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REVIEW PAPER

Species concepts and speciation factors in cyanobacteria, with connection to the problems of diversity and classification

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Abstract The cyanobacteria are the most important prokaryotic primary producers on Earth, inhabiting a great diversity of aquatic and terrestrial environments exposed to light. However, the evolutionary forces leading to their divergence and speciation remain largely enigmatic compared to macroorganisms due to their prokaryotic nature, including vast population sizes, and largely asexual reproduction. The advent of modern molecular techniques has facilitated an understanding of the important factors shaping cyanobacterial evolution, including horizontal gene transfer and homologous recombination. We review the forces shaping the evolution of cyanobacteria and discuss the role of cohesive forces on speciation. Further, while myriad species concepts and definitions are currently used, only a limited subset might be applied to cyanobacteria due to their asexual reproduction. Additionally, concepts based solely on phenotypes provide insufficient resolution. A monophyletic species concept which is universal may be ideal for cyanobacteria. Actual identification of the cyanobacteria is difficult due to cryptic diversity, lack of morphological variability, and frequent convergent evolutionary events. Thus, applied molecular techniques such as DNA barcoding will be useful for identifications of environmental samples. Lastly, we show that the real biodiversity of the cyanobacteria is widely underestimated, due in part to low sampling efforts, sensitivity to the molecular markers

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employed, and the species definitions employed by researchers. In conclusion, we anticipate a rapid increase in cyanobacterial taxa described and large revisions of the system in the future as scientists adopt a common approach to cyanobacterial systematics.

Keywords Cyanobacteria · Species concept · Evolution · Speciation · Biodiversity

Introduction

The Cyanobacteria (also known as the Cyanophyceae, Cyanophyta, cyanoprokaryota, blue-green algae or blue-green bacteria) are prokaryotes possessing oxygenic photosynthesis, while sharing similar habitats to eukaryotic algae (Kauff and Büdel 2011). Moreover, cyanobacteria can live in some of the most extreme habitats on earth (Seckbach 2007). On the basis of fossil records, Schopf (2000) estimated that cyanobacteria may have evolved 3.5 BYA, making them the oldest oxygen producing photosynthetic microbes, and significant contributors to the sudden increase in atmospheric oxygen during the Great Oxidation Event (Bekker et al. 2004; Kauff and Büdel 2011).

The cyanobacteria exhibit remarkable variability in morphology and ultrastructure, from unicellular to filamentous forms (Figs. 1, 2). They may also possess intercellular connections or microplasmodesmata, considered a sign of multicellularity (Nürnberg et al. 2014). Moreover, some genera exhibit morphological and functional cell differentiation such as heterocytes (adapted to nitrogen fixation) and akinetes (resting stage cells) (Whitton and Potts 2000).

The purpose of this paper is three-fold. First, we will review the most recent literature relating to the evolutionary processes forming bacterial (and cyanobacterial in particular) species. Second, we will evaluate their application in cyanobacterial taxonomy, distribution, species concepts and species definitions. Third, we will discuss some practical aspects of cyanobacterial taxonomy and systematics.

Species concept in (cyano)bacteria

Some authors postulate that all prokaryotes are species-less or fuzzy (e.g. Hanage et al. 2005; Konstantinidis and Tiedje 2005; Hanage 2013), because they lack ecologically or genetically coherent groups. Such "fuzziness" might be apparent in ambiguous ecological boundaries among species, which was suggested by Cohan and Perry (2007) and Kopac et al. (2014). For cyanobacteria, the most obvious phenotypic features (i.e. cell morphology) may at times be phylogenetically uninformative when compared to phylogenies generated by 16S rRNA gene data, which is the currently accepted "Gold-standard" in bacterial systematics (e.g. Honda et al. 1999; Robertson et al. 2001; Kim et al. 2014).

Moreover, species identifications might be complicated by the analyses used, with some traditional methods lacking species-level resolution. For example, Hanage et al. (2005) showed that multilocus sequence analysis is required to distinguish highly recombinant species of the human inhabiting bacterium *Neisseria* spp. Similarly, marine picoplanktic *Synechococcus* is composed of several ecological and geographical lineages, which may be recognized only based upon multilocus sequence analysis (Mazard et al. 2012).

Fig. 1 Illustration of morphological diversity in cyanobacteria. Groups (orders) follow Rippka et al. (1979). *I.* **Chroococcales**: **a** *Chroococcus subnudus*, **b** *Ch. limneticus*, **c** *Cyanothece aeruginosa*, **d** *Snowella litoralis*, **e** *Microcystis aeruginosa*. *II.* **Pleurocapsales**: **f** *Pleurocapsa minor*. *III.* **Oscillatoriales**: **g** *Planktothrix agardhii*, **h** *Limnothrix redekei*, **i** *Arthrospira jenneri*, **j** *Johanseninema constricum,* **k** *Phormidium* sp., **l**, **m** *Oscillatoria* sp., **n** *Schizothrix* sp., **o** *Tolypothrix* sp., **p** *Katagnymene accurata., IV.* **Nostocales**: **q** *Dolichospermum planctonicum,* **r** *Dolichospermum* sp., **s** *Nostoc* sp., **t** *Nodularia moravica. V.* **Stigonematales**: **u, v** *Stigonema* sp. *Scale bar* **a**–**u** = 10 μm, **v** = 20 μm. (Color figure online)

