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Cyanocohniella calida gen. et sp. nov. (Cyanobacteria: Aphanizomenonaceae) a new cyanobacterium from the thermal springs from Karlovy Vary, Czech Republic

JAN KAŠTOVSKÝ [1]*, ESTHER BERRENDERO GOMEZ [1], JINDŘICH HLADIL[2] & JEFFREY R. JOHANSEN [1,3]
[1] *Department of Botany, Faculty of Science, University of South Bohemia, 31 Branišovská, 370 05 České Budějovice, Czech Republic.*
[2] *Institute of Geology AS CR, v. v. i., Laboratory of Geological Processes, Rozvojová 269, 165 00 Prague 6, Czech Republic.*
[3] *Department of Biology, John Carroll University, University Heights, OH 44118 USA.*
* *Corresponding author (hanys@prf.jcu.cz)*

Abstract

The thermal springs of Karlovy Vary (Carlsbad) is the *locus classicus* of the well-known thermal cyanobacterium *Mastigocladus laminosus*. In addition to the nominate variety, several other varieties and forms were described based on differences in morphology (true-branching *versus* non-branching) or ecology (thermal *versus* non-thermal). The cyanobacterial strain Kaštovský 1996/2, which was provisionally identified as *M. laminosus* f. *nostocoides*, was also isolated from this locality and discussed in previous work. Based on both morphological and molecular (SSU) analyses, this strain was found to not belong to *Mastigocladus*, but rather to an undescribed genus, presumably within the Nostocaceae. This strain was subsequently lost, and absence of type materials prevented the description of the genus. The species was successfully re-isolated in 2012. The new strain is identical in morphology, life cycle, and 16S rRNA sequence to the lost strain 1996/2. It is herein described as *Cyanocohniella calida gen. et sp. nov.* The genus differs from all other Nostocaceae and Aphanizomenonaceae by the unique combination of these characteristics: 1) thermal-tolerant ecology, 2) life cycle that includes Pseudanabaenaceae-like, *Nostoc*-like and *Chlorogloeopsis*-like stages, 3) absence of aerotopes, and 4) phylogenetic placement in the Aphanizomenonaceae. The sister taxa, based on 16S rRNA gene sequence phylogenetic analysis, are *Cyanospira* and *Anabaenopsis*, both spiral, planktonic, aerotope-bearing, tropical genera and species clearly distinct from *C. calida.*

Key words: Cyanobacteria, new genus, polyphasic approach, thermal springs, Karlovy Vary

Introduction

Cyanobacteria are one of most ecologically important groups of microorganisms in the history of the Earth. Our knowledge about cyanobacterial diversity is still poor, although, with the widespread implementation of the polyphasic approach to taxonomy in the last decade, the scientific community has discovered many new genera and species of cyanobacteria. Many newly described genera are tropical (e.g. *Brasilonema* Fiore *et al*. (2007: 794), *Cyanoaggregatum* Werner *et al*. (2008: 493), *Geminocystis* Korelusová *et al*. (2009: 933), *Cronbergia* Komárek *et al*. (2010: 329), *Ophiothrix* Sant'Anna *et al*. (2010: 218), *Streptostemon*, Sant'Anna *et al*. (2010: 220), *Oxynema* Chatchawan *et al*. (2012: 50), *Calochaete* Hauer *et al*. (2013: 38), *Chakia* Komárková *et al*. (2013: 228), *Limnoraphis* Komárek *et al*. (2013: 45), but Europe or North America have also been sources of genera new to science (e.g. *Spirirestis* Flechtner *et* Johansen in Flechtner *et al*. (2002: 6), *Rexia* Casamatta *et al*. (2006: 23), *Mojavia* Řeháková *et* Johansen in Řeháková *et al*. (2007: 488), *Coleofasciculatus* Siegesmund *et al*. (2008: 1575), *Nodosilinea* Perkerson *et al*. (2011: 1404), *Oculatella* Zammit *et al*. (2012: 351), *Anathece* Komárek *et al*. (2011: 321)). However, genera and species are being described from other climates, continents, and habitats and the present is certainly a time of biodiversity discovery in the cyanobacteria that has not been paralleled in the past 60 years.

One of the more complicated questions in cyanobacterial taxonomy is the taxonomy and phylogenetic placement of *Mastigocladus laminosus* (Schwabe 1837: 124) Cohn (1863: 42) ex Kirchner (1898: 81). Ferdinand Cohn described the genus and species from thermal water in the main spring of Karlovy Vary. This is the same locality from which *Fischerella thermalis* Gomont (1895: 52), the type species of that genus, was described, and these two important genera likely share a single population of one species as a common type (Kaštovský & Johansen 2008). Furthermore,

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the genus *Hapalosiphon* Nägeli ex Bornet & Flahault (1887: 53) is taxonomically confused with both genera, a subject dealt with in some detail in Kaštovský & Johansen (2008). During the past century, *M. laminosus* was found to occur all over the world in thermal springs of similar chemical and physical composition, most often in temperatures of 30–55 °C.

During the last century, the species concept of *M. laminosus* was significantly expanded to include forms both ecologically and morphologically distinct. Non-thermal varieties were proposed (e.g. *M. laminosus* var. *indicus* Desicachary (1959: 581)) as well as thermal, but non-branching forms such as *M. laminosus* var. *phormidioides* (Petersen 1923: 300) Copeland (1936: 92) and *H. laminosus* f. *nostocoides* Frémy (1936: 186). This broader morphological variety was summarized by Anagnostidis (1961).

Kaštovský & Johansen (2008) examined some of the taxa in this expanded concept of the genus from cultured material (including two isolated from the type locality in Karlovy Vary) using 16S rRNA sequence data to determine phylogenetic relationships among the taxa. They found that all thermal strains of true-branching cyanobacteria form a well-supported clade that includes the population presently inhabiting the type location. The non-thermal strains (including *M. laminosus* var. *indicus*) belong to a separate clade that contains non-thermal species of *Fischerella* (Bornet & Flahault 1887: 64, 66) Gomont (1895: 52). The non-branching thermal taxon was found to be distantly placed from all true-branching taxa in the Nostocaceae. They were able to correct one taxonomic problem by forming the new combination *Fischerella indica* (Desikachary 1959: 581) Kaštovský & Johansen (2008: 309), but the non-branching culture had been lost, and it could not be described as a new taxon without type material or reference strain. After repeated efforts to find and isolate the taxon again, a new strain was isolated by Jindřich Hladil in 2012 from Karlovy Vary, and subsequent morphological and molecular work on this taxon confirmed its conspecificity with the lost culture. We here report expanded analysis of the morphology and phylogeny of this new genus, which will hereafter be referred to as *Cyanocohniella calida gen. et sp. nov.*

