraction was performed using the protocol of Doyle & Doyle (1990). The integrity and quality of DNA was checked on 1.8% agarose gel. Concentrations of DNA samples were assessed by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, USA). The PCRs with 16S rRNA primers CYA106F, CYA359F, CYA781R(a) and CYA781R(b) (Nübel et al. 1997) were performed using a FastStart PCR Master Kit (Roche) following the PCR protocol of Nübel et al. (1997). The PCR products were checked by agarose electrophoresis, purified using GeneElute PCR Clean up Kit (Sigma-Aldrich Co., USA) or cloned into the pGEM®-T vector (Promega Corporation, Madison, USA) and sequenced.

Table 2. – List of primer sets used in the reactions with the total number of scored and polymorphic bands.

Amplification Primer Sets Sequences	Number of bands	Number of polymorphic bands
EcoRI primer E-CAG / MseI primer M-CAAC	77	77
EcoRI primer E-CAG / MseI primer M-CAAT	86	85
EcoRI primer E-CAG / MseI primer M-CGAT	65	65
EcoRI primer E-ACC / MseI primer M-CAAC	74	72
EcoRI primer E-ACC / MseI primer M-CAAT	50	50
EcoRI primer E-ACC / MseI primer M-CGAT	77	76
EcoRI primer E-ATC / MseI primer M-CGAT	72	72
EcoRI primer E-ACA / MseI primer M-CGAT	42	42
lEcoRI primer E-ACT / MseI primer M-CGAT	67	67
EcoRI primer E-ACG / MseI primer M-CAAC	48	48
EcoRI primer E-ACG / MseI primer M-CTT	53	53

Total number of bands: 711
Polymorphic bands: 99.43%

AFLP analysis

The original procedure published by Vos et al. (1995) with the modification proposed by Kitner et al. (2008) was used for AFLP analysis of six *Nodularia* strains. In total eleven selective primer combinations were chosen to generate the AFLP profiles (Table 2). Products of amplification were separated on a 6%, 0.4 mm thick denaturing polyacrylamide gel (0.5× TBE buffer) using the T-REX (Thermo Scientifis Owl Separation Systems, Rochester, NY, USA) sequencing gel electrophoresis apparatus. Subsequent silver staining was used for visualizing the AFLP patterns.

Phylogenetic analyses

The 16S rRNA sequences of selected *Nodularia* strains (see Table 1 for access numbers) obtained above and from EMBL Nucleotide Sequence Database were aligned with ClustalW2 (EMBL Sequence Analysing tool; available from http://www.ebi.ac.uk/Tools/clustalw2/). The phylogenic tree was obtained using Bayesian MCMC (Markov chain Monte Carlo) analysis, which also enables a molecular clock analysis (Drummond & Rambaut 2007). The 95% confidence interval for the divergence of *Nodularia* isolates was inferred by an analysis with BEAST 1.4.2. (available from http://beast.bio.ed.ac.uk). The analysis was run for 10M generations and the burn-in was set to 100K generations. Then program TreeAnnotator summarized a tree sample from BEAST annotating it with posterior probabilities, HPD node heights and rates. This tree was viewed in the program FigTree (http://tree.bio.ed.ac.uk/software/figtree). Minimum evolution and maximum likelihood bootstrap analysis was carried out using PAUP* (Swofford 2001). Visualized AFLP gels were scored for presence (1) or absence (0) of bands. The binary matrix was constructed from primary data and subjected to FreeTree for cluster analysis (Pavlíček et al. 1999; method UPGMA, Jaccard similarity coefficient). The resulting cluster was visualized in TreeView (Page 1996). To validate how consistently the AFLP data support given isolate bipartitions, bootstrap analysis was carried out using 1000 replicates (Felsenstein 1985).

Results

Investigation of natural population of Nodularia sphaerocarpa

The morphology of *N. sphaerocarpa* accords with the description in Komárek et al. (1993) and differences between the species studied are compared in Table 3. *Nodularia sphaerocarpa* was initially observed in December 2007 when it formed an important part of the benthic cyanobacterial assemblages (25%) on the surface of sandy bottom sediments. Filaments were usually straight, bent or occasionally wavy, attenuated or not, 5–8 µm wide (Figs 1–5). Sheaths were colourless and diffluent. Cells without gas vesicles were short, barrel-shaped, 2–5 × 5–7 µm. Heterocytes were elliptical, rectangularly-rounded or barrel-shaped, 5–9 × 6–10 µm. Akinetes were spherical or elliptical, 6–10 µm in diameter, with wart-like incrustations on their surface (Figs 6–9) and often in chains (more than four cells). Occasionally, short hormogonia (up to 20 cells), which had germinated from akinetes, were found.

Table 3. – Comparison of the morphology of the *Nodularia* species included in this study.

	N. sphaerocarpa	*N. moravica*	*N. harveyana*	*N. spumigena*
Vegetative cells	2–5 × 5–7	2–6 × 7–15	1.5–3.5 × 4–5	2–5.6 × 6.8–12
Heterocytes	5–9 × 6–10	4–11 × 8.5–11	3–6 × 4–7	2–5 × 9–13.7
Akinetes	6–10 in diam.	6–10 × 8–12	4–8 × 5–7	5.7–15 × 8–12
Gas vesicles	not present	not present	not present	present
Habitat	periphytic, benthic, alkaline waters	alkaline littoral	periphytic, benthic, saline or mineral water	planktonic, marine
Reference	this study	Hindák et al. 2003	Komárek et al. 1993	Komárek et al. 1993

Effect of salinity on morphology of Nodularia strains

Five strains of *Nodularia,* from culture collections, were compared with the isolate (Table 1, Figs 10–43). Growth and filament shape were variable and depended on salinity. *Nodularia sphaerocarpa* (both strains), *N. harveyana* (both strains) and *Nodularia* sp. were mostly long and straight (Figs 1–30) or slightly bent. Short filaments and hormogonia of *N. sphaerocarpa* (strain Dvořák 2009) were recorded in standard BB medium (Fig. 10).

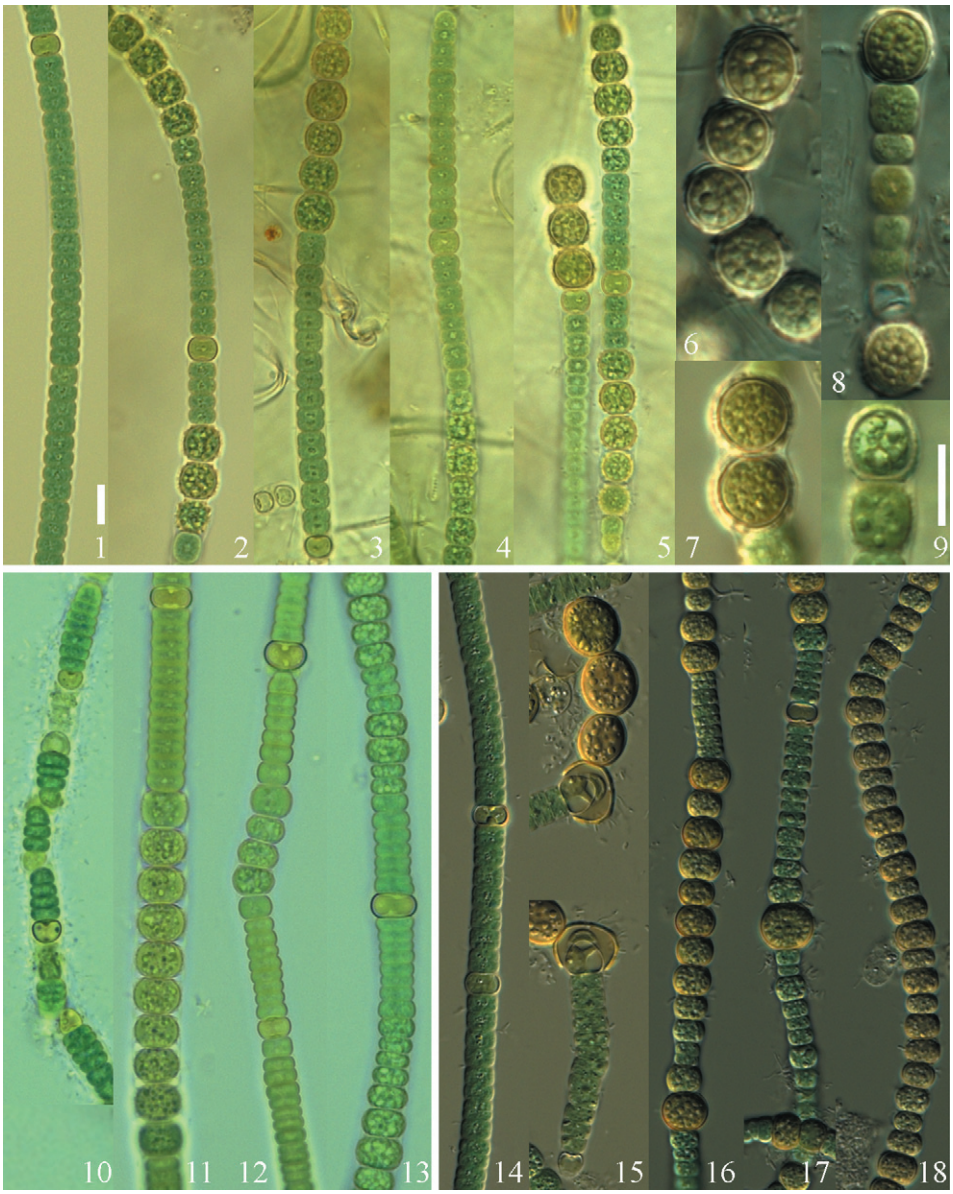
Growth of *N. sphaerocarpa* (strain Dvořák 2009) was best when the salinity was 10–20‰ (Table 4) and inhibited when it was 40‰, whereas the second strain (SAG 50.79) grew well only in basic BB medium and at a salinity of 10‰ (1920000 cells per ml). *Nodularia moravica* (Hindák 2000/15) grew well in media with salinities of up to 40‰ (with the highest abundance of 4,138,000 cells per ml at 10‰ salinity). Strain *N. harveyana* (CCAP 1452/1) grew in basic BB medium with salinities up to 50‰, but the growth was similar over the entire range. Strains of *N. harveyana* (SAG 44.85) and *Nodularia* sp. (CCAP 1452/6) grew in basal BB medium with salinities up to a 40‰ and *N. harveyana* and *Nodularia* sp. grew best at salinities of 30‰ and 10‰, respectively (Table 4). Growth parameters (abundance and chlorophyll-a) were significantly correlated with gradient of salinity, except for strain *N. harveyana* CCAP 1452/1 (Table 5).

Table 4. – Culture growth parameters on salinity gradient: abundances (thousands·ml^{-1}) of vegetative cells, heterocytes, akinetes and the chlorophyll *a* concentration (μg·l^{-1}) of *Nodularia* species when kept at particular salinities; n.d. – no data (no akinetes appeared or strain was not growing); salinity gradient: BBM – Bristol-Bold medium without addition of NaCl (= 0‰) and salinity range (10–50‰).

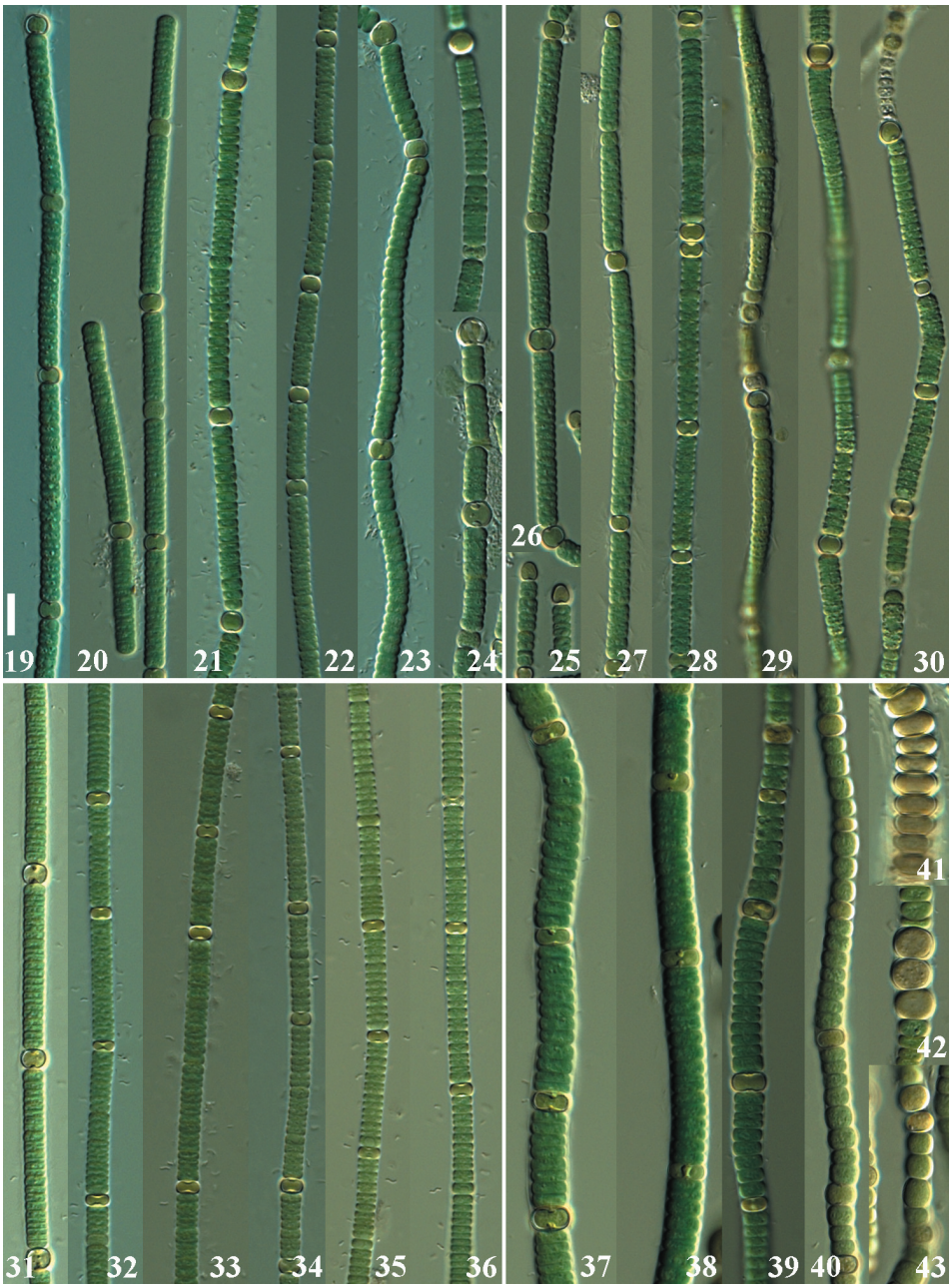
Species (strain)	Abundance	Salinity					
		BBM	10‰	20‰	30‰	40‰	50‰
N. sphaerocarpa	cells	2904	13737	18205	2558	n.d.	
(strain Dvořák 2009)	heterocytes	149	290	349	49	n.d.	
	akinetes	4	27	37	4	n.d.	
	total cells	3058	14053	18592	2611	n.d.	
	chlorophyl *a*	11.89	31.39	22.63	5.72	n.d.	
N. sphaerocarpa	cells	5893	1920	n.d.	n.d.	n.d.	
(SAG 50.79)	heterocytes	209	43	n.d.	n.d.	n.d.	
	akinetes	n.d.	130	n.d.	n.d.	n.d.	
	total cells	6102	2093	n.d.	n.d.	n.d.	
	chlorophyl *a*	37.43	8.89	n.d.	n.d.	n.d.	
N. moravica	cells	3222	4138	3940	3247	320	
(strain Hindák 2000/15)	heterocytes	471	347	273	210	27	
	akinetes	n.d.	n.d.	n.d.	n.d.	63	
	total cells	3693	4484	4213	3457	410	
	chlorophyl *a*	110.62	76.58	54.10	13.08	3.43	
N. harveyana	cells	19653	13871	13113	12573	19431	10071
(CCAP 1452/1)	heterocytes	1327	809	847	740	870	542
	total cells	20980	14680	13960	13313	20306	10613
	chlorophyl *a*	25.02	40.90	40.37	31.66	52.96	16.00
N. harveyana	cells	3849	2960	6422	9600	3871	
(SAG 44.85)	heterocytes	658	596	556	756	191	
	total cells	4507	3556	6978	10369	4076	
	chlorophyl *a*	19.56	19.94	17.91	15.24	5.97	
Nodularia sp.	cells	15607	18853	8044	10449	4787	
(CCAP 1452/6)	heterocytes	747	900	484	716	223	
	total cells	16353	19753	8529	11164	5010	
	chlorophyl *a*	49.07	32.54	24.54	16.90	10.31	

Table 5. – Coefficient of determination (r^2) of the correlation between the growth parameters and salinity. Salinity ranges as in Table 4; the strain *N. harveyana* CCAP 1452/1 growth of which was not significantly correlated with salinity is not shown. * significance level 0.05.

Growth parameters	*N. sphaerocarpa* (strain Dvořák 2009)	N. moravica (strain Hindák 2000/15)	*N. sphaerocarpa* (SAG 50.79)	*N. harveyana* (SAG 44.85)	*Nodularia sp.* (CCAP 1452/6)
Vegetative cells	0.949*	0.987*	0394	0.214	0.711*
Heterocytes	0.930*	0.972*	0.549	0.694*	0.649*
Total cells	0.949*	0.979*	0.369	0.241	0.710*
Chlorophyll–a	0.948*	0.984*	0.980*	0.440	0.993*



Figs 1–18. – Morphological variability of *Nodularia sphaerocarpa*. **1–9** seminatural population of *N. sphaerocarpa* from a sand pit at Olomouc: **1–5** filaments; **6–9** akinetes; **10–18** changes in the filaments recorded at different salinities. **10–13** strain Dvořák 2009: **10** standard BB medium; **11** salinity 10‰; **12** salinity 20‰; **13** salinity 30‰; **14–18** strain SAG 50.79: **14–15** standard BB medium; **15** hormogonia production; **16–18** salinity 10‰. Scale bars 10 µm (1–5, 10–18; 6–9)



Figs 19–43. – Morphological variability of *Nodularia harveyana* and *Nodularia moravica*. **19–24** strain CCAP 1452/1: **19** standard BB medium; **20** salinity 10‰; **21** salinity 20‰; **22** salinity 30‰; **23** salinity 40‰; **24** salinity 50‰; **25–30** strain SAG 44.85: **25–26** standard BB medium; **27** salinity 10‰; **28** salinity 20‰; **29** salinity 30‰; **30** salinity 40‰; **31–36** strain CCAP 1452/6: **31** standard BB medium; **32** salinity 10 ‰; **33** salinity 20‰; **34** salinity 30‰; **35–36** salinity 40‰; **37–43** strain Hindák 2000/15: **37** standard BB medium; **38** salinity 10‰; **39** salinity 20‰; **40** akinete formation in standard BB medium; **41–43** salinity 10 and 30‰. Scale bar 10 µm.

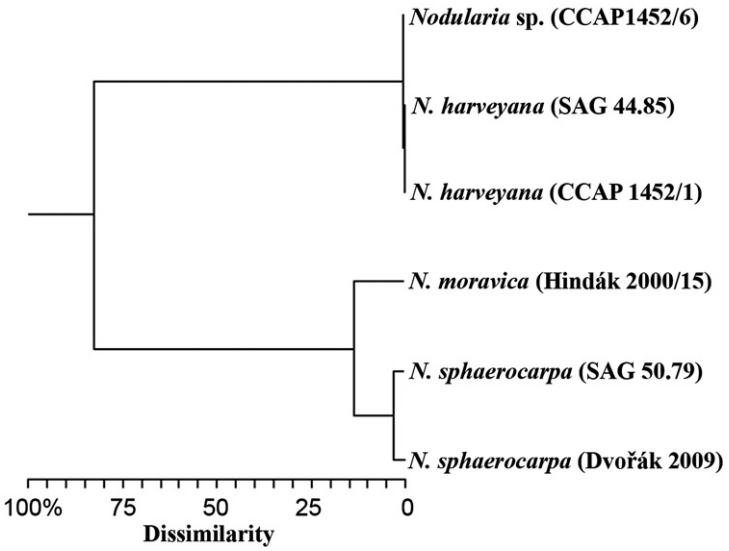


Fig. 44. – Hierarchical clustering based on morphology of vegetative cells and heterocytes (Ward's minimum variance method).

Vegetative cells were always wider than long. Barrel-shaped cells occurred occasionally. Cell morphology was significantly influenced by the salinity gradient (Table 6). Hierarchical clustering analysis based on cell morphology clearly separated the marine group of *N. harveyana* and *Nodularia* sp. from the freshwater group of *N. sphaerocarpa* and *N. moravica*. *Nodularia moravica* was also slightly separated from *N. sphaerocarpa* (Fig. 44). There was no significant influence of salinity on cell size within the *N. harveyana* group. A wide variation in cell dimensions was observed in *N. moravica*, but without any general trend relative to salinity. Cells, akinetes and heterocytes seemed to be wider at the higher salinities in *N. sphaerocarpa* (Table 6). Cells and heterocytes were the widest in a medium adjusted to a salinity of 30‰. Cell dimensions of the strain (SAG 50.79) of *N. sphaerocarpa* were similar to those of strain Dvořák 2009. There were no significant differences in cell dimensions in the standard medium with a salinity of 10‰ (except for that of the heterocytes). Cells of *N. moravica* were wider than long, occasionally barrel-shaped or elliptical. Cell and heterocyte width in the *N. harveyana* group were not as variable as in *N. sphaerocarpa* and *N. moravica*. Cells and heterocytes were always wider than long and the minimum and maximum values were similar (for details see Table 6).

Within the first 10 days of the experiment vegetative cells and heterocytes were observed in cultures of all strains. No akinetes were formed by *N. harveyana* and *Nodularia* sp. After 20 days akinetes occurred in the cultures of *N. sphaerocarpa* and *N. moravica*, particularly at salinities between 10–30‰ and sporadically in the basic BB medium. Akinetes of *N. sphaerocarpa* (strain Dvořák 2009) were not of the same shape as in the natural population and were usually wider than long and lacked wart-like incrustations on their surfaces (Figs 11–13). Low salinities stimulated *N. sphaerocarpa* to produce short hormogonia from filaments by means of necridic cells (Fig. 10). Akinetes of *N. sphaerocarpa* (SAG 50.79) occurred in long chains (0–10‰ salinity) and those in stan-

Table 6. – Changes in morphology of *Nodularia* strains kept at different salinities. Salinity gradient as in Table 4; n.d.– no data. Effects of salinity on the length (μm) and width (μm) of each cell type were tested using one-way Anova; * significance level 0.05–0.01; ** significance level < 0.01, n = 100 cells for each salinity.