On the other hand, since the frequency of horizontal gene transfer (HGT) and homologous recombination (HR) decreases with the genetic distance suggests coherence within evolutionary lineages and thus the existence of prokaryotic species. However, this assertion might be complicated by the methods used or by the stage of speciation. Models of speciation presented by Polz et al. (2013) and Dvořák et al. (2014b) showed mixed phylogenetic signals based on different loci at the beginning of speciation caused by HGT and HR. A stronger phylogenetic signal comes later during speciation and is balanced until coherent species units are evident (Shapiro et al. 2012). Conversely, Cohan (2011) argues that cohesion is not maintained by barriers of recombination, but rather concerned with ecological diversification, which precedes barriers of recombination (Wiedenbeck and Cohan 2011).

Cohesion might not necessarily be the key factor for the existence of species delimitations, and Kopac et al. (2014) proposed ecological differences among ecotypes as key features. They suggest that ecotypes exist indefinitely, but lineages within ecotypes are changing. In conclusion, regardless mechanisms of coherence, there seem to be coherent evolutionary lineages in cyanobacteria, which might be called species.

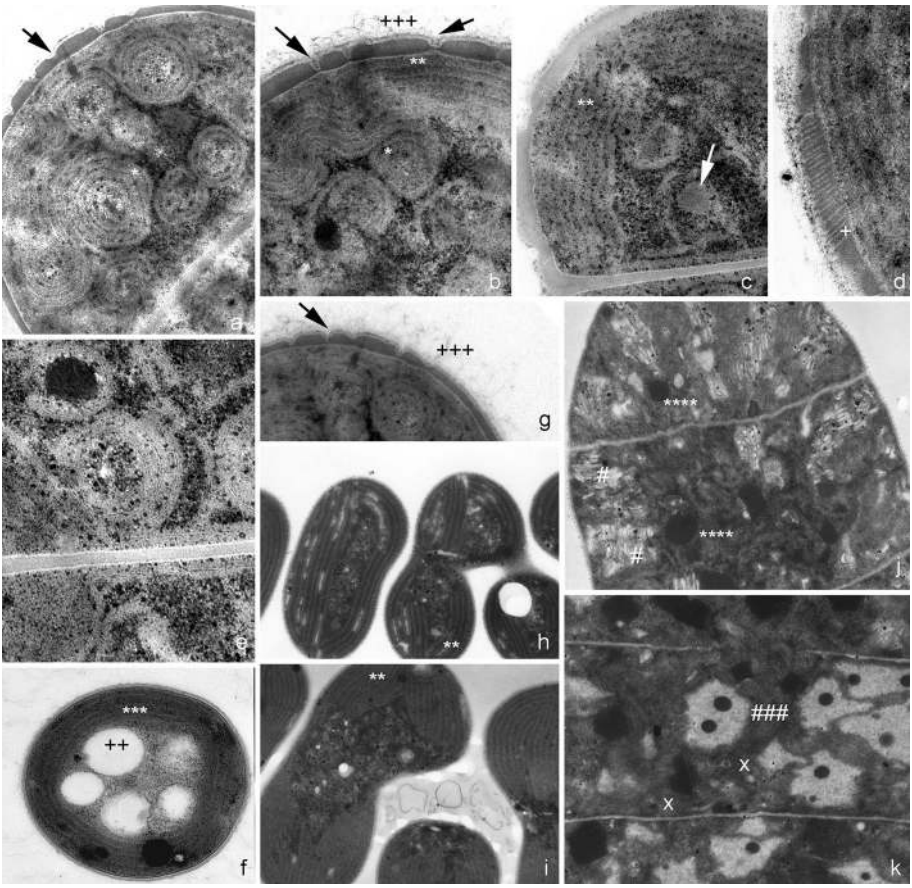

Fig. 2 Transmission electron images of cyanobacteria—illustration of diversity in ultrastructure; **a**–**e**, **g** *Hormoscilla pringsheimii*; **f** *Neosynechococcus sphagnicola*; **h**, **i** *Spirulina*; **j**, **k** *Arthrospira*; **a** The cell wall depressions (wall pores, *black arrows*) are the passages through which mucilage crosses the wall. A large part of the cytoplasm is occupied by thylakoids often coiled to form roundish structures formed by circular thylakoids (*asterisk*) **b** detail of **a**. The *black arrows* indicate the cell wall depressions (mucilage pores). Abundant sheath fibrillar mucilage (+++) is evident along the wall. Some wavy thylakoids (**) run along the cytoplasmic membrane as single lamellae, while more internally they coil to form roundish bodies (*single asterisk*). Roundish electron dense bodies (****) represent polyphosphate granules. **c** *Grey* spherical bodies (*white arrow*) in the cytoplasm represent cyanophycin bodies. **d** Junction pores (+) through the cell wall appear as channels orthogonal to the cytoplasmic membrane surface. **e** Detail of a polyphosphate granule (****). Many ribosomes can be observed in the cytoplasm, particularly close to the thylakoids. **f** In unicellular species, thylakoids are typically arranged parietally (***), along the cytoplasmic membrane. Polyhydroxybutyrate bodies (PHB, ++) are visible. **g** Detail of the cell wall depressions (*black arrow*) and their relationships with the fibrillar component of mucilage. **h** Image of the spirally arranged filament of *Spirulina*, whence the frequent observation of double flanked cells. The wavy thylakoids are clustered in bundles of lamellae. **i** Detail of **h**. A large part of the cytoplasm is occupied by wavy thylakoids, while the "free" cytoplasm appears lectron dense and containing many different bodies at very variable level of electron density. **j** In a filament of *Arthrospira* the apical cell appears to have a different shape with respect to the other cells. Many heterogeneous cytoplasmic structures are visible, among which polyphosphate bodies (****) and gas vesicles (aerotopes, #). **k** Detail of **j**. Apparently even spaces possibly enclosed by membranes ### and containing electron dense bodies and fibrillar material can be observed, such bodies are interpreted as assembling carboxysomes. Cylindrical bodies (x) can be observed in the cytoplasm. Material can pass through cell wall pores (microplasmodesmata) from one cell to another in filamentous genera