Materials and methods

Origin and morphological characterization of strains:—The two strains used in this study have a diffrent history. Strain Kaštovský 1996/2 was isolated from the hot water spring (temperature 47 °C, Na-HCO_3-SO_4-Cl type of mineral water, 6.4 g L^{-1} dissolved matter) in Karlovy Vary, *locus classicus* of *Mastigocladus laminosus* (Kaštovský & Komárek 2001) in 1996. This strain (originally determined as *M. laminosus* var. *nostocoides*) was studied in some detail previously (Kaštovský & Johansen 2008). In 2012, it was in the context of another experiment collected in 1 m^3 of thermal water from an underground pipeline, 0.5 km SSE from the original locality of strain Kaštovský 1996/2. This water was then deliberately exposed to ambient atmosphere for 1 hour at a temperature of about 55 °C. Subsequently it was closed in a sterile plastic tank and was exposed in the laboratory to a daily rhythm of light intensity. During the 105 days of the experiment (up until 19 September 2012) about 100 g of wet cyanobacterial biomass formed in the tank. When examined, this cyanobacterium was morphologically very similar to the original Kaštovský 1996/2 strain, and was immediately isolated for subsequent research. For isolation and cultivation of unialgal strains of cyanobacteria, we used agar-solidified BG-11 medium (Stanier *et al*. 1971) and BG11-N. Cultures were maintained in a growth chamber with 16:8 h light:dark cycle at 17–19 °C. To investigate strain morphology throughout the cyanobacterial life cycle, observations of cultures were made each ten days for three months using an Olympus BX 51 microscope with Nomarski DIC optics equipped with a digital camera (Olympus DP71). Measurements of filament, trichome, cell sizes were taken, and characteristic properties were noted. Images were composited into a photographic plate using CorelDRAW® X6.

Molecular characterization:—Genomic DNA was extracted from approximately 100 mg of fresh biomass using the Invisorb ™ Spin Plant Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) according to the manufacturer's instructions. For amplification of the 16S rRNA gene (bp 325-1487) and associated 16S–23S internal transcribed spacer (ITS) region, the following primers were used: primer 1 (5'-CTC TGT GTG CCT AGG TAT CC-3') after Wilmotte *et al*. (1993) and primer 2 (5'-GGG GAA TTT TCC GCA ATG GG-3') after Nübel *et al*. (1997), as previously described in detail by Boyer *et al*. (2001). PCR amplification was performed with a Thermal XP Cycler model TC-XP-D (BIOER Technology, Hangzhou, P.R. China) as follows: an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of DNA denaturation at 94 °C for 1 min, primer annealing at 55 °C for 45 s, strand extension at 72 °C for 2 min, and a final extension step at 72 °C for 10 min. DNA bands were examined on a 1.5% agarose gel. The target amplified

DNA fragment was purified by electrophoresis in a 1.5% low melting point (LMP) agarose gel. Ligation reaction was done right in the LMP gel using the Promega Easy-Vector Cloning kit (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Five colonies were chosen at random and PCR amplified using primers T7 and SP6. PCR consisted of initial denaturation at step at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min 45 s, followed by final extension step at 72 °C for 10 min. DNA sequencing was performed by the chain-termination method using Applied Biosystems BigDye™ Terminator Cycle Sequencing Kit v.3.1 (Life Technologies), following the manufacturer's instructions (Life Technologies). The sequences were obtained for both strands independently.

Phylogenetic Analyses:—Nucleotide sequence obtained from DNA sequencing was compared with sequence information available in the National Center for Biotechnology Information database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). For the phylogenetic analysis, 16S rRNA gene sequences of heterocytous cyanobacteria longer than 1200 bp available through GenBank were downloaded into fasta files. The sequences were aligned by MAFFT v. 7.1 (Katoh & Standley 2013; http://mafft.cbrc.jp/alignment/server/) together with sequence obtained in this study and 2 suitable outgroup taxa (*Chrococcidiopsis thermalis* Geitler 1933: 625 (FJ805841) and *Blennothrix* sp. (EU586735)) and the alignments were corrected manually. The alignment was submitted to FindModel (http://www.hiv.lanl.gov) which determined that the generalized time-reversible (GTR) substitution model with a gamma distribution of rate variation was the most appropriate model (Tavare 1986). Phylogenetic calculations were run employing Bayesian inference in MrBayes 3.2.2 (Ronquist *et al.* 2012), Maximum Likelihood analysis (ML) and Maximum Parsimony analysis (MP) in the MEGA 6.06 program (Tamura *et al.* 2013). For the Bayesian analysis, two runs of eight Markov chains were executed for 1 million generations with default parameters, sampling every 100 generations (the final average standard deviation of split frequencies was lower than 0.01), and first 25% of sampled trees were discarded as burn-in. ML tree was conducted using the GTR model and a discrete gamma distribution in six categories (GTR+I+G model). MP tree was generated using a heuristic search constrained by random sequence addition (1000), steepest descent and tree bisection-reconnection (TBR) branch swapping. All bases and base changes were weighted equally, and gaps were coded as missing data. For ML and MP, one thousand bootstrap replicates were performed to evaluate the relative support of branches, and only bootstrap values above 50% were indicated at the nodes of the trees. A distance matrix was also calculated on the alignment file using the Jukes & Cantor (JC69) model by MEGA 6.06 (Tamura *et al.* 2013). To analyze intrageneric variability among our strains, the ITS secondary structures—D1-D1', Box-B, V2, and V3 helices, were estimated using M-fold (Zuker 2003), choosing secondary structure predictions with minimum free energy under default conditions in M-fold. We also estimated secondary structures for related taxa for which the ITS region was available and compared these structures to those in *Cyanocohniella*. The secondary structures together with the 16S tree were re-drawn in Adobe Illustrator CS5.1.

Herbarium specimens and accession numbers:—Herbarium specimens were placed in the Herbarium for Nonvascular Cryptogams at the Department of Botany, Faculty of Science, University of South Bohemia, Czech Republic, with accession numbers CBTS A-023. The strain was deposited in the Culture Collection of Autotrophic Organisms (CCALA) at the Institute of Botany, Academy of Sciences of the Czech Republic, Třeboň, CZ (strain CCALA 1049)**.** Sequences obtained as part of this work were submitted to the GenBank database with number KJ 737427-737428 (CCALA 1049) and EU116036 (strain Kaštovský 1996/2).

Results

Both investigated strains were found to be identical based on ecology, morphology and molecular sequence. Because the combination of diacritical features associated with this species does not correspond with any described genus or species, we here name it as both a new genus and species.

