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Taxonomy and species delimitation in cyanobacteria

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Abstracts: Cyanobacteria (also Cyanoprokaryota) are prokaryotes whose taxonomy follows the rules of the International Botanical Nomenclature Code, IBNC, built mainly for eukaryotic photosynthetic organisms. Hence, accepted names of cyanobacteria follow the same rules and are assigned to biological entities (species) that should correspond, taxonomically, to eukaryotic species. The main difficulty with that is that the species concept in eukaryotes is based theoretically mainly on the biological species concept, that is centered on genetic exchange through sexual reproduction within species or lack of exchange between different species. However, as here shown, this difference is important from a theoretical point of view, but also in eukaryotes, the boundaries between different species are very rarely checked experimentally by direct observation of sexual barriers and hybridization events. The main tool for species delimitation is hence morphological description and, more recently and always in relation to morphology, DNA sequences. Unfortunately, macromorphology provides an insufficient amount of useful characters in cyanobacteria and in prokaryotes in general, while micromorphological data are yet largely insufficient for giving a sufficiently extended framework. On the contrary, the introduction of distances obtained from matrices of aligned sequences in the framework of a barcoding project provides a quantitative interpretation of species delimitation in relation to genetic distance that can be used both in eukaryotes and prokaryotes. However, the introduction of quantitative criteria needs the definition of distance thresholds to identify the boundaries between different species and, for doing that, it is necessary to test those thresholds in models of traditionally defined and recognized species. An alternative approach may be to experimentally investigate the capability of strains/species to establish barriers to genetic information exchange with respect to other species/strains. Data about this last question is still insufficient. The adoption of molecular criteria to check species boundaries based on morphological characters has proved particularly challenging in cyanobacteria: a known example is provided. In conclusion, the only possible approach appears to be the association of molecular data to the increase of available data about the cell structure and the variation thereof in different physiological situations, particularly at the ultrastructural level. A further necessity is the check of the nomenclatural typus for a large number of cyanobacteria species, often based on old basionyms and old material. For many old names the typus is often a drawing and more rarely a herbarium specimen or a microscope slide. In many cases an epitypification or a neotypification appears to be necessary.

Keywords Cyanobacteria; *Arthrospira*; species concept; typus; species concept in prokaryotes

1. Introduction

Cyanobacteria, more recent denomination Cyanoprokaryota [1-2], and earlier Cyanophyta, Cyanophyceae, blue-green algae, or blue-green bacteria, depending on the level of similarity to (eukaryotic) algae implied in the term, are prokaryotes capable of oxygenic photosynthesis and hence containing chlorophyll a. A clade of cyanobacteria owns also chlorophyll b, as green algae do, but without a direct phylogenetic relationship with these last organisms [3]. Cyanobacteria share the same environment of eukaryotic algae [4], even if the first have normally higher temperature optimum [5] and high capability of resistance to desiccation and water stress [6]. Moreover, cyanobacteria are widely

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distributed and can live in some extreme habitats on earth [7] and are one of the most diversified clades of bacteria [8]. On the basis of fossil records, Schopf [9] estimated for Cyanobacteria an age of 3.5 billion years, hence they are probably the oldest organisms capable to perform oxygenic photosynthesis, which caused a sudden increase in atmospheric oxygen [4,10].

Eukaryotic algae themselves evolved because of intracellular symbioses of cyanobacteria with eukaryotic heterotrophic hosts. The capability of forming symbioses appears to be a hallmark of cyanobacteria [see for instance 11-14].

Cyanobacteria show species delimitation issues similar to those of other prokaryotes. Species delimitation in prokaryotes is a more difficult task with respect to eukaryotes, since a common theoretical ground about the species concept such as the biological species concept in eukaryotes is of difficult application due to the lack of sexual reproduction processes [15,16]. As in other prokaryotes, cyanobacteria diversification occurred by adaptation to different environmental contexts thanks to their high phenotypic plasticity and niche adaptability [17]. The genetic exchange occurred via asexual reproduction and homologous recombination, also with horizontal gene transfer between distinct lineages/species. Selection pressures on the ecotypes led to speciation [17].

Cyanobacteria taxonomy is ruled by the International Botanical Nomenclature Code (IBNC, https://www.iapt-taxon.org/nomen/main.php [18], being the only group of prokaryotes here considered. In the article we will refer to the above-cited version of the code, that is the Shenzen code of 2017 [18] unless otherwise indicated. The consequence is that the taxonomy of a group of prokaryotes (such as cyanobacteria are) is ruled by a code otherwise dedicated to eukaryotes, where speciation and species delimitation occur mainly through mechanisms involving sexual reproduction.