Before we begin a discussion of species concepts in bacteria, we would like to emphasize the differences between a species concept and species definition, which is often confused. A species concept is a theoretical demarcation of the species, which would be ideally applicable to all organisms. Conversely, species definitions are a set of rules used for practical identification of species (Hanage 2013). For instance, most bacteriologist use distance among genes or genomes (DNA–DNA hybridization, average nucleotide identity; Richter and Rosselló-Móra 2009) as a species definition while not considering an actual species concept. It does not take into account a phase of speciation or phylogenetic position, and thus it does not show the true evolutionary history of the species.

A possible concept of a bacterial species may be a "genomically and phenomically cohesive cluster" to which a possible concept of species may be applied (Doolittle and Zhaxybayeva 2009). However, the same authors pointed out that there would be "no principled way in which questions about prokaryotic species, such as how many there are, how large their populations are, or how globally they are distributed, can be answered". Thus, the question remains: how to evaluate biodiversity among prokaryotes?

Is there a quantitative threshold of genetic difference sufficient to describe a prokaryotic species in order that eukaryotes-centered biological species concept might work (sensu Mayr 1942, 1946)?

The recognition of the prokaryotic species problem eventually led to a partial consensus about species delimitations (Gevers et al. 2005, 2006; Staley 2006). According to these authors, a prokaryotic species should be recognized primarily on the basis of genotypic similarity and hence mainly on genetic distances. Stakebrandt et al. (2002) proposed that two isolates may be assigned to the same species in case of a value higher than 70 % in a standardized DNA–DNA hybridization experiment. Other distances based on the small subunit (SSU, or 16S) rRNA, could be used to exclude the belonging to the same species in case of a > 97.5 % similarity (Fox et al. 1992; Stackebrandt and Goebel 1994). Another threshold range 98.7–99 % has been proposed by Strackerbrandt and Ebers (2006). Goris et al. (2007), and Richter and Roselló-Móra (2009) proposed 95–96 % average nucleotide identity (ANI) of homologous genomic regions as a gold standard for species delimitation and also as an alternative to DNA–DNA hybridization. Most recently, Kim et al. (2014) combined previously mentioned approaches and proposed 98.65 % similarity in 16S rRNA as a threshold for species delimitation. Unfortunately, these are all similarity based criteria, and not in line with modern systematics approaches which emphasize broader tools of reconstruction of evolutionary relationships (Castenholz and Norris 2005; Johansen and Casamatta 2005; Komárek 2010 and many others).

The main problem with bacterial species concepts is that they do not fit well into the requirements of the classical species concept used for eukaryotes. Staley (2006) proposed the genomic-phylogenetic species concept, while Achtman and Wagner (2008) adapted the de Queiroz (2005, 2007) general lineage concept to a prokaryote-limited metapopulation lineages concept, requiring only that "members" of a species (lineage) evolve separately from other lineages. Such separation would provide the cohesive force that eventually forms a species. However, they observed that such a concept does not provide sufficient detection and quantification of cohesive forces.

One of the main issues in bacterial systematics is whether or not lineages necessarily represent a genetic continuum (Konstantinidis et al. 2006). For example, a simple computational model of randomly replicating lineages will produce groups of genetically related individuals separated by genetic gaps (Zhaxybayeva and Gogarten 2004; Mes 2008; Doolittle and Zhaxybayeva 2009). Hence a "good" species should have deeper gaps with respect to what happens with a random model. The possibility of a failed recognition of

intermediate forms may also arise due to sampling or difficult cultivability of many bacterial strains, because most bacterial species are unculturable (reviewed in Stewart 2012).