Class **Cyanophyceae**
Subclass **Nostocophycideae**
Order **Nostocales**
Family **Aphanizomenonaceae** (sensu Komárek *et al*. 2014)

Cyanocohniella **Kaštovský, Berrendero, Hladil *et* Johansen, *gen. nov.* (Fig. 1).**

Thallus blue-green to dark green, filaments hypervariable in the course of the life cycle. Motile hormogonia germinating from akinetes, composed of 4–8 cells. Filaments developing from hormogonia with cylindrical cells, not constricted or slightly constricted at cross-walls, without sheath or with fine colorless or slightly yellowish, unlamellated sheath, slightly attenuated towards apices, with apical cells longer than wide, oval to conical, lacking calyptra. Filaments of next phase with marked constrictions at the cell walls, attenuated towards apices, with cells becoming oval to spherical, producing intercalary oval to spherical heterocytes and one- to two-celled apical hormocytes, with apical cells similar in form to common cells of the filament. Filament of mature phase with cells spherical, oval or irregularly shaped, with cells irregularly arranged in ensheathed filaments, sometimes evidencing cell division in two planes, producing partly biseriate filaments. Akinetes single or in series, with cell walls often yellowish. Necridia absent during entire life cycle.

Type species:—*Cyanocohniella calida* Kaštovský, Berrendero, Hladil *et* Johansen*, spec. nov*.

Habitat:—Thermal mineral water and atmophytic localities around thermal springs.

Etymology:—Genus is named in honor of Ferdinand Cohn, phycologist, who described *Mastigocladus laminosus*.

Description:—Population with a polymorphic life cycle. Hormogonia and young filaments with isodiametric to cylindrical cells, not constricted or slightly constricted at cross-walls, (1)1.5(2) µm wide, with intercalary cells (1)1.5–2.5 µm long, with apical cells longer than wide, oval to conical, 1.5–2.5 µm long. Sheath absent or thin, fine, colorless to slightly yellow (Figs. 1A–C). Intercalary cells gradually become constricted at the cross-walls and oval to spherical shape, (2)3.0–4.5(5) µm in diameter. Intercalary heterocytes rarely present, oval or spherical, 5–6(7) µm in diameter. Apical cells remain similar in form to intercalary cells, but may become less wide as filaments become attenuated towards apices (Figs. 1D–H). Mature filaments with cells spherical, oval or irregularly shaped, 2–7(8) µm in diameter, with cells irregularly arranged in ensheathed filaments, sometimes evidencing cell division in two planes, partly biseriate, with heterocytes rarely present, sheath colorless to slightly yellow, usually thin, unlamellated (Figs. 1K–N). Akinetes single or in series, spherical to oval, often with yellowish cell walls, 4–8(10) µm in diameter.

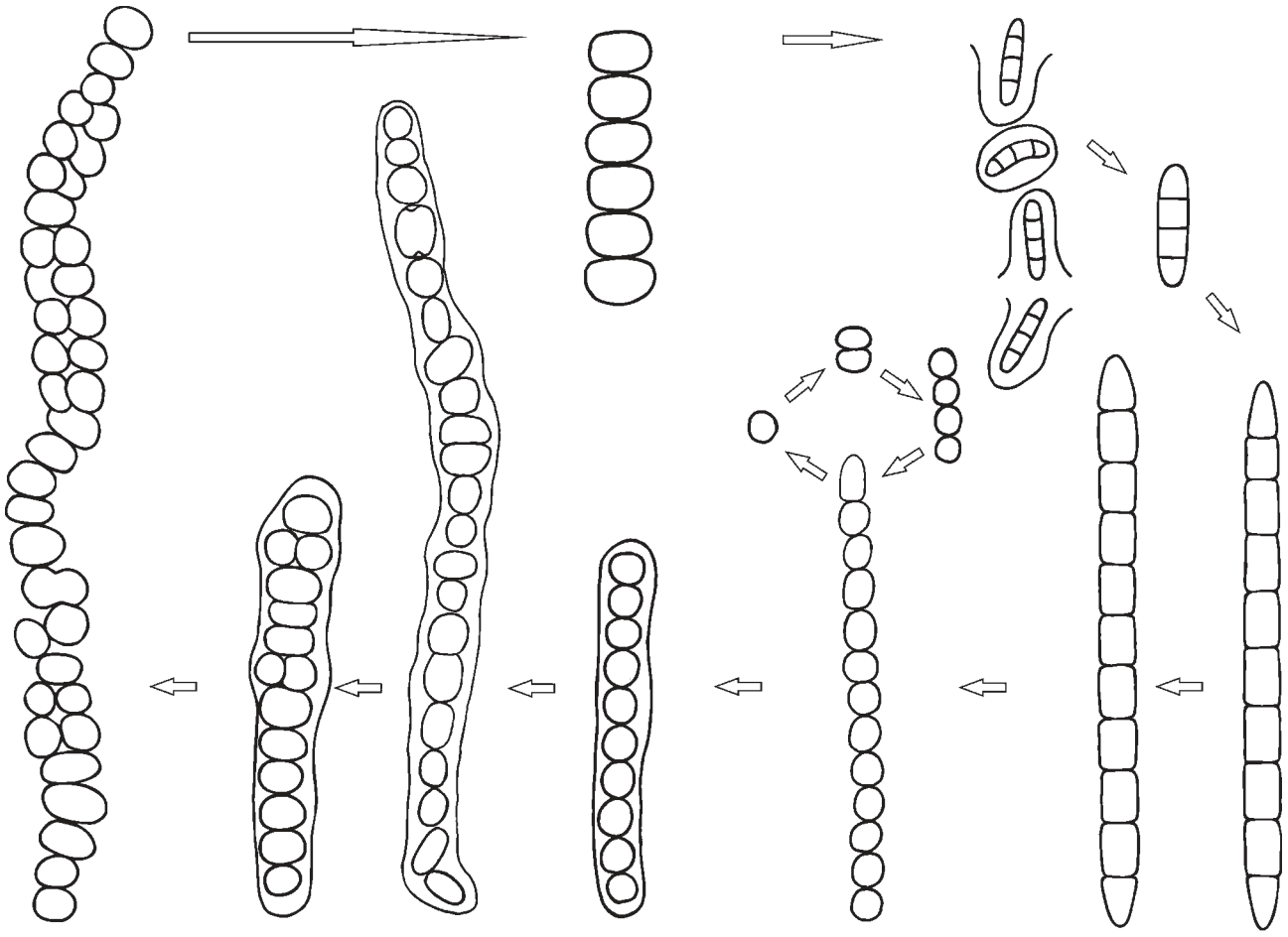

FIGURE 1. Diagram of diagnostic life cycle for *Cyanocohniella gen. nov.*

***Cyanocohniella calida* Kaštovský, Berrendero, Hladil *et* Johansen, *sp. nov.* (Fig. 2).**

Type:—CZECH REPUBLIC. Karlovy Vary, 50°13'06.0"N, 12°53'08.6"E 445 m a.s.l., J. *Hladil, 2012* (holotype CBTS! A-023, Herbarium for Nonvascular Cryptogams at the Department of Botany, Faculty of Science, University of South Bohemia, Czech Republic). Reference Strain: *Cyanocohniella calida* CCALA 1049 (Culture Collection of Autotrophic Organisms at the Institute of Botany, Třeboň, CZ).