Principle I of the IBNC states that the nomenclature of algae (cyanobacteria included), fungi, and plants is independent of zoological and prokaryotic nomenclature. The principle II of the same code states that the application of names of taxonomic groups is determined by means of nomenclatural types. Hence, the type (typus) is a fundamental cornerstone for taxon naming and delimitation, since the name is attached to a specific type. For filamentous species, it is possible to prepare desiccated herbarium samples, that are not informative as samples of land plants, but provide relatively stable material for future investigations (for instance in [19]). However, for many types of cyanobacterial species, no herbarium sample is available, while for unicellular species only drawings and slides (rarely used) could be considered as valid types.

For this reason, also species of relevant commercial interest are sometimes of uncertain nomenclature and typification even if the species name is always the starting point for the organization of data contained in databases of DNA, RNA and protein sequences, such as Genbank or more specifical ones, as, for instance of fatty acid content [20].

However, even in prokaryotes DNA exchange is possible with effects on genome recombination that are comparable to those caused by sexual reproduction in eukaryotes, and hence the building of species boundaries may have something in common with eukaryotes [17].

As in other organisms, species in cyanobacteria were described mainly on the basis of macro-morphological character or, as in general in the case of microscopic organisms by observation with the light microscope. This method is able to identify the thallus characters in the case of filamentous species and even some cell character such as shape and dimension, but at has a low level of definition. As a consequence also the systematic knowledge and the phylogenetic reconstructions of cyanobacteria were largely based on simple morphological characters. The applications of ultrastructural and molecular investigations

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provided larger datasets, in many cases largely changing the knowledge about relationships between different groups and hence influencing taxonomy, both at the species higher-order taxonomic level [21]. Particular relevance had the identification of monophyletic groups containing both filamentous and unicellular species [21], that would not have been imagined on the basis of macro-morphological characters only.

The Automatic Barcode Gap Detector (ABGD), a tool developed for animal DNA taxonomy, was able to test for the existence of a barcoding gap in a dataset of genetic sequences of a single marker obtained from different individuals from closely related species [22].The application of this tool revealed that a barcoding gap could actually be found in many tested datasets of cyanobacteria DNA sequences [23]. The method postulates that, if a barcoding gap exists, the genetic distances within each species would be low, while genetic distances between species would be high, while no intermediate distances should be present. Surprisingly, the identification of units of diversity through this method provided results that were not always compatible with those obtained with the identification of OTUs with the threshold of similarity in genetic distances of 97% or 99%, hence the authors proposed caution in the estimate of diversity from 16S sequences [23].

The aim of this work is to try to answer the questions: are there right types for cyanobacteria taxa? Are the recognized species boundaries in cyanobacteria solid?

2. Materials and methods

The cells of cyanobacteria *Dolichospermum lemmermannii* (Richter) Wacklin, Hoffmann & Komárek (strain C101N2 from Como Lake, Italy), *Arthrospira platensis* (Gomont) a commercial strain (strain ORB-4, by Lagunafarm, Orbetello, Italy https://www.spirulinafarm.it/) and *Arthrospira fusiformis* (Voronichin) Komárek & Lund (strain CHITU-2 from Chitu lake, Ethiopia, Africa) were cultivated in Zarrouk's medium [24]. The light was provided with LED, PAR about 150 µE, one of the samples was exposed only to blue light whose peak was 435nm.

Samples were collected from the culture with the growth medium and fixed overnight in 1.25% glutaraldehyde in filtered sea water at 4°C, then post-fixed in 1% OsO4 in 0.1 M phosphate buffer (pH 6.8) for 1 hour. After dehydration in an ethanol series and a propylene oxide step, the samples were embedded in Spurr's epoxy resin [25]. At each step the cells were sedimented with a 5 minutes centrifuge step at 1500 rpm and only the sediment was used for the following step in order to substitute safely the various solvents with micropipettes without losing cells, as previously shown in [26-28].

Transverse sections approximately 80 nm thick were cut with a diamond knife and a Reichert-Jung ULTRACUT ultramicrotome. The sections were stained with uranyl acetate [29], lead citrate [30], and then examined with a Philips EM201 TEM at 80 kV. Living samples and semi-thin sections were observed with a Leica microscope DM RB Fluo.

3. Results and discussion

3.1. The example of the two genera Spirulina and Arthrospira and the influence of the physiological status on ultrastructure of cyanobacteria

Many genera of cyanobacteria are simply trichal (a single filament) and four of them are described as with thalli forming helicoidally coiled filaments: *Spirulina* Turpin ex Gomont (1892), *Halospirulina* Nübel, Garcia-Pichel et Muyzer 2000, *Arthrospira* Stizenberger ex Gomont (1892), and *Limnospira* Nowicka-Krawczyk, Mühlsteinová & Hauer 2019. The last two names are

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traditionally associated with species cultivated as food source, for production of local dishes as dihé in Africa or tecuitlatl in Mexico [31-33]. *Arthrospira* and *Limnospira* together account for 23 species, most of them of freshwater [33,2].