		Vegetative cells		Heterocytes		Akinetes		Cells HTC-HTC
		Length	Width	Length	Width	Length	Width	
Nodularia sphaerocarpa	BBM	2.51±0.64	6.90±0.58	4.96±0.77	7.54±0.81	5.67±0.71	9.43±1.15	31±7
(strain Dvořák 2009)	10‰	2.40±0.70	8.02±0.73	4.98±0.90	9.06±1.00	5.03±1.31	10.00±0.81	95±15
	20‰	2.51±0.62	7.57±0.67	5.29±0.90	9.28±0.96	6.09±0.94	9.81±1.29	69±20
	30‰	2.24±0.46	9.19±1.02	6.08±1.29	11.06±1.44	5.73±0.64	10.89±1.53	70±31
	F	3.83**	141.71**	25.15**	160.04**			
	r^2	0.50	0.74	0.69	0.93			
Nodularia sphaerocarpa	BBM	3.28±0.92	6.97±0.47	6.43±1.16	8.75±0.88	9.77±1.76	10.56±1.31	17±7
(SAG 50.79)	10‰	2.93±0.76	7.13±0.94	5.62±1.80	7.92±0.95	7.96±1.42	10.40±0.98	23±4
	F	2.64	0.70	3.32	7.78**			
Nodularia moravica	BBM	3.13±0.85	9.81±0.65	5.48±1.03	9.67±0.60	n.d.	n.d.	13±4
(strain Hindák 2000/15)	10‰	3.00±0.89	8.90±0.60	4.52±0.68	9.16±0.64	n.d.	n.d..	13±5
	20‰	3.58±0.92	7.71±0.59	5.29±0.78	8.26±0.68	8.00±0.00	12.00±0.00	9±3
	30‰	3.06±0.89	7.00±0.68	5.55±0.76	8.03±0.60	n.d..	n.d..	11±4
	40‰	3.81±0.75	8.69±1.25	6.06±1.18	10.00±0.63	7.32±2.04	9.84±1.60	14±8
	F	3.88**	69.05**	10.31**	47.78**			
	r^2	0.39	0.36	0.38	0.01			
Nodularia harveyana	0‰	2.29±0.59	5.42±0.51	5.16±0.58	5.93±0.44	n.d.	n.d.	18±6
(CCAP 1452/1)	10‰	2.29±0.46	5.29±0.46	4.58±0.81	5.61±0.50	n.d.	n.d.	17±5
	20‰	2.29±0.46	5.16±0.27	4.90±0.83	5.55±0.57	n.d.	n.d.	15±4
	30‰	2.58±0.50	4.90±0.60	4.74±0.58	5.45±0.57	n.d.	n.d.	13±5
	40‰	2.09±0.65	5.00±0.51	4.77±0.92	5.58±0.62	n.d.	n.d.	17±6
	50‰	2.45±0.63	5.37±0.49	5.09±0.88	6.27±0.65	n.d.	n.d.	15±7
	F	2.73*	6.89**	2.38*	9.22**			
	r^2	0.02	0.13	0.01	0.07			
Nodularia harveyana	0‰	2.44±0.62	5.25±0.57	5.03±0.53	5.53±0.88	n.d.	n.d.	12±6
(SAG 44.85)	10‰	2.06±0.56	5.19±0.40	4.94±0.80	5.62±0.61	n.d.	n.d.	17±9
	20‰	2.03±1.18	5.19±0.57	4.72±0.77	5.81±0.64	n.d.	n.d.	15±5
	30‰	2.16±0.37	5.16±0.37	4.60±0.71	5.88±0.55	n.d.	n.d.	14±7
	40‰	2.16±0.57	5.31±0.47	4.69±0.78	5.91±0.47	n.d.	n.d.	11±4
	F	1.61	0.63	2.03	2.06			
	r^2	0.20	0.06	0.79	0.93			
Nodularia sp.	0‰	2.06±0.57	4.87±0.50	5.13±0.62	6.10±0.60	n.d.	n.d.	26±9
(CCAP 1452/6)	10‰	2.06±0.68	5.26±0.68	3.94±0.81	5.94±0.73	n.d.	n.d.	22±8
	20‰	2.00±0.63	4.98±0.48	4.19±0.75	5.58±0.50	n.d.	n.d.	24±9
	30‰	1.68±0.60	5.39±0.56	3.58±0.81	6.03±0.60	n.d.	n.d.	26±12
	40‰	1.94±0.57	5.16±0.45	3.71±0.94	6.00±0.77	n.d.	n.d.	25±12
	F	2.13	4.68**	18.73**	3.04*			
	r^2	0.39	0.29	0.68	0.01			
Pooled data	F	17.26**	233.43**	20.13**	200.98**			

dard medium were more spherical than in the 10‰ medium (Figs 16–18). Germination was observed in the standard BB medium (Fig. 15). Akinetes of *N. moravica* occurred throughout the whole salinity gradient and were the widest of all of the cultivated strains (Table 6). Their shape changed from flatly elliptical to round (Figs 41–43). Unlike in

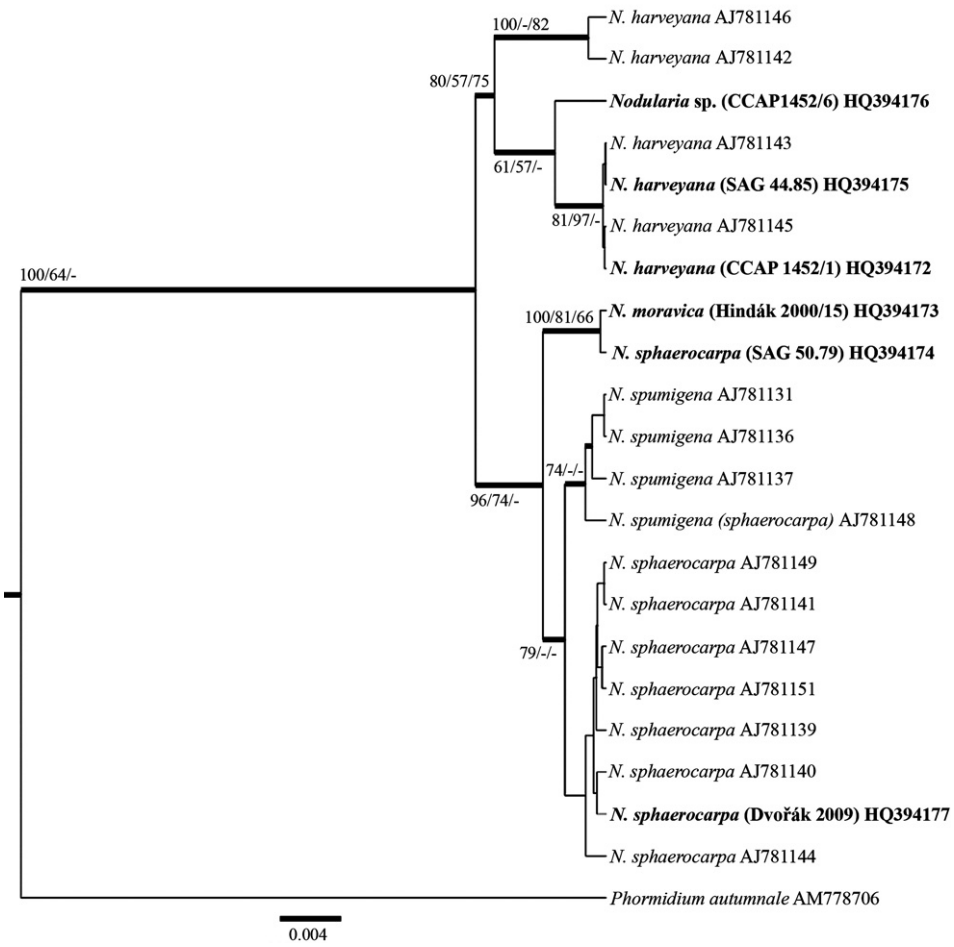


Fig. 45. – Phylogram of single tree generated from Bayesian MCMC (Markov chain Monte Carlo) analysis based on 16S rRNA sequences (length = 421bp) and with *Phormidium autumnale* as the outgroup. Labelled branches indicate Bayesian posterior probabilities ≥ 95%. Bootstrap support values (≥ 50%) are shown above the branches (posterior probability from Bayesian analysis/minimum evolution/maximum likelihood). Strains studied are in bold.

N. sphaerocarpa, the content of the akinetes was less granulated, rather fine granulated to nearly homogenous and pale brown or green-brown. Akinetes of *Nodularia moravica* germinated to produce hormogonia in media with salinities of between 10 and 20‰.

Analysis of 16S rRNA gene sequences

The 16S rRNA sequences from the *Nodularia* strains studied were compared with those available from the GenBank and one *Phormidium* isolate (outgroup). Following Lyra et al. (2005), only well-defined sequences were used. This analysis clearly divided the genus *Nodularia* into two major groups: *N. harveyana* and a group represented by *N. moravica*,

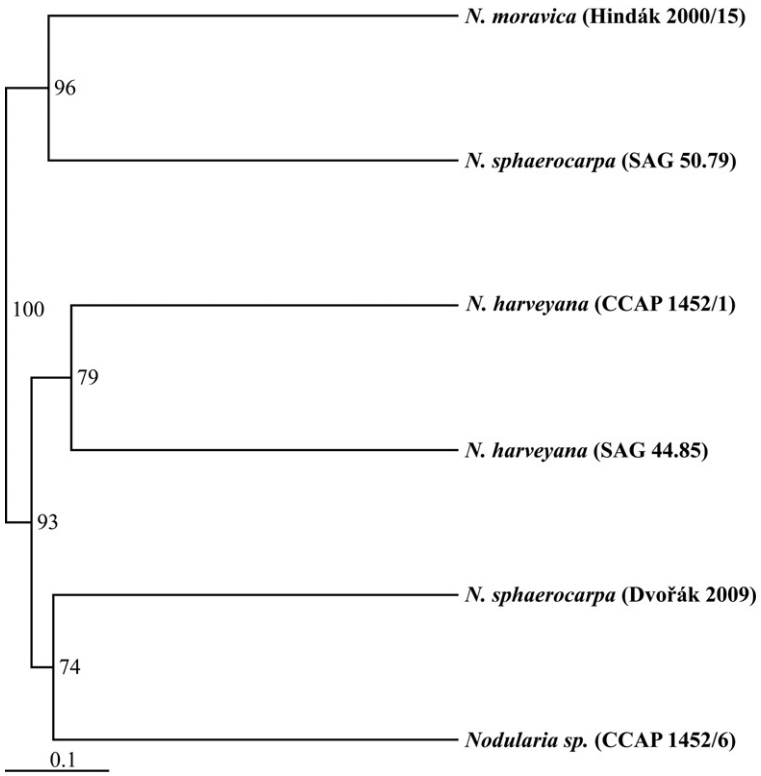


Fig. 46. – UPGMA analysis dendrogram (Jaccard's similarity coefficient) based on 711 AFLP fragments. Significant values of bootstrap analysis are shown on branches.

N. spumigena and *N. sphaerocarpa* (Fig. 45). The *N. harveyana* clade included the strains CCAP 1452/1 and SAG 44.85 (*N. harveyana*) and strain CCAP 1452/6 (*Nodularia* sp.). There was not a high degree of similarity within the *N. harveyana* clade.

The second group is represented by *N. moravica*, *N. spumigena* and *N. sphaerocarpa* and its inner separation is supported by high bootstrap values (Fig. 45). The most dissimilar group inside this clade is *N. moravica,* which is separate from the *N. sphaerocarpa* and *N. spumigena* groups.

AFLP analysis

In total six *Nodularia* spp. strains were analysed using eleven AFLP primer combinations, which generated 711 unambiguously scored fragments, of which 99.4% were polymorphic. The statistical analysis (Fig. 46) supported the results of the 16S rRNA sequencing (Fig. 45). The only difference was the co-segregation of *Nodularia* sp. (CCAP 1452/6) strain together with *N. sphaerocarpa.* Nevertheless, the co-segregation of *N. moravica* and *N. sphaerocarpa* support the theory that *N. moravica* has diverged significantly from other *Nodularia* species. Moreover, *N. harveyana* is considered to be a monophyletic species clearly diagnosed both by molecular and morphological characters.

Discussion

Cyanobacteria play a key role in the functioning of many ecosystems and because they produce toxins they are potentially harmful organisms. Despite their importance, however, many aspects of their biodiversity and ecology are poorly understood. Routine species identification, mostly using morphology-based classifications, may not provide sufficient taxonomic resolution as cyanobacteria with similar or identical morphology can differ significanly in their physiology. In recent years, the analysis of 16S rRNA gene sequences has demonstrated that the morphological classification of cyanobacteria in some cases corresponds to phylogenetically coherent taxa (Garcia-Pichel et al. 1996), whereas in other cases the traditional classification greatly underestimates extant diversity (Ferris et al. 1996). For example, in bacteriology, the tolerance and requirements for high salt concentrations and high temperatures are recognized as important phenotypic properties that are correlated with phylogeny (Imhoff et al. 1998).

The genus *Nodularia* consists of apoheterocytic nostocalean cyanobacteria, for which filament and cell morphology can be used for intraspecific taxonomy (e.g. Geitler 1932, Kondrateva 1968, Nordin & Stein 1980, Komárek et al. 1993). Altogether 28 species, varietes and forms were revised by Nordin & Stein (1980) and only *N. harveyana* and *N. spumigena* were considered as valid species. They hypothesized that the other morphological species may only be a result of adaptation to growth conditions, e.g. salinity and pH values, which are the most important factors influencing the distribution of *Nodularia*. Thus these authors do not consider cell and heterocyte dimensions and morphology in general to be reliable taxonomic characters.

However, hierarchical cluster analysis based on morphological data of five *Nodularia* strains clearly separated the species *N. sphaerocarpa*, *N. harveyana* and *N. moravica*. While *N. harveyana* differs in trichome width, shape and number of chained akinetes (2–4), *N. sphaerocarpa* forms spherical akinetes and has chains of high numbers of akinetes (Komárek et al. 1993). These features were probably overlooked by previous authors (Geitler 1932, Elenkin 1938, Starmach 1966, Kondrateva 1968, Bourrelly 1970, Nordin & Stein 1980). Similarly, *N. moravica* differs from both of the species mentioned above (Hindák et al. 2003) in terms of its morphology (vegetative cells, heterocytes and akinetes) and ecology.

The classification based on morphology accords with molecular data. *Nodularia harveyana* is clearly separated from other *Nodularia* species. Although the validity of *N. sphaerocarpa* is confirmed by molecular studies (Bolch et al. 1999, Laamanen et al. 2001, Lyra et al. 2005) it is heterogeneous. *Nodularia sphaerocarpa* (strain Dvořák 2009) belongs to the main cluster of *N. sphaerocarpa*, however, *N. sphaerocarpa* (SAG 50.79) is separated from this group and has a high similarity with *N. moravica*. An identical strain of *N. sphaerocarpa* from a thermal spring (strain PCC 7804; Dax, France) studied by Bolch et al. (1999) has the same dissimilarities in the nucleotide sequences of its cpcBA-IGS as *N. sphaerocarpa*. However, the position of strain PCC 7804 is closer to *N. sphaerocarpa* than the other species included in his study.

Nodularia moravica was described by Hindák et al. (2003) and currently there are no molecular studies on this species. This author provided us with the type strain of *N. moravica* (Hindák 2000/15) for DNA analysis. The molecular, morphological and ecophysiological data support the claim of Hindák et al. (2003) that *N. moravica* differs significantly from

the other *Nodularia* species. The taxonomic position and/or determination of the thermophilic *N. sphaerocarpa* (SAG 50.79) strain remains unresolved and it is proposed to keep this strain as *Nodularia* sp.

A similar situation exists for *N. harveyana*, whose separation from other species was supported by morphology and 16S rRNA sequences. Strain *Nodularia* sp. CCAP 1452/6 belongs to the *N. harveyana* cluster, although it was not supported by AFLP profiles, due to the fact that the few strains tested could not be compared with database data. Based on the 16S rRNA sequencing results it is proposed to identify *Nodularia* sp. CCAP 1452/6 as *N. harveyana*.

Nordin & Stein (1980) studied *Nodularia* isolates kept in various salinity and pH gradients and concluded that although this genus can tolerate low salinities and pH values, it does not have the features of a "true freshwater" species. Warr et al. (1984) record a negative influence of increasing salinity on the growth of *N. harveyana*. The results presented here support the idea that halotolerance in *Nodularia* is species-specific. Unlike species isolated from marine environments (*N. harveyana*) those from freshwater (*N. sphaerocarpa*, *N. moravica*) grew better at low salinities. The highest range of salinities tolerated by species in the study was recorded for *N. harveyana* and *Nodularia* sp. and the narrowest for *N. sphaerocarpa* isolated from thermal water. However, its extremely narrow range compared to that for *N. sphaerocarpa* (strain Dvořák 2009) may be because of the age of the culture and the length of time it was maintained under laboratory conditions.

The dependence of the growth rate of genetically variable strains on salinity and species-specific halotolerance is also reported for *Spirulina* spp. by Nübel et al. (2000). Their data do not support the traditional opinion that a few closely related species of cyanobacteria with *Spirulina* morphology have a broad ecological euryvalence and ubiquitous distribution (Anagnostidis & Golubić 1966). Three of the isolates originated from hypersaline water and were similar in their high halotolerance and broad euryhalinity. Phylogenetic analysis placed them in a monophyletic cluster apart from all the other species (Nübel et al. 2000).

The ecophysiological characteristics of *Nodularia* strains studied support the hypothesis, that ecologically distinct organisms thriving in different habitats have different physiological capabilities and different evolutionary histories, which are reflected in their genetic divergence.

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Souhrn

Studie se zabývá morfologií, taxonomií a ekofyziologií sinic rodu *Nodularia,* který zahrnuje sladkovodní, brakické i mořské zástupce. Experimentální práce spočívala v hodnocení morfologické variability a růstových parametrů v gradientu salinity. Ekofyziologické a morfologické charakteristiky velmi dobře korespondují s výsledky molekulárních metod (16S rRNA, AFLP) a mohou vysvětlit rozdělení bentických druhů rodu *Nodularia* na dva sladkovodní druhy (*N. sphaerocarpa a N. moravica*) a jeden mořský (*N. harveyana*).

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Paper II

Morphological and molecular study of epipelic filamentous genera *Phormidium*, *Microcoleus* and *Geitlerinema* (Oscillatoriales, Cyanophyta/ Cyanobacteria)

Petr Hašler [1*], Petr Dvořák [1], Jeffrey R. Johansen[2], Miloslav Kitner [1], Vladan Ondřej [1] & Aloisie Poulíčková [1]

[1] *Department of Botany, Faculty of Science, Šlechtitelů 11, CZ–783 71 Olomouc, Czech Republic; *Corresponding author e–mail: petr.hasler@upol.cz*
[2] *Department of Biology, John Carroll University, 20700 North Park Blvd., University Heights, Ohio 44118, USA*

Abstract: Filamentous epipelic cyanobacteria were isolated from ponds and lakes in the Czech Republic, Austria and Italy. Morphological and genetic variation of 20 isolated strains within the genera *Geitlerinema, Microcoleus* and *Phormidium* were studied. Partial sequences of the 16S rRNA gene were used for phylogenetic analyses, and secondary structure of the 16S–23S ITS region was used to additionally define clades. Morphological and molecular were congruent, and we were able to identify the majority of strains correctly to species on the basis of morphological features. Overall diversity and morphological/genetic variation of epipelic species is not as high as described from other benthic habitats, possibly due to the relative microhabitat uniformity of lake/pond bottom sediments. The *M. vaginatus* clade is well defined by an 11 bp insert in 16S rRNA gene (bp 423–433) and populations from different ecological conditions differ in secondary structure in the 16S–23S ITS regions, particularly in Box–B helices. *Ph. autumnale* and the genus *Geitlerinema* appear to be polyphyletic as presently defined.

Key words: 16S rRNA, cyanobacteria, ecology, ITS, morphology, phylogeny, Oscillatoriales

Introduction

During most of the 19th and 20th centuries cyanobacterial taxonomy was based almost entirely on morphology (Geitler 1932; Elenkin 1938; Desikachary 1959; Starmach 1966; Kondrateva 1968). The taxonomic position of many morphologically–defined species is unclear and some genera urgently need revision (e.g. Komárek & Anagnostidis 1998; Komárek & Anagnostidis 2005). Moreover, the situation is complicated by a conflict between bacteriological and botanical nomenclatural rules and taxonomic practices (Stanier et al. 1978; Rippka et al. 1979; Castenholz 2001). The most progressive system utilizes a polyphasic approach (Anagnostidis & Komárek 1985; Komárek & Anagnostidis 1986; Anagnostidis & Komárek 1988; Komárek & Anagnostidis 1989; Anagnostidis & Komárek 1990; Komárek 1994, 2003; Komárek 2011), which includes a combination of morphological, ecological and molecular character sets. Recent molecular data support the validity of many genera, e.g. *Planktothrix*, *Pseudanabaena* (Willame et al. 2006), *Microcystis*, and *Spirulina* (Komárek 2003, 2010) as defined by Komárek & Anagnostidis (1998, 2005), but at the species level we often have insufficient morphological, ecological and molecular data for reliable recognition of species–level diversity. In recent years, the analysis of the 16S rRNA gene sequences has demonstrated that morphological classification of cyanobacteria in some cases corresponds to phylogenetically coherent taxa (Garcia–Pichel et al. 1996), whereas in other cases the traditional classification drastically underestimates extant diversity (Ferris et al. 1996).

The assemblages of autotrophic micro-oranisms (cyanobacteria, algae) on bottom sediments of stagnant and running waters are called epipelon. These microorganisms perform a range of ecosystem functions including biostabilisation of sediments, regulation of

benthic–pelagic nutrient cycling, and primary production (POULÍČKOVÁ et al. 2008a). Although epipelic eukaryotic algae were previously studied, e.g. diatoms (reproductive biology, cryptic speciation, geographic biodiversity and bioindication; POULÍČKOVÁ et al. 2008a, 2008b, 2009), epipelic cyanobacteria have been largely overlooked. The ecology of epipelic cyanobacteria is poorly understood. Species distribution is probably influenced by numerous environmental variables such as temperature, light irradiation, oxygen concentration, pH, sediment structure and chemical composition (e.g. ROUND 1953, 1957, 1961; HAŠLER et al. 2008). Autochthonous epipelic assemblages typically include 20 – 80% filamentous motile cyanobacteria during some seasons of the year, particularly *Komvophoron, Oscillatoria, Phormidium, Geitlerinema* and *Pseudanabaena* (ŠPAČKOVÁ et al. 2009; HAŠLER & POULÍČKOVÁ 2010).

We isolated 20 strains of filamentous epipelic cyanobacteria from ponds and lakes of different trophic status in three EU countries (Czech Republic, Austria and Italy). This project aims at taxonomic evaluation of the epipelic filamentous cyanobacteria (*Geilterinema, Microcoleus* and *Phormidium*) based on morphological and molecular characters.

Materials and Methods

Strain isolation and morphological study. Altogether 48 sediment samples were taken during May 2007 using methods described by HAŠLER et al. (2008). The geographic position and environmental variables of the Czech sites were published by HAŠLER et al. (2008). Italian localities (Monbino, GPS: 46°7‘28.191“N, 11°3‘30.647“E; Lago di Tovel, GPS: 46°15‘40.775“N, 10°56‘57.851“E) were situated in Trento, near the border between Italy and Austria. The locality in Austria (Untersee) is situated at Lunz am See (GPS: 47°51‘11.602“N, 15°3‘3.256“E), southwest of Vienna. Strains of filamentous morphospecies were isolated following standard methods (ANDERSEN et al. 2005). Cultures were maintained in 100 ml Erlenmeyer flasks under our standard laboratory conditions (temperature 22 ± 1 °C, illumination 20 $mmol.m^{-2}.s^{-1}$, light regime 12h light/12h dark, liquid Zehnder medium (STAUB 1961). All strains were studied using a Zeiss AxioImager light microscope (objectives EC Plan–Neofluar 40×/1.3 N.A., oil immersion, DIC; Plan–Apochromat 100×/1.4 N.A., oil immersion, DIC); with images taken with a high resolution camera (AxioCam HRc 13MPx). During morphological evaluation we focused on these characters: trichome shape and width, presence of sheath, cell dimensions, cell wall constrictions, shape of apical cell, presence or absence of calyptra, and granulation of cells. At least 30 filaments of each strain were characterized.

DNA extraction. DNA extraction was performed using the protocol of DOYLE & DOYLE (1990). The integrity and quality of DNA was checked on 1.8% agarose gels. Concentrations of DNA samples were assessed using a NanoDrop ND–1000 Spectrophotometer (NanoDrop Technologies, Delaware, USA).

DNA amplification and sequencing. PCR amplification of the partial 16S rRNA gene and full 16S–23S ITS region was performed using a combination of two primers P1 (5’–CTCTGTGTGCCTAGGTATCC–3’) and P2 (5’–GGGGAATTTTCCGCAATGGG–3’) described previously in BOYER et al. (2002). These primers produce a ~1180 bp segment of the 16S rRNA gene (bp 325–end) as well as the complete 16S–23S ITS region and 30 bases of the 23S rRNA gene. Total volume of the PCR reaction was 20 µl and it contained: 8.5 µl of sterile water, 0.5 µl of each primer (concentration 0.01 mM), 10 µl FastStart PCR master (Roche Diagnostics GmbH, Mannheim, Germany) and 0.5 µl of template DNA (50 $ng.\mu l^{-1}$). Conditions of the PCR reaction were: 1) initial denaturation for 4 min at 95 °C, 2) 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 57 °C, and extension for 1 min 50 s at 72 °C, and 3) a final extension for 7 min at 72 °C. PCR product was checked on 1.5% agarose gels stained with ethidium bromide. Finally, PCR product was purified using GenElute™ PCRClean–Up Kit (Sigma–Aldrich, Co., Saint Louis, Mo, USA) and sent away for commercial sequencing. Sequencing primers were same as primers for amplification.

Phylogenetic analyses. The sequences were assembled in BioEdit v 7.0.5 (HALL 2005) and gene sequence anomalies (e.g. chimeras) were detected using Mallard software (ASHELFORD et al. 2005). All sequences investigated in this study were deposited in GenBank (see accession numbers in Table 1). Additional sequences for further phylogenetic analysis were acquired from GenBank (http://www.ncbi.nlm.nih.gov/) using the following criteria: sequences had to be sufficiently long (at least 1013 bp) and freshwater species of Oscillatoriales *sensu lato* (Pseudanabaenales, Phormidiales, Oscillatoriales in newer taxonomy). Moreover, we tried to avoid poorly determined sequences (marked with sp.). Using these criteria, 78 sequences were chosen for analysis, a data set that was as large as possible given the time restraints of the phylogenetic analyses used. All sequences were initially aligned in Clustal X (LARKIN et al. 2007) and manually corrected in BioEdit version 7.0.5 (HALL 2005). *Gloeobacter violaceus* PCC 8105 was selected

as the outgroup taxon.

Phylogenetic analysis was carried out in Mr. Bayes 3.1 (Ronquist & Huelsenbeck 2003), PAUP* version 4.0b10 (Swofford 2001) and MEGA 5.02 (Tamura et al. 2007). Evolutionary models were selected on the basis of the BIC (Bayesian Information Criterion) model test implemented in MEGA 5.02. The evolutionary model used in Mr. Bayes was the GTR model with gamma–distributed rate variation across sites and a proportion of invariable sites. The analysis was run for 10 000 000 generations with sampling every 100th generation. Minimum evolution (ME) and maximum likelihood (ML) analyses were performed in MEGA 5.02 and maximum parsimony (MP) in PAUP*, gaps were treated as missing data. GTR+Γ model was used in ML analysis. Bootstrap resampling was performed using 1000 replications (ME, MP) or 500 replications (ML), respectively.

The secondary structures of different ITS regions (D1–D1′ helix and Box–B helix) were predicted with the Mfold web server version 3.2 (Zucker 2003) with temperature set to default conditions (37 °C) and draw mode at untangle with loop fix. Secondary structures were then drawn in Adobe Illustrator (CS–3).