Recent papers have employed multi-locus DNA sequences analyses (MLSA) for species definition, which often yield results that fit with traditionally delimited species (Gevers et al. 2005; Hanage et al. 2005, 2006). MLSA has been developed originally for identification of pathogenic strains of bacteria due to lack of resolution of traditional genetic markers, mainly the 16S rRNA gene (Maiden et al. 1998). For example, Melendrez et al. (2011) used three protein coding genes and found 4–14 times more ecotypes in the thermophilic *Synechococcus* sp. inhabiting Mushroom Spring in Yellowstone National Park than based solely on 16S rRNA and 16S-23S ITS sequence. Thus MLSA provides significantly higher resolution. Similar considerations may be obtained from the investigations on the marine planktic genera *Synechococcus* and *Prochlorococcus* (Johnson et al. 2006) or fine-scale distribution of marine *Vibrionaceae* (Preheim et al. 2011). On the other hand, Kopac et al. (2014) analyzed all orthologous genes within *Bacillus subtilis* and showed that MLSA was insufficient to distinguish ecotypes, which are considered as species in this paper.

Recently, DNA barcoding has been proposed as a possibility for cyanobacteria. Eckert et al. (2015) tested barcoding gaps in cyanobacteria and found that barcoding gaps among species were identified in a half of investigated cases. Thus, this approach has to be further investigated before it takes place in practical identification of species.

Speciation factors in (cyano)bacteria

Bacteria and Archaea are evolutionarily intriguing as they are asexual, and possess extensive populations with relatively short generation times (for review see Cohan 2001, 2002). Cohesive or disruptive forces shaping bacterial species have remained enigmatic for a long time. However, the development of modern molecular methods has shown significant differences between prokaryotic and eukaryotic evolutionary trajectories. For example, some of the non-consistent phylogenetic signals of different gene families within the same bacterial species have been explained by HGT and HR (e.g. Hanage et al. 2005; Lodders et al. 2005; David and Alm 2011). It has been suggested that a part of the bacterial genome usually referred to as the core genome is more stable with less evolutionary changes. The core genome is usually defined as a portion of genes shared by some group of bacteria coding for essential metabolic pathways (Daubin et al. 2002; Shi and Falkowski 2008; Polz et al. 2013). The shell or flexible genome refers to a less stable part of bacterial genome which undergoes substantial evolutionary changes including HGT (Hess 2011). It often contains genes specific to some environment with a large portion of unannotated gene families without any known function (e.g. Shi and Falkowski 2008), which putatively plays an important role in rapidly changing environments (Rodriguez-Valera et al. 2009) and niche partitioning among close relatives (Kopac et al. 2014). The shell genome genes do not seem to be randomly dispersed over the chromosome, but rather concentrated within genomic islands with frequent HGT and HR events (Hacker and Carniel 2001; Rodriguez-Valera et al. 2009). However, Narechania et al. (2012) showed that many core genes have an identical phylogenetic signal as shell genes, which denotes their common evolutionary history. Narechania et al. (2012) defined core genome as orthologs with the same phylogenetic tree topology and the shell genome as composed of the rest of orthologs. Core genes may also exhibit evidence of HGT events, which might be identified by comparing scenarios of gene phylogenies with individual species trees (David and Alm 2011; Nakhleh

2013). The question remains, though, do these changes provide enough force to diverge evolutionary lineages with subsequent cohesion to form an analogue of the eukaryotic "sexual" species?

The most extensively studied HGT events are concerned with the human microbiome (e.g. Smillie et al. 2011) and marine picoplankton, mostly of the genera *Synechococcus* and *Prochlorococcus* (e.g. Marston et al. 2012). In terms of cyanobacteria, it has been further suggested that most HGT are mediated by phages (cyanophages) (e.g. Sullivan et al. 2010; Sabehi et al. 2012). These phages often contain genes important in photosynthesis (Zheng et al. 2014). While HGT events may occur between phylogenetically divergent lineages, they are most frequent among individual species within the same environment and decrease with the overall genetic distance of genomes (Popa et al. 2011). A very similar phenomenon has been observed in HR (Smillie et al. 2011). Fraser et al. (2007) modeled HR within bacteria, showing that if HR exceeds mutation rate, a species evolves in a similar manner as sexually reproducing eukaryotes, and with low HR the populations are clonal. Polz et al. (2013) suggested in their synthesis that rather than genetic isolation of emerging lineages, there exist local genetic innovative gene pools (i.e. local metagenomes), which are constantly changing by HGT within a pool and by input of incoming genotypes.

