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Elucidating the *Cronbergia* (cyanobacteria) dilemma with the description of *Cronbergia amazonensis* sp. nov. isolated from Solimões river (Amazonia, Brazil)

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ABSTRACT

The settlement of the genus *Cronbergia* as novel taxa has been frequently questioned among cyanobacterial taxonomists. Its validity is uncertain mainly because of the high similarity (97.4%) found between the 16S rRNA gene sequences of the type-species *Cronbergia siamensis* SAG 11.82 and *Cylindrospermum stagnale* PCC7417, despite their substantial morphological differences. In the present study, one filamentous heterocytous cyanobacterial strain isolated from the Solimões river (Brazilian Amazonia) was studied in detail by means of morphology and molecular phylogeny as well by ITS (internal transcribed spacer) secondary structures. According to the results, this novel strain showed all the morphological features recognized for the *Cronbergia* genus and its 16S rRNA gene sequence clustered with *C. siamensis* SAG 11.82, leading to the description of *C. amazonensis* as a novel species. However, this cluster did not group to the 16S rRNA gene sequence used in the description of the genus *Cronbergia*. Additionally, this study demonstrated the existence of multiple 16S rRNA gene sequences retrieved from the type-species, *Cronbergia siamensis* SAG 11.82, which fall into two distinct phylogenetic lineages. Herein, we suggest considering the *C. amazonensis*-cluster as the typical *Cronbergia* lineage. Meanwhile, the sequence originally adopted to describe the genus *Cronbergia* should be designated as a member of the genus *Cylindrospermum*. This study also emphasizes that both morphologic and genetic data must be generated with caution in order to avoid more misunderstandings in cyanobacterial systematics.

1. Introduction

The genus *Cronbergia* [1] is a controversial taxon within classification systems of Cyanobacteria. It was erected after a polyphasic re-evaluation of *Anabaena siamensis* and was described under the provisions of the International Code of Nomenclature for Algae, Fungi and Plants (ICN) [1] without correspondence in the International Code of Nomenclature of Prokaryotes (ICNP).

Cronbergia is a nitrogen-fixing and free-living genus with only three described species (*C. siamensis*, *C. paucicellularis* and *C. planctonica*) [1]. Ecologically, this group is found in aquatic and terrestrial ecosystems, such as moist soil and paddy fields in Bangkok (Thailand), metaphyton from pools and backwaters in Slovakia and in plankton from brackish water in Sweden [1].

Morphologically, this genus is characterized by uniseriate, isopolar, metameric, short and non-branched trichomes with the ability to differentiate akinetes and heterocytes. Akinetes are apo- and para-heterocytically formed, while heterocytes are found at both terminal positions of the trichomes. The terminal heterocytes are formed after rupture of two intercalary and unipored heterocytes, which originated from a symmetrical division of a single, elongated intercalary vegetative cell. This characteristic is the diacritical feature to separate *Cronbergia* morphotypes from *Anabaenopsis* (terminal heterocytes developed from the asymmetrical division of two intercalary vegetative cells). Furthermore, the typical terminal heterocyte always formed from a terminal vegetative cell in *Cylindrospermum*, and the symbiotic association found in *Richelia* distinguished their morphotypes from *Cronbergia* ones [1]. Taking into account these morphological divergences, Komárek et al. [1] reclassified *Anabaena siamensis* into the new genus *Cronbergia*, and defined *C. siamensis* as the type-species type and *C. siamensis* SAG 11.82 as the reference strain. Although these authors detected high levels of 16S rRNA similarity (97.4%) between *C. siamensis* SAG 11.82 and *Cylindrospermum* sequences, they justified the erection of the genus *Cronbergia* based only on those morphological differences. More recently, Jonhansen et al. [2] demonstrated that the 16S rRNA gene sequence of *C. siamensis* SAG 11.82 was strongly

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clustered with the sequence of the type-species of the genus *Cylindrospermum* (*C. stagnale*), revealing their close genetic relatedness. These authors concluded that *Cronbergia* should not have been described solely on morphologic characteristics and, therefore, they transferred *C. siamensis* SAG 11.82 to the genus *Cylindrospermum*, as *Cylindrospermum siamensis* SAG 11.82. Although doubts have emerged about this genus as presented, Komárek et al. [3] considered it a valid and phylogenetically supported generic entity in their most recent taxonomic classification system.

Given that other *Cronbergia* species do not have 16S rRNA gene sequences available to test the validity of *Cronbergia* as a stable genus, the phylogenetic position of this cyanobacterial genus remains questionable. In this study, we demonstrated that one heterocytous strain, isolated from the Solimões river (Brazilian Amazonia), morphologically resembling the genus *Cronbergia* does not cluster with the type-species *C. siamensis* SAG 11.82 (*sensu* Komárek et al. [1] and *sensu* Jonhansen et al. [2] corresponding to *Cylindrospermum siamensis* SAG 11.82) in the phylogenetic reconstruction. This study will shed some light on the misconception regarding this problematic genus, as has been observed for many other cyanobacterial genera. To confirm this statement, the specific objectives were (i) to morphologically characterize the isolated strain, (ii) to investigate its phylogenetic relationship using the 16S rRNA gene sequence, and (iii) to compare its ITS (internal transcribed spacer) sequence with other published sequences for deeper phylogenetic resolution.