Results

Morphology of investigated strains

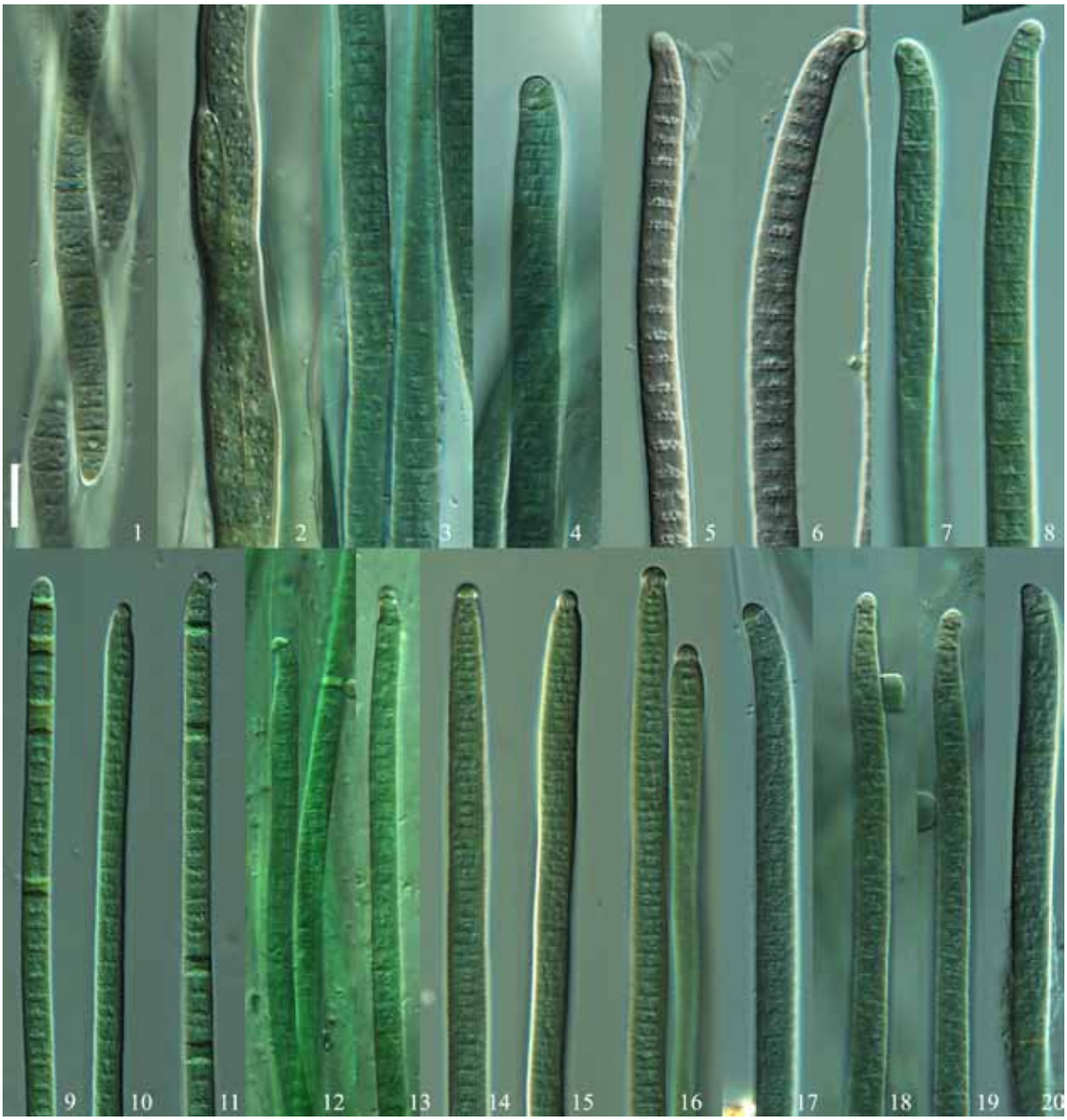
Morphological variability was studied in natural samples as well as in isolated strains. We did not observe extensive variability in filaments in studied morphospecies, especially in natural samples (Table 1). All strains produced single–trichome filaments, only seldom forming filaments of up to five trichomes (e.g. typical for *M. vaginatus*). Isolated strains usually formed fine mats (*Phormidium*, *Geitlerinema carotinosum*, *G. pseudoacutissimum*), macroscopic/microscopic fasciculated colonies (*M. vaginatus*, *G. carotinosum*, *G. pseudacutissimum*) or spherical colonies (*G. splendidum*). *M. vaginatus* often loses its fasciculated filaments in culture, and is then morphologically difficult to separate from *Ph. autumnale* given the similarities in cell dimensions, type of cell division, absence of constrictions at cross–walls, and presence of tapering and calyptra in mature trichomes. However, *M. vaginatus* (Figs 1–8) was distinguishable from *Ph. autumnale* (Figs 9–20) in the frequent presence of conspicuous granules at the cross–walls and generally wider trichomes. Trichomes of *Ph. formosum* (Figs 21–27) were intensely motile (gliding, rotating), constricted slightly at cross–walls, tapered towards apices which possessed rounded to rounded–conical apical cells lacking calyptra. Granulation was fine, if present. A strain of *G. carotinosum* (P013, Fig. 33) was isolated from Lunzer Untersee, from the same watershed as Geitler´s type material (Lunzer Untersee is hydrologically connected with the type locality Lunzer Obersee, Austria). Apical cells were rounded and conspicuous carotenoid granules were present at cross–walls in this strain. *G. pseudacutissimum* (Figs 28–32) contained fine carotenoid granules at cross–walls but to a lesser extent than in *G. carotinosum*. Apical cells were hooked or rounded–acuminate. Both strains of *G. splendidum* (Figs 34–39) did not differ from each other. They both possessed intensely motile attenuated trichomes, and were bent or screw–like at the ends with capitate or rounded apical cells.

Analysis of 16S rRNA and secondary structures of ITS

The PCR reactions yielded a partial 16S rRNA gene (size ~1100 bp) from every strain. Phylogenetic analysis included also comparable long sequences available in GenBank, particularly well defined freshwater strains of filamentous cyanobacteria (Fig. 40) from the families Pseudanabaenaceae, Phormidiaceae and Oscillatoriaceae. Positions of isolated species in the consensus Bayesian tree were in good agreement with their morphology. The Phormidiaceae formed a distinct clade, but members of the Pseudanabaenaceae formed a paraphyletic cline below the Phormidiaceae (Fig. 40). This made clear separation of Pseudanabaenaceae from the Phormidiaceae difficult.

M. vaginatus, as defined by both morphology and the 11 bp insert (bp 423–433), formed a distinct well–supported clade (Fig. 40, clade A). A single filamentous strain identified initially as *Ph. autumnale* P007 due to its slightly narrower trichome diameter had the 11 bp insert as well and was subsequently redesignated *M. vaginatus*. Three strains of *Ph. autumnale* in clade A (strains EU196619–21) had the same 11 bp insert and were isolated from puddles in the Czech Republic by other workers (Lokmer 2007). We conclude that they belong to *M. vaginatus*, and should be considered as such in future studies. All strains in this clade were 98% or more similar in their 16S rRNA gene sequence similarity.

Phormidum autumnale sensu stricto (lacking the 11 bp insert) fell into two lineages sister to *M. vaginatus*, and included a GenBank



Figs 1–20. Variability of filamentous epipelic cyanobacteria: (1–2) *M. vaginatus*, strain P006; (3–4) *M. vaginatus*, strain P0R1; (5–6) *M. vaginatus*, strain P09; (7) *M. vaginatus*, strain P0B; (8) *M. vaginatus*, strain P0C; (9–11) *Ph. autumnale*, strain P00; (12–13) *M. vaginatus*, strain P007; (14–16) *Ph. autumnale*, strain P019; (17–20) *Ph. autumnale*, strain P012. Scale bar 10 mm.

sequence designated as *Phormidium* cf. *subfuscum*. The branch of *Ph. autumnale* including *Ph*. cf. *subfuscum* did not have good support. However, the clade with clearly calyptrate taxa (Fig. 40, clade B) had good bootstrap support. *Oscillatoria sancta* and *Oscillatoria* cf. *curviceps* do not have the capitate apices with calyptra, but both can have a thickened end cap which has been interpreted to be a calyptra (KOMÁREK & ANAGNOSTIDIS 2005). The clade that includes these two *Oscillatoria* and clade B, (*Ph. autumnale* and *M. vaginatus*) is also well supported.

The clade of *Ph. formosum* had high bootstrap support and 16S rRNA sequence data showed at least two lineages corresponding to their geographic origin. Both lineages had high bootstrap support.

The branch containing the calyptrate taxa and non–calyptrate *Phormidium*, along with a mixture of taxa including some *Geitlerinema*, *Microcoleus*, *Coleofasciculus, Wilmottia* and *Phormidium* species had good bootstrap support (Fig. 40, clade C).

Analysis of the 16S rRNA gene separated

Figs 21–39. Variability of filamentous epipelic cyanobacteria: (21) *Ph. formosum*, strain P0010; (22–23) *Ph. formosum*, strain P07; (24) *Ph. formosum*, strain P0A; (25) *Ph. formosum*, strain P001; (26–27) *Ph. formosum*, strain P010; (28) *G. pseudacutissimum*, strain P03; (29–30) *G. pseudacutissimum*, strain P004; (31–32) *G. pseudacutissimum*, strain P005; (33) *G. carotinosum*, strain P013; (34–36) *G. splendidum*, strain P014; (37–39) *G. splendidum*, strain P017. Scale bars 10 mm [(21–32, 34–39), (33)].

G. carotinosum P013 (Austria) from morphologically similar strains of *G. pseudacutissimum* originating from Italian Lakes Tovel and Monbino (Italy). The internal sequence similarity of the 16S rRNA gene in the *G. pseudoacutissimum* clade was 98.6–99.4% (Fig. 40, clade D). *G. carotinosum* had very low similarity to the taxa we place in *G. pseudoacutissimum* (including *"G. carotinosum"* AICB 37), with 16S rRNA similarity to each of those strains ranging 93.1–93.6% similar. *G. splendidum* formed a separate clade, which was strongly supported, although our two strains shared only 97.0% similarity. All our strains of *Geitlerinema* were more related to Phormidiaceae than to Pseudanabaenaceae. *Geitlerinema* sequenced by others were clearly

Table 1. List of isolated strains of epipelic filamentous cyanobacteria [morphology: (L/W) length width ratio, (C) calyptra, (S) sheath, n=30; origin: (A) Austria, (CZ) Czech Republic, (I) Italy]. Molecular characteristics of isolated strains [length of 16S rRNA for all strains ~1031 bp; Gen Bank access number (16S rRNA+ITS), genes of $tRNA^{Ala}$ and $tRNA^{Ile}$ in all strains]. Measured environmental variables are shown in Hašler et al. (2008).

Strain	Trichome end	Apical cell	Width mm	Cell L/W	C	S	GenBank access number	ITS length	Origin
Ph. autumnale									
P00	Attenuated	Rounded, conical, capitate	4–6	0.3–1	+	+	JQ712616 JQ347244	560	CZ Obectov
P012	Attenuated	Rounded, conical, capitate	5–7	0.5–1	+	+	JQ712612 JQ347240	556	CZ Chropyně
P007	Attenuated	Rounded, conical, capitate	4–5	0.5–1	+	+	JQ712604 JQ347232	547	CZ Vrah
P019	Attenuated	Rounded, conical, capitate	4–6	0.3–0.75	+	+	JQ712607 JQ347235	525	CZ Buková
Ph. formosum									
P0010	Shortly attenuated	Rounded, conical	4–5	0.5–1	–	–	JQ712600 JQ347228	645	CZ Naděje
P07	Shortly attenuated	Rounded, conical	4–6	0.5–1	–	–	JQ712606 JQ347234	635	CZ Velký Tisý
P0A	Shortly attenuated	Rounded, conical	4–5	0.5–1	–	–	JQ712603 JQ347231	642	CZ Tovačov
P001	Shortly attenuated	Rounded, conical	4–5	0.5–1	–	–	JQ712611 JQ347239	644	CZ Záhlinice 2
P010	Shortly attenuated	Rounded, conical	4–6	0.3–1	–	–	JQ712613 JQ347241	642	CZ Chropyně
M. vaginatus									
P006	Attenuated	Rounded, conical, capitate	6–7	0.5–1	+	+	JQ712615 JQ347243	566	CZ Obora
P0R1	Attenuated	Rounded, conical, capitate	6–7	0.3–1	+	+	JQ712610 JQ347238	557	CZ Buková
P09	Attenuated	Rounded, conical, capitate	5–7	0.5–1	+	+	JQ712605 JQ347233	553	CZ Rožmberk
P0B	Attenuated	Rounded, conical, capitate	6–7	0.5–1	+	+	JQ712609 JQ347237	577	CZ Horní Ves
P0C	Attenuated	Rounded, conical, capitate	6–7	0.5–1	+	+	JQ712601 JQ347229	581	CZ Bezedník
G. pseudacutissimum									
P004	Not attenuated	Rounded, pointed	1.5–2	1.5–3	–	–	JQ712617 JQ347245	447	I Lake Monbino

Table 1 Cont.

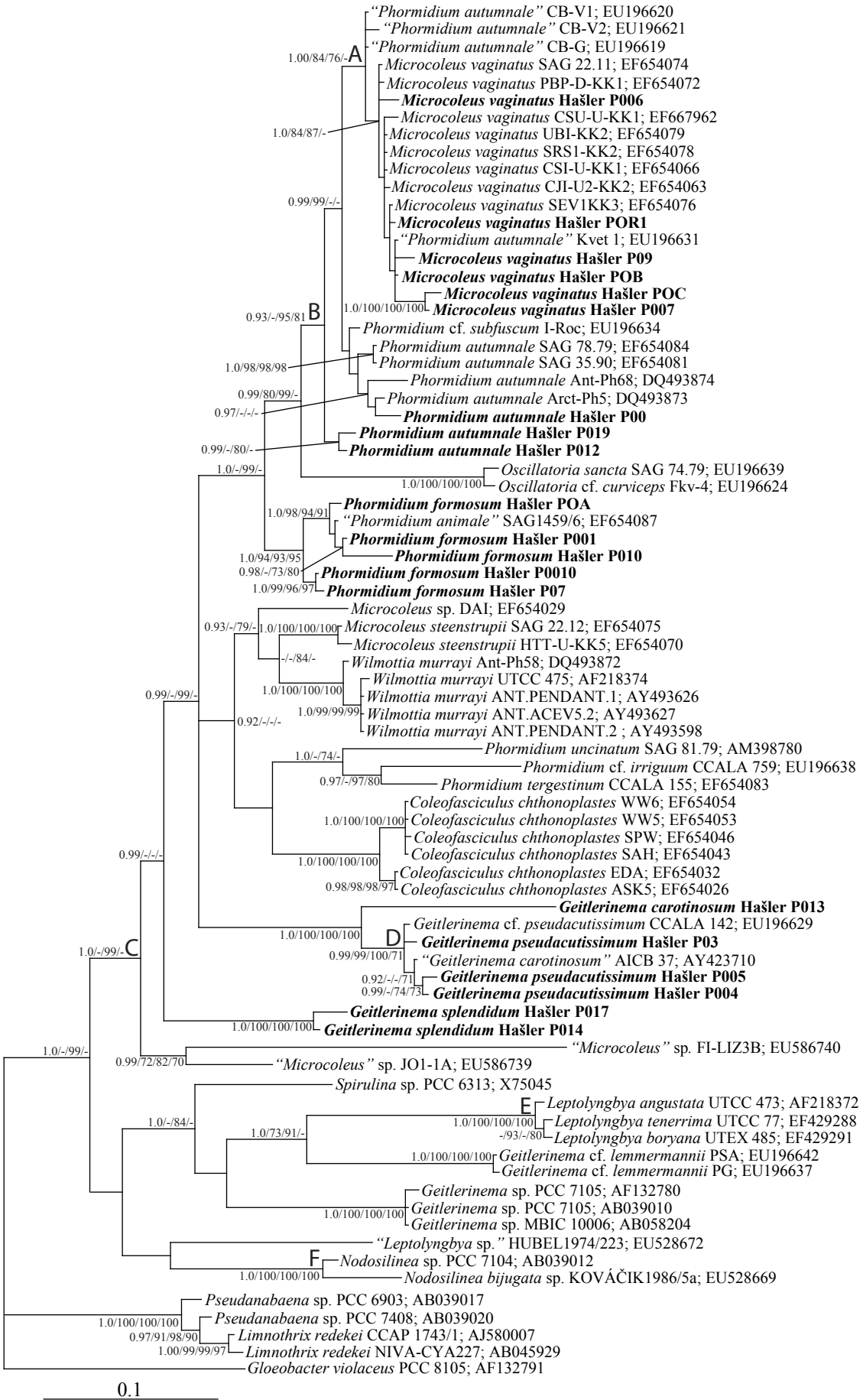
P005	Not attenuated	Rounded, pointed	1.5–2	1.5–3	–	–	JQ712608 JQ347236	451	I Lake Monbino
P03	Not attenuated	Rounded, pointed	1.5–2	1.5–3	–	–	JQ712614 JQ347242	461	I Lake Tovel
G. carotinosum									
P013	Not attenuated	Rounded, pointed	1.5–2	1.5–3	–	–	JQ712598 JQ347226	477	A Lake Untersee Lunz
P014	Attenuated, bent, screw-like	Rounded, capitate	2–3	1.5–3	–	–	JQ712602 JQ347230	493	I Lake Monbino
P017	Attenuated, bent, screw-like	Rounded, capitate	2–2.5	1.5–3	–	–	JQ712599 JQ347227	491	I Lake Tovel

in the Pseudanabaenaceae sister to *Leptolyngbya sensu stricto* (Fig. 40, clade E). *Nodosilinea* (Fig. 40, clade F) is part of a group of strains that were recently described as a new genus (Perkerson et al. 2011).

Analyses of secondary structures in 16S–23S ITS regions (size 447–645 bp) demonstrated both similarity and heterogenity in D1–D1´and Box–B helices of *Ph. autumnale*, *Ph. formosum*, *M. vaginatus*, *G. carotinosum*, *G. pseduacutissimum* and *G. splendidum*. Basal parts of all D1–D1´ helices in *Phormidium* and *Microcoleus* were formed by identical 5 bp basal helices (5'–GACCA–UGGUC–3'), followed by a unilateral bulge on the 3' side (Figs 41–48). Generally, secondary structures of D1–D1´ helices of *Ph. autumnale*, *Ph. formosum* and *M. vaginatus* were also similar in the formation of a large terminal loop (Figs 41–48). This is also consistent with our observations of this structure in isolates from desert soils. The region which was variable in the Phormidiaceae clade was the central helix, which contained various and differing small bilateral and unilateral bulges (Figs 41–48). D1–D1´helices of *Ph. autumnale*, *Phormidium formosa* and *Microcoleus vaginatus* were strikingly similar in structure, demonstrating a close phylogenetic relationship between the three taxa (Figs 41–48). Secondary structure of the D1–D1' helices in *Ph. formosum* demonstrated two lineages. One represented by strains P0010 and P07 (isolated from South Bohemia, Fig. 47) and the second represented by strains P00, P010, P001 (isolated from Central Moravia, Fig. 48), a result consistent with the 16S rRNA phylogeny (Fig. 40).

The genus *Geitlerinema* was quite variable in structure of D1–D1´. *G. carotinosum* (strain P013 isolated from Lunzer Untersee) differed in structure (Fig. 49) from *G. pseudoacutissimum*, in which D1–D1´ helices demonstrated two lineages (Fig. 50–51). However, both species did have the typical 5'–GACCU–AGGUC–3' basal helix characteristic of most cyanobacteria. Both strains of *G. splendidum* had an identical D1–D1´ helix, but these structures were very unique. They lacked the 3'–unilateral bulge found in almost all D1–D1' helices in prokaryotes (Fig. 52). Furthermore, they had a small branch on the 3' side of the central helix (Fig. 53).

Analysis of secondary structures in Box–B helices demonstrated a pattern similar to that obsrved for the D1–D1´helices. All lineages had a conserved basal helix with sequence 5'–CAGCA–UGCUG–3'. *M. vaginatus* generally had longer helices than *Ph. autumnale* (Fig. 54–59). Dissimilarity was evident in the terminal loops, which varied in size and sequence. We found that structures of strains of *M. vaginatus* isolated from Central Moravia were different from those originating from Bohemia. We found some difference between strains of *P. formosum* originating from Bohemia (Fig. 60) and those isolated from Moravia (Fig. 61). Box–B helices differed widely among studied species of *Geitlerinema*. Two structures of Box–B helices were found in *G. pseudoacutissimum* but they differed only by one base (Fig. 62–63). The structures of Box–B helices in all three species of *Geitlerinema* were different (Fig. 62–65).


"Phormidium autumnale" CB-V1; EU196620
"Phormidium autumnale" CB-V2; EU196621
"Phormidium autumnale" CB-G; EU196619
1.00/84/76/-A
Microcoleus vaginatus SAG 22.11; EF654074
Microcoleus vaginatus PBP-D-KK1; EF654072
***Microcoleus vaginatus* Hašler P006**
Microcoleus vaginatus CSU-U-KK1; EF667962
1.0/84/87/-
Microcoleus vaginatus UBI-KK2; EF654079
Microcoleus vaginatus SRS1-KK2; EF654078
Microcoleus vaginatus CSI-U-KK1; EF654066
Microcoleus vaginatus CJI-U2-KK2; EF654063
0.99/99/-/-
Microcoleus vaginatus SEV1KK3; EF654076
***Microcoleus vaginatus* Hašler POR1**
"Phormidium autumnale" Kvet 1; EU196631
***Microcoleus vaginatus* Hašler P09**
***Microcoleus vaginatus* Hašler POB**
***Microcoleus vaginatus* Hašler POC**
1.0/100/100/100
***Microcoleus vaginatus* Hašler P007**
0.93/-/95/81B
Phormidium cf. *subfuscum* I-Roc; EU196634
Phormidium autumnale SAG 78.79; EF654084
1.0/98/98/98
Phormidium autumnale SAG 35.90; EF654081
Phormidium autumnale Ant-Ph68; DQ493874
0.99/80/99/-
Phormidium autumnale Arct-Ph5; DQ493873
0.97/-/-/-
***Phormidium autumnale* Hašler P00**
***Phormidium autumnale* Hašler P019**
0.99/-/80/-
***Phormidium autumnale* Hašler P012**
1.0/-/99/-
Oscillatoria sancta SAG 74.79; EU196639
1.0/100/100/100
Oscillatoria cf. *curviceps* Fkv-4; EU196624
***Phormidium formosum* Hašler POA**
1.0/98/94/91
"Phormidium animale" SAG1459/6; EF654087
***Phormidium formosum* Hašler P001**
1.0/94/93/95
***Phormidium formosum* Hašler P010**
0.98/-/73/80
***Phormidium formosum* Hašler P0010**
1.0/99/96/97
***Phormidium formosum* Hašler P07**
Microcoleus sp. DAI; EF654029
0.93/-/79/-
1.0/100/100/100
Microcoleus steenstrupii SAG 22.12; EF654075
Microcoleus steenstrupii HTT-U-KK5; EF654070
-/-/84/-
Wilmottia murrayi Ant-Ph58; DQ493872
1.0/100/100/100
Wilmottia murrayi UTCC 475; AF218374
0.99/-/99/-
Wilmottia murrayi ANT.PENDANT.1; AY493626
1.0/99/99/99
Wilmottia murrayi ANT.ACEV5.2; AY493627
0.92/-/-/-
Wilmottia murrayi ANT.PENDANT.2 ; AY493598
Phormidium uncinatum SAG 81.79; AM398780
1.0/-/74/-
Phormidium cf. *irriguum* CCALA 759; EU196638
0.97/-/97/80
Phormidium tergestinum CCALA 155; EF654083
Coleofasciculus chthonoplastes WW6; EF654054
1.0/100/100/100
Coleofasciculus chthonoplastes WW5; EF654053
Coleofasciculus chthonoplastes SPW; EF654046
0.99/-/-/-
Coleofasciculus chthonoplastes SAH; EF654043
1.0/100/100/100
Coleofasciculus chthonoplastes EDA; EF654032
0.98/98/98/97
Coleofasciculus chthonoplastes ASK5; EF654026
***Geitlerinema carotinosum* Hašler P013**
Geitlerinema cf. *pseudacutissimum* CCALA 142; EU196629
1.0/100/100/100
D
***Geitlerinema pseudacutissimum* Hašler P03**
1.0/-/99/-C
0.99/99/100/71
"Geitlerinema carotinosum" AICB 37; AY423710
0.92/-/-/71
***Geitlerinema pseudacutissimum* Hašler P005**
0.99/-/74/73
***Geitlerinema pseudacutissimum* Hašler P004**
***Geitlerinema splendidum* Hašler P017**
1.0/100/100/100
***Geitlerinema splendidum* Hašler P014**
1.0/-/99/-
"Microcoleus" sp. FI-LIZ3B; EU586740
0.99/72/82/70
"Microcoleus" sp. JO1-1A; EU586739
Spirulina sp. PCC 6313; X75045
E
Leptolyngbya angustata UTCC 473; AF218372
1.0/-/84/-
1.0/100/100/100
Leptolyngbya tenerrima UTCC 77; EF429288
1.0/73/91/-
-/93/-/80
Leptolyngbya boryana UTEX 485; EF429291
1.0/100/100/100
Geitlerinema cf. *lemmermannii* PSA; EU196642
Geitlerinema cf. *lemmermannii* PG; EU196637
Geitlerinema sp. PCC 7105; AF132780
1.0/100/100/100
Geitlerinema sp. PCC 7105; AB039010
Geitlerinema sp. MBIC 10006; AB058204
"Leptolyngbya sp.*"* HUBEL1974/223; EU528672
F
Nodosilinea sp. PCC 7104; AB039012
1.0/100/100/100
Nodosilinea bijugata sp. KOVÁČIK1986/5a; EU528669
Pseudanabaena sp. PCC 6903; AB039017
1.0/100/100/100
Pseudanabaena sp. PCC 7408; AB039020
0.97/91/98/90
Limnothrix redekei CCAP 1743/1; AJ580007
1.00/99/99/97
Limnothrix redekei NIVA-CYA227; AB045929
Gloeobacter violaceus PCC 8105; AF132791
0.1

