

Peer-Review Record:

Does a Barcoding Gap Exist in Prokaryotes? Evidences from Species Delimitation in Cyanobacteria

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Reviewer 1: Anonymous

Reviewer 2: Anonymous

Editors: John C. Meeks and Robert Haselkorn (Guest Editors of Special Issue “Cyanobacteria: Ecology, Physiology and Genetics”)

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First Round of Evaluation

Round 1: Reviewer 1 Report and Author Response

The main sense of taxonomy is to offer the method for description and orientation in organismal diversity. However, the only one universal method is quite insufficient in microorganisms, and the combination of all possible classification criteria is desirable. Of course, the complex of all these methods must be corrected with the progress of science, and especially for cyanobacteria is necessary to apply the so called “polyphasic approach” for their classification. The molecular methods must be evidently preferred in this work, but other approaches (biochemical, ecophysiological, ecological, morphological) must be included into the final evaluation.

The manuscript of Eckert *et al.*, submitted into the journal Life, concerns in principle again only one, but very important genetic criterion for evaluation of prokaryotic diversity. It should be a serious part of this complex evaluation. It is very important and useful and publication of the article is surely commendable. However, the connection, coincidences and relations to other criteria should be better expressed.

Response: The concern the reviewer mentions here was also one of our main conclusions for the article. Apparently we did not formulate it well enough. It has thus been reformulated to: “However an accurate taxonomy can never be achieved by the use of a barcoding method only, since it is based on nucleotide substitutions of a single gene. Accurate classification always requires an integrative taxonomy effort

including characteristics from ecology, morphology and physiology, as already previously suggested for prokaryotes [9,28,29,45], and as it is becoming common also for animal taxonomy (e.g., [53,54]).”

Moreover, it is necessary to mention the following aspects of this manuscript:

- (1) If we accept also strains and populations from literature or from databases to the evaluation, there is always questionable the taxonomic determination (scientific names) of various species and strains, which are based on the old nomenclature and morphology. The critical revision of identification of these strains and cited materials is necessary.
- (2) It follows from the whole article that the authors accepted the names (and also concepts) of taxa (mostly genera) from older literature, without necessary criticism. There are included revised genera according to modern methods together with taxonomically very problematic groups. As examples is possible to mention that *Aphanizomenon ovalisporum* and *Anabaena bergii* are really very related taxa, but they were already re-classified in one special and separate genus *Chrysosporum*. On the other hand, the complex “*Prochlorococcus/Synechococcus*” is still very unclear and surely is distant from the typical genus *Synechococcus*. etc.

*Response: (1) We understand that our choice of naming as in the database needed clarification. We have thus added the following paragraph. We thank the reviewer for the useful example and have included it: “The names of genera and species used in this study were copied from the names given in the database. This choice was made for the reason that cyanobacterial phylogeny underlies continuous changes and genera and species are often reclassified (e.g., [34]). Thus a whole different type of work would be required in order to use all state-of-art classification of cyanobacteria. Moreover, for the aims of this study not so much the phylogenetic classification of the bacterial species, but the genetic structure of the cyanobacterial 16S rRNA genes was of importance. The use of the names provided in the database on the other hand enables other researchers to conduct a similar analysis using the same sequences. Thus some old and revised names are used, for example the species name *Anabaena bergii* is used throughout the study despite its revised taxonomy in the genus *Chrysosporum* [35].”*

*(2) We understand that particularly the case of *Synechococcus* needs clarification and have thus extended the explanation there: “Sequences termed *Synechococcus* on the other hand were analysed together with sequences of the monophyletic clade *Prochlorococcus*, since *Synechococcus* is known to be a polyphyletic group and if all sequences (including a minimum of five lineages [36–38]) are taken together, *Prochlorococcus* has to be included, too”.*

Numerous cited taxa (on the generic level) were already controlled and revised by most modern methods, and examples in the manuscript should be used especially and selected from these genera. It is true, that majority of the cited genera were already revised (*Microcystis*, *Planktothrix*, *Aphanizomenon*), but just these taxa are important as examples and should be preferred. From the presented results follows only the confirmation of the present methodological principles and a modern system. This fact should be emphasized.

Response: We agree with the reviewer that we have to avoid misunderstandings of this kind and added a very clear statement to the discussion: “Moreover it has to be emphasised that a close analysis of the actual properties of the cyanobacterial groups tested is limited by the fact that we used the provided

names for the sequences and groups. Thus this study intends only to verify the existence of a barcoding gaps within the 16S rRNA sequences of certain cyanobacterial groups, and by no means revise or confirm the complex cyanobacterial taxonomy.”

It is necessary to stress the fact that the numerical criteria (e.g., exactly limited percentage similarity) never are valid uniformly for large groups of organisms and cannot be applied as one main criterion in large groups.