Spirulina is the commercial name commonly used for the same cyanobacteria cultivated for human use (food, nutraceuticals, pigment production, industrial production of cosmetics). The result is an evident overlapping between the commercial name and the scientific name of genus *Spirulina* Turpin ex Gomont (notice the use of italics), which is not used for cultivation. The species name of the most commonly cultivated spirulina is generally considered to be *A. platensis* Gomont. After Nowicka-Krawczyk et al. [33], however, this species would be a benthic organism first described in Uruguay, while most commonly cultivated spirulina would be only the planktonic *A. fusiformis* (Voronichin) Komárek & Lund and *A. maxima* Setchell & Gardner (both later assigned to genus *Limnospira* by Nowicka-Krawczyk et al. [33]). Genus *Spirulina* Turpin has been shown to be separated from *Arthrospira* already by Komárek and Anagnostidis [2], Castenholz et al. [34] and Komárek and Lund [35] -among others-, despite some common morphological feature (namely, the coiled shape of the thallus) shared by many species belonging to one or the other of the two genera.

Some species (we will discuss later the use of this term) of *Arthrospira* with clearly different morphology, such as *A. maxima* Setchell & Gardner and *A. fusiformis* (Voronichin) Komárek & Lund are similarly cultivated. However, a phylogenetic analysis with 16S, a commonly used molecular marker for prokaryotes barcoding, was not able to discriminate these species one from the other [32].

Nevertheless, the commonly cultivated *A. platensis* (Fig. 1A) can be easily distinguished with light microscopy with respect to *A. fusiformis* (Fig. 1B), while *A. maxima* is described with a thallus width about the double of *A. platensis* [36]. Recently, Nowicka-Krawczyk et al. [33] reevaluated the type of genus *Arthrospira*, that is *A. jenneri* Stizenberger ex Gomont, arriving at the conclusion, on the basis of molecular data, that the cultivated species of *Arthrospira* (*A. platensis*, *A. fusiformis* and *A. maxima*) show enough genetic distance with respect to *A. jenneri*, to deserve the inclusion in a newly described genus, *Limnospira*, containing the species *L. fusiformis* and *L. maxima,* with the type species being *L. fusiformis*. A few months later, Papapanagiotou and Gkelis [37], in order to determine the taxonomic relationships among the existing *Arthrospira* strains (including in it *Limnospira*), investigated their phylogenetic relationships with a combined analysis of molecular (16S rRNA, 16S-23S rRNA ITS, and cpcBA-IGS sequences) and phenotypic (13 morphological and morphometric characters), arriving at the conclusion that *Arthrospira* strains may be divided into three clusters/taxa as subspecies of a single species of genus *Arthrospira*. Papapanagiotou and Gkelis [37], in their phylogenetic analysis, used also a sequence from a strain of *A. jenneri*, but the provenience was not indicated.

A noteworthy effect on the morphology of the cultivated spirulinas can be caused by physiological and ecological conditions as already observed in several experiments [6,38]. Quite well known is the linearization of the thallus occurring in a depleted medium (Fig. 1C, with Fig. 1D showing the chlorophyll fluorescence), as originally described by Van Eykelenburg and Fuchs [39]. Later, the reversion from linear to coiled thallus was described by Wang and Zhao [40].

It is remarkable that the linear forms of *Arthrospira* and *Limnospira* spp. would have been surely inserted in a different genus with respect to the spiralized form on the basis of the structure of its thallus observed with light microscopy. It is also possible that in some cases, currently recognized different species of cyanobacteria may actually be the same species in different physiological status

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since in many cases the diagnosis is based on the shape and dimension of the thallus. This hypothetical occurrence would be analogous to the well-known case of the erroneous description of the red alga *Conchocelis rosea* Batters as an independent species, later reinterpreted as the sporophyte of *Porphyra umbilicalis* Kützing [41].

Another modification caused by the physiological condition is the high concentration of salt in the medium, leading to a strong modification of the thallus; or the use of blue light for growth, causing an increase in carboxysomes (Fig. 2A) with respect to white light (Fig. 2B). Even if in higher number, carboxysomes maintained here in average the same dimension (0.5 µm in diameter) and shape in *Arthrospira* (now *Limnospira*) spp., while in other species, belonging to a different order (Nostocales), such as *Dolichospermum lemmermanni* (Aphanizomenonaceae, Fig. 3A) and *Tychonema* sp. (same family of *Arthrospira*, Microcoleaceae, Fig. 3B), shape (more roundish) and dimension (0.8-1 µm in diameter in average) of carboxysomes appeared different from those of *Arthrospira*. Hence, some ultrastructural characters (dimension and shape of carboxysome, but not the number) appear to be stable within a recognized species (the cultivated *A. platensis sensu lato*), while the general shape and even the width of the thallus (observed with light microscope) can vary within the same species if exposed to different physiological conditions. A decrease in proteins due to saline stress and the linearization effect in case of stress was investigated by Sili et al. [42]. The change in morphology may be due to the increase in respiration rate in the case of salinity stress [43,44] and in general by environmental stress for the production of the linear form [45], such as nutrient depletion, as in our case.