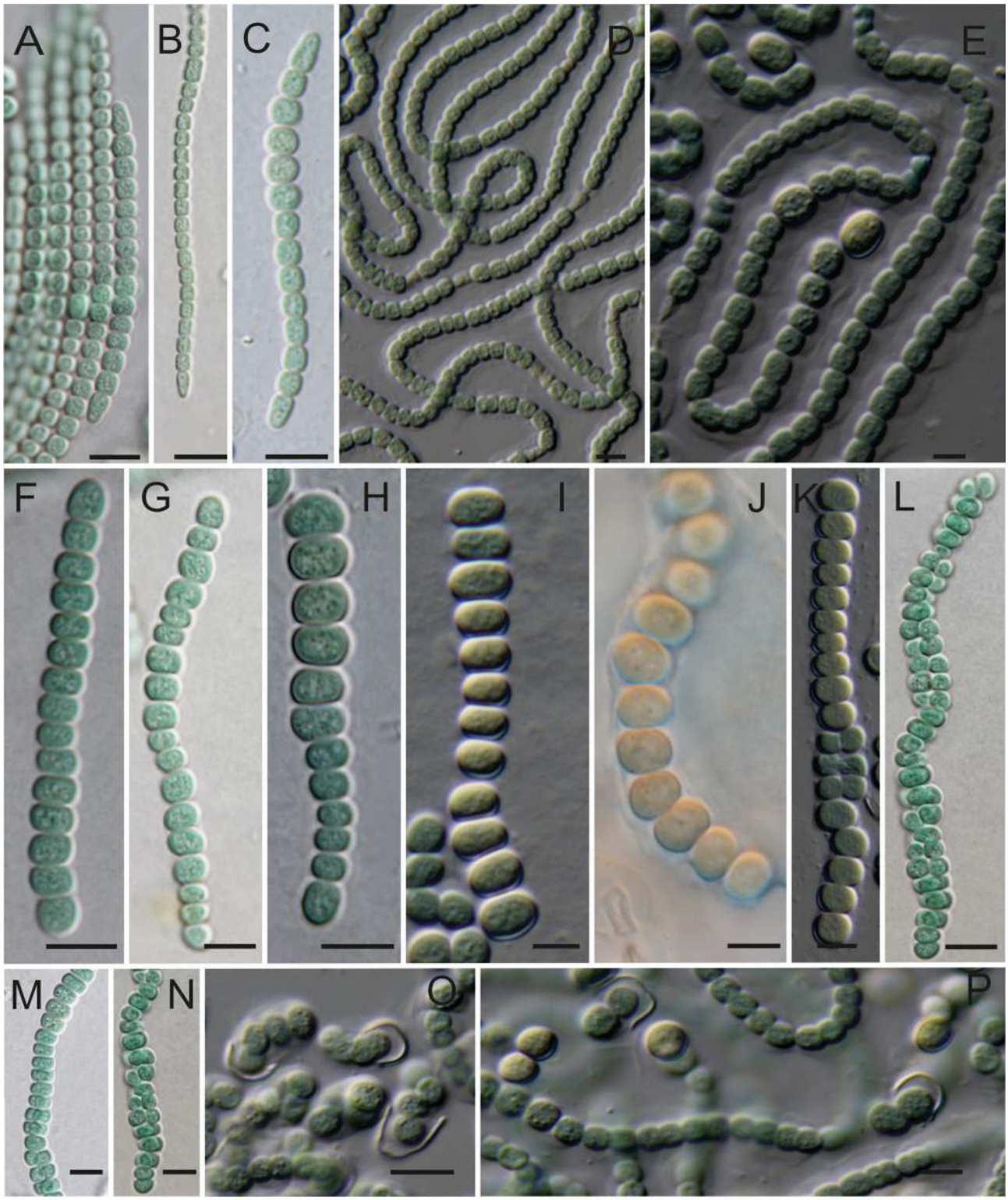

FIGURE 2. Morphological variability of the type strain of *Cyanocohniella calida sp. nov*. A. Hormogonia (*Pseudanabaena*-like stadium). B. Intermediate stage between *Pseudanabaena* and *Nostoc*-like stages. C–G. *Nostoc*-like stage. H. Different sizes of cells in one trichome. I–J. Akinetes. K–N. *Chlorogloeopsis*-like stage, multiple brachning. O, P. Germination of akinetes. Scale = 5 µm

Etymology:—'calida' (L.) = warm or hot, named for its thermotolerance.

Observations:—Without examination of the entire life cycle, the stages of *Cyanocohniella* are similar to several previously described cyanobacterial taxa.Young filaments resemble *Leptolyngbya* Anagnostidis & Komárek (1988: 390) or *Pseudanabaena* Lauterborn (1915: 437). For the majority of its life cycle filaments resemble *Nostoc* Vaucher ex Bornet & Flahault (1888: 181), while the late stage biseriate filaments with irregular cells are similar to *Chlorogloeopsis* Mitra & Pandey (1967: 111). At times, different life cycle stages are evident in single filaments (see Figs. 1C and H). It is possible that *Hapalosiphon laminosus* f. *nostocoides* and *H. laminosus* var. *oscillarioides* Frémy (1936: 182) are conspecific with *C. calida*. However, we list these only as possible synonyms. They are taxa at a different rank than species so have no nomenclatural standing in the naming of this taxon (Art. 11.4 in McNeill *et al*. 2012). They are poorly known taxa from African hot springs, and even if material were available, it would be nearly impossible to say if they were or were not identical to the Karlovy Vary material based on morphology alone. We recommend that these taxa be considered in need of study and revision, and if they are again recovered from African warm springs molecular characterization could determine if they belong to *Cyanocohniella* or some other genus. In natural conditions this species lives in hot water or atmophytically close to the hot spring. The type locality is periodically flooded with cold stream water, and populations must re-establish after these events. It consequently appears that the taxon is thermotolerant rather than obligately stenothermal. In culture, the species does not require elevated temperatures and grows without difficulty at room temperature. We hypothesize that the thermotolerance allows this taxon to escape competition with the Pseudanabaenales and Oscillatoriales that grow in thick mats on the periphery of the springs.