2. Materials and methods

2.1. Cyanobacterial isolation and morphological evaluation

Environmental water samples collected in the Solimões River (03°14′ 34.4″ S and 60° 00′ 28.6″ W, Amazonas State, Brazil) were used for cyanobacterial isolation. Four litres of surface water were collected and successively filtered using membrane filters with three distinct pore sizes (8–0.8; 0.8–0.6 and 0.22 μm). The membranes were kept chilled during transportation to the Environmental Microbiology laboratory (EMBRAPA) in Jaguariúna, São Paulo State.

Under aseptic conditions, the membrane filters were sliced, followed inoculation in 250-mL Erlenmeyer flasks containing 50 mL of liquid BG-11_0 medium (BG-11 without a nitrogen source) [4], containing cycloheximide (70 mg·L^{-1}) to inhibit eukaryotic cell growth [5]. After detecting cyanobacterial growth on the submerged membrane filters, the colonies were spread on the agar BG-11_0 medium (1.2% w/v). Monospecific cultures were obtained by successive streaking on fresh solid media followed by optical microscope observations. In all of the isolation steps, the cultures were grown in a 14:10 h light:dark cycle under white fluorescent irradiance (40 μmol photon·$\mu m^{-2}·s^{-1}$) at 24 ± 1 °C. Morphological identification of the isolated cyanobacteria strain was made using a Zeiss Axioplan2 optical light microscope equipped with an AxioCamMR3digital imaging system (Carl Zeiss, Jena, Germany) and the classification system devised by Komárek [6], Komárek et al. [3] and recently published literature.

The isolated cyanobacteria (CMAA1598) was cultured and maintained in 125-mL Erlenmeyer flasks filled with 50 mL of BG-11_0 medium. The cyanobacterial strain was also cryopreserved at − 80 °C freezer with glycerol (20% v/v), methanol (5% v/v) and DMSO (5% v/v) as cryoprotective agents at the Culture Collection of Agriculture and Environmental Importance (CMAA) of Brazilian Agriculture Research Corporation (EMBRAPA), Jaguariúna, SP, Brazil. In addition a herbarium preparation of cultured material was deposited at the Herbarium of Institute of Botany in São Paulo, SP, Brazil.

2.2. DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from the cultured cyanobacterial cells using the Ultraclean® Microbial DNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA, USA), following the manufacturer's instructions. The 16S rRNA gene plus the 16S-23S ITS was PCR-amplified in a Veriti thermocycler (Applied Biosystems, Life Technologies, Singapore) using the primer set 27F1 [7] and 23S30R [8] (*E. coli* position 27 of the 16S rRNA gene until position 30 of the 23S rRNA gene). The PCR amplifications, cloning and sequencing were performed as described by Genuário et al. [9]. The sequenced fragments were assembled into one contig using the software Phred/Phrap/Consed (Philip Green, Univ. of Washington, Seattle, USA), and only bases with a quality > 20 were considered [10,11,12].

2.3. Phylogenetic analyses and ITS folding

The nucleotide sequences obtained in this study and the reference sequences retrieved from GenBank were aligned using *ClustalW*, trimmed (matrix with a 1499-base-pair length) and used to generate phylogenetic trees. The 16S rRNA gene phylogenetic trees were inferred using maximum likelihood (ML) and Bayesian (B) methods. The ML tree was reconstructed using the MEGA programme package, version 5 [13], applying Kimura's two-parameter model of sequence evolution with gamma-distributed evolutionary rates and with an estimated proportion of invariable sites (K2 + G + I). The robustness of the phylogenetic tree was estimated *via* bootstrap analysis using 1000 replications. The Bayesian inference was conducted using MrBayes 3.2 [14] applying two separate runs with four chains each and 50,000,000 Markov Chain Monte Carlo generations. The tree was viewed in FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree). The general time-reversible evolutionary model of substitution with gamma-distributed evolutionary rates and with an estimated proportion of invariable sites (GTR + G + I) was selected as the best according to MrModeltest version 2.3 [15]. Given that the ML and Bayesian methods resulted in nearly identical topologies, only the ML tree is presented, with assignment of bootstrap values and Bayesian probabilities for branch support. To provide a taxonomic resolution at the species level, the 16S-23S ITS region of the studied sequence was used for secondary structure folding. The complete 16S-23S ITS sequence was aligned and the regions (D1-D1′, D2, Box-B, Box-A, D4 and V3) were found using LocARNA-Alignment & Folding [16,17,18] taking into account its closest relative sequences. The tRNA genes were found using the tRNAscan-SE Search Server (1.21) [19]. The secondary structures (D1-D1, Box-B and V3) were folded using the Mfold WebServer with the default conditions, except for the application of the structure draw mode with an untangle loop fix [20].

The 16S rRNA and the 16S-23S ITS obtained from the studied strain were deposited in the NCBI GenBank database under the accession number MF002129.

2.4. PCR screening for microcystin

Three microcystin synthetase genes (*mcyD*, *mcyE* and *mcyG*) were investigated in the isolated strain. The PCR amplifications were performed as described by Genuário et al. [9].

3. Results

3.1. Isolation and morphological evaluation

One filamentous heterocytous strain was isolated from water samples collected in the Solimões River. In culture, macroscopic benthic mats were observed at the beginning of cultivation, followed by the formation of a fine green layer on the surface of the liquid culture medium. Microscopic analyses revealed straight and coiled trichomes (without sheath) that were solitary or enclosed by diffluent mucilage. Trichomes are uniseriate, isopolar, short (34 cells, 120 μm long) and non-branched with both intercalary (rare) and terminal (very frequent) heterocytes. Terminal heterocytes were observed at both ends after a