Besides genetic isolation resulting from genome differentiation, which takes place in population without geographical isolation (sympatric speciation) often observed in bacteria (e.g. Friedman et al. 2013; Koeppel et al. 2013), there are geographical and ecological factors affecting bacterial speciation. The speciation of macroorganisms is often driven by geographical isolation (allopatry) due to their limited dispersal capabilities. This has also been in, e.g., asexual rotifers, but on a larger geographical scale (Fontaneto et al. 2008). However, how these processes relate to microorganisms is still subject to broad debate (see Martiny et al. 2006; Ramette and Tiedje 2007 for review). Baas Becking (1934) postulated that all microbes can spread everywhere and only the specific local environmental conditions would select actual species composition. However, recent analyses of different molecular markers reveal an ambiguous signal. For example, thermophilic cyanobacteria *Mastigocladus laminosus* and *Synechococcus* spp. showed geographical difference based on 16S rRNA analysis (Papke et al. 2003; Miller et al. 2007). However, it should be noted that in the case of *Synechococcus* (Papke et al. 2003), the clusters were genetically very distant, which might be because they belong to different taxa (even genera) and there is not sufficient variation within a species to elucidate meaningful patterns. Dvořák et al. (2012) showed that episodic genetic isolation of the mat-forming cyanobacterium *Microcoleus vaginatus* may have led to the speciation events. On the other hand, 16S-23S ITS phylogenies of the freshwater, planktic cyanobacterium *Microcystis aeruginosa* revealed no connection between geographic position and a placement in phylogeny (van Gremberghe et al. 2011). Further, no geographical patterning has been observed in polar cyanobacteria based on 16S rRNA (Jungblut et al. 2010). Taken together, the role of geographical isolation as it relates to the speciation of microbes should be further investigated using whole genome data or using more variable genome regions (Ramette and Tiedje 2007). A whole genome approach has been used in thermophilic archeon *Sulfolobus islandicus*, which has shown clear geographical patterning (Reno et al. 2009). This may reveal very recent events of genetic exchange leading to speciation as in case of marine picoplanktic *Synechococcus* (e.g. Mazard et al. 2012) because the geographical isolation may be important in a very short time frame (Ramette and Tiedje 2007; Dvořák et al. 2012) rather than in relatively long times as observed in macroorganisms.

Cohan (2001) advocates a bacterial speciation model by ecological diversification. When a new niche is introduced the stable ecotype is periodically overgrown by new, "fitter" ecotypes, which are able to effectively exploit the new niche. Periodic selection events decrease the overall genetic diversity and after some time a new ecotype can be sufficiently diversified to form a new stable ecotype, which may be non-competing with the parental genotype. This also results with the coherence within evolutionary linages.

The ecotype model of prokaryotic speciation proposed by Cohan (2001, 2002, 2006) and Cohan and Perry (2007) treat bacteria as asexual clones, where homologous recombination rates are low. Thus, many crucial questions still remain as unanswered concerning the mechanism of bacterial speciation.

Particular problems of species definitions and concepts in cyanobacteria

The previously mentioned concepts and definitions of species are also applicable to cyanobacteria, but in the following paragraphs, we will emphasize some important considerations pertaining to cyanobacteria.

A classic, phenetic species concept using only morphological or ecological data has been shown to be insufficient to describe the real biodiversity within cyanobacteria. Morphology alone in cyanobacteria often lacks resolution on the species level, while completely ignoring cryptic species (e.g. Johansen and Casamatta 2005; Hašler et al. 2012, and many others, see further). Cyanobacterial species have traditionally been distinguished based on the similarity of morphological markers, which might be very subjective. Moreover, some morphological characters, such as sheath formation or presence of heterocytes, may be lost in cultures and environmentally plastic. For example, *Microcoleus vaginatus*, which is usually found in soil crusts, puddles and other aerophytic habitats, has multiple filaments enclosed in common sheath. However, strains isolated from epipelon (fine lake sediment) produce no sheath in nature or culture. An analysis of morphology, 16S rRNA-based phylogeny and 16S-23S ITS secondary structure revealed very close relations with soil crust *M. vaginatus* strains (Hašler et al. 2012). 16S rRNA of all strains also contained an 11 bp insert typical for this species (Boyer et al. 2002). Phenotypic characters (i.e. cell dimension, division type, color) provided insufficient resolution for discerning these lineages. The employment of new characters (mostly 16S-23S ITS region) have allowed researchers to recognize finer differences among taxa with coherent morphology, leading to the idea of cryptic speciation (Boyer et al. 2001; Siegesmund et al. 2008; Komárek 2010, 2011; Hašler et al. 2012). Cryptic taxa are unrecognizable using solely morphological characters. Cryptic species have been identified or suggested in almost all traditional genera (Komárek 2010) such as with the mat-forming cyanobacteria *Microcoleus* (Siegesmund et al. 2008), *Oculatella* (Osorio-Santos et al. 2014), *Trichocoleus* (Mühlsteinová et al. 2014) and *Phormidium* (Casamatta et al. 2003; Hašler et al. 2012). This topic is discussed in great extent elsewhere (e.g. Johansen and Casamatta 2005; Komárek 2010). It should be noted that genus *Oculatella* consists of 7 cryptic species (Osorio-Santos et al. 2014), which were able to be resolved based on 16S-23S ITS sequence, which has higher resolution under the genus level.

A majority of recent taxonomic revisions and descriptions use a combination of morphological, ecological, and genetic observations, referred to as a polyphasic approach (Castenholz 1992; Castenholz and Norris 2005; Komárek 2003, 2010; Komárek et al. 2014). It has already been employed to recognize separate evolutionary lineages and for description of new species. A polyphasic approach is commonly used in taxonomic works

in combination with a monophyletic species concept sensu Johansen and Casamatta (2005), if phylogenetic analyses of 16S rRNA or other genes are used.