Discussion

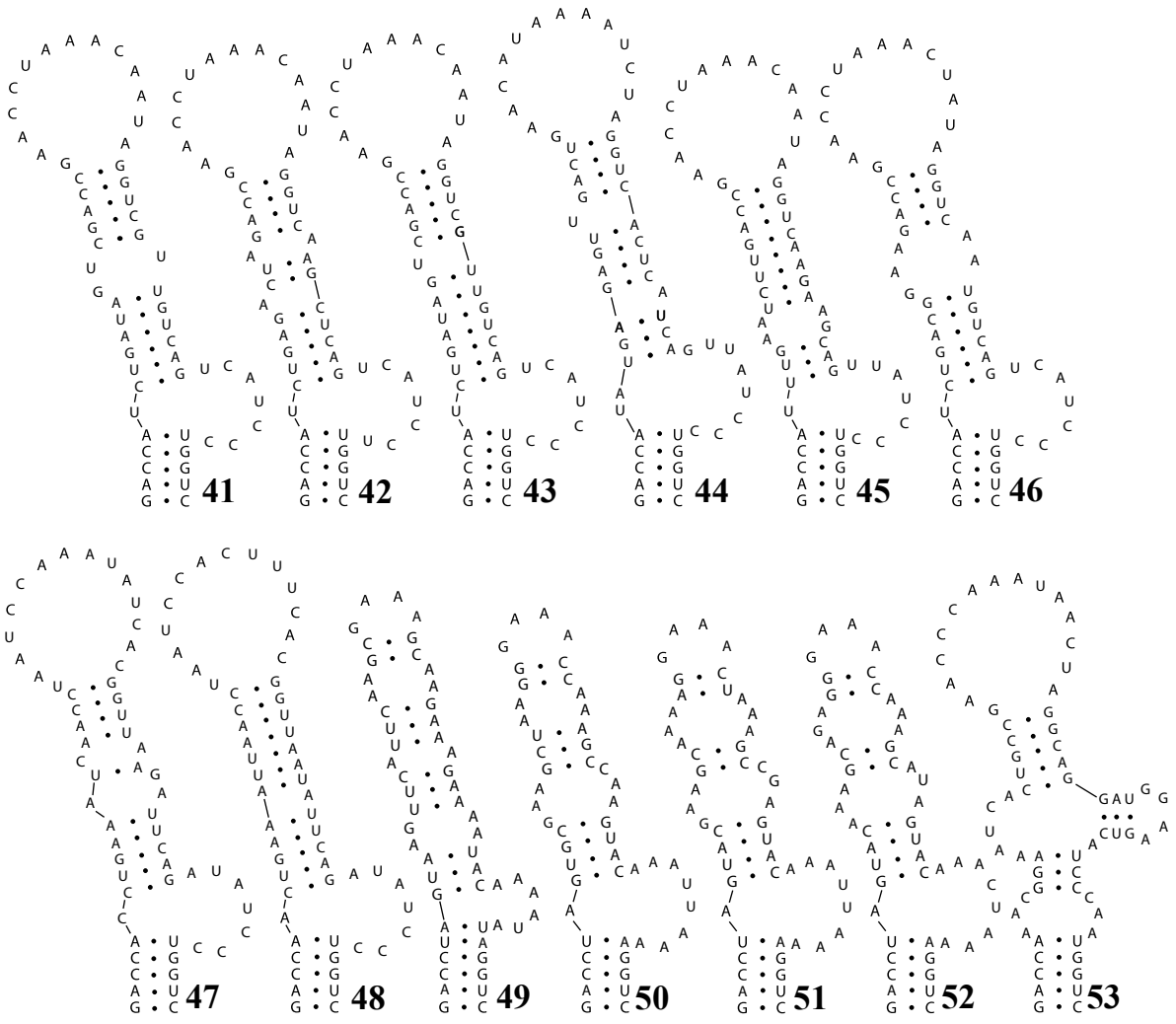
Morphological variability

We studied morphological variability of filamentous cyanobacteria from the families Phormidiaceae (*Phormidium*, *Microcoleus*) and Pseudanabaenaceae (*Geitlerinema*), which were collected and isolated from the bottom sediments. The distribution of epipelic species has been found to be influenced primarily by sediment quality (Hašler et al. 2008). The proportion of fine mud tends to be higher at more eutrophic sites, sandy sediments are characteristic for oligo/dystrophic sites. Muddy or sandy–muddy sediments were inhabited by *Ph. autumnale* [Agardh] Trevisan ex Gomont, *Ph. formosum* (Bory ex Gomont) Anagnostidis et Komárek, *M. vaginatus* Gomont ex Gomont and *G. splendidum* (Greville ex Gomont) Anagnostidis. Sandy sediments were inhabited by *G. carotinosum* (Geitler) Anagnostidis and *G. pseudacutissimum* (Geitler) Anagnostidis.

Some of the species, *Phormidium autumnale* and *Microcoleus vaginatus*, seem to be widely distributed among sampling sites and exhibit overlapping morphological variation. A typical feature of *M. vaginatus*, fasciculate filaments, was consistently observed except in strain P007, which kept a single trichome per filament mode of life in culture. The similarity between *Ph. autumnale* and *M. vaginatus* was first discussed by Drouet (1962). He considered *Ph. autumnale* as a special single filament stage (ecophene) of *M. vaginatus*. The author studied eleven *Phormidium*–like species (*Lyngbya aerugineo–caerulea*, *Ph. autumnale*, *Ph. favosum*, *Ph. incrustanum*, *Ph. setchellianum*, *Ph. subsalsum*, *Ph. toficola*, *Ph. umbilicatum*, *Ph. uncinatum*, *Oscillatoria amoena*, *Os. beggiatoiformis*) and postulated that all of them represented natural variability of *M. vaginatus* under different ecological conditions. Recent studies on *Ph. autumnale* and *M. vaginatus* have not supported Drouet´s opinion (e.g. Casamatta et al. 2005; Siegesmund et al. 2008). Our epipelic strains of *M. vaginatus* showed a narrow morphological variability under laboratory conditions in contrast to descriptions by Drouet (1962) or Komárek & Anagnostidis (2005). The strain P006 was the most representative of epipelic *Microcoleus* and we consider this strain as epitypic. *Ph. autumnale* did not exhibit high morphological variability in contrast to previous reports (e.g. Gomont 1888; Geitler 1932; Desikachary 1959; Starmach 1966; Kondrateva 1968; Komárek 1972; Anagnostidis & Komárek 1988, 2005). Cells were usually wider than long and granulation at cross–walls was fine. The single trichome per filament mode of life was typical. However, old cultures formed flat leathery mats. *Ph. formosum* represents *Phormidium* group No. III (following the classification published by Komárek & Anagnostidis 2005; p. 423, fig. 602). All strains were characterized by shortly narrowed and bent trichome ends with conically–attenuated or rounded apical cells without calyptra. *Ph. formosum* shows similarity to another species, e.g. *Ph. animale*, which belongs to group No II, having gradually narrowed trichome ends in contrast to *Ph. formosum*. Our strains of *Ph. formosum* and strain *Ph. animale* SAG 1459–6 (identical strains: CCAP 1459/6; UTEX 1309) were placed in the same cluster. *Ph. animale* was isolated before 1972 and morphology has been influenced by long–term cultivation. However, it seems to be similar to our epipelic strains of *Ph. formosum*. With respect to similar morphology and position in the same cluster, we conclude that the strain of *Ph. animale* should be referred to as *Ph. formosum* in future studies. In the case of *Ph. formosum*/ *Ph. animale* morphological features may be insufficient to separate them as the key diagnostic feature (long vs. short trichome attenuation) appears variable.

Members of the genus *Geitlerinema* were originally described within the genus *Phormidium*. However, morphology, ultrastructure and physiology differ significantly (Anagnostidis & Komárek 1988; Anagnostidis 1989). We isolated two strains of *G. splendidium* with low morphological variability in contrast to variation described previously (e.g. Anagnostidis 1989). We had occasion to study populations of *G. carotinosum* quite close to the type locality (Austria, Lunz am

Fig. 40. Phylogram (Consensus Bayesian tree) based on 16S rRNA sequences (size ~1000 bp) originated from 20 strains of epipelic cyanobacteria (in bold). Bootstrap values are shown (from left to right) as follows: posterior probabilities ≥ 0.9 and for ≥ 70% minimum evolution, maximum parsimony, maximum likelihood. Sequences from GenBank which appear to us to be misidentified are in quotation marks.



Figs 41–53. ITS secondary structures of D1–D1′ helices: (41–43) *M. vaginatus*, (41) strain P006, (42) strain P09, P007, (43) strain P0B, 0C, P0R1; (44–46) *Ph. autumnale*, (44) strain P00, (45) strain P019, (46) strain P012; (47–48) *Ph. formosum*, (47) strain P0010, strain P07, (48) strain P0A, P010, P001; (49) *G. carotinosum*, strain P013; (50–52) *G. pseudacutissimum*, (50) strain P03, (51) strain P005, (52) strain P004; (53) *G. splendidum*, strain P014 and P017.

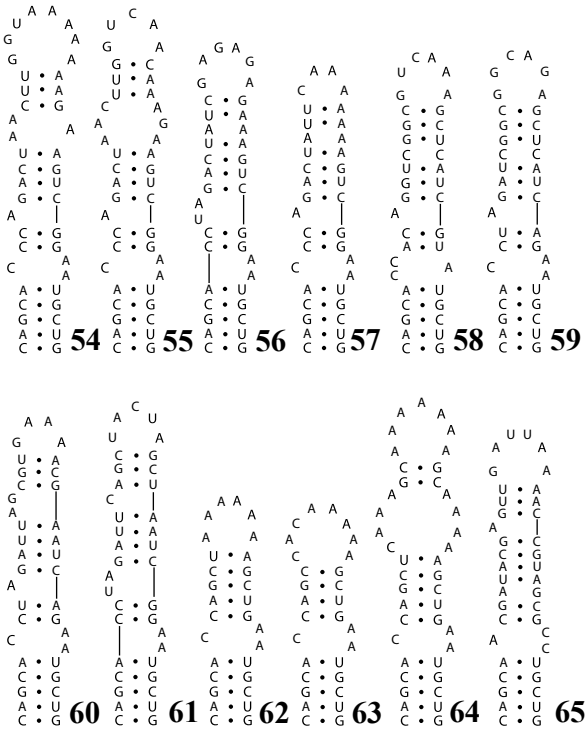
See, Lake Untersee, strain P013). The species was originally described as *Oscillatoria carotinosa* (GEITLER 1956), later combined as *Phormidium carotinosum*, subg. *Geitlerinema* (ANAGNOSTIDIS & KOMÁREK 1988). The diagnostic feature of *G. carotinosum*, carotenoid granules, was found in *G. pseudoacutissimum* as well. Morphology of both species is very similar. However, from our study it seems that trichome ends and type of thallus differ. While *G. pseudoacutissimum* from Italy formed fascicles and resembled *Microcoleus*–like thalli, *G. carotinosum* from Austria created single filaments. Trichome ends of *G. carotinosum* were usually rounded in contrast to *G. pseudoacutissimum* with conical apical cells. However, separation of these two taxa based on type/shape of apical cells can be a fairly subjective decision if used as a sole criterion.

In summary, it seems to be rather characteristic that within species, epipelic populations within this study are morphologically very similar, and consequently populations from different ponds and lakes can be reliably placed within the same species. We tried to verify this hypothesis using molecular methods (see below).

16S rRNA and secondary structures of 16S–23S ITS

Molecular data for the epipelic species under study were congruent with morphology. However the autecology and distribution of individual species shows the patterns discussed below.

Numerous papers focusing on phylogeny of *Phormidium*–like taxa have been published during



Figs 54–65. ITS secondary structures of Box B helices: (54–56) *M. vaginatus*, (54) strain P006, (55) strain P0B, P0C, (56) strain P09, P007; (57–59) *Ph. autumnale*, (57) strain P00, (58) strain P019, (59) strain P012; (60–61) *Ph. formosum*, (60) strain P0010, (61) strain P0A, P010, P001; (62–63) *G. pseudacutissimum*, (62) strain P03, (63) strain P004, P005; (64) *G. carotinosum*, strain P013; (65) *G. splendidum*, strain P014, P017.

the last decade (e.g. Boyer et al. 2002; Marquardt & Palinska 2007; Palinska & Marquardt 2008; Siegesmund et al. 2008). The majority of authors considered the *M. vaginatus–Ph. autumnale* complex as polyphyletic, although a high degree of morphological and genetic similarity between the two taxa was found. Important diacritical features overlap (trichome structure, cell dimensions, successive cell division, presence of calyptrae and sheaths, autecology). Analysis of the 16S rRNA gene presented here confirmed the relationship between *M. vaginatus* and *Ph. autumnale* recorded previously (Siegesmund et al. 2008). A number of workers have noted an 11 bp insert in the 16S rRNA gene (bp 423–433) of *M. vaginatus* (Garcia–Pichel et al. 2001; Boyer et al. 2002; Siegesmund et al. 2008), and this has been identified as an important synapomorphic feature defining the species. It was surprising to find this marker in our aquatic, epipelic strains, as *M. vaginatus* has been thought to be a soil species in arid soils in the past. Both phylogenetic analysis and the presence of the 11 bp insert distinguished all strains of *M. vaginatus* from *Ph. autumnale*. *M. vaginatus* P007 was morphologically similar to *P. autumnale* and was identified by us as that taxon at first due to its narrower trichome width. In previous studies, *M. vaginatus* was recorded as cosmopolitan, occurring mainly in subaerophytic habitats, soils, moist walls, stones, etc. (e.g. Garcia–Pichel et al. 2001; Komárek & Anagnostidis 2005). Our epipelic strains from the Czech Republic clustered together with desert soil strains from the USA (Boyer et al. 2002; Siegesmund et al. 2008). It seems possible that cryptic diversity is present in the clade we currently call *M. vaginatus*, and this diversity is not resolved in the 16S rRNA phylogeny. Morphological differences between strains are evident in our work (Figs 1–8). More detailed study of these aquatic strains (ITS, rbcL, physiology) may allow taxonomic recognition of these strains in the future. Secondary structures of 16S–23S ITS regions were different in epipelic and desert soil strains, the highest variation being found in Box–B helices (cf. Siegesmund et al. 2008; fig. 4). We conclude that differences in 16S–23S ITS regions show at least two lineages, one adapted for short periods of desiccation in contrast to a second lineage adapted for long hot periods. In general, genetic variation in the ITS region seems to be a useful feature for distinguishing populations of cyanobacteria with respect to geographical and habitat preferences.

On the other hand our results support the purported cosmopolitanism of *Ph. autumnale*. Comte et al. (2007) did not find any genetic or morphological differences between Arctic and Antarctic *Phormidium*–like strains, and their sequences belong to the same clade as our epipelic strain P00. We postulate that one worldwide–distributed genotype might exist, which co–occurs with genotypes adapted for particular geographical and environmental conditions, as in the case of genetically different strains Hašler P012 and P019. Secondary structures in the ITS region are considered as informative (Boyer et al. 2001, 2002; Řeháková et al. 2007; Perkerson et al. 2011), and can serve as an additional taxonomic character. As with previously mentioned authors, we did not find high variability in D1–D1′ helices, but Box–B helices showed divergent patterns, which corresponded to the topology of our tree. Differences found between clones from Moravia (P00, P010, P001) and Bohemia (P0010 and P07) cannot be explained by ecology, as all

localities are eutrophic fishponds with large bird colonies causing organic pollution. However the geographical distance between both regions is approximately 400 km and the ponds belong to different watersheds and geological units.

Ph. formosum has not been sufficiently studied by molecular methods. Only three sequences of 16S rRNA have been submitted to GenBank. Our strains formed a well supported clade with *Ph. animale* SAG 1459/6 which may have been misidentified. Secondary structures of Box–B helices in *Ph. formosum* had a specific pattern, different from *Ph. autumnale* and previously described similar filamentous cyanobacteria (cf. SIEGESMUND et al. 2008).

In the first molecular studies on *Geitlerinema* (e.g. MEYERS et al. 2007; BITTENCOURT–OLIVEIRA et al. 2009), the authors did not discuss the position of the genus within the order Oscillatoriales. In a more recent study (and the most thorough on this genus), the authors indicated that *Geitlerinema* was polyphyletic, with *Geitlerinema sensu stricto* (including the freshwater *G. splendidum*) in the Pseudanabaenaceae (PERKERSON et al. 2010). However, their phylogeny included no *Microcoleus* or *Phormidium* taxa, and consequently the familial placement of *Geitlerinema* remains uncertain. Our strains of *G. carotinosum*, *G. pseudacutissimum* and *G. splendidum* were in an uncertain position between the Phormidiaceae and Pseudanabaenaceae. While some *Geitlerinema* strains were clearly close to *Leptolyngbya* in the Pseudanabaenaceae, others were sister to the Phormidiaceae (clade containing *Microcoleus, Phormidium, Wilmottia* and *Coleofasciculus*). Two problematic strains originally assigned to *Microcoleus* (FI–LIZ3B and JO1–1A) by BOYER et al. (2002) are certainly not in that species, and this further confuses the placement of our *Geitlerinema* strains. The most interesting result of our phylogenetic analysis is that our *Geitlerinema splendidum* strains (Hašler P014, Hašler P017) are sister to the clade that includes the remainder of our *Geitlerinema* strains (under 2 μm in diameter), as well as all of the Oscillatorineae (Phormidiales and Oscillatoriales). *Geitlerinema* is currently very problematic as it occupies three clades, two between Pseudanabaenaceae (Synechococcineae) and Phormidiaceae (Oscillatorineae) and one clade within the Pseudanabaenaceae. Studies conducted thus far suggest that *Geitlerinema* has a thylakoid structure belonging to the Pseudanabaenaceae (KOMÁREK & ANAGNOSTIDIS 2005). More study on the taxa transitional between the two families (indeed between two subclasses – Synechococcineae and Oscillatorineae! – see HOFFMANN et al. 2005) is certainly needed.

We suggest the revision of the genus *Geitlerinema* based on material collected from more localities and ecological conditions. Our data show that the genus is not a monophyletic group. This would certainly be consistent with the conclusions of PERKERSON et al. (2010) who looked at more putative *Geitlerinema* than us. Sequences of 16S rRNA from *G. carotinosum* and *G. pseudacutissimum* confirmed the validity of recognizing these as separate species. Description of both species based on morphology is almost identical (KOMÁREK & ANAGNOSTIDIS 2005). However, both species are clearly separated with strong bootstrap support. This finding is supported by analysis of secondary structures in D1–D1′ and Box–B helices. It seems that *G. carotinosum* has been observed only in the type locality and connected lakes in Lunz am See. By contrast, *G. pseudacutissimum* is known from the Czech Republic (Lužnice River, strain CCALA 142) and from Italy (Lakes Tovel and Monbino). Despite some limitation (number of strains under study) we do not agree with WILLAME et al. (2006) that *G. splendidum* and *G. carotinosum* are closely related. Our results are supported by differences in secondary structures in ITS and have a high bootstrap support.

This study showed that for a number of species good agreement between morphology and phylogeny existed at the species level. *M. vaginatus, P. autumnale, P. formosum, G. pseudoacutissimum,* and *G. carotinosum* all formed monophyletic groups consistent with their morphology. What was surprising was that aquatic members of the *M. vaginatus* clade were found, and these were fairly indistinguishable morphologically from *P. autumnale*. These two taxa differ primarily in sheath and filament characteristics, and these are very variable depending on environmental cues. The sheaths tend to disappear in culture, and actually are not very evident in aquatic populations. The fasciculation clear in soil populations of *M. vaginatus* was only weakly expressed in the epipelon. The strong difference in biotopes (desert soil, Czech lakes) suggests separate lineages, but these lineages were not separable by phylogenetic analysis of the 16S rRNA gene sequence. More study of these populations is certainly of interest,

as it is at the center of the physiological variability possible in multiple populations of a single species, or the alternative, cryptic species within a genus.

Finally, this study shows that taxonomic revision is almost certainly inevitable in the group of taxa currently encompassed in *Phormidium* and *Microcoleus*. These two taxa share the same starting point (Gomont 1892). *Microcoleus vaginatus* has cell division similar to the Oscillatoriaceae, and is very different from the majority of species in the genus which have cell division similar to Phormidiaceae. The type species of *Phormidium* is *P. lucidum,* which also has cell division closer to Oscillatoriaceae than Phormidiaceae. Thus, the types for both *Microcoleus* and *Phormidium* are in the Oscillatoriaceae as presently defined in Komárek & Anagnostidis (2005), leaving the vast majority of species in both genera needing revision. *Phormidium* and *Microcoleus* are also confused, and a recommendation has even been made to retypify *Phormidium* with *P. autumnale* (Komárek & Anagnostidis 2005), which would place both types in a highly supported monophyletic clade. Clearly, this problematic group of species, genera, and even families is in need of further study and revision!

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Paper III

Johansenia, a new genus among filamentous epipelic cyanobacteria

Johansenia, nový rod mezi vláknitými epipelickými sinicemi

Petr Hašler, Petr Dvořák & Aloisie Poulíčková

Department of Botany, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-78371 Olomouc, Czech Republic, e-mail: petr.hasler@upol.cz

Abstract

Epipelic cyanobacteria of the genus *Komvophoron* (Cyanobacteria/Oscillatoriales /Borziaceae) were sampled in stagnant freshwater bodies in the Czech Republic. Single filament PCR technique was carried out and unique sequences of 16S rRNA gene and ITS region were obtained. Bayesian interference and maximum likelihood methods confirmed that the genus *Komvophoron* is not monophyletic. The validity of recently described species of *K. hindakii* was confirmed using molecular methods. Two cryptic species were distinguished within traditional morphospecies *K. constrictum*, based on the polyphasic approach and both differ from all other representatives of the genus *Komvophoron*. This was the reason for introducing a new genus *Johansenia* gen. nov. This genus contains to date, two species – *J. constricta* comb nov. and *J. pseudoconstrica* sp. nov. Morphological differences between *J. constric*ta and *J. pseudoconstricta* were found particularly in cell shape and filament width. Further, analysis of the secondary structure of ITS region of *J. constricta* and *J. pseudoconstricta* supports the separation of the species.

Keywords: cyanobacteria, 16S rRNA, ITS, epipelic, new species

Introduction

The notion of species based entirely on morphology (Geitler 1932) has today been replaced by a complex approach to the taxonomy of cyanobacteria, respecting all aspects of their biology,

genetics, physiology and ecology under botanical classification (Anagnostidis & Komárek 1985, 1988, 1990, Komárek & Anagnostidis 1986, 1989, Komárek 2011).

Recent progress in molecular techniques has revealed the high cryptic diversity within cyanobacteria and new genera/species have been described (e.g. Boyer et al. 2002, Casamatta et al. 2003, Řeháková et al. 2007, Siegesmund et al. 2008, Perkerson III et al. 2011). However, many cyanobacterial genera have not been revised yet because they do not grow in cultures and the molecular data were lacking. Single filament/cell PCR and DNA cloning techniques were involved in the taxonomy of unculturable cyanobacteria (Hayes & Barker 1997, Hayes et al. 2002, Nakayma et al. 2011, Yanagihara et al. 2011).

The order Oscillatoriales represents a problematic group of ubiquitous filamentous non-heterocytous cyanobacteria. The taxonomy of oscillatorean cyanobacteria is complicated and needs revision based on the polyphasic approach sensu Komárek (2011). Thin motile oscillatorean cyanobacteria with constrictions at cross-walls were usually identified as *Pseudanabaena* (e.g., Geitler 1932, Skuja 1948, 1956, Starmach 1966,). However, Anagnostidis & Komárek (1988) noticed differences in morphology within the genus *Pseudanabaena* and combined a few of them into the genus *Komvophoron.* The generic features of *Komvophoron* include filament length (brevitrichomy), cell shape (spherical, hemispherical, barrel-like), shape of apical cell (broadly conical, wart-like protrusions), thylakoid arrangement (fasciculate type known only for *K. bourrellyi*), autecology - usually benthic in freshwaters, epiphytic or epizoic in marine environments; e.g. Turon et al. (1991), Willame et al. (2006), Garbary et al. (2007), Matuła et al. (2007), Hašler et al. (2008), Kirkwood et al. (2008), Turicchia et al. (2009), Hašler & Poulíčková (2010). The genus includes two different subgenera, *Alyssophoron* (filaments up to 3.5 μm; type: *K. minutum*) and *Komvophoron* (filaments above 3.5 μm; type: *K. schmidlei*) and several unclear and unrevised taxa (Komárek & Anagnostidis 2005). The majority of natural populations show broad morphological variation and similarity with ,the life stages (hormogonia) of other cyanobacteria (Hašler et al. 2008, Špačková et al. 2009, Hašler & Poulíčková 2010). Detailed knowledge of the biology, ecology and genetic variation has also been lacking for two main reasons: 1. many species inhabit bottom sediments (epipelon), which are overlooked in comparison to other attached or planktic niches (Hašler et al 2008, Špačková et al. 2009, Hašler & Poulíčková 2010); 2. they are unculturable organisms. Epipelic populations of *Geitlerinema splendidum*, *G. carotinosum*,

Microcoleus vaginatus, *Phormidium autumnale* and *Ph. formosum* growing in cultures have been studied in detail across the Europe (Hašler et al. 2012). The method of single filament PCR necessary for molecular work with the genus *Komvophoron* (*K. minutum*, *K. constrictum*, *K. schmidlei* and *K. hindakii)* inhabiting bottom sediments (Špačková et al. 2009, Hašler & Poulíčková 2010) was optimized exclusively in this study. As epipelic representatives of the genus *Komvophoron* do not grow in cultures, strains are not available in culture collections and molecular data for comparison are severely limited. Three short sequences of *Komvophoron* spp. (496-622 bp, 16S rRNA) are available in GeneBank: (Willame et al. 2006, Kirkwood et al. 2008) (http://www.ncbi.nlm.nih.gov/nuccore/?term=komvophoron). Other molecular markers within the genus have not been studied.