Evidence of phylogenetic separation:—*Cyanocohniella* is very distant from the thermal *Mastigocladus laminosus* strains that have been sequenced (Fig. 3), and in our larger phylogeny still places sister to several genera in the Aphanizomenonaceae. The newly isolated strain CCALA 1049 clusters tightly with strain Kaštovský 1996/2, and both strains are certainly conspecific. In our phylogeny, *Cyanocohniella* was resolved as a sister taxon to the clade containing *Anabaenopsis* Miller (1923: 125) and *Cyanospira* Florenzano *et al*. (1985: 305), with bootstrap support in the maximum likelihood and probability in Bayesian analysis; bootstrap of maximum parsimony is only 60 (Fig. 3). It was also closely related to *Nodularia* Mertens ex Bornet & Flahault (1888: 243) and *Chrysosporum* Zapomělová *et al.* (2012: 359), with similar support. The remainder of the genera in the Aphanizomenonaceae was not resolved with bootstrap support with regards to their relationship with the in-group taxa containing *Cyanocohniella,* or even among other taxa in the Nostocales. The Aphanizomenonaceae is problematic because 16S rRNA sequence similarity exceeds 97.6% for all taxa in the family, which contains very morphologically and biochemically diverse species and genera. Most members of the family are capable of producing aerotopes. However, some benthic taxa (certain *Nodularia* species, *Anabaena* Bory de Saint-Vincent ex Bornet & Flahault (1888: 180, 224) *sensu stricto*, and *Trichormus variabilis* (Kützing ex Bornet & Flahault 1888: 226) Komárek & Anagnostidis (1989: 304) lack aerotopes. *Cyanocohniella* lacks aerotopes, and this is evidence of its evolutionary separation from the other members of its clade which are obligately planktonic (Fig. 3). It is further separated from *Anabaenopsis* and *Cyanospira* by the complete absence of spiraling trichomes. The *Mastigocladus* strains demonstrating true branching were all contained in a clade of Fischerellaceae/Mastigocladaceae/Hapalosiphonaceae distant from the Aphanizomenonaceae. The strains of *M. laminosus* from thermal habitats were grouped in the same clade together with exclusively thermal strains ascribed to *Fischerella*. *Fischerella indica* CALU987 (EU116033) from soil grouped with *Fischerella* sp. CENA 19 (AY039703), a strain also isolated from soil. There was a complete phylogenetic divide between the thermal true-branching strains and the soil-inhabiting true-branching strains. *Cyanocohniella* was very distant from all true-branching taxa. According to the comparison of the 16S rRNA sequence similarities, the two strains of *Cyanocohniella calida* had a similarityof 99.9% (Table 1). *Cyanospira ripkkae* (FR774774), *Anabaenopsis elenkinii* Miller (1923: 125) (AM773308) and *Nodularia harveyana* Thuret ex Bornet et Flahault (1888: 243) (AF268019) were the most similar taxa to *C. calida* (98%, 97.1% and 97.2%, respectively). The *Mastigocladus*/*Fischerella* thermal strains clade had very low similarities of 89.8–90.5% with *C. calida* CCALA 1049. *Fischerella indica* CALU987 and *Fischerella* sp. CENA19 were also more distantly related, with similarity values of 92.0 and 91.2% to *C. calida,* respectively (Table 1).

Secondary structure of the 16S–23S ITS:—Comparison of the secondary structure of the 16S–23S ITS conserved domains provides further evidence of genetic separation from other genera in the Aphanizomenonaceae. The D1–D1' helix of *Cyanocohniella* is diffrent from all D1–D1' helices in related genera in the Nostocales (Fig. 4). The closest relatives (based on 16S rRNA gene phylogeny) for which ITS sequence is available are *Dolichospermum* (Ralfs ex Bornet & Flahault 1888: 228) Wacklin, Hoffmann & Komárek (2009: 60), *Nodularia*, *Chrysosporum*, and *Anabaenopsis* (Figs. 4B–E). These structures are notably different, especially in the basal part of the helix which has two bases (5'-AU-3') on the 5' strand opposite the basal unilateral bulge in contrast to the single cytosine residue in this position in *Cyanocohniella* (Figs. 4A–E). The apices of the members of Aphanizonenonaceae are variable, but all are

different from the apical region of *Cyanocohniella*. The central portion of the helix has fairly high sequence similarity in all these strains, but differs in structure in notable ways (Figs. 4A–E). *Camptylonemopsis* Desikachary (1948: 46), which is much more distant based on 16S rRNA gene phylgeny, actually had the D1–D1' helix most similar