16S rRNA sequencing and progress in phylogenetic reconstruction have allowed researchers to employ the evolutionary species concept sensu Simpson (1953). This has facilitated further derived species concepts like the monophyletic species concept. Johansen and Casamatta (2005) used this to define a species as the smallest monophyletic group with recognizable autapomorphy (a trait unique only for particular taxon). They also designed a concrete species definition based on the mentioned concept, which is suitable for cyanobacteria and may be used under the International Code of Botanical Nomenclature. It is probably the most widely accepted concept with cyanobacteria (according to the number of taxonomic papers using the concept under the Botanical Code), although sometimes not precisely followed (Siegesmund et al. 2008; Perkerson et al. 2011; Dvořák et al. 2014a; Hašler et al. 2012, 2014a, b; Osorio-Santos et al. 2014 and many others). The most important advantage of the monophyletic species concept is the general applicability to asexual organisms. However, it might be problematic when a monophyletic lineage lacks sufficient morphological, ecological or physiological differentiation. Moreover, monophyletic species concept is not accepted in the International Code for Nomenclature of Bacteria, which uses species concepts mentioned above.

Synechococcus sensu lato is a group of cyanobacteria with cosmopolitan distribution inhabiting almost all environments (Komárek and Anagnostidis 1998), including thermal and aerophytic habitats (Honda et al. 1999; Robertson et al. 2001). Although some cyanobacteria, such as *Synechococcus* sensu lato, lack phenotypic variability, great ecological and genetic diversity suggest that polyphyletic complexes of cryptic taxa might exist (Honda et al. 1999; Robertson et al. 2001; Dvořák et al. 2014a, b). Many traditional cyanobacterial genera (Geitler 1932) are polyphyletic (Komárek 2010; Engene et al. 2011; Hašler et al. 2012; Engene et al. 2013; Dvořák et al. 2014a, b; Hašler et al. 2014a) and need extensive revisions, which will be very difficult particularly in the case of *Synehococcus* sensu lato due to extreme polyphyly within this genus. We suggest that this extreme polyphyly (cryptogenera sensu Komárek et al. 2014) should be distinguished from polyphyly in the original sense, since in extreme polyphyly a large number of lineages derived over very long time period (over 3 billion years), as shown in Dvořák et al. (2014b).

Such extreme polyphyletic groups with little morphological distinction, in which similar morphotypes may belong to polyphyletic lineages and hence different genera, suggest that taxonomic revisions based solely on morphological data must be performed with great caution. Thus, stable molecular markers such as 16S rRNA should be used for taxonomic revisions (see Komárek 2010 for a review). However, even revisions of genera without molecular support for all studied species have been recently proposed. For instance, after recent revisions of polyphyletic genera with molecular markers, some authors have added new species based on morphological similarity as new combinations (Strunecký et al. 2014). However, these species might be polyphyletic taxa in a manner similar to *Synechococcus*. Therefore, we recommend the use of molecular data in all cases to increase the certainty of taxonomic revisions.

A growing number of polyphyletic genera recently identified might be connected with frequent convergent evolutionary events in cyanobacteria. Convergent evolution is a phenomenon that occurs when similar features have evolved in independent lineages. It seems to be very frequent in cyanobacteria and is evidenced by several phenotypic traits (e.g. Shishido et al. 2013; Dvořák et al. 2014b). We have chosen the example of the prochlorophytes to show another case of convergence in cyanobacteria.

Prochlorococcus, *Prochlorothrix*, and *Prochloron* are cyanobacteria that additionally produce chlorophyll *b* (the typical pigment of green algae and land plants) and lack phycobilisomes (Giddings et al. 1980; Burger-Wiersma et al. 1986; Miller et al. 1988; Chisholm et al. 1992; Hess et al. 1996; Pinevich et al. 1997; Kauff and Büdel 2011). On this basis and due to a *psb*A gene based phylogenetic analysis, they were considered strictly associated with the chloroplast of green algae and terrestrial plants (Morden and Golden 1989). Successive analyses (e.g., Litvaitis 2002) showed that prochlorophytes actually nested within cyanobacteria, and are polyphyletic. The conclusion is that the appearance of chlorophyll *b* and the loss of phycobilisomes evolved multiple times in different lineages, and hence these characters are subjected to convergent evolution and reversals, probably in connection to environmental pressures.

We note that *Prochlorococcus marinus* appears to cluster quite clearly apart from the other cyanobacteria on the basis of the analysis of all the tRNA sequences, considering the isoacceptor variation for each codon and the number of copies for each type of tRNA (Fig. 3). This suggests convergent or parallel evolutionary events leading to similar phenotypic traits, because it contradicts phylogenomic analyses in Shih et al. (2013). Such convergent events might be explained by HGT within the environment and therefore environmental pressures (Litvaitis 2002). It is also likely that it represents a frequent trend in cyanobacterial evolution, since other morphological traits, such as multicellularity, have evolved repeatedly (Honda et al. 1999; Robertson et al. 2001; Schirrmeister et al. 2013; Dvořák et al. 2014a, b). Dvořák et al. (2014b, Fig. 4) also suggested a model of serial convergence in cyanobacteria, where frequent convergent events might be explained by constant genetic changes via HGT and HR within local habitat gene pools as proposed by Polz et al. (2013).