An increase of data about physiological variation causing morphological changes is needed. As shown earlier, a specific ultrastructure may be related to the difference at the species level or rather to different physiological situations. As a matter of fact, electron microscopy was the technique that made it clear that cyanobacteria were prokaryotes, by revealing the circular DNA and the lack of membrane-bound organelles, together with cell walls that are typical of Gram-negative bacteria [46].

Currently, more weight is given to molecular data. Nevertheless, overlooking the analysis of the morphological characters risks to produce results of insufficient value for the purpose of identification of morphological variation in the field and recognition of the ecological variation due to genotypic variation [13]. For this reason, several authors proposed a combination of methods, the so-called polyphasic approach, that would provide in-depth knowledge of the organism [47].

[13] suggested 6 steps. 1) analysis of DNA sequences for the evaluation of the phylogenetic position and, possibly, a genotypic variation of the strain. Currently, also genomic data have been available at reduced cost and laboratory time. 2) morphological characters including variation thereof, possibly in nature and in culture. Normally only light microscopy is intended here. 3) ecological and ecophysiological investigation, also for phytogeographical distribution. 4) ultrastructural studies. 5) biochemical characters in general as optional data. 6) Formal description after the bacteriological and/or botanical rules of nomenclature. This last point, which is fundamental, will be treated in the last chapter.

However, a polyphasic approach as proposed by Castenholz and Norris [48], Komarek [49] and Papapanagiotou and Gkelis [37] may be insufficient in case of large morphological variation due to an insufficiently known physiological property of the investigated strain.

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3.2. Do the nomenclatural entities that we recognize as different species in cyanobacteria correspond to unitary genetic groups potentially able to exchange genetic material within but not outside the entity? Is there a genetical barrier between different cyanobacterial species?

The organization of biodiversity in discrete entities as species, in opposition to a vision with which diversity represents a continuum, is a matter of debate in prokaryotes [17,50-52]. The extreme position of researchers who retain the continuum as the best way to describe diversity in bacteria suggests we should simply use the strain as the basic unit [53,54] and, maybe, its phylogenetic position. However, following this direction, we would have a strain code as a basic taxonomic unit, which would not be informative at all in the context of a phylogenetic tree containing hundreds of thousands of other strains. Probably, the consequence would be that names would be given to higher-order branches and inevitably some of these branches would correspond to the currently used species name, returning to the starting point.

A key point appears to be the lacking of sexual reproduction in prokaryotes, which is considered the cornerstone of the biological species concept in eukaryotes [55,56]. For this reason, different species concepts have been proposed, such as the Ecotypic Species Concept, the Evolutionary Species Concept, the Monophyletic Species Concept and the Phylogenetic Species Concept [15,57], trying to relax the importance of sexual reproduction as a mean for genetic exchange between lineages.

In bacteria the Horizontal Gene Transfer (HGT) and related phenomena may partially substitute sexual reproduction in order to delimit the lineage boundaries [58,59]. HGT in prokaryotes has been extensively investigated in the human microbiome [59] and, in cyanobacteria, in genera Synechococcus and Prochlorococcus [61]. The HGT events, more frequent than expected, would be caused or facilitated by cyanophages [62,63]. Such phages would often transport genes important for cyanobacteria adaptation, such as photosynthesis-related genes [64]. The integration of many cyanobacterial genes into cyanophages shows that a genetic transfer occurs between cyanobacteria and phages in both directions, driving microevolution and, in some cases, speciation. After Shestakov and Karbysheva [63], the strict cyanophage–host interactions support even the concept of coevolution between cyanophages and cyanobacterial genomes, hence species delimitation in cyanobacteria may be related to the specificity of cyanophages for some strains and not for others.

HGT events would be more frequent between species sharing a common particular environment and higher phylogenetic affinity, while the frequency of HGT events would be proportionally inverse with respect to the genomes genetic distance [16,60]. Therefore, the differential capability of DNA exchange through HGT may be the most important factor for species delimitation in cyanobacteria.

Homologous recombination (HR) at a frequency exceeding the mutation rate would lead a species to evolve in a mode similar to that of sexual species, while with low HR the populations would maintain a clonal status [65].

Horizontal gene transfer was one of the key selection pressures leading to cyanobacteria diversification [17] and hence a certain degree of genetic interchange is apparently possible also between distantly related strains/species. However, the same can occur also in strictly related different species iof angiosperms (introgression in the sense of Briggs and Walters [66]), without affecting the species concept and the consequential taxonomy. Moreover, genome-level investigations led to the concept of “stable core” and “variable shell” within a genome [67], with the stable core being the set of genes with a common evolutionary history (the most important from the point of view of

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metabolism control), and the variable shell the group of genes that may be present or not or be present in a varying number of copies. The genes of this last group would be those more implied in horizontal gene transfer and homologous recombination [68,69].