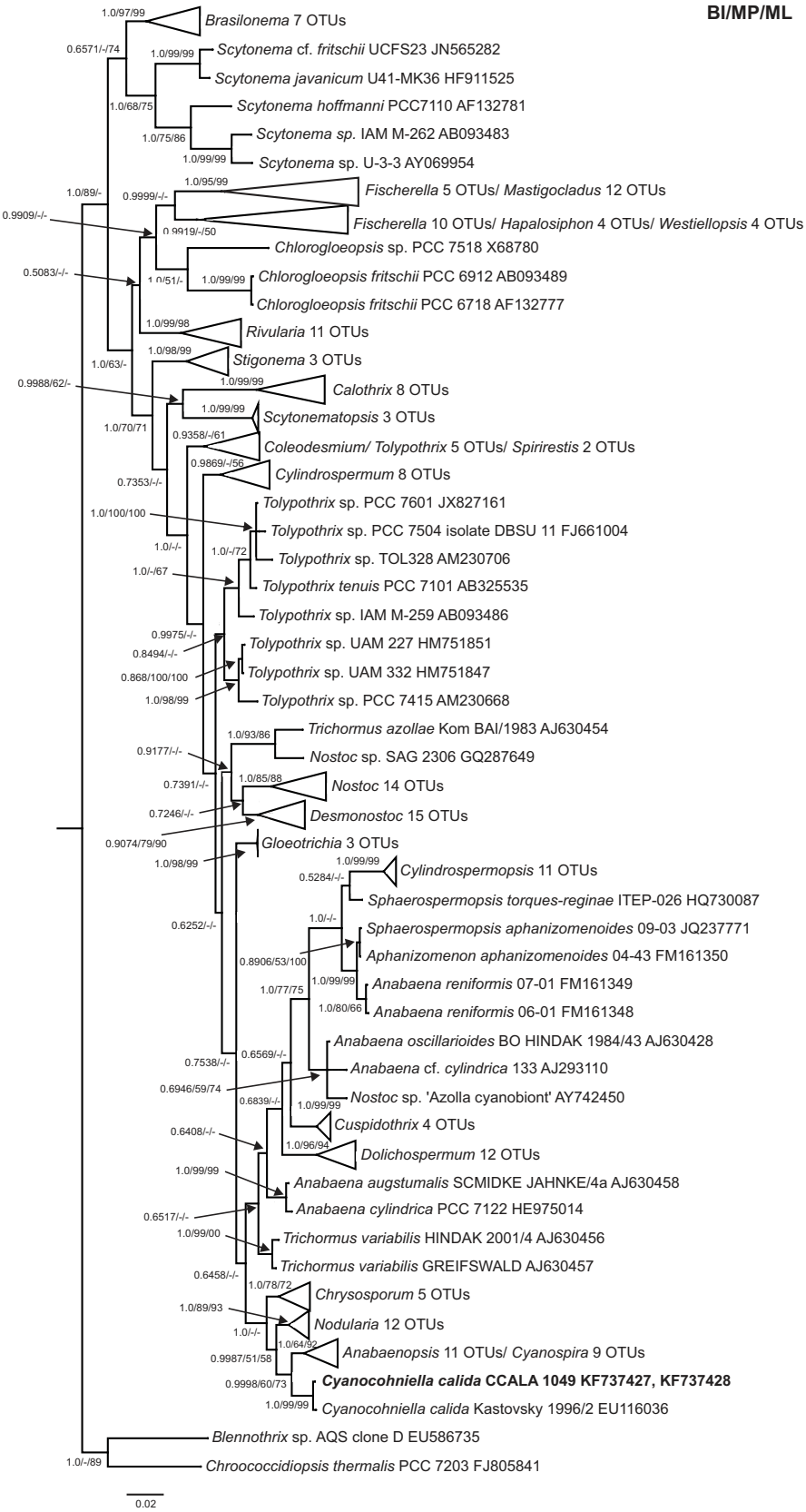

FIGURE 3. Phylogenetic position of the genus *Cyanocohniella* in the order Nostocales based on Bayesian analysis with 16S rRNA gene sequence data. Bootstrap support from Bayesian, maximum parsimony and maximum likelihood analysis reported above nodes respectively. Sequences generated in this study are in bold font.

TABLE 1. Comparison of the 16S rRNA gene sequence similarity among *Cyanocohniella* and its related taxa.

	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*C. calida* CCALA 1049																	
2	*C. calida* Kastovsky 1996/2 EU116036	99.9																
3	*C. rippkae* CR86F7 FR774774	98.0	98.3															
4	*A. elenkinii* NIVA-CYA 494 AM773308	97.1	97.4	98.7														
5	*N. harveryana* PCC 7804 DQ185243	97.2	97.5	97.5	96.7													
6	*Anabaena bergii* AF160256	96.4	96.8	97.1	97.1	97.2												
7	*Nostoc commune* AB721392	94.6	94.4	93.0	92.7	94.9	94.3											
8	*M. laminosus* CALU 987 EU116033	92.0	91.9	91.5	91.1	91.6	90.6	90.8										
9	*M. laminosus* CCAP 1447/3 JX316764	90.4	91.3	92.0	92.2	91.1	91.4	91.7	92.4									
10	*M. laminosus* Ono AB607199	89.8	90.7	91.0	90.88	91.1	91.2	91.5	91.9	97.6								
11	*M. laminosus* Greenland 8 DQ431003	89.9	90.6	91.3	90.7	91.0	91.2	91.6	91.8	96.6	97.9							
12	*M laminosus* SAG 4.84 EU116035	90.1	90.0	90.8	90.0	90.4	90.9	91.9	91.2	96.2	97.2	99.5						
13	*M. laminosus* Kovacik 1987-7B EU116034	90.6	90.4	91.1	90.6	91.2	91.5	92.4	91.4	97.8	98.9	97.4	96.9					
14	*M. laminosus* Oni II AB607195	90.1	90.9	91.2	91.0	91.3	91.4	91.3	92.1	97.8	99.6	98.1	97.4	99.1				
15	*Fischerella* sp. CENA19 AY039703	91.2	92.3	92.2	92.1	92.0	92.3	90.7	94.0	93.7	93.5	92.8	92.1	93.1	93.7			
16	*Fisherella* sp. MV11 DQ786170	90.6	91.1	91.7	91.4	91.6	91.6	92.1	92.1	97.5	98.8	97.8	97.5	99.2	99.0	94.2		
17	*W. prolifica* SAG 23.96 AJ544087	92.9	92.8	92.9	92.6	91.6	92.8	92.2	93.6	94.5	94.6	94.2	93.7	94.9	94.9	94.0	95.3	
18	*H. delicatulus* IAM M-266 AB093484	92.7	93.1	93.7	93.1	93.0	92.8	92.1	94.4	95.2	94.9	94.4	93.9	95.1	94.1	94.6	95.2	98.2