Response: We agree with the reviewer that we have not stressed this fact enough and have added more sentences explaining why OTUs are used and the problem: “Enormous progress happened in prokaryote taxonomy in the recent years [8,12,24]. However, the data produced with novel methodologies, such as next generation sequencing, often requires a high throughput taxonomic classification of sequences such as fixed threshold to identify OTUs. This kind of fixed numeric classifications can always only be a vague approximation to the actual structure of relatedness of organisms.”

As concerns the delimitation of taxa on the level of cyanobacterial species, there exists a lot of valuable literature, which was not cited. The characterization and separation of species was not satisfactory solved, but it is particularly important (criteria are not unique for all genera) and need better discussion.

Response: our aim was not to start from known and named sequences and test whether species from DNA taxonomy matched them or not; our aim was to test whether a barcoding gap existed at all. To do so, we did not download named sequences from GenBank, but used a carefully annotated and checked database with a built-in phylogeny that would allow us to obtain monophyletic clades, regardless of their names. We thus only briefly discuss names and taxonomy in our analysis. Given that both reviewers commented on this issue, we tried to clarify our aims and included a sentence at the end of the introduction, in order to clarify the point. We hope that now both reviewers, and all other potential readers, will not be misled by our approach. We do not deal with taxonomy at all, only with testing the presence of a barcoding gap.

In Line 40 is cited only DeQueiroz (2007) to the problematic of species concept. It would be useful to evaluate and mention many other authors, who discussed this question more complexly. It concerns also several other problems, e.g., problematics of horizontal gene transfers (Line 43), “boundaries between taxon units” (Line 45), species concept (Line 48), *etc.* About species concept in cyanobacteria exist particularly many studies from last years.

Response: we now include new citations in relationship to different problems: Butlin et al. (2009), Cohan (2011), Cohan (2013), Cohan & Aracena (2012), Diekmann et al. (2004), Nosil (2012), Vos (2011), Wiedenbeck & Cohan (2011).

I am not sure that cyanobacteria can be accepted as a typical example of all prokaryotes. On the contrary, they have very special position (phylogenetic, metabolic, function in nature, ultrastructural, they grow often in multicellular and differentiated thallus) and their diversity must be evaluated respecting these specificities. It is clear that the taxonomic classification must be different in different groups of organisms.

Response: We agree with the reviewer's concern. In fact, this is exactly the reason why we chose Cyanobacteria for this analysis. We reformulated this part of the introduction, to make this statement more clear, which now reads: "We choose Cyanobacteria as an example of prokaryotes, not because they are representative for all prokaryotes, but because there is ample phenotypic, ecological, physiological ultrastructural, and biochemical evidence of the existence of independently evolving units in this group [20,21]. Thus, the expectation is that, if a barcoding gap exists in prokaryotes, this should be more easily seen in taxa where groups can be identified also with other methods, as in Cyanobacteria."

The correct citation of scientific names is the obvious request of serious scientific publications. Few unnecessary mistakes are in the manuscript, e.g., in the whole text and also in Figures is written "*Arthospira*" instead the correct *Arthrospira*, on other places must be *Leptolyngbya* (Table 1, Line 191), *Fischerella* (Line 207), *Aphanizomenon* (Line 218), *Cylindrospermopsis* (Line 219).

Response: We apologize for our carelessness in double-checking the naming of the organisms and hope that we now eliminated all errors.

I do not understand why *Leptolyngbya* and *Chamaesiphon* could be "closely related genera". It is in contraversion to the whole up to date results and taxonomic classification. The authors really found the "close" relation just between these two genera? By way, they both are polyphyletic and will be surely divided in several different taxa after the following precise studies.

Response: We agree with the reviewer, this part was formulated clumsily. What we meant was actually exactly what the reviewer means. The two groups were polyphyletic and we could only chose a monophyletic group when analysing them together. We changed the sentence to: "... by two genera that were monophyletic only when taken together in the database used, e.g., Leptolyngbya and Chamaesiphon ..." In accordance we did not check for relatedness of the taxa, neither in this one nor for any other one.

Summary: The manuscript is surely useful and, in principle, it presents a good contribution to the recent methodological trends (polyphasic approach) in cyanobacterial taxonomy, this conclusion should be the main result from this work. More citations of modern literature would be expectable and useful.

Round 1: Reviewer 2 Report and Author Response

The authors tested novel approach to identification of species in cyanobacteria based on 16S rRNA based on identification of barcoding gaps previously used in animals. It is overall well-written and innovative work, but there are few things to consider in methods and results, which should be revised, and some formal flaws.

When you refer to the *Synechococcus*, it is important to specify, which clade you have in mind. Honda *et al.* (1999) and Roberston *et al.* (2001) showed *Synechococcus* is polyphyletic genus composed of a least 5 lineages. There are more recent works showing even more clades. From an amount of sequences and relationship to *Prochlorococcus*, I can assume, it is a marine pelagial picoplanktic clade. However, it should be specified for unexperienced reader in the field of cyanobacteria. The other polyphyletic genera should be also specified—*Phormidium*, *Leptolyngbya*, *Microcoleus* and others. Moreover, although the sequences might have same name (*i.e.*, *Synechococcus*), they belong to polyphyletic groups, thus they probably belong to different genera, which have not yet been described.