Diversity of cyanobacteria and their current classification

Taxonomy is usually defined as an operative version of systematics. Both the taxonomy and systematics of cyanobacteria have undergone substantial changes in the last two

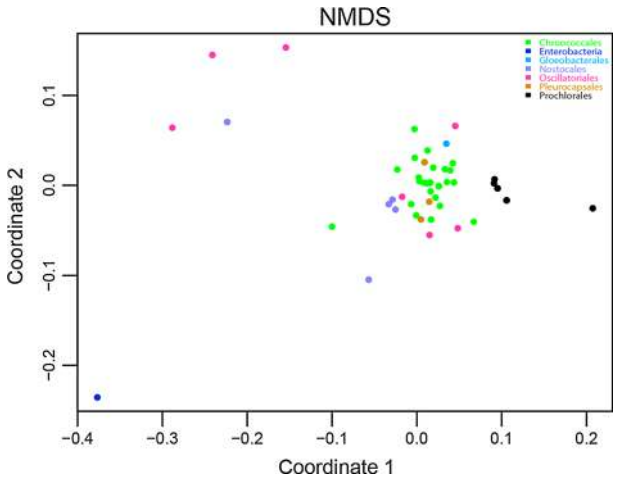

Fig. 3 Plot derived from MDS analysis representing the diversity of cyanobacterial strains and lineages on the basis of variation in tRNA isoacceptors (tRNA targeting considering also different tRNAs but with the same anticodon) for each amino acids types and copy number calculated on the known complete genomes of cyanobacteria. Only 5 of 9 accessions of prochlorophytes are visible, since 4 accessions are completely overlapping with the others. (Color figure online)

decades. Previously, the cyanobacteria were placed into three botanical orders, the number of which has changed with respect to the state of investigation of morphological variability and ecology of the species. Geitler (1932) revised the systematics of cyanobacteria established in the nineteenth century and proposed three orders: Chroococcales (coccoidal species reproducing by binary fission), Chamaesiphonales (a heteropolar type of binary fission), and Hormogonales (the filamentous species). Other authors of the twentieth century usually followed Geitleŕs botanical system. However, their systems changed as additional characters were uncovered and additional taxa included, e.g. Desikachary (1959) distinguished five orders (Chroococcales, Chamaesiphonales, Pleurocapsales, Nostocales, and Stigonematales), and Starmach (1966) split the system of cyanobacteria into four classes (Chroococcophyceae, Chamaesiphonophyceae, Pleuastrophyceae, and Hormogoniophyceae).

Later, in the 1970s, a bacteriological approach was used in the classification of cyanobacteria (Stanier et al. 1978). Five subgroups, corresponding to the orders Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales, and Stigonematales, were classified with respect to the type of cell reproduction, cell differentiation, and molecular/biochemical attributes (Rippka et al. 1979; Boone and Castenholz 2001). This classification concept facilitated substantial progress in the research on cyanobacteria because of the new methods advocated.

The most comprehensive studies on the classification of cyanobacteria in the modern era were made by Anagnostidis and Komárek (1985, 1988, 1990; Komárek and Anagnostidis 1986, 1989). The authors combined both botanical and bacteriological approaches, integrating traditional cyanobacterial morphology, physiology, and ecology in a total evidence synthesis. They established four orders: Chroococcales (non-filamentous), Oscillatoriales (filamentous, lacking specialized cells), Nostocales (filamentous, facultative specialized cells), and Stigonematales (filamentous, obligatory specialized cells, and division in multiple planes). During the 1990s, analysis of the 16S rDNA gene elucidated and supported phylogenetic relationships among morphologically similar genera, and, surprisingly, among genera from different orders as defined by Anagnostidis and Komárek. Hoffmann et al. (2005) proposed a new system of classification where members of the Chroococcales and Oscillatoriales formed two subclasses, the Synechococcophycideae and the Oscillariophycidae. Members of the Nostocales and Stigonematales belonged to a separate monophyletic subclass, the Nostochophycideae. Terminal taxonomic units (genera and species) represent a crucial element in the world of cyanobacteria. Numerous new genera are being erected because molecular methods usually show a higher diversity than the traditional botanical (morphological) approach by providing additional character sets (cryptic species). This topic is discussed in greater extent above. This system of higher taxonomic ranks has been recently re-evaluated in a review by Komárek et al. (2014). These authors proposed a subdivision of cyanobacteria based on phylogeny and morphology in the following orders: Gloeobacterales, Synechococcales, Spirulinales, Pleurocapsales, Chroococcales, Chroococcidiopsidales, Oscillatoriales, and Nostocales.

The higher level systematic classification of cyanobacteria needs more investigation based on revised genera. A complete revision should include morphological description of natural populations based on light and electron microscopy, habitat characterization, molecular analysis of 16S rRNA gene and other markers such as ITS region and information about stored strains or DNA. Moreover, important consideration should be given to biochemical/bioorganic data, e.g. fatty acids composition of cyanobacterial cell wall, which seems to be applicable for species identification (Caudales et al. 2000; Řezanka et al. 2003; Li and Watanabe 2004).