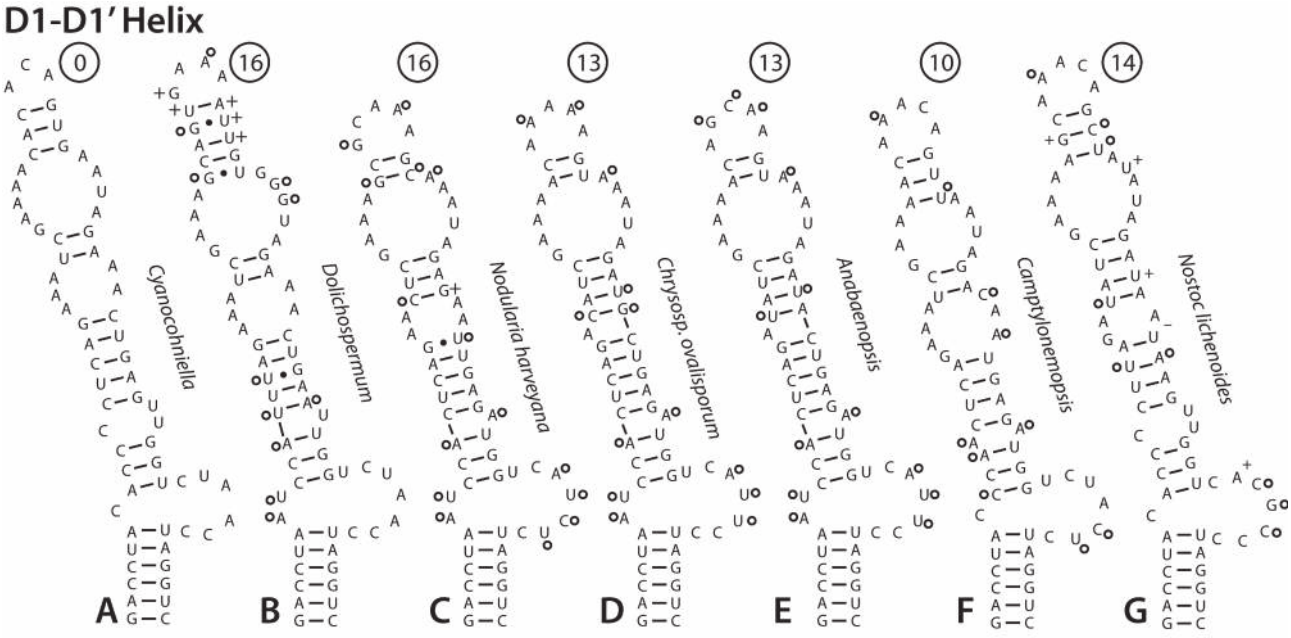

FIGURE 4. D1–D1' helix of the 16S-23S ITS for *Cyanocohniella* and comparison taxa. A. *Cyanocohniella calida* CCALA 1049 (KJ737427). B. *Dolichospermum circinale* 33-10 (EF634474). C. *Nodularia harveyana* Hubel 1983/300 (AF367159). D. *Cylindrospermum ovalisporum* ILC-164 (JF768743). E. *Anabaenopsis* Oleksovice (KF010323), F. *Camptylonemopsis* sp. HA4241-MV5 (JN385292). G. *Nostoc lichenoides* CNP-AK1 (AY579894). Sequences of all comparison taxa are compared to sequence of *Cyanocohniella calida* and minimum number of mutations to achieve the *C. calida* sequence are given in circles above terminus of each structure. Possible substitutions (hollow circles) and insertions (plus signs) are shown at positions in helix where they likely occurred if differences observed are explained parsimoniously through just comparison to *C. calida*.

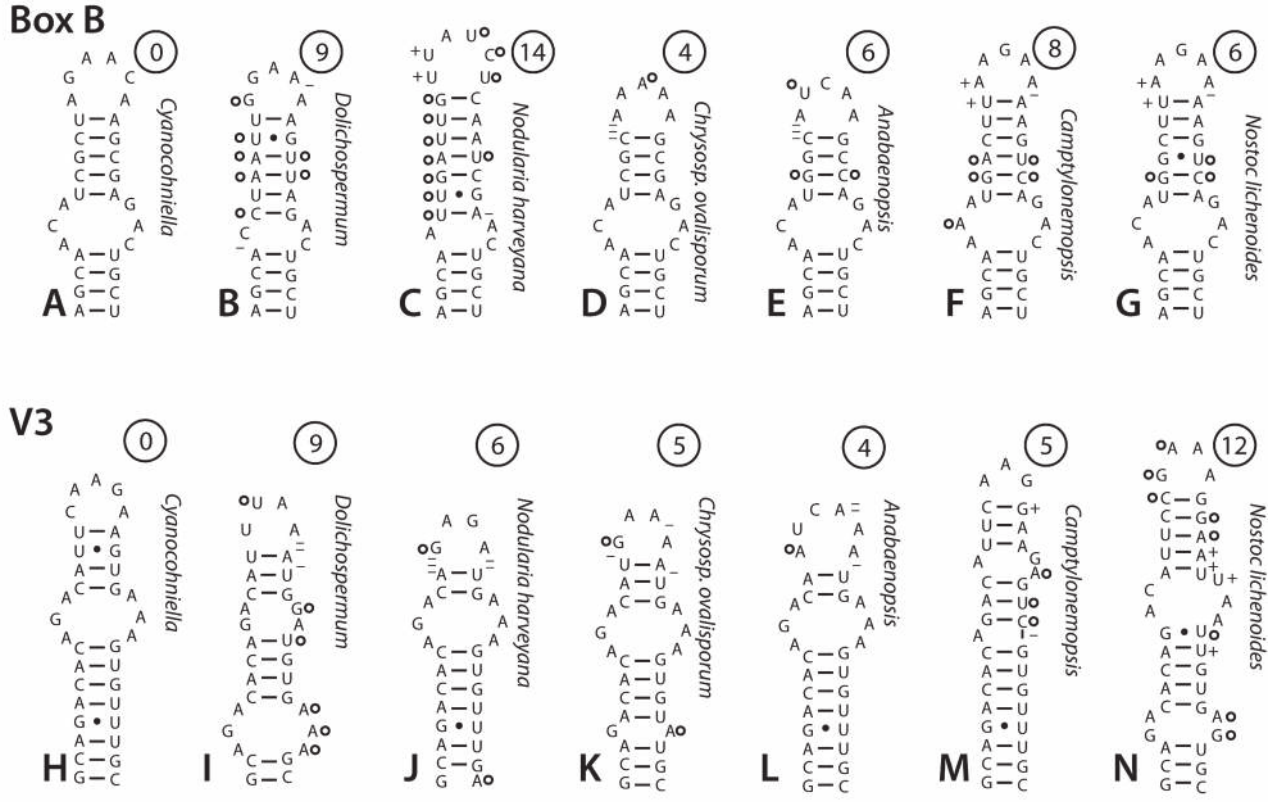

FIGURE 5. Box B and V3 helices of the 16S-23S ITS for *Cyanocohniella* and comparison taxa (full citations to taxa and accession numbers given in legend for Fig. 4). A–G. Box B helix. H–N. V3 helix. Sequences of all comparison taxa are compared to sequence of *C. calida* and minimum number of mutations to achieve the *C. calida* sequence are given in circles above terminus of each structure. Possible substitutions (hollow circles), deletions (minus signs), and insertions (plus signs) are shown at positions in helix where they likely occurred if differences observed are explained parsimoniously through just comparison to *C. calida*.

to *Cyanocohniella* (Fig. 4F). *Nostoc lichenoides* Vaucher ex Řeháková, Johansen, Casamatta, Xuesong & Vincent (2007: 484) is as similar in sequence and structure as most of the Aphanizomenonaceae (Fig. 4G). The most conserved regions in terms of sequence and structure across all taxa compared was the basal stem of 6 bp and the subterminal adenine-rich bilateral bulge (Fig. 4). For ease in comparison of sequence differences with *Cyanocohniella*, minimum numbers of nucleotide substitutions and insertions required to obtain the same sequence as *Cyanocohniella* are given in circles above each helix (Fig. 4). The position of substitutions and insertions shown are hypothetical, and represent the most parsimonius solution. The actual course of sequence change likely required more steps in order to maintain structure in intermediate stages. Furthermore, identical bases in the ITS could be homoplasies, which would also give an impression of fewer steps than actually occurred. Homoplasies have beeen documented in the ITS regions of green algae (Caisová *et al.* 2011) and very likely occur in cyanobacteria as well. The Box B helices available were also all notably different from the Box B helix of *Cyanocohniella*, the only species to have 5 bp in the upper helix (Fig. 5 A–F). *Chrysosporum ovalisporum* (Forti 1911: 3) Zapomělová *et al.* ex Zapomělová *et al*. (2013: 201), *Anabaenopsis*, *Nostoc lichenoides* and *Camptylonemopsis* shared identical basal secondary structure with *Cyanocohniella,* and showed the least separation in number of nucleotide substitutions, insertions, and deletions required to match the sequence of *Cyanocohniella* (Figs. 5D–G). *Nodularia harveyana* was markedly different in sequence and structure (Fig. 5C). The V3 helices of the Aphanizomenonaceae all shared a mid-helix bilateral bulge of identical sequence flanked on either side by highly similar nucleotides, but its position in the helix varied (Figs. 5H–N). *Anabaenopsis, Chrysosporum ovalisporum, Camptylonemopsis,* and *Nodularia harveyana* had minimal sequence divergence from *Cyanocohniella* (Figs. 5J–M). *Nostoc lichenoides,* which is typical of the genus *Nostoc*, was very different in sequence in the V3 helix. The V2 region (not shown) was only available for four strains, as *Nodularia* and *Anabaenopsis* lacked tRNA genes, and *C. ovalisporum* lacks the V2 even though both tRNA genes are present. When all ITS conserved domains are taken into consideration, *Cyanocohniella* is most similar among the Aphanizomenonaceae strain structures to *Chrysosporum ovalisporum* and *Anabaenopsis*. However, *Camptylonemopsis* which clearly places in the Nostocaceae based on 16S rRNA gene phylogeny, was also quite similar. *Cyanocohniella* is unique among all Nostocales for which we have presently examined ITS structures.