Recently, an ecogenomic approach proposed a connection between taxonomy and ecological adaptations [70], but the pattern worked only at high-level taxonomy since only three groups of cyanobacteria were identified on ecogenomic basis: i. Low Temperature; ii. Low Temperature Copiotroph; iii. High Temperature Oligotroph.

After Willis and Woodhouse [17], currently the delimitation of a bacterial species would require >70% DNA–DNA hybridization, less than 5°C DTm, and less than 5% mol GþC difference of genomic DNA. Identity in 16S rRNA should be more than 97% after Stackebrandt et al. [71], or 95–96% of the whole-genome average nucleotide identity (ANI) [72]. Despite remarking the lack of a generally accepted quantitative threshold of genetic distance to describe a prokaryotic species, Willis and Woodhouse [16] showed that species delimitation in the cyanobacterial genera *Raphidiopsis* and *Microcystis* could be assessed with species boundaries occurring at ~96% average nucleotide identity at the genomic level, together with a constrained homologous recombination capability.

Eckert et al. [11] showed the existence of a barcoding gap (after the method of ABGD, Automatic Barcode Gap Detection) in only half of the tested datasets of cyanobacteria genomes. However, the delimitation of a set of biological entities (strains) interpretable as species was not always compatible with the units of diversity obtained with the threshold of similarity in genetic distances of 97% or 99%. The ABDG method is promising, but it shows some limitations for a general application to cyanobacteria species delimitation. The first concern is that the method is based on a priori identification of "real" species [22] as test dataset (for parameter calibration), and this a priori choice is somehow subjective. The second point is that the recognized "gap" that should identify the species boundary, appears to be quite subjective too. As a matter of fact, the method was tested by Puillandre et al. [22] on metazoans, that may show more evident species "gaps" with respect to cyanobacteria (and prokaryotes in general).

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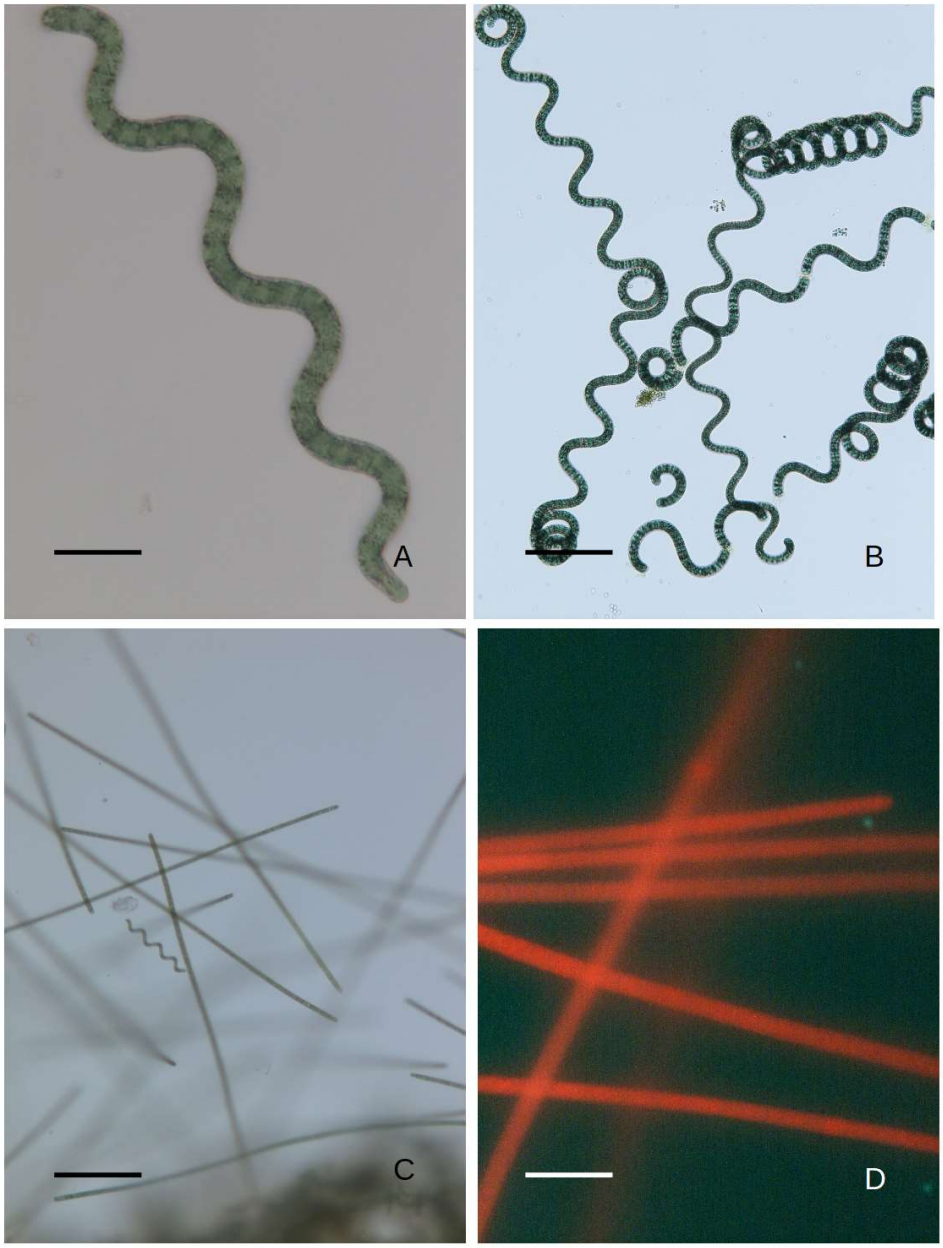