Whenever a wide agreement on species concepts in cyanobacteria is reached, some practical identification of species and other taxa open another ample array of problems. For instance, cyanobacteria may be described under both the International Code for Algae, Fungi and Plants (ICN, http://www.iapt-taxon.org/nomen/main.php) and the International Code for Nomenclature of Prokaryotes (ICNP), although the vast majority of cyanobacterial taxa are described under the Botanical Code (Oren 2011). The reason for that are the strict requirements of the ICNP, i.e. axenic culture and DNA–DNA hybridization etc. Detailed values, description and discussion may be found on the website of the International Committee on Systematics of Prokaryotes (http://icsp.org/; Starkerbrandt et al. 2002; Oren and Garrity 2014). Some additional problems in the application of the ICNP to cyanobacteria are discussed in Oren (2004, 2011), and Oren and Tindall (2005). An attempt to develop a special code valid only for cyanobacteria has been proposed at the Meeting of the International Association for Cyanophyte Research in Luxembourg in 2004 (http://www.cyanodb.cz/files/CyanoGuide.pdf). However, it is an unofficial document that has not yet been accepted. Thus, a schism among cyanobacteriologists still continues, but a number of authors largely favor the Botanical Code, because new taxa might be described without cultures (e.g. Hašler et al. 2014a).

Estimate of the total cyanobacterial biodiversity

Culture-independent estimates of prokaryotic biodiversity fall between millions and billions of species (e.g., Dykhuizen 1998; Gans et al. 2005). Estimates of the current cyanobacterial biodiversity range from 2000 (SantAnna et al. 2006) to 8000 (Guiry 2012). Nabout et al. (2013) applied a discovery curve to cyanobacteria utilizing the CyanoDB database (http://www.cyanodb.cz/) with three asymptotic models, yielding from 3166 to 6280 species, depending on the model of choice. A total of 453 authors have described cyanobacterial taxa, and two of them (J. Komárek and K. Anagnostidis) have described 30.9 % of the total described species (Nabout et al. 2013). However, the real number of species can be barely assessed by statistics. It requires extensive observation of the species diversity and distribution in nature (Foissner 2006) with subsequent quantification. In the future, genetic and molecular data will be increasingly helpful. For example, the last decade of polyphasic studies brought tens of newly erected or revised cyanobacterial genera (e.g. see Komárek 2010 for review of older works, afterwards e.g. Strunecký et al. 2011; Komárek et al. 2013; Komárková et al. 2013; Dvořák et al. 2014a). During the 19th Symposium of the International Society for Cyanophyte Research in 2013, 16 new genera were presented (Komárek et al. 2014). Thus, the great atomization of cyanobacterial systematics is now in progress, which is a result of species definition, concept used, and introduction of molecular methods into cyanobacterial systematics. Moreover, with higher resolution abilities, we can expect a further expansion of the number of described taxa. For example, *Oculatella* erected with single species (Zammit et al. 2012) now contains seven species, which have been described by different researchers. Thus, even the most liberal estimates may be undervalued.

Conclusions and future directions

Great challenges lie ahead in regards to the taxonomy and systematics of cyanobacteria. Fortunately, molecular techniques have facilitated a renaissance in describing and

elucidating cyanobacterial biodiversity. In this review, we showed that although cyanobacteria lack sexual reproduction, we are able to apply, in terms of evolutionary-lineage coherence, a species concept similar to that one used for eukaryotic macroorganisms, even though it might be considered "fuzzy" due to the molecular markers applied, homologous recombination or horizontal gene transfer. However, many questions remain regarding cyanobacterial species definitions and concepts. Caution must be maintained, though, as morphology is sometimes in conflict with molecular markers, or has limited resolution. Therefore, cryptic species and extremely polyphyletic genera caused by serial convergence represent problematic phenomena resulting with uncertainty of proper morphological identification. We suggest that more attention should be paid to the use of molecular markers in taxonomy and practical identification of taxa. On the other hand, ecological and morphological criteria are also important, which should be taken into consideration. Thus, deposited sequences in GenBank and other databases should also be completed with such data or they should be made easily accessible by providing the original papers. These data may be afterwards a source for a barcoding database, which would provide correct and fast identification workflow, and would resolve cryptic taxa and polyphyletic genera problems.

The rapidly growing number of described taxa signifies large gaps in our current knowledge of cyanobacterial biodiversity and distribution. Although the total biodiversity of any microbial lineage is probably unknown, all estimations suggest a significant increase of described taxa. Moreover, the selected species definition will impact on how many species are identified and will be recognized in the future. It also largely influences possible patterns of distribution. Thus, evidently, we are now in a period of important changes in taxonomy, and knowledge of cyanobacterial biodiversity is amplified by novel techniques, and increasing sampling effort.

Methods of analysis

Multivariate statistics have been carried out by using the R software 3.0 (R Development Core Team 2013) and some functions included in Vegan and MASS packages (Venables and Ripley 2002; Oksanen et al. 2013). Transfer RNA data of all the analyzed organisms have been imported in R as dataframe. The distance matrix has been computed using the "vegdist" function and selecting "jaccard" as method. The quantitative form of the Jaccard distance in Vegan actually is the Ruzicka index and has been preferred over the Euclidean distance for its better performances in presence of species containing missing tRNA (counts equal to zero). In order to visualize the distances between organisms, data have been statistically explored through unconstrained ordination by computing a non-metric multidimensional scaling (NMDS) using the "metaMDS" function included in the Vegan package. Multidimensional Scaling helps to visualize the distance between samples through a low-dimensional spatial map. The non-metric scaling methods are able to map non-Euclidean distances.

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