This study aims at the molecular characterization of frequent epipelic species of the genus *Komvophoron* and their phylogenetic position based on two genes (16S rRNA and ITS - internal transcribed spacer), using single filament PCR technique. New genus and species have been described.

Methods

Sampling and study of the morphology

The samples of sediments were collected over the years 2010-2011 in fishponds across the Eastern part of the Czech Republic (Líšnice: (A) 49°45'42.480"N, 16°51'37.783"E, (B) 49°45'17.333"N, 16°52'35.100"E, Loštice: 49°43'38.772"N, 16°55'43.526"E, Moravičany: 49°44'41.812"N, 16°59'35.503"E, Chropyně: 49°21'21.320"N, 17°22'7.744"E, Bezedník: 49°17'58.709"N, 17°43'27.083"E) using the method introduced by Round (1953). The morphology of epipelic cyanobacteria was studied in seminatural populations incubated under laboratory conditions: temperature t=22 °C, photoperiod L/D=16/8 hrs, irradiation 20 μmol. cm^{-2}. s^{-1}, liquid medium according to Zehnder (Staub 1961). The light microscope Zeiss AxioImager with objectives: EC Plan-Neofluar oil obj. 40×, NA 1.3 DIC; Plan-Apochromat oil obj. 100×, NA 1.4 DIC) was used for observations. Images were taken using Zeiss HRc camera 12MPx; and digital image processing

software AxioVision 4.7). Thirty filaments per species were measured and statistically analysed using NCSS software (Hintze 2000).

Single filament PCR and sequencing

A previously published protocol for the PCR amplification from single filament (Boyer et al. 2002) was modified in this study. *Komvophoron* filaments were firstly examined and characterized under a light microscope to achieve correct identification of subsequently isolated filaments. Biomass was harvested to fresh sterile water. Using micromanipulation, a single filament was transferred to a drop of sterile water. This step was repeated until there were no contaminants. Afterwards, the filament was transferred into 0.2 ml PCR tubes with 9 µl of PCR grade water. To extract genomic DNA, tubes were 3 times frozen in liquid nitrogen, thawed and vortexed for 10 seconds.

PCR amplification of partial 16S rRNA and complete 16S-23S ITS sequence was performed using cyanobacteria specific primers described in Boyer et al. (2002): forward P2 (5'-GGGGAATTTTCCGCAATGGG-3'), and reverse P1 (5'-CTCTGTGTGCCTAGGTATCC-3'). Premix composed of 0.5 µl of each primer (0.01 mM) and 10 µl FastStart PCR Master (Roche Diagnostics GmbH, Mannheim, Germany) was added to the mixture. The PCR amplification was carried out under the following conditions: initial denaturation for 4 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 57 °C, extension for 1 min 50 s at 72 °C, and finally the reaction was finished with an extension for 7 min at 72 °C. The PCR product was checked on 1.5% agarose gel with 0.5× TBE buffer, stained with Ethidium Bromide. Expected PCR product length was ~1600 bp. Subsequently, all positive bands were isolated using GenElute^{TM} Gel Extraction Kit (Sigma-Aldrich, Co., Saint Louis, MO, USA). Extracted PCR products were cloned using pGEM-T Easy Vector System (Promega Corporation, Madison, WI, USA) following the manufacturer's manual. Transformed competent *Escherichia coli* cells were spread on ampicilin 1.5% agarose plates with Luria Bertani medium. After white-blue selection, at least 4 colonies were isolated and placed into 4 ml of fresh Luria Bertani medium and cultured overnight in 37 °C. Plasmid DNA from all clones was isolated using High-Speed Plasmid Mini Kit (Geneaid, Sijhih City, Taiwan) and sent for commercial sequencing.

The plasmids were sequenced using the following primers: M13f and M13r, with the additional internal primers P5 (5'-TGTACACACCGCCCGTC-3') and P8 (5'-AAGGAGGTGATCCAGCCACA-3') after Boyer et al. (2001), and Boyer et al. (2002). Rough sequences were processed (assembled, proofread and trimmed plasmid sequences) in a Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA), and deposited into GenBank (http://www.ncbi.nlm.nih.gov/). Chimeras and other anomalies were checked in the program Mallard 1.02 (Ashelford et al. 2005).

Phylogenetic analysis

The most closely related sequences to the studied strains were indentified using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Of these, only sufficiently long sequences (at least 1000 bp) were chosen for analysis avoiding uncultured strains. For broader taxonomical context, additional sequences from whole Oscillatoriales, Nostocales and Stigonematales were added (Altogether 96 sequences). Multiple sequence alignment was performed in MEGA 5.05 (Tamura et al. 2011) by implemented Muscle algorithm (Edgar 2004), manually corrected in text editor implemented in the MEGA software and exported in different formats for further analyses.

An evolutionary model for the maximum likelihood analysis was selected based on both Akaike Information Criterion and Bayesian Information Criterion. The analysis was performed in the jModelTest 0.1.1 (Posada 2008) and both criterions revealed General Time Reversible model with gamma distributed rate variation across sites (GTR+G) as the most suitable model. The phylogenetic tree was inferred in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) via CIPRES Science Gateway web server (Miller et al. 2010). Two parallel Markov chain Monte Carlo (MCMC) were simultaneously run for 10 000 000 generations, each one with one cold and three heated chains. MCMC chain was sampled every 1000^{th} generation. The first 2500 trees were burned-in. The GARLi (Zwickl 2006) web server (Bazinet & Cummings 2011) was used to infer bootstrap analysis under the maximum likelihood optimality criterion. Neighbor joining bootstrap analysis was performed in the MEGA 5 using Kimura 2 parameter model (Kimura 1980). Both analyses were carried out with 1000 replications.

Secondary structures of the D1D1' and Box-B helices were predicted using Mfold Web server (Zucker 2003) with temperature set to default (37 °C).

Results

Molecular characterization of Komvophoron species

Using single filament PCR technique unique sequences of members of the genus *Komvophoron* were obtained. These sequences showed a low similarity to sequences available in GenBank (91 – 97% of maximal identity in BLAST search). The most closely related are members of the family Pseudanabaenaceae and Gomontiellaceae. The analysis of 16S rRNA sequences based on Bayesian inference, maximum likelihood and neighbour joining revealed that the genus *Komvophoron* is not monophyletic. The tree topology indicates that three distinct species exist within the sampled epipelic populations: *K. hindakii* and two cryptic species within *K. constrictum sensu lato* (sequence similarity 90%). Moreover the dissimilarity of both *K. constrictum* morphospecies of *K. hindakii* (sequence similarity 88%) allows their separation and description of a new genus *Johansenia*. The first clade (Fig. 1A) includes species described here as *J. pseudoconstricta* sp. nov., which is related to the family Pseudanabaenaeceae (especially to genera *Geitlerinema* and *Spirulina*). *J. pseudoconstricta* clade consists of two subclades indicating existence of two genetically different populations. However, division into these subclades has no bootstrap support. Clade of *J. constricta* (previously *K. constrictum* possesses molecular similarity to Pseudanabaenacean cyanobacteria as well (Fig. 1B, posterior probability 0.97). This species has been selected as a type of the genus *Johansenia*. The existence of two species within the genus *Johansenia* is also supported by secondary structures of ITS region, both D1-D1´ helices and B-box helices (Fig. 2). Interestingly, D1-D1´ and B-box helices of *J. pseudoconstricta* differed in the two populations. The D1-D1´ helix of *J. pseudoconstricta* (population from Bezedník) contained a large hairpin and lower bulge loops, while the D1-D1´ helix contained a small hairpin loop, large upper and small lower internal loops. A different architecture of B-box helices in the two populations was found as well. The B-box helix of *J. pseudoconstricta* (population from Líšnice) contained two small internal

loops and one bulge loop in contrast to one small internal loop found in the population from Bezedník. *J. constricta* possessed higher similarity to *J. pseudoconstricta* (population from Bezedník), however it differed in the size of the hairpin loop and also included one small internal loop in the lower part of helix stem (sequence similarity of D1-D1´ 52%, B-box 69%). *Komvophoron hindakii* belongs to third clade (Fig 1C, posterior probability 1.0) and is related to the family Gomontiellaceae. The secondary structure of D1-D1´ and B-box ITS helices of *K. hindakii* were very different from other analysed structures of *J. pseudoconstricta* and *J. constricta* (sequence similarity D1-D1´ 51-57%, B-box 35%). D1-D1´ included small hairpin loop, large multi-branched loop and large lower bulge loop. The shape of the hairpin loop was similar to *J. pseudoconstricta* (population from Líšnice) and *H. pringsheimii*. B-box helices of *K. hindakii* and *H. pringsheimii* had similar shape, however *K. hindakii* included a large upper bulge loop. Sequence similarity of B-box helices in the two species was very low (D1-D1´48%, B-box 20%).

Morphological and ecological remarks

Molecular data are congruent with the morphology. Both species within *Komvophoron constrictum sensu lato* separated by molecular methods were distinguished morphologically and the description of a new genus *Johansenia* is justified. Overall similarity of both morphotypes (Figs 1-17) lies in filament morphology, both form long, unattenuated and deeply constricted filaments with prominent granules at cross-walls. However, they differ in the shape of the vegetative and apical cells. Filaments of the first morphotype (*J. constricta*) consisted of isodiametric to cylindrical vegetative cells, usually with rounded apical cells. The second morphotype (*J. pseudoconstricta*) consisted of rectangular isodiametric vegetative cells, usually with conical apical cells. Both morphotypes possessed greater similarity to Pseudanabaenaceae than to Borziaceae. Statistical analysis showed significant differences in filament width in the two morphotypes (One Way ANOVA: $F= 32.44$, $p< 0.01$, Fig. 20). Filaments of *J. constricta* were usually up to 5 μm, on the other hand filaments of *J. pseudoconstricta* were over 5 μm. The parietal arrangement of thylakoids was evident in both species using DIC contrast. The pseudanabaenacean character of *Johansenia* is in good agreement with molecular data. The mentioned morphological features possessed a high stability among natural populations and in incubated material. Moreover, the two species differ in

autecology. *J. pseudoconstricta* usually inhabits sandy sediments with a lower proportion of fine mud. In contrast *J. constricta* occurs on muddy sediments with a high portion of organic detritus or rarely on black, anoxic sediments.

The morphology of *K. hindakii* (population from Kvasice) was congruent with the original description by Hašler & Poulíčková (2010). The filaments were usually short, up to 50 cells, straight or bent, motile (gliding), vegetative cells spherical or hemispherical with typical whole like structures near deep cross-walls constrictions, the cell content was usually fine homogenous, pale green or blue-green. The population inhabits fine muddy sediment with a high portion of organic particles in Kvasice pond accompanied by *J. constricta*.

Johansenia Hašler, Dvořák & Poulíčková, gen. nov.

Diagnosis: single filaments or fine mats, filaments disintegrate without the help of necridic cells, filaments short to long (more than 50 cells), motile (gliding), flexible, not attenuated at the ends, deeply constricted at cross walls, cells without gas vesicles, usually barrel-shaped, rectangular or cylindrical, apical cells rounded or conical, cell content divided into visible chromatoplasma and nucleoplasma, thylakoids in peripheral arrangement.

Type species: *Johansenia constricta*.

List of species

Johansenia constricta (Szafer) Hašler, Dvořák et Poulíčková, comb. nov. [basionym: *Oscillatoria constricta* Szafer, Bull. Int. Acad. Sci. Cracovie, Mat-Nat Sci, ser. B, 1910: 164; synonym: *Komvophoron constrictum* (Szafer) Anagnostidis et Komárek, Algological Studies 50-53: 327-472].

Johansenia pseudoconstricta Hašler, Dvořák & Poulíčková sp. nov.

Diagnosis: single filament or seldom fasciculate thallus. Filaments long, straight, seldom bent, motile, deeply constricted at cross-walls (visible mucilaginous bridges). Cells green, pale green or blue-green, usually isodiametric (rectangular shape) or shorter than wide 5.4 ± 0.4 μm, with granulated content (dark and bright granules), especially at cross-walls. Apical cells broadly conical. Filaments divide into short parts (hormogonia) without the help of necridic cells.

Habitat: epipelic species in stagnant freshwaters on sandy to muddy sediments

Holotype: Figs 11-15

Paratype: Figs 10,16,17

Isotype: preserved sample is stored at the Department of Botany, Palacky University, Olomouc, Czech Republic

Type locality: small forest pond near Líšnice, Olomouc Region, Czech Republic; 49°45'42.480"N, 16°51'37.783"E

Etymology: the species name reflects similarity with *J. constrictum*

Discussion

The genus *Komvophoron* (Borziaceae) is an overlooked group of oscillatorean cyanobacteria possessing cryptic diversity. Historically, the first study on the genus *Komvophoron* was carried out by Anagnostidis & Komárek (1988), who combined pseudanabaenacean cyanobacteria with respect to their morphology. Later, a few new species were described based on the International Code of Botanical Nomenclature and polyphasic approach in cyanobacterial research (Turon et al. 1991, Turicchia et al. 2009, Hašler & Poulíčková 2010). Although the genus *Komvophoron* comprises few species in contrast to, "wide genera" such as *Phormidium*, its taxonomy and position remained unclear and cryptic diversity occurs within the genus. Especially thin species of the subgenus *Alyssophoron* are complicated and molecular data are needed to confirm their position.

The phylogenetic relationships of the genus *Komvophoron* have not been discussed in detail, because sufficient number of sequences have been lacking. Recently published molecular studies based on 16S rRNA gene include either sequences which are too short (*Komvophoron* sp., 520 bp, Willame et al. 2006) or entities whose sequences are not available in GenBank (*K. apiculatum* and *K. rostratum*, Turcchia et al. 2009). Under this study was analyzed the largest number of *Komvophoron* sequences ever collected before and our analysis strongly supports morphological incongruence in recently defined genus (Komárek & Anagnostidis 2005). Our data show that the

genus *Komvophoron* is not monophyletic because it appears in two distinct clusters. Similarities of 16S rRNA with known sequences in GenBank are lower than 95%, which supports genus validity.

The group of *Johansenia* gen. nov. is related to the Pseudanabaenaceae (*Geitlerinema*, *Spirulina*), and this is congruent with the morphology. This group does not cluster with the *Leptolyngbya* group as reported earlier (Willame et al. 2006). *Komvophoron* sp. (strain 0RO36S1) sharing 90.1% similarity with strain *Leptolyngbya* 0ES31S2 is not comprehensively characterized. In our view, *Komvophoron* sp. 0RO36S1 was misidentified and does not represent the genus *Komvophoron*.

Johansenia constricta is one of the most frequent epipelic morphospecies across Europe (e.g. Hašler et al. 2008, Hašler & Poulíčková 2010). Its taxonomy was originally discussed (as *Komvophoron constrictum*) in detail by Anagnostidis and Komárek (1988) who combined this species with similar types, and separated them from the genus *Pseudanabaena*. Pairs of large black granules were reported as an important diagnostic feature (Komárek & Anagnostidis 2005). We observed that these granules do not occur in all cases or can be poorly visible, even under Nomarski differential contrast. Filament width should range between 3-7 μm (Komárek & Anagnostidis 2005). However, filaments from 4 to 5 μm (4.6 ± 0.2 μm) have been found across Europe (Hašler et al. 2008, Špačková et al. 2009, Hašler & Poulíčková 2010). Thus the former *Komvophoron constrictum* sensu lato consists of two cryptic species (*J. constricta* and *J. pseudoconstricta*) and with respect to wide morphological variability and with respect to molecular analysis it should be combined into the family Pseudanabaenaceae. Morphological differences useful for distinguishing this genus from *Komvophoron* sensu stricto include filament length and shape of vegetative cells. This is supported by molecular differences. *Johansenia* usually forms longer filaments than *Komvophoron* and usually contains angular cells in contrast to the spherical to hemispherical cells of *Komvophoron*.

The *K. hindakii* group is related to the family Gomontiellaceae, which forms a strongly supported clade consisting of the genera *Hormoscilla*, *Crinalium* and *Starria*. Two strains of *Hormoscilla* were out of this clade. The strain *Hormoscilla* sp. (described as *Hormoscilla* sp.nov., figs 3, S17, Pereira et al. 2011, access no JF262062) is not a validly described taxon either under the International Code of Botanical Nomenclature or under the International Code of Nomenclature of Bacteria and morphologically does not represent the genus *Hormoscilla*. On the other hand, the

strain *Hormoscilla* sp. LCR-OSC2 (access no HQ012544), correctly described as *H. irregularis* Novis & Visnovsky (2011), possesses all features of the genus *Hormoscilla* and should be studied in detail, to explain its phylogenetic position. The strain is still available, but it cannot leave New Zealand (Phill Novis, personal communication). The position of *K. hindakii* and its phylogenetic relationship to Gomontiellaceae is based on logic. Filaments of *K. hindakii* and cyanobacteria of the family Borziaceae are more similar to Gomontiellaceae than to other families, even if they do not form necrotic cells. The cell content does not show differentiation into chromatoplasm and nucleoplasm as in *J. constricta*. The cell wall of *K. hindakii* incorporates hole-like structures near cross wall constrictions. These structures correspond to the wall depressions known in layer L-II of *Hormoscilla pringsheimii* (Rosowski & Lee 1991). *K. schmidlei* (type species) is morphologically similar to *K. hindakii*. However it is extremely rare across Europe (Hašler & Poulíčková 2010) and no sequence exists in Gene Bank. Sequence no. AF355398, misinterpreted as *K. schmidlei* in Turcchia et al. (2011), in GenBank is originally identified as *Leptolyngbya schmidlei.* It morphologically corresponds to *Leptolyngbya* or it may represent a new undescribed species (Johansen, pers.com.).

Historically, the 16S rRNA gene symbolizes the basic molecular marker in the taxonomy of prokaryotic organisms. During last two decades, many genera of cyanobacteria were revised using 16S rRNA sequences (Komárek 2010). However the importance of this marker has been discussed many times. Its low sensitivity was proven at species level (sequences similarity >97%). Use of additional molecular markers in species phylogeny produces more precise results (e.g. Ludwig 2011). More recently, 16S-23S rRNA internal transcribed spacer (ITS) region has been found to be a suitable marker capable of differentiating species or lower levels. The ITS regions of *Johansenia* and *Komvophoron* were not sequenced before this study. Our data indicate high variability of ITS regions within *J. pseudoconstricta,* exceeding the normal range of structural variability as found e.g. in *Microcoleus* or *Coleofasciculus* (Boyer et al. 2002, Siegesmund et al. 2008).

In summary, epipelic cyanobacteria represent an extraordinary group, dominated by specialized filamentous, motile species such as *Johansenia constricta*, *J. pseudoconstricta* and *K. hindakii*. This study gathered critical evidence that the genus does not represent a monophyletic lineage and has to be divided into two genera: *Johansenia* (Pseudanabaenaceae) and *Komvophoron* (Borziaceae). This study (based on molecular, morphological and ecological data) confirmed the validity of *K. hindakii*

and its phylogenetic relation to the family Gomontiellaceae. The new genus *Johansenia* was described and its phylogenetic position was discussed.

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Souhrn

Epipelické populace vláknitých sinic rodu *Komvophoron* představují velmi komplikovanou skupinu, ve které není zcela jasné vymezení některých druhů. Ze studií a znalostí morfologické variability doposud vyplývá, že některé druhy (*K. schmidlei*, *K. constrictum*) byly definovány poměrně široce a vykazují známky kryptické diverzity. S ohledem na komplikace při kultivaci a izolaci těchto sinic nebyly dosud získány vhodné kmeny, které by mohly být využity při molekulární analýze. Metodou single cell/filament PCR a následným sekvenováním jsme získali 13 unikátních sekvencí 16S rRNA genu a ITS oblasti dvou významných druhů *K. hindakii* a *K. constrictum*. Z našich výsledků vyplývá, že rod *Komvophoron* netvoří monofyletickou jednotku a musí být rozdělen do dvou rodů. *Komvophoron hindakii* je nejblíže typovému druhu *K. schmidlei*, proto druhy podobné těmto typům a s podobnou sekvencí by měly být označovány jako *Komvophoron*. Tato skupina patří do čeledi Borziaceae a je blízce příbuzná sinicím čeledi Gomontiellaceae. Na druhou stranu druhy morfologicky a geneticky podobné *K. constrictum* náleží do nového rodu *Johansenia*. Tyto druhy vykazují morfologickou a molekulární podobnost se sinicemi čeledi Pseudanabaenaceae. S ohledem na molekulární podobnost s rodem *Geitlerinema* a morfologickou podobnost s rodem *Pseudanabaena* řadíme rod *Johansenia* do podčeledi Pseudanabaenoideae.

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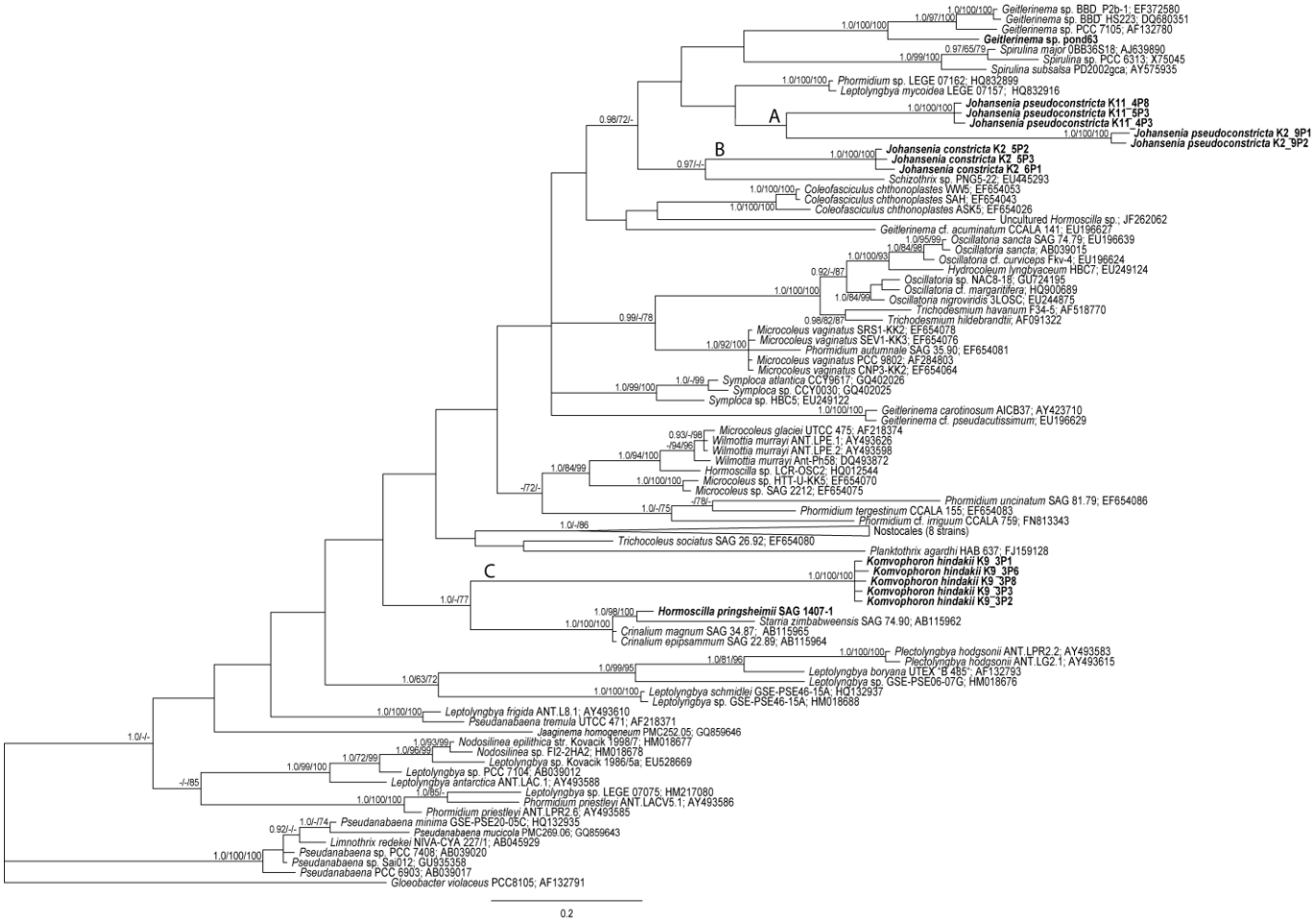


Fig. 1. Concensus Bayesian tree based on 16S rRNA (size 1000 bp), 96 species of filamentous cyanobacteria were added to the analysis, original sequences from 13 isolates of epipelic *Komvophoron*, *Johansenia* species and *Hormoscilla pringsheimii* SAG 1407.1 (in bold). Node supports are shown in the following order: Bayesian posterior probabilities, bootstrap values of maximum likelihood and neighbour joining . **A** clade of *Johansenia pseudoconstricta*, **B** clade of *J. constricta*, **C** *Komvophoron hindakii*.