Response: We agree that this might be confusing to the reader. We have therefore clarified this choice and added this part to the first M&M section and the literature suggested has been cited: “Sequences termed Synechococcus on the other hand were analysed together with sequences of Prochlorococcus, since Synechococcus is known to be a polyphyletic group and if all sequences (including a minimum of five lineages [36–38]) are taken together, Prochlorococcus has to be included too.”

They might also appear to be monophyletic, because there are missing taxa between them.

Response: The sequences we used were clustering monophyletically a-priori in the tree provided in the database used, and we chose them independently of the name. We now clarified this in the first section of M&M: “Thereby a group was considered monophyletic if it was monophyletic in the tree provided with the database, regardless of the taxonomy and the nomenclature of the organisms included in the clade (Supplementary Figure 1), and only secondarily if there was a correspondence with known taxa.”

This might also cause false positive barcoding gaps, because there would be low similarity among sequences with same name. Therefore it may largely affect results.

Response: As mentioned above, we did not only choose sequences that had the same name, but groups of sequences that were monophyletic in the database tree. Thus we should not have artificially introduced a barcoding gap by sequence selection.

I suggest that the paper should be expanded with a discussion, whether barcode gaps are able to delimit described species. For example, are there two species within *Cylindrospermopsis*? There seems to be a barcoding gap within this clade. In this particular case, it would be beneficial to use a species epithet too to avoid confusion. Is it *Cylindrospermopsis raciborskii*? Does a barcoding gap in the *Planktothrix* clade correspond to the described species?

*Response: Similarly to the replies for the previous reviewer, our aim was not to start from known and named sequences and test whether species from DNA taxonomy matched them or not; our aim was to test whether a barcoding gaps existed at all. To do so, we did not download named sequences from GenBank, but used a carefully annotated and checked database with a built-in phylogeny that would allow us to obtain monophyletic clades, regardless of their names. We thus only briefly discuss names and taxonomy in our analysis. Given that both reviewers commented on this issue, we tried to clarify our aims and included a sentence at the end of the introduction, in order to clarify the point. We hope that now both reviewers, and all other potential readers, will not be misled by our approach. We do not deal with taxonomy at all, only with testing the presence of a barcoding gap. Given this rationale and the kind of data we used, no clear statement towards the two barcoding groups within *Cylindrospermopsis* can be made. Nearly all sequences within the database are termed *Cylindrospermopsis raciborskii* by the people who deposited the sequences. However the clade does not contain the type-strain (which was not in the database). So we would rather not speculate too much on the taxonomic naming of these and other barcoded groups. However, we can clearly say that what is deposited in database SILVA 111 as *Cylindrospermopsis* contains two species with a barcoding gap. For *Planktothrix* we added a sentence: “Considering the naming of *Planktothrix* sequences in the database, some OTUs and ABGD units seem to correspond not only to monophyletic groups but also to named species such as *P. mougeotii* or similarly in the case of *Fischerella muscicola* (Figure 3).”*

However we wish to remain careful on these kinds of statements since we are not sure if the underlying naming of the species used is correct or not.

Why did you use Phormidium as an outgroup (Line 139)? Would not be more beneficial to use Gloeobacter or some other bacterium?

Response: We understand why the reviewer would think that that would be beneficial. However, one has to keep in mind that we were not constructing a phylogenetic tree for all cyanobacteria but for single genera. In this case it is preferential to use a sister group that is more closely related to gain resolution within that group. e.g., Hedtke, S.M.; Townsend, T.M.; Hillis, D.M. Resolution of phylogenetic conflict in large data sets by increased taxon sampling. Syst. Biol. 2006, 55, 522–529. Moreover, for the sake of the barcoding analyses, the choice of the outgroup will not affect the results, given that it will not change the relationships towards the tips of the tree.

Is there any way to use barcoding gaps in a taxonomy of cyanobacteria? This might be added to discussion.

Response: The last paragraph of the discussion already included some comments on this issue. Now the paragraph has been expanded to provide a more detailed discussion on the possibility of using a DNA barcoding gap in cyanobacteria.

Minor point: There are errors in nomenclature throughout the text, figures, and supplements, e.g. Leptolyngbya, Aphanizomenon, Arthrospira. You might check: <http://www.cyanodb.cz/>. It is an updated database of names of cyanobacteria.

Response: We apologize for our carelessness in double checking the naming of the organisms and hope that we now eliminated all errors.

Second Round of Evaluation

Round 2: Reviewer 1 Report

I have had the more critical remarks to the manuscript; however, I think that the presented data are useful and should be published. My critics should inform authors about more complicated problematics of this whole group, and several principles should be respected and accepted for future cyanobacterial research. I believe that the article with proposed changes will be useful for future research.

Round 2: Reviewer 2 Report

I accept the paper in the present form. All comments are answered satisfactorily.

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