Figure 1. *Arthrospira*, light microscope. A: *A. platensis* grown in fresh medium. Bar = 20 µm. B: *A. fusiformis* from Chitu Lake, Ethiopia and grown in fresh medium. Bar = 50 µm. C: *A. platensis* grown in depleted medium. Most thalli are in linearized form. Some normal spiralled (helicoidal) form is still present (arrow). Bar = 100 µm. D: *A. platensis* grown in depleted medium. Blue light, fluorescence microscope. Bar = 30 µm.

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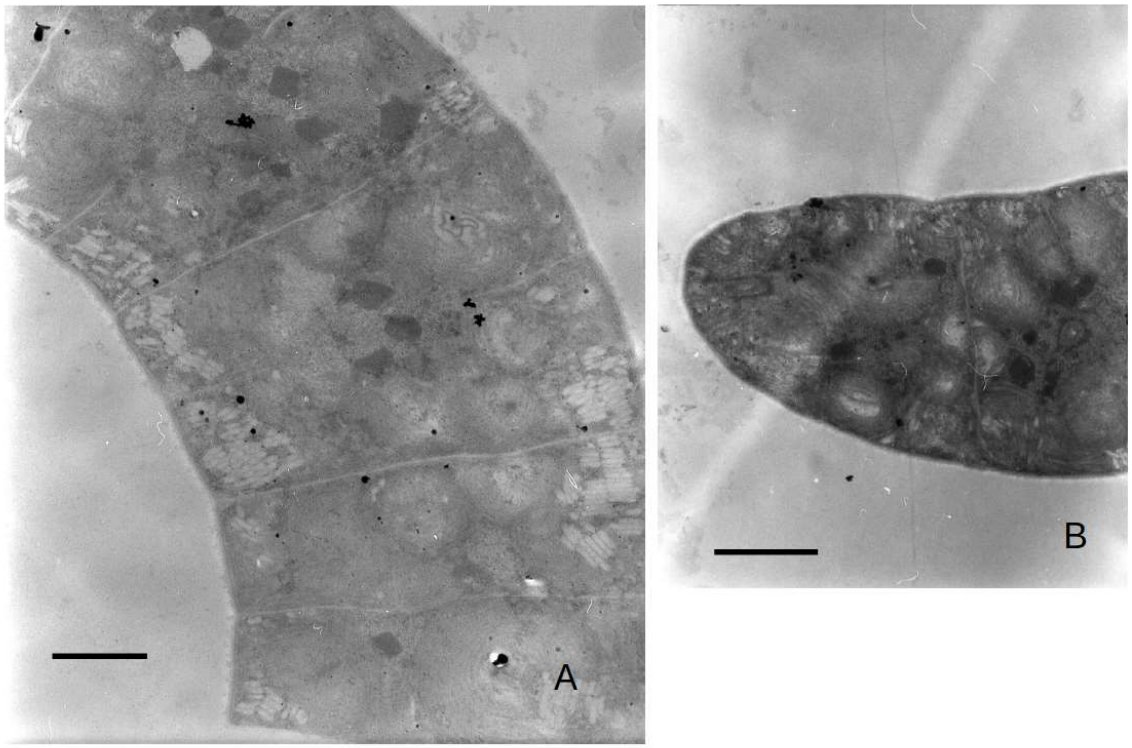

Figure 2. *Arthrospira*, transmission electron microscope. A: *A. platensis* grown in blue. Bar = 1 µm. B: *A. fusiformis* from Chitu Lake, Ethiopia and grown in white light. Bar = 3 µm.