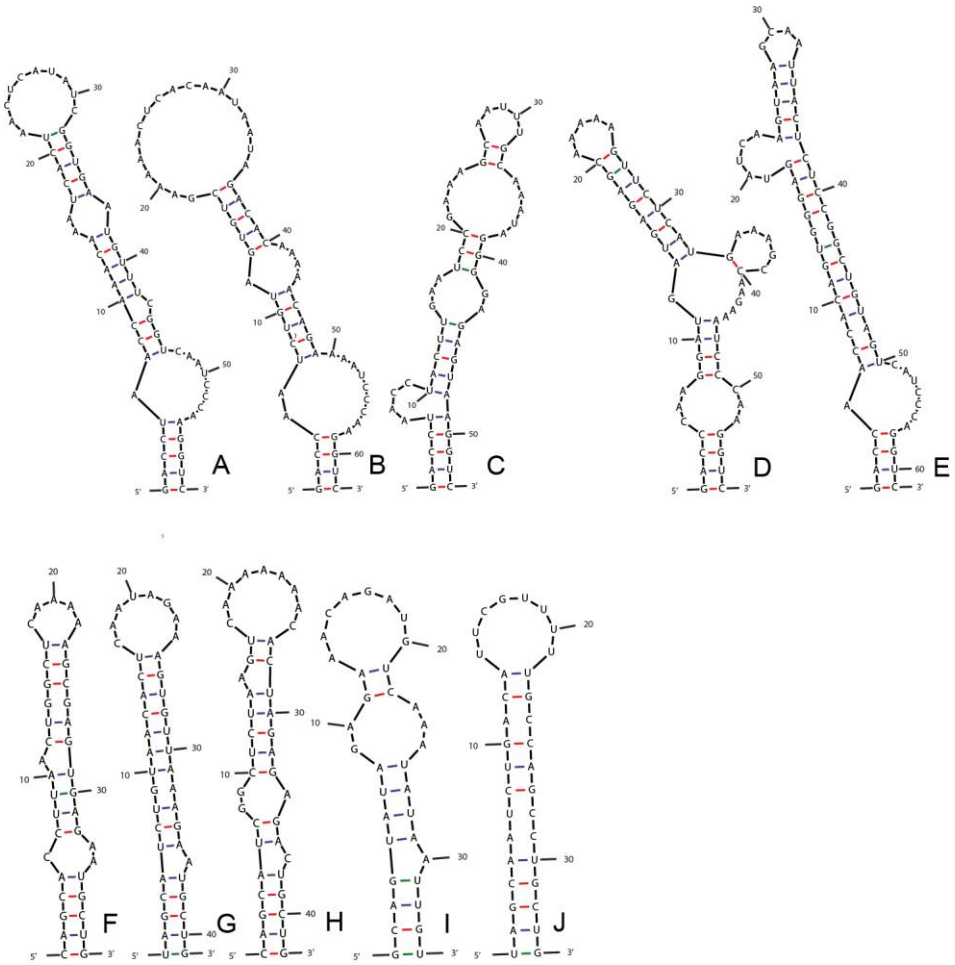
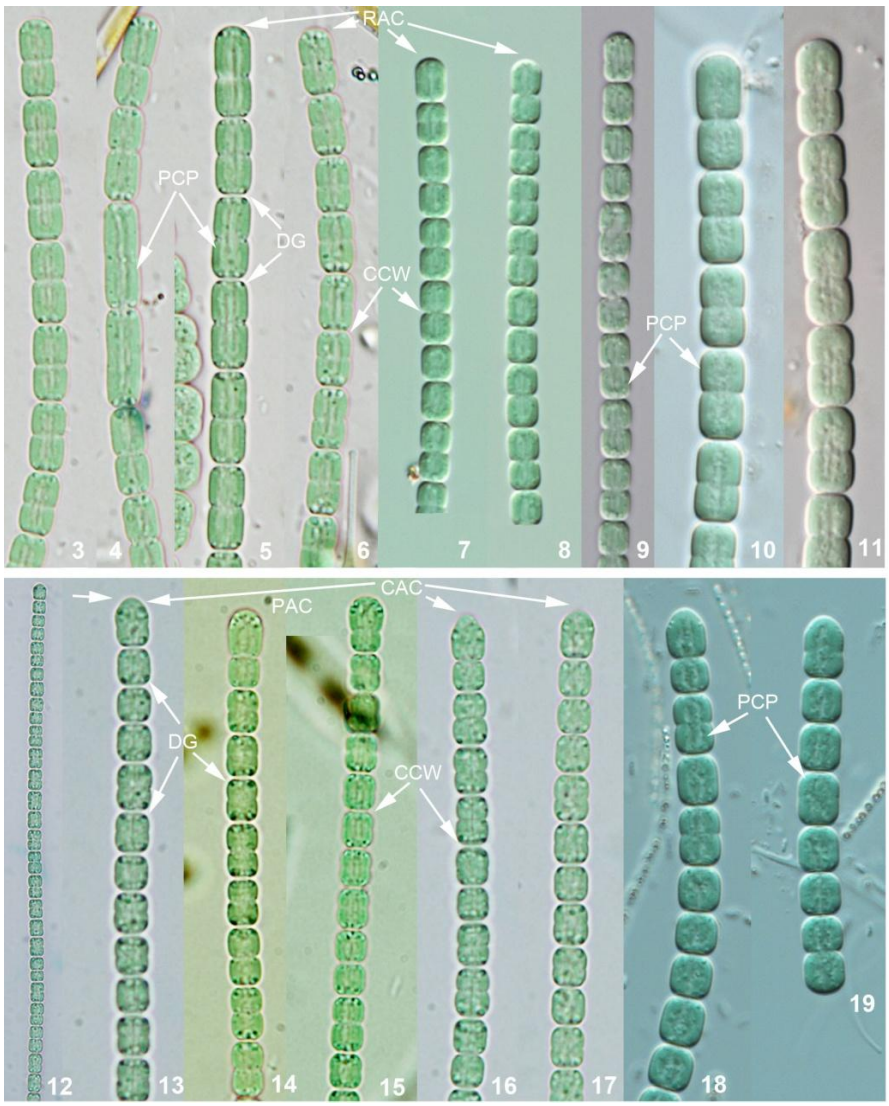


Fig. 2. 16S-23S rRNA secondary structures. D1-D1´helices: **A** *J. constricta*, population from Líšnice; **B** *J. pseudoconstricta*, population from Bezedník; **C** *J. pseudoconstricta*, population from Líšnice; **D** *K. hindakii*, population from Kvasice; **E** *H. pringsheimii*, strain SAG 1407.1. B-box helices: **F** *J. constricta*, population from Líšnice; **G** *J. pseudoconstricta*, population from Bezedník; **H** *J. pseudoconstricta*, population from Líšnice; **I** *K. hindakii*, population from Kvasice; **J** *H. pringsheimii*, strain SAG 1407.1.



Figs 3–19. Morphological variability of *Johansenia*. **3-11** *Johansenia constricta*, **3-6** population from Chropyně pond, **7-11** population from Líšnice pond; **12-19** *Johansenia pseudoconstricta*, **12** filament from Moravičany, **13-17** population from Líšnice, **18-19** population from Bezedník. Abbreviations: **RAC** rounded apical cell, **CAC** conical apical cell, **PCP** parietal chromatoplasm, **CCW** constricted cross-walls, **DG** dark granules.

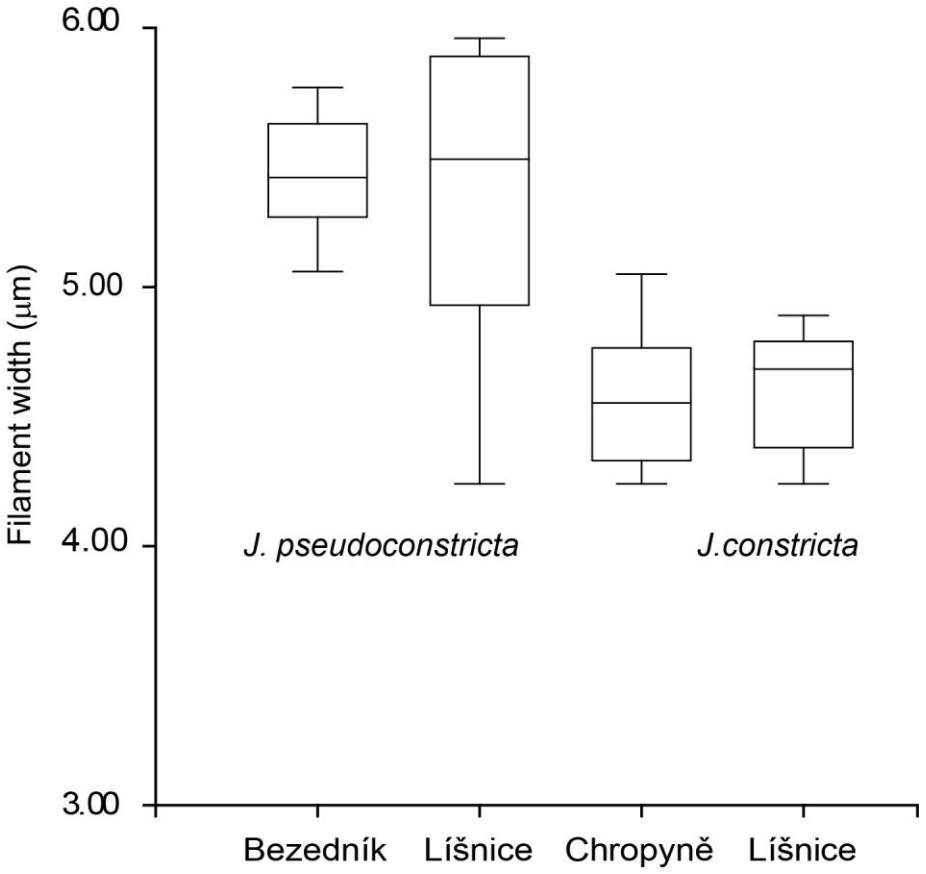


Fig. 20. Box plot diagram based on filament width of *J. pseudoconstricta* and *J. constricta*. One Way ANOVA shows significant difference between both species ($F= 32.44$, $p << 0.01$).

Paper IV

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PLoS one

Phylogeography of the *Microcoleus vaginatus* (Cyanobacteria) from Three Continents – A Spatial and Temporal Characterization

Petr Dvořák*, Petr Hašler, Aloisie Poulíčková

Department of Botany, Palacký University Olomouc, Olomouc, Czech Republic

Abstract

It has long been assumed that cyanobacteria have, as with other free-living microorganisms, a ubiquitous occurrence. Neither the geographical dispersal barriers nor allopatric speciation has been taken into account. We endeavoured to examine the spatial and temporal patterns of global distribution within populations of the cyanobacterium *Microcoleus vaginatus*, originated from three continents, and to evaluate the role of dispersal barriers in the evolution of free-living cyanobacteria. Complex phylogeographical approach was applied to assess the dispersal and evolutionary patterns in the cyanobacterium *Microcoleus vaginatus* (Oscillatoriales). We compared the 16S rRNA and 16S-23S ITS sequences of strains which had originated from three continents (North America, Europe, and Asia). The spatial distribution was investigated using a phylogenetic tree, network, as well as principal coordinate analysis (PCoA). A temporal characterization was inferred using molecular clocks, calibrated from fossil DNA. Data analysis revealed broad genetic diversity within *M. vaginatus*. Based on the phylogenetic tree, network, and PCoA analysis, the strains isolated in Europe were spatially separated from those which originated from Asia and North America. A chronogram showed a temporal limitation of dispersal barriers on the continental scale. Dispersal barriers and allopatric speciation had an important role in the evolution of *M. vaginatus.* However, these dispersal barriers did not have a permanent character; therefore, the genetic flow among populations on a continental scale was only temporarily present. Furthermore, *M. vaginatus* is a recently evolved species, which has been going through substantial evolutionary changes.

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* E-mail: p.dvorak@upol.cz

Introduction

Having been intensively studied over the past two decades, biogeography is one of the crucial factors necessary for an understanding of the ecological, evolutionary, and diversity patterns of prokaryotes [1,2].

Generally, two different approaches toward the biogeography of free-living microorganisms have recently been discussed. (1) Historically, an older hypothesis claims that the occurrence of free-living organisms is driven by the environment, which selects the composition of a microbial community. The dispersal is then considered without any barriers (ubiquity); therefore, allopatry does not affect speciation [3,4]. (2) To the contrary, some authors have recently advocated the existence of dispersal barriers and even endemic taxa within free-living microorganisms [2,5–12]. The existence of some of the desmids' distributional areas resembling the phytogeographical patterns of vascular plant taxa has been noted by some authors [13,14]. If the biogeography patterns of prokaryotes are closely related to those in eukaryotes [1], the existence of allopatric speciation can be expected [15].

The idea of cosmopolitanism is supported in some cyanobacteria by molecular markers, e.g. *Coleofasciculus* (*Microcoleus*) *chthonoplastes* [16], *Microcystis aeruginosa* [17]. However, van Gremberghe *et al.* [17] suggested the existence of a globally distributed population, which locally undergoes repeated events of bottleneck and selective sweeps [18,19]. This drives speciation without any specific biogeographical pattern and allopatry. Arguments against ubiquity have recently been suggested in situations of geographical isolation on the continental level in thermophilic cyanobacteria such as *Synechococcus* spp. [20], *Mastigocladus laminosus* [21]. The inconsistency among the findings (mentioned above) implies a poor understanding of the overall mechanisms involved in cyanobacterial biogeography.

The cyanobacterium *Microcoleus vaginatus* (Vaucher) Gomont appears to be a suitable model organism for the evaluation of the biogeography and evolutionary patterns within free-living cyanobacteria, due to its world-wide distribution as well as its relatively easy identification, isolation, and culturing. *M. vaginatus* is an important primary producer within soil crusts and other subaerophytic environments all around the World.

[22–24]. However, *M. vaginatus* has also been isolated from freshwater epipelon [25], and from periodically dry puddles (this study); thus, indicating that it is not strictly aerophytic. Its taxonomy has been sufficiently studied [23,26] and it has been genetically well characterized by the presence of an 11-bp insert in its 16S rRNA gene, which is a molecular autapomorphy for this

species [22,23]. However, practical identification of cultured strains is problematic because some important morphological features are missing in cultured materials, particularly the multiple filaments in a common sheath (e.g. [23]).

The 16S rRNA gene is a molecular marker, frequently used in the taxonomy and ecology of cyanobacteria, particularly on the genus level; additionally, there are a huge number of sequences available in GenBank (e.g. [27]). By contrast, 16S-23S ITS (internal transcribed spacer) is a variable region, which seems to be suitable for investigation on (and below) the species level, even for population genetics [28,29].

Evolutionary relationships on different taxonomical levels are usually visualized graphically using phylogenetic trees. Nevertheless, when such mechanisms as recombination, horizontal gene transfer, or hybridization are taken into account, phylogenetic networks are more appropriate [30]. Accordingly, the network construction approach is also advantageous for the phylogeny of prokaryotic organisms (e.g. [31]).

The present study focuses on the evolutionary dispersal and distributional patterns of *M. vaginatus*, isolated from different continents, based on the 16S rRNA gene and 16S-23S ITS region, using phylogeographic methods combining both the tree and network, as well as PCoA analysis. Molecular clocks were applied in order to put the spatial distribution of *M. vaginatus* into a temporal framework.

Materials and Methods

Ethics statement

No specific permits were required for the described field studies. No specific permission was required for any locations and activity. The locations are not privately owned or protected in any way. No activity during field study involved any endangered species or protected species.

Sample collection and cultivation

Altogether, 21 strains of *M. vaginatus* and 7 strains of *Phormidium* spp. (only used for the 16S rRNA analysis) were obtained either from natural samples or from the Culture Collection of Autotrophic Organisms (CCALA; http://www.butbn.cas.cz/ccala/index.php).

The samples were collected from different habitats (e.g. puddles, moistened soil) and geographic sites (See Figure 1 and Table S1). Unialgal cultures were isolated following standard techniques [32]. The identification of all strains was based on their morphology using a light microscope, and following the system *sensu* Komárek & Anagnostidis [24]. The cultures were maintained in 100 mL Erlenmeyer flasks under the following conditions: temperature $22\pm1^{\circ}C$, illumination 20 $\mu mol/m^2/s$, light regime: 12h light/12h dark, and liquid Zehnder medium [33].

DNA extraction, PCR, and sequencing

Genomic DNA was extracted using an UltraClean Microbial DNA Isolation Kit (MOBIO, Carlsbad, CA, USA) from approximately 30 mg of fresh biomass, harvested during the log phase of the culture growth. 1.5% agarose gel, stained with ethidium bromide, was used to check DNA quality.

Partial 16S rRNA genes and the whole 16S-23S ITS region were PCR amplified using primers: forward P2 (5′-GGGGAATTTTCCGCAATGGG-3′), and reverse P1 (5′-CTCTGTGTGCCTAGGTATCC-3′). The combination of primers was previously described in Boyer *et al.* [23]. The PCR reaction, with a total volume of 20 μL, contained: 8.5 μL of sterile water, 0.5 μL of each primer (0.01 mM concentration), 10 μL FastStart PCR master (Roche Diagnostics GmbH, Mannheim, Germany), and 0.5 μL of template DNA (50 $ng.\mu L^{-1}$). The PCR reaction was performed under the following conditions: initial denaturation for 4 min at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 57°C, extension for 1 min 50 s at 72°C, and lastly the reaction was completed with an extension for 7 min at 72°C. Quality PCR products (~1600 bp) was examined on 1.5% agarose gels, stained with ethidium bromide. The PCR products, amplified from newly obtained strains, were cloned using a StrataClone PCR Cloning Kit (Agilent Technologies, Stratagene Product Division, La Jolla, CA, USA), following the manufacturer's instructions. After the white-blue selection on ampicillin 1.5% agarose plates with Luria Bertani medium, at least 4 positive colonies were transferred into fresh liquid Luria Bertani medium and cultured overnight at 37°C. The plasmid was isolated using a QIAGEN Plasmid Mini Kit (QIAGEN Inc., Valencia, CA, USA). The PCR product, amplified from culture collection strains, was purified using a GenElute™ PCRClean-Up Kit (Sigma-Aldrich, Co., Saint Louis, MO, USA).

Both the plasmid (all positive clones) and purified PCR product were sent for commercial sequencing. The plasmids were sequenced using primers M13f and M13r, with the additional internal primers P5.

(5′-TGTACACACCGCCCGTC-3′), and P8 (5′-AAG-GAGGTGATCCAGCCACA-3′), which have been previously described [23,29]. The PCR products were sequenced using the same primers as used for amplification, with the additional internal primers P5 and P8 (see above). The sequences were assembled and proofread in a Sequencher 4.10 (Gene Codes Corporation, Ann Arbor, MI, USA); then they were deposited in GenBank (http://www.ncbi.nlm.nih.gov/). Accession numbers of the 16S rRNA sequences are JQ712618 to JQ712645, and 16S-23S ITS JQ712646 to JQ712666. All of the clones which were generated from each strain were aligned (ClustalX 2.0.11) [34]. All clones from all individual strains were found to be completely identical. Therefore, each strain is represented by one sequence.

Phylogenetic and statistical analyses

The 16S rRNA Sequences were checked against chimeras and other anomalies within Mallard 1.02 software [35]. Multiple sequence alignment of both the 16S rRNA gene and 16S-23S ITS was performed by the ClustalW [34] algorithm, implemented in MEGA 5.05 [36], and corrected manually in a MEGA software alignment editor; following, were then exported in different formats for further analyses. The 16S-23S ITS sequences were used to construct the phylogenetic tree, as well as the network; further, to conduct the P-test and the PCoA analysis.

All available sequences, with their known geographical origin in GenBank (containing both genes $tRNA^{Ile}$ and $rRNA^{Ala}$ and with a known geographical origin) of *M. vaginatus* 16S-23S ITS, were added to the studied strains for analysis. Those sequences which had originated from desert soil crusts in the USA were well defined and had been previously published in Boyer *et al.* [23] and Siegesmund *et al.* [26]. Maximum likelihood and neighbour joining analyses were conducted in MEGA. Bayesian Information Criterion [37] was employed to achieve the most appropriate substitution model for maximum likelihood, and was determined as HKY+G (sample size: 647). The substitution model used in the neighbour joining analysis was the Kimura 2-parameter model [38]; with gaps treated as missing data. In both cases, bootstrap resampling was performed using 1000 replications.

A Neighbour-net phylogenetic network was constructed in SplitsTree4 4.11.3 [30], and all of the parameters were set at the

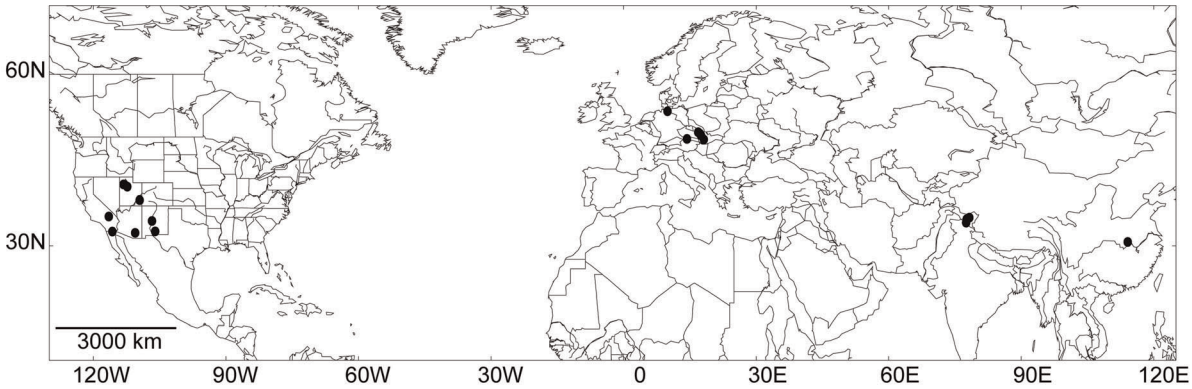

Figure 1. Location of *M vaginatus* sampling sites. The locations of the North American strains were adopted from Boyer *et al.* [23] and Siegesmund *et al.* [25].
doi:10.1371/journal.pone.0040153.g001

defaults. The bootstrap test was performed using 1000 replications.

The Mantel test (9999 permutations) implemented in GenAlEx 6.4.1 [39] was performed in order to test the relationships between the geographic and genetic distances. The genetic distance matrix was inferred in MEGA, and the geographic distance matrix in GenAlEx 6.4.1.

A parsimony P-test [40] for strains which had originated from each continent, and the unweighted principal coordinate analysis (PCoA) were carried out in Fast UniFrac [41]. The best-scoring maximum likelihood tree, inferred in MEGA, was used for the input tree.

Molecular clocks

The partial 16S rRNA gene was used to estimate the dates of divergence of *M. vaginatus*. Sufficiently long sequences (at least 1000 bp) with known geographical origins of *M. vaginatus* were selected from GenBank. Additional sequences from the entire spectrum of cyanobacteria (including partial 16S rRNA sequences of *Phormidium* spp. from the CCALA culture collection) were added to the analysis in order to achieve a broader taxonomic context, as well as more accurate results (total of 146 sequences). *Escherichia coli* was selected as the outgroup. To test the molecular clock hypothesis, a likelihood ratio test implemented in MEGA was used. The null hypothesis of equal substitution rates throughout the entire tree was rejected. Therefore, the relaxed uncorrelated clocks were selected for analysis [42]. The most suitable evolutionary model was presented using Bayesian Information Criterion [37] implemented in MEGA (sample size: 1010). The molecular clocks were calibrated based on the evolutionary distance between sequences of 16S rRNA obtained from fossil DNA samples and the closest recent descendant that could be identified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

All clones (16S rRNA fragments isolated from a 5.8–5.9 Ma late Miocene gypsum crystals) except the two presented in Panieri *et al.* [43] were used. One of the excluded clones was not determined in the study, as there is no sequence deposited in GenBank (see [43]). The second (FJ809895) had the most related recent descendant among eukaryotic chloroplasts. A pairwise distance (in substitutions per site) between each ancestor/descendant sequences was calculated using p-distance model in MEGA. Subsequently, the final substitution rate per site per million years was determined as the mean of all individual pairwise distances per million years. The standard deviation and 95% confidence interval (CI) were calculated. Specific values are shown in the Table S2. The mean substitution rate per million years (0.001861) and 95% CI (0.000643–0.003079) with uniform distribution was set for further analysis, carried out in BEAST 1.6.1 [44]. The analysis was set with the following parameters: GTR+G+I substitution model, MCMC chain length of 6.0004×10^7 generations, sampled each 1.4×10^4 generation, and relaxed uncorrelated lognormal clock [42]. The BEAST.xml file was created in BEAUTi [44]. Due to the temporal demands of the computation, the analysis was carried out on the web portal CIPRES Science Gateway (specialized in phylogeny), where BEAST is implemented [45]. The effective sample size (ESS) was evaluated using TRACER 1.5 [46]. The final maximum credibility tree was annotated using TreeAnnotator 1.6.1 [44], with the first 100 trees burned-in.

Results

Species identification

All of the strains that were under investigation showed the characteristic features according to Komárek & Anagnostidis [24]. *M. vaginatus* strains CCALA 757, 143, and 152 had originally been incorrectly identified and assigned as different species of the genus *Phormidium* within the culture collection. Our re-identification to *M. vaginatus* is based on light microscopy morphology as well as the presence of 11-bp insert within the 16S rRNA. All of the strains were coherent in their important morphological characteristics (cell dimension, shape, cell division, and the presence of calyptra).

16S-23S ITS phylogeographical analysis

Altogether, 32 sequences obtained from strains having originated from three continents (Europe, Asia, and North America) were analysed using two phylogenetic approaches (tree and network), and PCoA analysis. All 16S-23S ITS sequences contained both genes for $tRNA^{Ile}$ and $rRNA^{Ala}$; therefore, the dataset did not exhibit large gaps which possibly could negatively influence the results. The Mantel test showed a very significant correlation between the geographic and genetic distances ($R = 0.184$, $P = 0.0001$).