Discussion

Cyanocohniella is unusually unstable in its morphology, with a life cycle that is among the most complex observed in all of the Cyanobacteria. The similarity of the various stages to existing genera in diverse subclasses is especially problematic, with *Pseudanabaena*-like stage, *Nostoc*-like stage, and *Chlorogloeopsis*-like stage. This variability in culture makes the taxon especially difficult to identify from environmental samples. It is possible with the definition given here that other workers will discover the genus in other thermal localities, but this will likely occur through phylogenetic analysis of isolated strains. *Mastigocladus laminosus*, which has the same type locality, has been found all over the world (Petersen 1923, Finsinger *et al*. 2007, Miller *et al*. 2007), so there is no reason to doubt that *Cyanocohniella* can be similarly distributed. *Aulosira bohemensis* Lukešová, Johansen, Martin & Casamatta (2009: 121) has a complexity in its morphology as well (akinete production, hormogonia, tapering trichomes, widened trichomes, unbranched, false branched), but apart from the akinete and hormogonia production the variation is not associated with life cycle (Lukešová *et al*. 2009). However, the morphology is sufficiently consistent such that no one would confuse the different stages with genera in other orders or families. *Nostoc*, *Asterocapsa*, and *Gloeocapsopsis* also have life cycles in which certain stages must be seen in order to diagnose the genera or species (Kantz & Bold 1969, Komárek 1993, Komárek & Anagnostidis 1998), but again, they are not so diverse that the forms would go into different orders or families. Most of the Nostocales are nearly constant in their morphology, including *Anabaenopsis*, *Chrysoporum*, *Dolichospermum*, *Nodularia*, *Aphanizomenon*, and *Sphaerospermopsis*, all members of the Aphanizomenonaceae in close relationship with *Cyanocohniella*.

Cyanocohniella bears greatest morphological similarity to members of the Nostocaceae such as *Trichormus* and *Nostoc* (Komárek 2013). This similarity is due to the absence of aerotopes, the smooth-walled and ungranulated nature of the akinetes, and the production of hormogonia. In contrast, it is morphologically distant from the genera of the Aphanizomenonaceae to which it is phylogenetically related. A similar situation exists for *Nodularia*, which contains benthic aerotope-lacking species with ungranulated akinetes. The difference is that *Nodularia* also contains planktonic aerotope-producing species, and these species are mixed with the benthic taxa together in a well-supported clade (Řeháková *et al*. 2014). Benthic *Anabaena* and *Wollea* likewise place with the Aphanizomenonaceae according to the

limited phylogenetic evidence available for these taxa (Rajaniemi *et al*. 2005, Kozhevnikov & Kozhevnikova 2011). In contrast, the members of the Nostocaceae that have been sequenced consistently lack aerotopes. Consequently, the presence of aerotopes in a heterocyte-producing genus likely indicates phylogenetic position within the Aphanizomenonaceae, but the absence of aerotopes is uninformative.

Cyanocohniella calida and *Mastigocladus laminosus* appear to be thermal species, but they are actually eurythermal, not stenothermal. Both taxa, collected from Karlovy Vary, occur at elevated (>55 °C) temperatures, but both also grow well at room temperature. It appears that the thermal tolerance gives them a competitive advantage among taxa that apparently overgrow them in less thermal waters. At Karlovy Vary, the springs are frequently flooded by the river, and small springs cool relatively quickly. Truly stenothermal taxa, such as *Thermosynechococcus* Katoh, Itoh, Shen & Ikeuchi (2001: 599, nom. inval.) are absent from this system of hot springs. The aggregation of all true-branching taxa occurring in warm and hot springs into *M. laminosus* suggests that thermal tolerance has taxonomic significance (Kaštovský & Johansen 2008). Only as more populations of cyanobacteria belonging to *Cyanocohniella* are collected and confirmed through molecular sequencing will it be possible to see if thermal tolerance has taxonomic significance in defining this genus.

Stackebrandt & Goebel (1994) suggested that in prokaryotic taxa, those strains with less than 97.5% 16S rRNA sequence similarity should be considered to be separate species, while those with less than 95% similarity should likely be considered to be separate genera. We have shown in this paper that *Cyanocohniella* can certainly not be congeneric with *Mastigocladus*, and report the low similarity in the 16S rRNA gene (<91%) as partial evidence of this. However, this guideline has been misused to group taxa which have highly similar 16S rRNA into the same species or genus (Otsuka *et al*. 1998, 1999). This only works as a recognition guideline, less than the similarity level indicates evidence of genetic separation roughly at these levels. It cannot be used as a grouping criterion to put strains into the same species or genus based on high similarity. The Nostocales in particular are highly similar in the 16S rRNA gene, and this is likely the reason that it is difficult to get bootstrap support along the backbone of phylogenies of heterocyte-producing cyanobacteria. When similarity is high, as it always is in the Nostocales, there are not enough phylogenetically informative sites to resolve relationships when taxon sampling is high. *Cyanocohniella* has 16S rRNA similarity above 95% for 29 different genera in the Nostocales, including taxa from five different families. If the 97.5% similarity cutoff were used to group taxa with *Cyanocohniella*, it would be the same species as *Anabaenopsis elenkinii*, *Cyanospira rippkae*, Florenzano, Sili, Pelosi & Vincenzini (1985: 305) *Trichormus variabilis*, *Nodularia baltica* Komárek, Hübel, Hübel & Šmarda (1993: 14) and *Nodularia harveyana*. This is clearly nonsensical, and we strongly discourage the further misuse of this guideline to group multiple morphospecies into a single species, or multiple genera into a single genus. Furthermore, as a separation guideline, in the Nostocales it is far more likely that strains less than 99% similar in 16S sequence are separate species, while the cutoff for closely related genera is possibly as high as 98%. However, even at these levels, similarity should not be used to group same taxa. It is always just part of the evidence.

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