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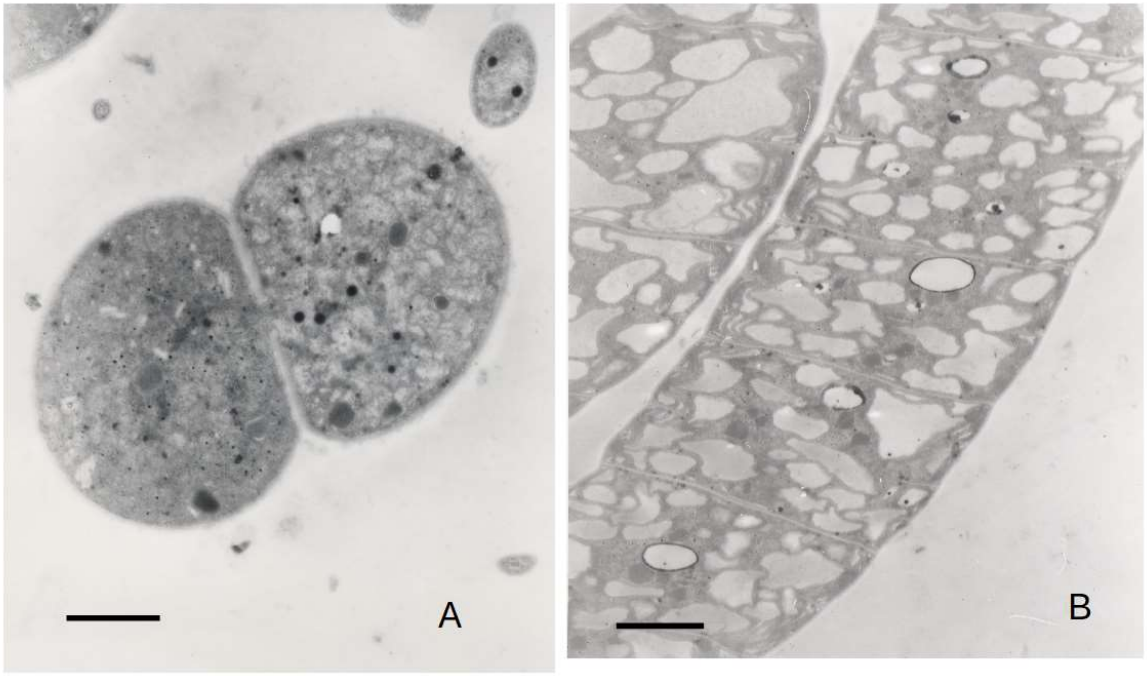

Figure 3. Transmission electron microscope. A: *Dolichospermum lemmermanni* grown in fresh medium. Bar = 2 µm. B: *Tichonema* sp. grown in fresh medium. Bar = 3 µm.

3.3. The problem of the nomenclatural typus in cyanobacteria

After the International Code of Nomenclature of Bacteria [73,74], 'The nomenclatural type, referred to in this Code as "type", is that element of the taxon with which the name is permanently associated' [75]. In many cases some confusion between the terms "type" in the sense of nomenclatural type (typus) and the same word used for indicating ecotypes, morphotypes, or phenotypes is frequent (for instance Fiore et al. [76]). This definition overlaps with that of the IBNC dedicated to plants and cyanobacteria [18]. Such element is normally a herbarium sample, which in many cases is not available or also difficult to produce in the case of small or unicellular organisms as many cyanobacteria. Other nomenclatural types accepted by the IBNC code are drawings, very common in cyanobacteria as, for instance for *A. jenneri*, whose basionym is *Spirillum jenneri* (Hassall 1845). Another possible typus material is dried or otherwise conserved material, even if normally a living typus (for instance a plant conserved in a botanical garden) is not admitted, since living samples may change. The IBNC, however, at article 8.4 allows the use of "cultures of algae and fungi if preserved in a metabolically inactive state (e.g. by lyophilization or deep-freezing to remain alive in that inactive state", as acceptable types. Cyanobacteria are not explicitly cited but surely belong to these categories by analogy. The use of microscope slides (light microscope) may be useful as typic material but it is rarely adopted. However, it is explicitly considered by article 8.2 of the IBNC, and a recent example is that of *Glaucospira laxissima* (G.S.West) Simic et al. [77].

Finally, the importance of Transmission Electron Microscopy (TEM) in the description of cyanobacteria [13] makes it necessary to maintain drawings and

pictures as cornerstones of typification in cyanobacteria. Unfortunately, most ancient descriptions are based on drawings obtained with light microscopy and hence, even if the drawing is of good quality, the level of detail is insufficient to give an account of the cellular structures such as presence and aspect of thylakoids and phycobilisomes, cyanophycin granules, carboxysomes, cell wall, gas vesicles, the ultrastructure of heterocysts (if present) and other structures. However, since the nomenclature typus should help to easily recognize the species, the description with the help of TEM should always be flanked by light microscope images, since the use of TEM needs a complex preparation that is not always available in all laboratories and, however, cannot be used for a high number of different samples.