The maximum likelihood tree (MEGA) revealed two clades: (A) European *M. vaginatus*, and (B) North American and Asian strains (Figure 2). Therefore, the European strains were distinguishable from the North American and Asian, with the exception of two strains with a transitional position between both clades (strains SLad22 and SL1plus, Figure 2). However, the North American and Asian strains clustered together within clade B, without any particular biogeographical pattern. Both clades (A and B) included

a couple of subclades (diversified genotypes), without any respect to the autecology of the strains. Strains S32 and 205-3F had an uncertain position within the tree, without any significant bootstrap support. Internal nodes within both clades A and B (Figure 2) had good bootstrap support; however, the clades themselves were very poorly supported. Thus, a phylogenetic network and the PCoA analysis approach were employed in order to achieve more accurate results.

The almost identical topology showed a neighbour-net network constructed using SplitsTree (Figure 3). The network exhibited groups A (European) and B (North America and Asian, Figure 3), containing almost the same taxa as did the phylogenetic tree. The problematic strains SLad22 and SL1plus (see above) belonged to groups of their biogeographical origin, with high bootstrap support. The position of strains S32 and 205-3F was better resolved. However, strain 205-3F also exhibited a very long branch, suggesting its enormous distance from the other strains.

A similar grouping pattern revealed the PCoA analysis carried out in Fast UniFrac (Figure 4) where the habitat type was taken into account. European strains (group A) formed a separate group from those strains which had originated from North America and Asia (Group B), without any respect to habitat type. Strains SLad22, SL1plus, S32, and 205-3F showed uncertain positions similar to the phylogenetic tree and network.

The clustering of strains in the phylogeny (tree, network) and PCoA analysis were also confirmed by corrected P-values (Fast

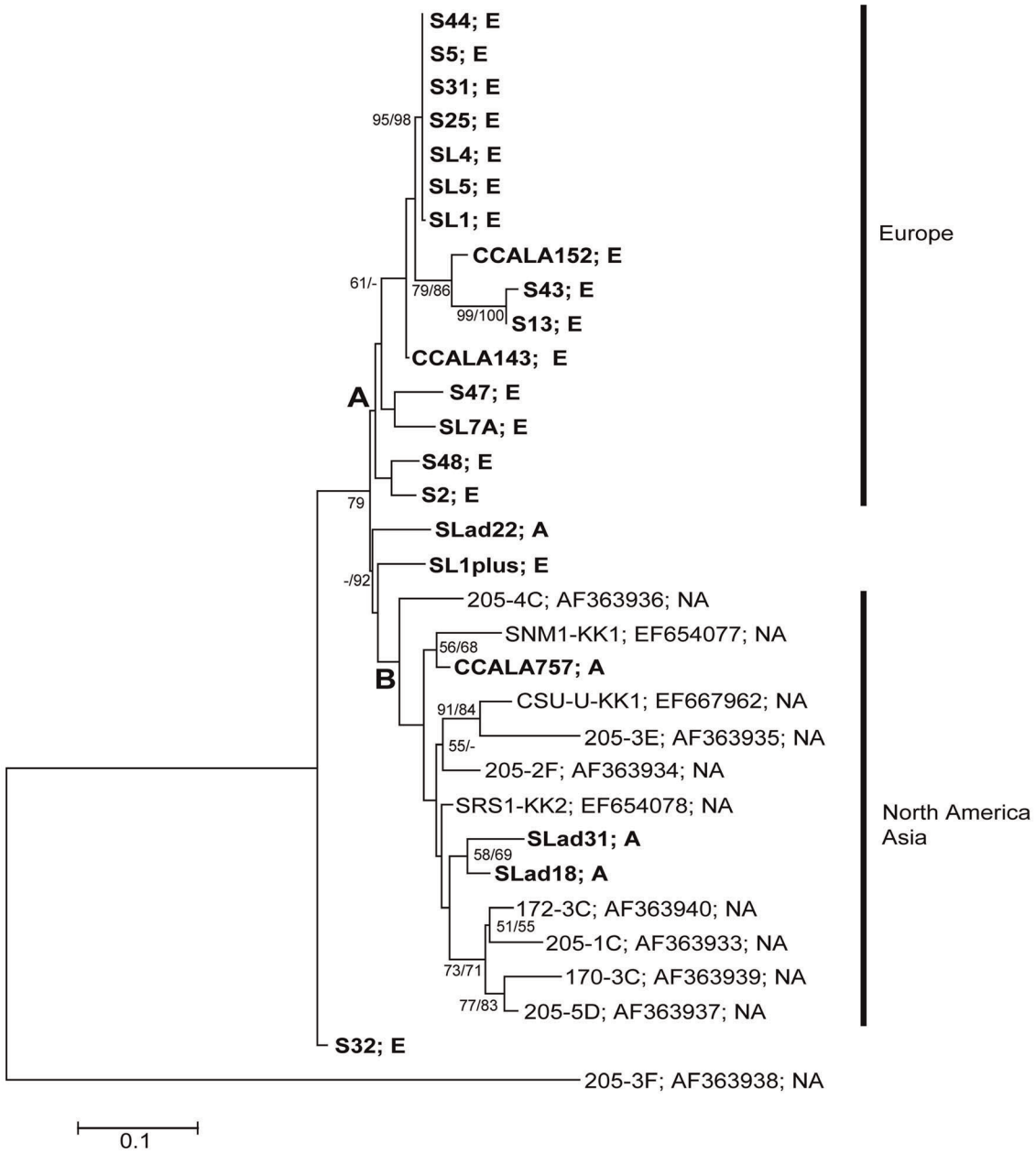

Figure 2. Maximum likelihood inferred phylogenetic tree based on the 16S-23S ITS of *M. vaginatus*. Maximum likelihood/neighbour joining bootstrap supports greater than 50% are shown at the nodes. The studied strains are in bold. The geographical origin of each strain is indicated as E – Europe, A – Asia, and NA – North America.
doi:10.1371/journal.pone.0040153.g002

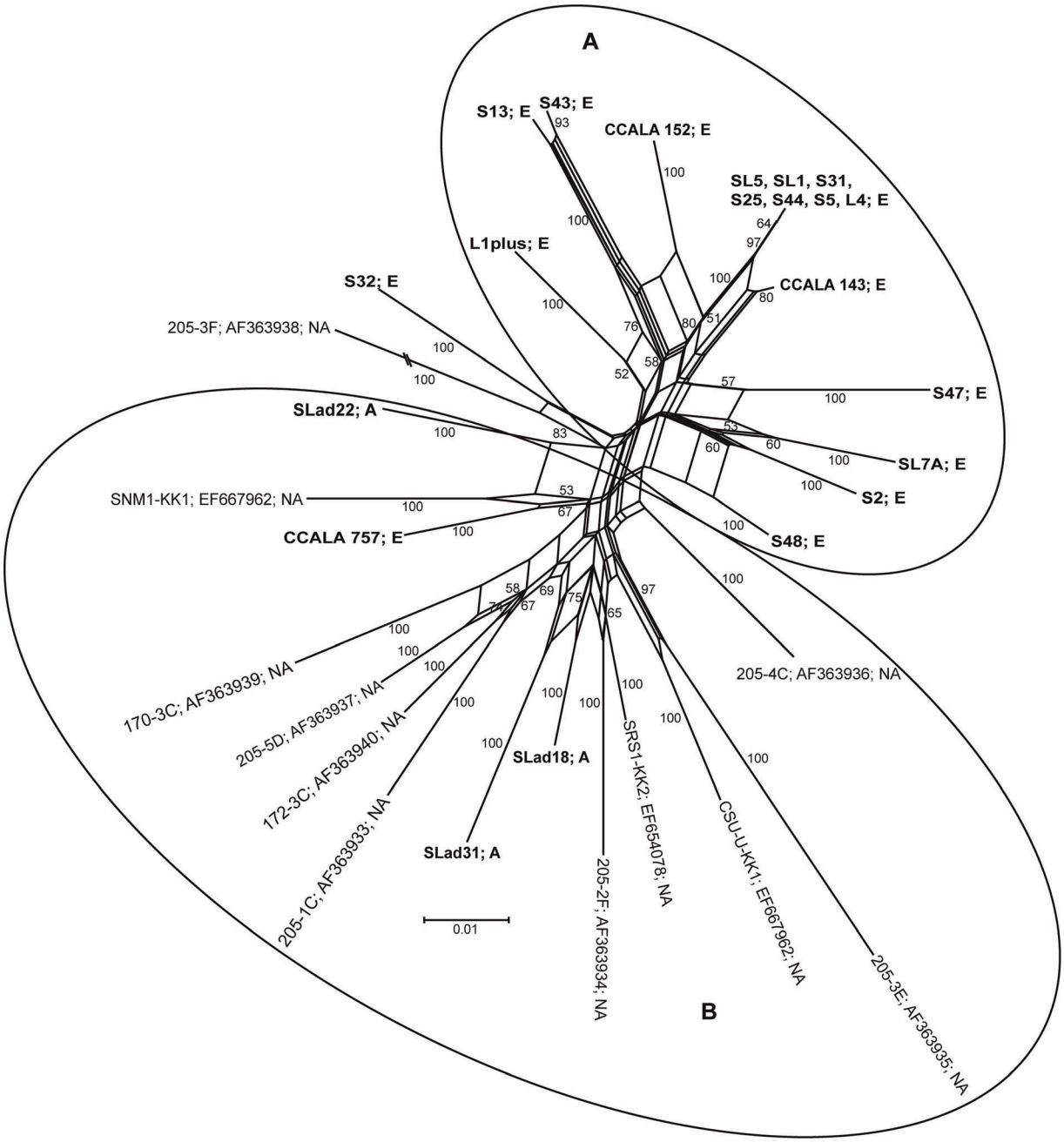

Figure 3. Neighbour-net phylogenetic network based on the 16S-23S ITS of *M. vaginatus.* Bootstrap supports greater than 50% are indicated. The studied strains are in bold. The geographical origin of each strain is indicated as E – Europe, A – Asia, and NA – North America.
doi:10.1371/journal.pone.0040153.g003

UniFrac) for those strains isolated from individual continents. European strains were significantly different from the North American and Asian ($P \leq 0.002$), and the difference between the North American and Asian were only marginally significant ($P \leq 0.096$).

All analyses suggest that strains of *M. vaginatus* which originated from the Europe are genetically different from those isolated from North America and Asia. Therefore, a dispersal barrier between Europe and Asia might well exist; with the speciation of these cyanobacteria also being driven by their geographical isolation. On the other hand, very close relationships, accompanied by an uncertain dispersal pattern between the North American and Asian strains, suggests frequent genetic exchanges between *M. vaginatus* populations on these two continents.

Divergence dating estimation

The dating analysis of the 16S rRNA gene in BEAST was calibrated at an evolutionary rate of 0.001861 substitutions per site per million years (95% CI = 0.000643–0.003079), which has only recently been determined for cyanobacterial 16S rRNA by the comparison of fossil and recent 16S rRNA sequences (see Materials and Methods). This approach gives a coherent image of the divergence times among recent living cyanobacteria; this is because there is a lack of convincing calibrating points and

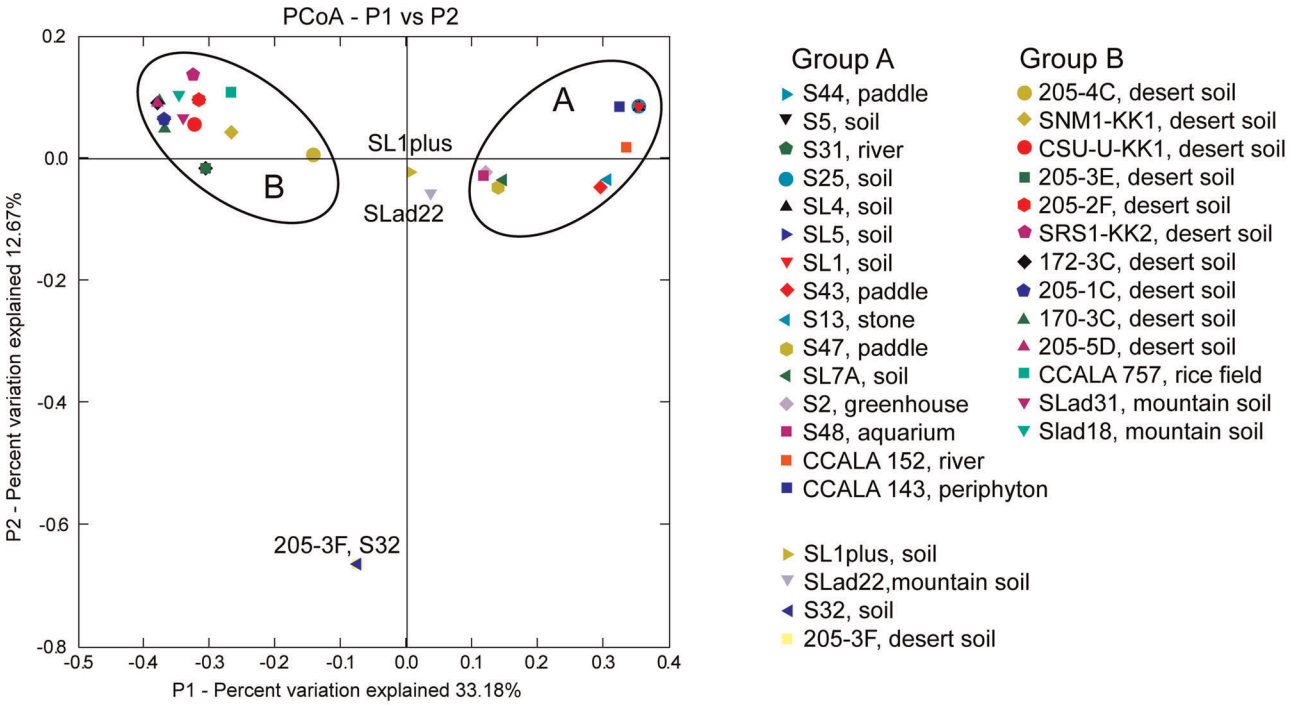

Figure 4. Principal coordinate analysis performed in Fast UniFrac based on the 16S-23S ITS of *M. vaginatus*. Principal coordinate 1 (P1) versus Principal coordinate 2 (P2) is shown. Group A consists of European strains, and group B of North American and Asian.
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dissimilar substitution rates among the different groups of bacteria [47].

The chronogram (Figure 5, 6), based on 16S rRNA, shows divergence times within all groups of cyanobacteria (Chroococcales, Oscillatoriales, Nostocales, and Stigonematales *sensu* Komárek & Anagnostidis [24]); however, focused on *M. vaginatus* (Figure 6). Recent unicellular cyanobacteria (order Chroococcales) diverged from Oscillatoriales before 184.3 Ma, 95% HPD (highest posterior density interval) 116.1–256.6 (clade 1, Figure 5), and formed a monophyletic group with the exception of two filamentous cyanobacteria *Spirulina* spp. Some of recent heterocystous cyanobacteria (Nostocales and Stigonematales; clade 2, Figure 5) diversified one time before 117.8 Ma (95% HPD 71.1–179.3) from filamentous cyanobacteria and formed a monophyletic group (clade 2, Figure 5).

M. vaginatus separated from the other filamentous cyanobacteria (order Oscillatoriales) before 39.5 Ma (95% HPD 22.5–61.8; clade 1, Figure 6) and formed a monophyletic clade with *Phormidium autumunale/Tychonema* spp., which is its sister clade 7 (Figure 6). The European strains were concentrated in clades 2 and 3; moreover, they formed other individual lineages of strains SL1plus, S48, S44, S32, and S47. Thus, the European strains have been derived at least twice: clade 2 (20.9 Ma; 95% HPD 12.8–31.6), and clade 3 (3.7 Ma; 95% HPD 1.2–7.5). On the other hand, clade 4 was composed of strains having originated from Asia, North America, and the S2 European strain, and diverged sometime before 4.4 Ma (95% HPD 1.5–9.8). Clade 5 was composed of two strains which originated from Asia and North America, having originated before 3.7 Ma (95% HPD 1.2–7.5). Similarly, Clade 6 included one North American and one European strain which originated before 9.3 Ma (95% HPD 4.6–15.5). Clade 7 (Figure 6) *Phormidium autumnale/Tychonema* spp. diverged before 16.6 Ma (95% HPD 8.8–26.5).

M. vaginatus appears to have diversified later than the other Oscillatoriales species. For instance, the newly described *Wilmottia murrayi* [48] (clade 3, Figure 5) diverged before 69.5 Ma (95% HPD 26–125.5) and *Coleofasciculus chthnoplastes* [26] (clade 4, Figure 5) before 65.2 Ma (95% HPD 22.9–115.2).

The 16S rRNA based tree exhibits a similar branching pattern for *M. vaginatus* as does the 16S-23S ITS based tree, network and PCoA analysis. European strains retained a geographical separation from North American and Asian strains. However, some minor discrepancies appeared. The European strains formed two separate clades with North American and Asian strains in between them (see details above). The North American and Asian strains showed a close phylogenetic relationship in all performed analyses. Some strains formed individual lineages, e.g. S48, S44 and S47. The position of these strains was better resolved in the 16S-23S ITS phylogeny, where they clustered together with the other European strains (Figure 2, 3, 4, group A). The European strains S2 and SL7A nested among the North American and Asian (clades 4–6, Figure 6) in comparison with the 16S-23S ITS phylogeny, where they retained the cluster of their geographical origin (Figure 2, 3, 4, group A). This suggests that in combination with the relatively long temporal distances among clade divergences the isolation of populations on a continental scale may have a temporary character.

Discussion

Although *M. vaginatus* is a common cyanobacterium that is distributed worldwide, its biogeography and possible dispersal patterns have yet to be sufficiently studied on a large scale. The species seems to be "cosmopolitan" and "ecologically euryvalent", inhabiting aerophytic and freshwater habitats (Table S1). A similar situation has been found with many other microalgae, which were also considered to be cosmopolitan before their cryptic

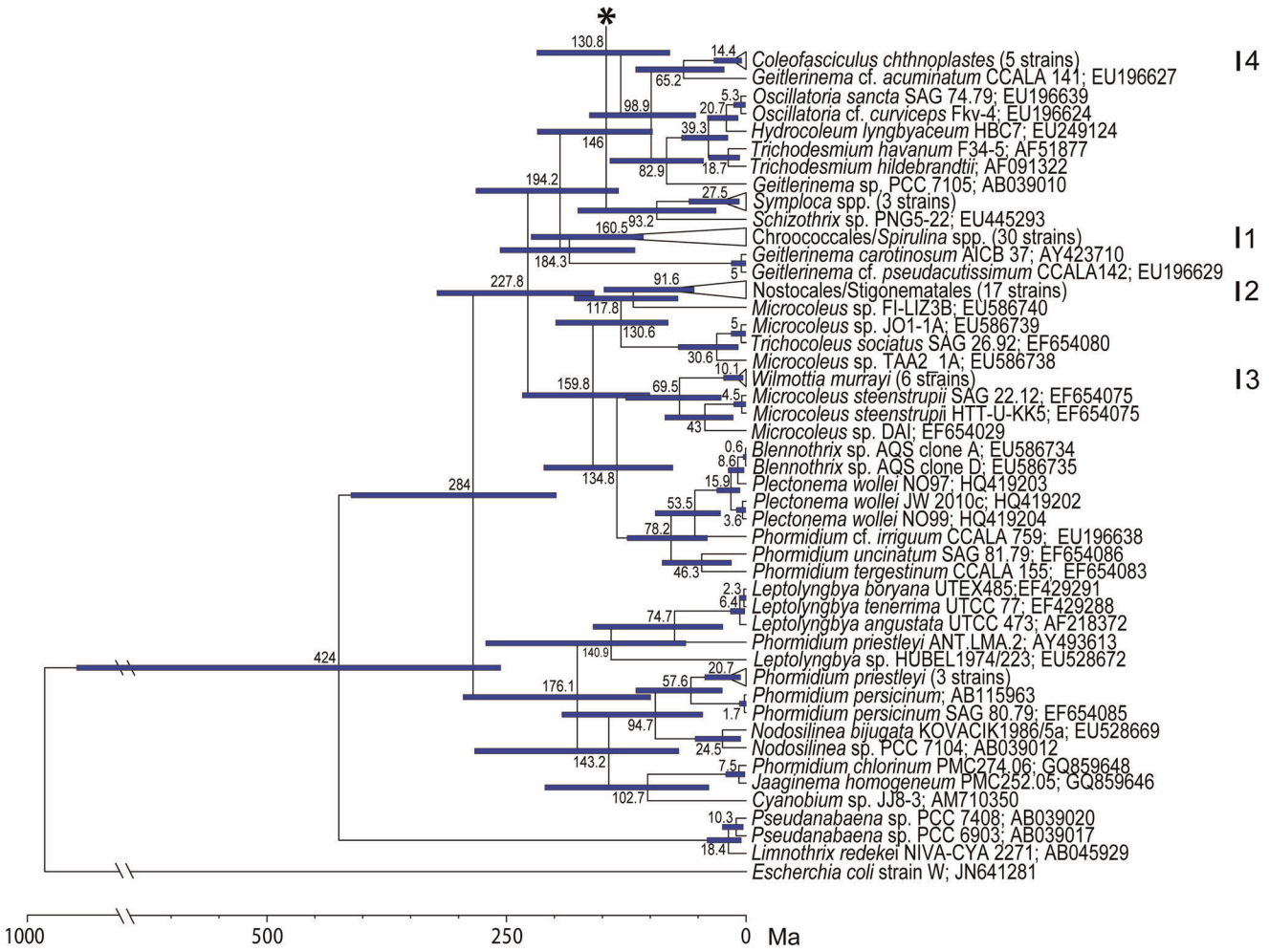

Figure 5. The dating of the divergence times among cyanobacteria. Maximum credibility chronogram based on 16S rRNA of cyanobacteria, with *Escherichia coli* as an outgroup. The mean ages and confidence intervals (95% HPD) are indicated at the nodes. An asterisk represents a node where Figure 5 and 6 were originally connected.
doi:10.1371/journal.pone.0040153.g005

diversity had been described [49–53]. Because microalgal speciation is not always accompanied by morphological change, the true number of species is likely to be greater than the current tally of nominal species, most of which are delineated on purely morphological grounds [54]. Previously, *M. vaginatus* had also been suggested as a complex, composed of several cryptic species [23,26]. However, *M. vaginatus* has diverged rather recently in comparison with the other species of filamentous cyanobacteria (e.g. *Wilmottia murrayi*, *Colofasciculus chthnoplastes*, Figure 5, clade 3, 4). It has been undergoing significant evolutionary differentiations, both spatial and temporal.

Recently, several species concepts have been proposed, applicable to the cyanobacteria. All of them treat the question of cosmopolitanism and endemism differently. The Evolutionary Species Concept describes a species as an entity, composed of organisms, which has its own historical and future evolutionary tendencies [55]. *M. vaginatus* would then possess several separate evolutionary lineages (Figure 2), each being characterized by geographic origin, as well. Thus *M. vaginatus* would not be considered as cosmopolitan. The Ecotypic Species Concept *sensu* Cohan [18] defines species (ecotype) based upon its ecological niche. Phylogenetic analysis (Figure 2) revealed various compositions of ecological features within a majority of the clades. Therefore, a true ecotype cannot be well defined. Johansen & Casamatta [56] proposed a modified Monophyletic Species Concept: species is a monophyletic clade, characterized by a unique apomorphy. There was no significance identified from either the morphological or molecular apomorphy for any particular clade. All of the studied strains only possessed their common synapomorphy (11-bp insert in 16S rRNA) [22,23]. Although there is a considerable genetic variability among different populations, we assume that *M. vaginatus* is an immature species, in the early stages of evolution, and that the existence of cryptic species is still unclear.