The holotype may be a dried sample, while the other descriptions (light microscope slide, light microscope image, TEM image, or the conserved culture should all be considered as isotypes (as correctly done in Fiore et al. [76]). Such an approach is normally not followed. For instance, in the case of the recently described two new species *Stenomitos kolaensis* and *S. hiloensis,* the designed (holo)types were two herbarium samples [78]. The authors provided also light microscope images and good quality drawings, that however were not designed as isotypes.

For the difficulty in identifying nomenclatural types, many researchers are using, also for nomenclature aims, directly the strains collected in public repositories such as EPSAG (https://www.uni-goettingen.de/de/45175.html). Such an approach is of course very practical, but of uncertain usefulness for nomenclature purposes [13], since there is normally no direct connection between the strains conserved by the repositories and the nomenclatural types. Often, also the reference to the locus classicus of the type in the description of new species is at least partially overlooked.

As an example, the recent separation of genus *Limnospira* from *Arthrospira* was based on the differences observed between the cultivated species of *Arthrospira* (then *Limnospira*) with respect to the type species of *Arthrospira*, that is *A. jenneri* [33]. However, the observations were neither done on a typus (herbarium sample or slide) of *A. jenneri,* either on a sample collected in the locus classicus, but rather on a collection of apparently the same organism in an urban reservoir in Poland. Nevertheless, the basionym of *A. jenneri* goes back to *Spirillum jenneri* Hassall, who named the species after the collector (Mr. Jenner) see Hassall [79], on page 277. The typus appears to be the figure drawn in plate LXXV, figure 5 by the same author and the locus classicus is declared to be Tunbridge (UK), quite far away from Poland. Moreover, the figure itself appears to be insufficient to be able to discriminate this species from other *Arthrospira* species. Hence, probably, it would be necessary an epitypification, defined as an "interpretative type when the holotype, lectotype, or previously designated neotype, or all original material associated with a validly published name, is demonstrably ambiguous and cannot be critically identified" after the article 9.7 of the IBNC, directly in the locus classicus, prior to accepting definitively taxonomical changes.

Another example is that by Mikhailyuk et al. [80] providing an etipyfication for *Crinalium magnum* Fritsch et John (Gomontiellaceae, Oscillatoriales). The species was originally described from a soil culture of the Irish Sea coast in Great Britain, namely Llandudno in North Wales [80]. Also, in this case, the typus appears to be the drawing Fig. 8A at the page 392 in Fritsch and John [81]. For this reason, the epitype by Mikhailyuk et al. [80] is correct and provides a lot of useful data, since the authors prepared a herbarium specimens and a liquid culture was preserved in 2% and 4% formaldehyde, while preserved material was then deposited in the Algotheca of the M.G. Kholodny Institute of Botany of the

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National Academy of Sciences of Ukraine. However, the epitype was not collected from the locus classicus (North Wales), but from coastal sand dunes in Mecklenburg-Vorpommern, Germany, with the reason that the authors' material corresponded to the diagnosis by Fritsch and John [81] and the locality of the collection showed the same ecological features of the *locus classicus*. The IBNC does not forbid to produce an epitype from a place different from the *locus classicus*, but it is certainly a risky choice, as suggested also by Dentant et al. [82].

Another interesting case is that of the neotypification of *Pleurocapsa fuliginosa* [83]. In this case, the authors verified that the holotype should possibly be in the herbarium of Paris and is not available for loan. However, all the available samples in the herbarium of Paris do not come from the locus classicus (Trieste, Italy, [84]) and hence the authors concluded that the holotype should be considered lost and proposed a neotype on the basis of material originally collected from freshwater in Hawaii [83].

Such cases are numerous in literature about cyanobacteria, also because the typus is often a drawing and more rarely a herbarium specimen or a microscope slide.

As a consequence, a retypification, often in the sense of an epitypification and in other cases a neotypification, is strongly needed for cyanobacteria taxa.

4. Conclusions

Species boundaries in cyanobacteria appear to be present in many groups/species, while in others the recognition of a species/taxon on the basis of molecular data is quantitative and related to the threshold at which a distance is considered sufficiently high to justify different specific ranks for different entities. Ultrastructural evidence can in many cases provide additional characters that are stable within the species or genus boundary.

In many cases, while describing a new taxon of cyanobacteria many authors use the term type interchangeably meaning the nomenclatural type or ecotypes, morphotypes or phenotypes, terms that are commonly used in prokaryotes. To avoid misunderstanding we suggest to use the term *typus* (Latin) for the nomenclatural type.

For a correct taxonomical classification a retypification (probably with the description in many cases of epitypes or even neotypes) is necessary for many of the old names.

Epitypes and types of new species should be preferentially formed by images, both with the light microscope and Transmission Electron Microscope, some biological samples as dried or otherwise conserved material. Slides and living cultures conserved in inactive form may be of help, possibly as epitypes.

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