The relationships for some microalgae to their ecological preferences [14] have not been confirmed in this study for *M. vaginatus*; however, this does not mean that ecology does not have any influence. Unfortunately, specific ecological data are only available for our isolates, not for most of the sequences obtained from GeneBank. Thus, the only "ecological parameter" used in this study is the biotope/habitat type. Both European and Asian strains originated from different biotopes (soil, puddles, and river; see Table S1 for details), The American strains have only been isolated from desert crusts [23]. Although strains from both clusters differ ecologically, they did not exhibit any particular clustering patterns, dependent on habitats (Figure 2, 3, 4). For

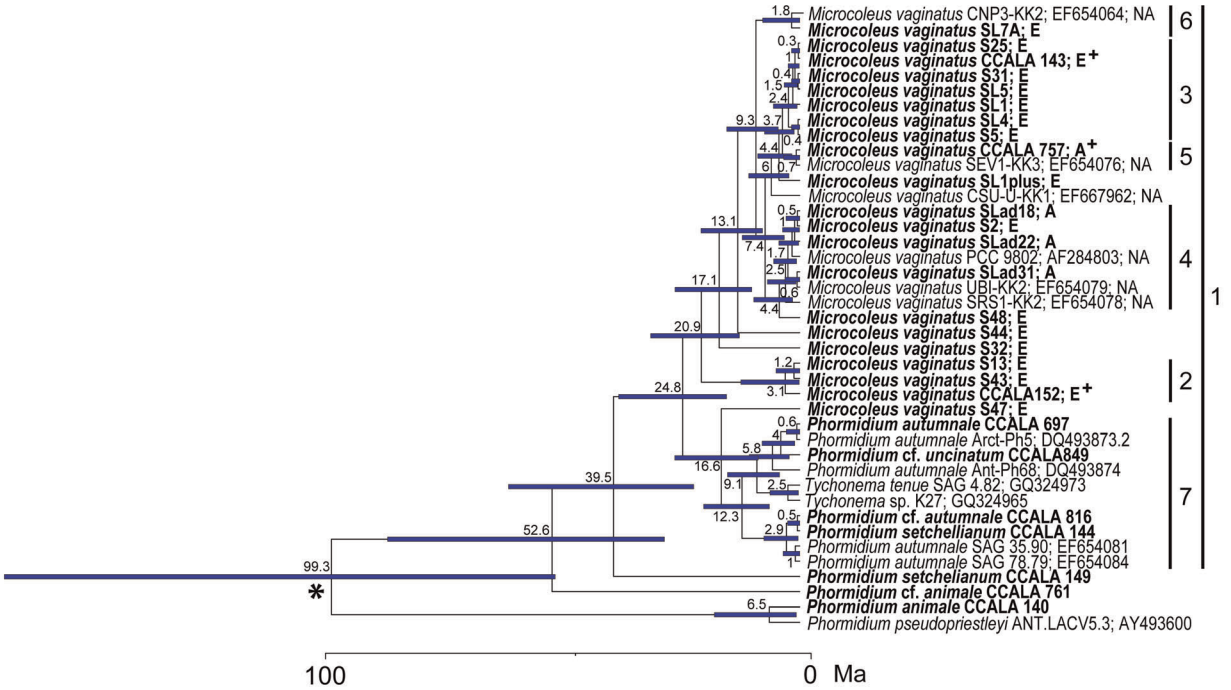

Figure 6. The dating of the divergence times among cyanobacteria. Maximum credibility chronogram based on 16S rRNA of cyanobacteria, with an emphasis on *Microcoleus vaginatus*. It is a continuation of Figure 5. The mean ages and confidence intervals (95% HPD) are indicated at the nodes. The studied strains are in bold. The geographic origin of each strain is indicated as E – Europe, A – Asia, and NA – North America. A plus mark indicates the strains which have been identified anew because of previous incorrect determinations in the culture collection. An asterisk represents a node where Figure 5 and 6 were originally connected.
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example, strain CCALA 757 (isolated from a rice field) was very close to strain SNM1-KK1 (isolated from desert crust). Indian strains (SLad 18, 22, and 31) were isolated from soil crusts in the Ladakh (Himalaya), where the average annual temperature is around −8.2°C [57]. Therefore, we assume that the clustering pattern within the tree, network, P-test, and PCoA analysis (Figure 2, 3, 4) is more likely the result of geographic differentiation, and not from the strains' autecology. This is also confirmed by the very significant correlation between the geographic and genetic distances in the Mantel test ($R = 0.184$, $P = 0.0001$), which should be a relevant support for the existence of phylogeography among the strains of *M. vaginatus*.

The aerophytic and subaerophytic habitats are optimal biotopes for studying the biogeographical and dispersal patterns of free-living microorganisms on the continental scale, since there are large potential barriers, which may prevent dispersal. Taton *et al.* [6] proposed the endemism of some Antarctic cyanobacteria investigated, combining morphology and analysis of the 16S rRNA. Similarly, Miller *et al.* [21] and Papke *et al.* [20] found dispersal barriers among extremophilic cyanobacteria. Later, Jungblut *et al.* [58] argued for the cosmopolitanism of cyanobacteria within the Polar Regions, having investigated large numbers of 16S rRNA sequences, and having found up to a 99.9% similarity among some individual Arctic and Antarctic isolates. Analysis of the polar *Phormidium autumnale* revealed an identical image [59]. Gracia-Pichel *et al.* [22] suggested a cosmopolitan occurrence of *M. vaginatus*, without any dispersal barriers. However, this statement was based on six 16S rRNA sequences as well as DGGE analysis. Our data showed geographical differentiation among *M. vaginatus*, which originated from different continents. The European strains differed from those which originated from North America and Asia. Surprisingly, the North American and Asian strains showed a very high similarity among themselves (Figure 2, 3, 4). This suggests that there were a greater genetic flow between the American and Asian populations. A possible explanation for this phenomenon is indicated by the global system of dust transport, where large regular dust flows are directed from Asian to the American deserts [60]. However, the European strains do not seem to be fully isolated. There appear some transitions such as strain S32, which may indicate that this particular strain is the result of a newly evolved genotype.

The mechanisms of speciation in prokaryotes differ from those in eukaryotes. Prokaryotic organisms do not exhibit sexual reproduction; they have extremely large populations and high dispersal abilities, small sizes of the individual, and the ability to produce resting stages. Therefore, the most important speciation mechanisms are considered horizontal gene transfer, homologous recombination, and periodic selection. Allopatry (geographical isolation) is not predominantly regarded as a crucial factor [17,61–63]. Our results revealed that *M. vaginatus* has certain dispersal barriers on the continental level. Thus, we suggest that allopatry is also an important speciation factor in *M. vaginatus*, although geographical isolation may only have a temporary character. This will be discussed further.

Divergence dating analysis (Figure 5, 6) uncovered unique evidence of temporal characterizations of *M. vaginatus*'s evolutionary and dispersal patterns. The chronogram revealed that recent European strains have diverged more than once, and that there were significantly long periods of time between events. Because of these long periods of time, we assume that while dispersal barriers existed, the gene flow among populations from Europe to other continents was not continuous. North American and Asian populations appear to have diverged almost simultaneously; additionally, there were no particular dispersal patterns found

(Figure 5, 6). Furthermore, dispersal of *M. vaginatus* does not seem to be dependent on continental drift, because the differentiation of the genotypes took place after the division of Euroasia and America, which occurred during the Cretaceous [64].

The molecular clocks for prokaryotes may be inferred based upon the fossil records, host fossil records, associations with ecological events, or molecular clocks derived from eukaryotes [65]. Because there is lack of convincing calibrating points, as well as significant differences among substitution rates within prokaryotes [47], we inferred a novel substitution rate for the cyanobacterial 16S rRNA gene from fossil DNA. Ochman & Wilson [66] proposed the universal 16S rRNA evolutionary rate of 1% change per 50 million years for bacteria. Moran *et al.* [67] suggested rates of 1–2% per 50 million years, from the relationships of aphids and its endosymbiont. Both of these universal calibrations ticked significantly slower than the rate determined in this study. One probable explanation is that these aforementioned rates were calculated for groups of bacteria other than cyanobacteria, which have unique physiological and ecological features among the other prokaryotes [68].

Our results show that dispersal barriers have played an important role in the evolution and ecology of *M.* vaginatus on the global scale; therefore, the speciation of *M. vaginatus* is also affected by allopatry. However, these dispersal barriers do not have a permanent character.

Supporting Information

Table S1 List of investigated strains.
(DOC)

Table S2 Identified evolutionary rates.
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: PD PH. Performed the experiments: PD. Analyzed the data: PD PH. Contributed reagents/materials/analysis tools: PD AP. Wrote the paper: PD PH AP.

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Petr Dvořák

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This Ph.D. thesis was carried out within the framework of internal Ph.D. studies at the Department of Botany, Faculty of Science, Palacký University Olomouc during the years 2009-2013.

Aspirant: **Mgr. Petr Dvořák**
Department of Botany, Faculty of Science, Palacký University Olomouc, Czech Republic

Supervisor: **Prof. RNDr. Aloisie Poulíčková, CSc**.
Department of Botany, Faculty of Science, Palacký University Olomouc, Czech Republic

Opponents: **Prof. RNDr. František Hindák, PhD. DSc**.
Institute of Botany, Slovak Academy of Sciences, Bratislava, Slovakia

Assoc. Prof. Dale A. Casamatta
Department of Biology, University of North Florida, Jacksonville, USA

RNDr. Bohuslav Uher, Ph.D.
Department of Botany and Zoology, Faculty of Science, Masaryk University, Brno, Czech Republic

The review of the Ph.D. thesis has been written by Department of Botany, Faculty of Science, Palacký University Olomouc.

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Prof. Ing. Aleš Lebeda, DrSc.
Chairman of the Commission for Ph.D.
Theses for Study Subject Botany
Faculty of Science, Palacký University Olomouc

Contents

1. Introduction

Cyanobacteria (Cyanophyta, Cyanophyceae, Cyanoprokaryota, Blue-Green Algae) represent a specific group of Gram-negative bacteria which has evolved very early during the Earth's history. It is morphologically, physiologically, and genetically diverse group of organisms inhabiting almost all aquatic and terrestrial environments (Castenholz 2001). Because of the unique features of their primary metabolism, particularly oxygenic photosynthesis and ability to actively fix atmospheric nitrogen, cyanobacteria have substantially transformed global ecosystem during the evolution (Kopp et al. 2005).

Cyanobacterial diversity was originally explored based entirely on morphology of isolated strains and specimens. The most important morphological traits were cell dimension, cell/filament morphology, type of cell division and presence of sheath/envelope (e.g. Bornet & Flahault 1886-1888, Gomont 1892, Geitler 1932). Afterwards, molecular markers (especially 16S rRNA) have revolutionized cyanobacterial systematics. However, it should be emphasized that an importance of the morphological characters is still eminent. Therefore, a combination of morphological, ecological and molecular data led to the concept of "polyphasic approach" which is today the most respected approach to the practical determination and description of cyanobacterial taxa (e.g. Johansen & Casamatta 2005, Siegesmund et al. 2008, Komárek 2010 In the light of recent research, the real biodiversity seems to be underestimated by morphological variability. There are numerous examples of species entities which are morphologically indistinguishable but do not share common evolutionary history, i.e. their molecular phylogeny is more diverse than morphological. This discrepancy resulted with a concept of "cryptic species" complexes which often occur in cyanobacteria (e.g. Boyer et al. 2002, Casamatta et al. 2003, Siegesmund et al. 2008).

Analysis of DNA (protein) sequences and other molecular markers have become key methods for understanding evolution of organisms. Similar trend took place also in molecular systematics and population genetics of cyanobacteria (e.g. Giovanonni et al. 1988, Boyer et al. 2001, Castenholz 2001, Komárek 2010). The most widely used gene is 16S rRNA (SSU) which codes small ribosomal subunit. Another useful marker in taxonomy of cyanobacteria is 16S-23S ITS region. Its applicability in taxonomy and population genetics has been tested in Boyer et al. (2001). 16S-23S ITS region is worth to be applied under the species level of in population genetics, because it possesses sufficient variability. It is advantageous compared to use of the 16S rRNA which seems to have more suitable resolution for genus level or above. Sequences of 16S-23S ITS region are used for reconstruction of phylogetic trees or for comparison of RNA secondary structures among studied strains (Boyer et al. 2001, Boyer et al. 2002, Siegesmund et

al. 2008).

The genus *Nodularia* Mertens ex Bornet & Flahault is composed of heterocystous cyanobacteria which mainly inhabit saline benthic and planktonic habitats (e.g. Laamanen et al. 2001, Lyra et al. 2005). Geitler (1932) recognized only two species of *Nodularia* based on filament width – *N. harveyana* was composed of species with narrower filaments (<8 μm) and *N. spumigena* had filaments wider than 8 μm. Komárek et al. (1993) and Hindák et al. (2003) recognized altogether 5 benthic species including new species *N. moravica* which was described using morphological approach.

Phormidium Kützing ex Gomont is filamentous, non-heterocytous cyanobacterium which represents morphologically very diverse genus composed of more than 100 species. Considering morphological features of a terminal part of the filament is *Phormidium* divided into 8 groups (Komárek & Anagnostidis 2005). Recently, genus *Phormidium* was partly revised mostly by establishing new genera from previously identified polyphyletic lineages (Marquardt & Palinska 2007, Palinska & Marquardt 2008, Siegesmund et al. 2008).

Genus *Microcoleus* Desmazieres ex Gomont was partly revised by establishing new genus *Colefasciculus* with one species *C. Chthnoplastes*, because it is polyphyletic genus. *M. vaginatus* also share same evolutionary lineage with *Phormidium autumnale* which probably place them to the different new genera out of both *Phormidium* and *Microcoleus* (Siegesmund et al. 2008).

Geitlerinema (Anagnostidis et Komárek) Anagnostidis was established by a separation of some species of *Phormidium* and *Oscillatoria* and assigned to the family Pseudanabaenaceae (Anagnostidis 1989). *G. splendidum* and *G. amphibium* occur often in epipelic assemblage of lakes (Hašler et al. 2008). However, taxonomy of *Geitlerinema* has not yet been properly revised based on molecular data. Perkerson et al. (2010) studied 5 strains of *Geitlerinema* sp. and recognized 4 different polyphyletic lineages.

Komvophoron (Skuja) Anagnostidis et Komárek is genus of filamentous cyanobacteria which are characteristic for a muddy or sandy sediments or less often for thermal springs (Komárek & Anagnostidis 2005). *Komvophoron* was established by Anagnostidis & Komárek (1988) and it was placed to the family Borziaceae. It is overlooked genus. The most extant revision of the *Komvophoron* was performed by Hašler & Poulíčková (2010). They described a new species *K. hindakii* which is morphologically similar to the type species *K. schmidlei*.

Microorganisms have small dimensions and enormous dispersal abilities which differentiate them from macroorganisms. Therefore potential structuring of geographical barriers and distributional patterns differs significantly (Martiny et al. 2002).

The first widely accepted idea describing the distribution of microorganisms was

published by Baas Becking (1934). He considered all microbial species as ubiquitous. Their distribution is only restricted by local environmental factors. Similar assumption suggested Finlay (2002) who claimed ubiquity for all organisms smaller than 1 mm.

Recently, molecular evidences brought new insights into study of biogeographical patterning in cyanobacteria. Jungblut et al. (2010) studied differences between Arctic and Antarctic cyanobacterial communities based on analysis of 16S rRNA. They found out that there are many almost identical (99.9% similarity in 16S rRNA) phylotypes in both polar regions.
Van Gremberghe et al. (2011) analyzed global distributional patterns of *Microcystis aeruginosa* species using 16S-23S ITS marker. Parsimony network revealed no particular dispersal pattern. Therefore authors assume that gene flow among population is very frequent and local events of bottleneck and selective sweep drive speciation.

On the other hand, some distributional patterns were identified in thermophylic cyanobacteria. Papke et al. (2003) described biogeographical patterns in cyanobacterium *Synechococcus* spp. on continental scale. The strains were isolated from 48 hot springs and showed correspondence between phylogeny of 16S rRNA and geographical origin. This study also brought an evidence of existence of allopatric speciation within cyanobacteria. Resembling pattern revealed investigation of stigonemetalean cyanobacterium *Mastigocladus laminosus* (Miller et al. 2007).

2. Aims

The principal goal of this thesis was to investigate filamentous cyanobacteria from different types of benthic and aerophytic microbial mats based on morphological and genetic variability (polyphasic approach). Using appropriate molecular markers challenge recent taxonomical findings leading to taxonomical revisions. Moreover, the thesis was also focused on finding spatial and temporal patterns in global cyanobacterial distribution. Particular aims are listed in points below:

- investigate taxonomical relationships within some benthic representatives of the genus *Nodularia* using polyphasic approach
- explore molecular and morphological diversity of some epipelic cyanobacteria
- revise genus *Komvophoron* by molecular markers using single filament PCR approach
- challenge an idea of the ubiquity within non-extremophylic cyanobacteria below the species level and reconstruct temporal dimensions of the cyanobacterial evolution using molecular clocks.

3. Results

3.1. Polyphasic characterization of the benthic *Nodularia*

A polyphasic characterization of *N. sphaerocarpa* isolated from epipelic sample in Olomouc, *N. moravica* (strain provided by Prof. F. Hindák), and another *N. sphaerocarpa* and *N. harveyana* obtained from culture collections was performed. Halotolerance and changes in morphology were assessed in the gradient of salinity. AFLP (Amplified Fragment Length Polymorphism) and sequences of 16S rRNA were analysed. 16S rRNA phylogram revealed *N. sphaerocarpa* and *N. harveyana* formed separated clusters. *N. moravica* formed common clade with another analysed strain of *N. sphaerocarpa*. However, these strains were clearly separated by morphology. *N. harveyana* exhibited higher tolerance to salinity in compare to *N. sphaerocarpa* and *N. moravica* which appears to be better physiologically adapted to the freshwater environments. Moreover, *N. harveyana* had more stable morphology (i.e. vegetative cell, heterocyst, and akinete width) across salinity gradient than *N. sphaerocarpa*.

3.2. Morphological and molecular diversity of some common epipelic cyanobacteria

P. autumnale, *P. formosum*, *M. vaginatus*, *G. splendidum*, *G. carotinosum*, and *G. pseudacutissimum* were isolated from epipelic assemblage originated from European lakes. 16S rRNA phylogeny revealed their monophyletic position of *P. autumnale* and *M. vaginatus* outside of Phormidiaceae. Moreover, there was found remarkable variability inside the clade which was also confirmed by analysis of 16S-23S ITS secondary structures and by different ecological origin of studied strains. Strains originated from very distinctive environments (desert crust and epipelon) were closely related based on 16S rRNA. They were distinguished only by the 16S-23S ITS secondary structures.

P. formosum formed separate mohophyletic clade among cyanobacteria. It was composed of two cryptic lineages which cannot be reliably distinguished by morphology or other traits.

16S rRNA phylogeny revealed three separate lineages within the genus *Geitlerinema*. Only one clade belonged to the family Pseudanabaenaceae (these strains were acquired from GenBank). The other two linages were closely related to Phormidiaceae. Moreover, *G. splendidum* lineage was separated from *G. carotinosum* and *G. pseudacutissimum* which are morphologically very similar. *G. carotinosum* and *G. pseudacutissimum* were convincingly separated by combination of phylogeny and 16S-23S ITS secondary structures.

3.3. Molecular diversity of *Komvophoron* based on Single Filament PCR

16S rRNA and 16S-23S ITS sequences of *Komvophoron* were obtained using Single Filament PCR which was optimized especially for epipelic samples. 16S rRNA phylogeny revealed two separate lineages within the genus *Komvophoron*. Sequences identified as *K. hindakii* formed monophyletic cluster with *Hormoscilla pringsheimii* and *Crinalium* spp. which belong to the family Gomontiellaceae. This is also in congruence with morphology of the *K. hindakii*. Sequences of *K. constrictum* clustered together with some members of the family Pseudanabaenaceae. Therefore *Komvophoron* is polyphyletic and new genus *Johansenia* was described based on polyphasic approach. Moreover, there appeared two lineages within *Johansenia* distinguished by 16S rRNA phylogeny, secondary structures of the 16S-23S ITS and morphology. One lineage was revised species as *J. constricta* and one newly described as species *J. pseudoconstricta*. These species were also derived based on 16S rRNA phylogeny, secondary structures of the 16S-23S ITS.

3.4. A phylogeography of the *Microcoleus vaginatus*

16S-23S ITS phylogeny was used for characterization of a phylogeography of the *Microcoleus vaginatus*. Genetic and geographical distance correlated significantly in the Mantel test. Phylogenetic tree, network and PCoA analysis (Principal Coordinate Analysis) exhibited similar clustering pattern. Geographical origin of strains was in congruence with their phylogenetic position. European strains diverged from North American and Asian strain which shared common clade. Molecular clocks calibrated by fossil DNA were used in order to put spatial differentiation of *M. vagitatus* to the temporal frame. Bayesian chronogram constructed from 16S rRNA sequences showed similar patterning to the 16S-23S ITS phylogeny. However, European population diverged at least two times. Thus, the geographical isolation of *M. vaginatus* has not been permanent during its evolution. The dating analysis also revealed a possible time period of evolution of the cyanobacterial species. For instance, a lineage *M. vaginatus* has diverged before 39.5 Ma.

4. Conclusions

In the presented thesis, I have explored morphological and genetical variability of cyanobacteria inhabiting benthic and aerophytic habitats, and made revisions in taxonomy based on polyphasic approach. Furthermore, using molecular clocks I have found evidences contradictory to the idea of universal ubiquity within prokaryotes and dated evolution of some cyanobacterial species.

1. There were recognized several benthic species of *Nodularia* with different species specific ecological demands. These species were confirmed by AFPL and 16S rRNA analyses. *N. moravica* was revised and a validity of species was confirmed. Salinity has a strong physiological effect and it is important trait which should be considered in taxonomy. In addition, phylogeny is in congruence with ecophysiology of strains. *N. sphaerocarpa*, *N. harveyana* and *N. moravica* have been separated based on different halotolerance, morphology changes in salinity gradient and molecular markers. It was also shown that AFLP analysis may be advantageous molecular marker with potential in taxonomy of cyanobacteria.

2. *P. autumnale* and *M. vaginatus* are complex of morphologically almost indistinguishable species. They form monophyletic group within 16S rRNA phylogenetic tree outside of their former genera. Epipelic strains of *M. vaginatus* had very close position in phylogenetic tree to the aerophytic strains isolated from desert crusts. A significant difference was found only based on analysis of secondary structures within 16S-23S ITS. This fact once again confirmed advantages of the 16S-23S ITS marker in taxonomy of cyanobacteria and importance of combination of the different molecular markers. Moreover, such a remarkable ecological, molecular, and geographical variability indicates existence of some cryptic species inside this lineage.

P. formosum is a valid species which existence was confirmed by polyphasic approach and it is probably composed of two cryptic species.

The 16S rRNA based phylogenetic analysis revealed that the genus *Geitlerinema* is polyphyletic conglomerate of species. Part of them does not belong to its former family Pseudanabaenaceae. A lineage including *G. carotinosum* and *G. pseudacutissimum* and a lineage including *G. splendidum* were closely related to Phormidiaceae. An existence of all these species was confirmed by 16S rRNA phylogeny and 16S-23S ITS secondary structures.

3. A synthesis of morphological features with phylogeny of the 16S rRNA sequences led

to a partial revision of the genus *Komvophoron* as it currently stays because it is polyphyletic group. Validity of a species description was confirmed. Moreover *K. hindakii* more likely does belong to the family Gomontiellaceae.

The genus *Johansenia* with two species – *J. constricta* and *J. pseudoconstricta* was described and placed to Pseudanabaenaceae based on 16S rRNA and morphology.

4. A reconstruction of *M. vaginatus* evolutionary history revealed that geographical barriers on a continental level may play an important role in evolution of the cyanobacteria. In fact, it is a first evidence of the specific distributional pattern resembling geographical isolation in non-extremophylic cyanobacteria. Therefore an allopatric speciation is probably an important factor of the speciation within cyanobacteria. However, dispersal barriers have not been persistent over a long time periods. Moreover, an application of molecular clocks showed unique evidence of long existence of the cyanobacterial species.

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8. Souhrn (Summary, in Czech)

Sinice jsou významnými primárními producenty v široké škále akvatických i terestrických ekosystémů. Nicméně diverzita sinic je natolik rozsáhlá, že většina z ní zůstává nepopsaná. V této dizertační práci byla zkoumána morfologická a molekulární diverzita bentických a aerofytických sinic. Navíc byly zkoumány globální prostorové a časové změny v rozšíření volně žijících sinic.

Kombinace sekvenovaní 16S rRNA, AFLP analýzy a ekofyziologických experimentů v gradientu salinity byla využita k získání nových poznatků z taxonomie druhů *Nodularia sphaerocarpa*, *N. harveyana*, a *N. moravica*. *N. sphaerocarpa* a *N. moravica* mají podle ekofyziologických experimentů vyšší senzitivitu ke zvýšené úrovni salinity. Oproti tomu *N. harveyana* je méně citlivá a navíc morfologie (šířka buňky) je v gradientu salinity stabilnější. *N. moravica* byla původně popsána pouze na základě morfologie. Validita tohoto druhu byla potvrzena s použitím molekulárních dat. Ekofyziologické experimenty také poskytly přesvědčivý důkaz o důležitosti ekologických parametrů v taxonomii sinic.

Byly analyzovány epipelické sinice rodů *Microcoleus*, *Phormidium* a *Geitlerinema* izolované z evropských jezer. *Microcoleus vaginatus* a *Phormidium autumnale* tvoří monofyletický komplex druhů, který by měl být podroben revizi, protože ani jeden z těchto druhů nenáleží do původního rodu. Kmeny *M. vaginatus* izolované z pouštních krust náležely do stejného kladu s epipelickými izoláty na základě analýzy 16S rRNA. Kmeny bylo možné rozlišit pouze na základě sekundárních struktur v 16S-23S ITS. Druh *P. formosum* byl potvrzen s použitím molekulárních dat. Navíc byly v rámci *P. formosum* klastru identifikovány dva pravděpodobně kryptické druhy. Rod *Geitlerinema* byl již v minulosti shledán polyfyletickým. Toto bylo potvrzeno fylogenetickou analýzou 16S rRNA, kde byly nalezeny tři separátní linie v rámci sinic. *G. splendidum*, *G. carotinusum*, a *G. pseudacutissimum* tvořily oddělené linie, které byly blízce příbuzné k čeledi Phormidiaceae, ačkoliv byly původně řazeny do Pseudanabaenaceaea.

Pomocí sekvencí 16S rRNA a 16S-23S ITS získaných Single Filament PCR optimalizovaných pro epipelické vzorky byl oddělen nový rod *Johansenia* od rodu *Komvophoron*. *Johansenia* náleží na základě fylogenetické analýzy do čeledí Pseudanabaenaceae. Původně morfologický popsaný druh *K. hindakii* byl potvrzen fylogenetickou analýzou 16S rRNA, podle které náležel k členům čeledi Gomontiellaceae. Rod *Komvophoron* je tedy polyfyletická skupina zasluhující větší pozornost.

Fylogenetická analýza a molekulární hodiny založené na 16S rRNA a 16S-23S ITS byly využity na charakterizování globálních prostorových a časových bariér rozšíření *M. vaginatus*. Tímto byl získán první důkaz pro existenci geografické izolace u volně žijících neextrémofilních sinic.

Nicméně geografické bariéry neměly permanentní charakter. Navíc tato studie přináší unikátní vyobrazení datování divergence druhů u sinic.