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Polyphasic characterization of *Trichocoleus desertorum* sp. nov. (Pseudanabaenales, Cyanobacteria) from desert soils and phylogenetic placement of the genus *Trichocoleus*

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Abstract

Little is known about the taxonomic diversity of cyanobacteria in deserts, despite their important ecological roles in these ecosystems. In this study, cyanobacterial strains from the Atacama, Colorado, and Mojave Deserts were isolated and characterized using molecular, morphological, and ecological information. Phylogenetic placement of these strains was revealed through Bayesian and parsimony-based phylogenetic analyses utilizing sequences of the 16S rRNA gene and the associated 16S–23S ITS region. Based on the combined evidence of this polyphasic approach, a new species from desert soils morphologically corresponding to the genus *Trichocoleus* was described. *Trichocoleus desertorum* sp. nov. Mühlsteinová, Johansen et Pietrasiak was used to obtain a phylogenetic reference point for *Trichocoleus*, a genus so far characterized by morphological description only. Through characterization of this new taxon in desert soils we hope to contribute to the general understanding of cyanobacterial diversity in extreme arid habitats.

Introduction

Arid lands represent biologically challenging but important ecosystems that extend over one third of the earth's land surface (Warren-Rhodes *et al.* 2007). Eukaryotic algae and cyanobacteria have been long recognized as crucial organisms playing a number of essential ecological roles in this harsh environment. Multiple studies have shown beneficial effects of microbiotic soil crusts as well as free living edaphic cyanobacteria, including increased soil stability, water penetration, and nutrient availability (e.g. Belnap & Gardner 1993, Evans & Johansen 1999). However, despite the ecological importance of algae and relatively long history of microbiotic soil crust research (*cf.* Phillipson 1935), we are still far away from a full understanding of cyanobacterial diversity in the soils of arid and semi-arid lands.

Our study took place in three different deserts—the Atacama Desert in Chile and the Colorado and Mojave Deserts in California. Whereas the research of North American deserts has resulted in description of several new cyanobacterial genera—such as *Mojavia* Řeháková & Johansen in Řeháková *et al*. (2007: 488) and *Spirirestis* Flechtner & Johansen in Flechtner *et al*. (2002: 7), our knowledge of algal communities inhabiting the Atacama Desert is still rather poor. Research in this desert has focused mainly on revealing how organisms are able to withstand extreme conditions in soils considered nearly Mars-like (Navarro-González *et al.* 2003), rather than investigating their finer taxonomic diversity (e.g. Wierzchos *et al.* 2006, Azúa-Bustos *et al*. 2011). In this work we hope to contribute not only to a further understanding of cyanobacterial diversity in general, but also to an understanding of the microflora in extreme desert environments.

Trichocoleus Anagnostidis (2001: 369) is a genus of filamentous cyanobacteria with parietal thylakoids, simple binary fission, and no specialized cells, distinguished from similar genera by the presence of multiple trichomes in a common sheath (Komárek & Anagnostidis 2005). This genus was created relatively recently (Anagnostidis

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2001). Anagnostidis separated *Trichocoleus* (Psedanabaenales) from the genus *Microcoleus* Desmazières ex Gomont (1892: 350) (Oscillatoriales)—a cyanobacterium also characterized by the presence of multiple trichomes in a common sheath (Komárek & Anagnostidis 2005), based primarily on the markedly thinner trichomes in *Trichocoleus*. Presently these two genera belong to different orders in two different subclasses (Pseudanabaenales in Synechococcophycidae and Oscillatoriales in Oscillatoriophycidae for *Trichocoleus* and *Microcoleus,* respectively), which were defined primarily by cellular ultrastructure (Hoffmann *et al*. 2005). Some ultrastructural features, such as thylakoid arrangement, are often visible in the light microscope (*cf.* Boyer *et al.* 2002, Casamatta *et al.* 2005, Flechtner *et al.* 2008, Johansen *et al.* 2011), and can consistently be used to place taxa in the correct order and subclass.

Whereas *Microcoleus* has received recent polyphasic study (Boyer *et al*. 2002, Siegesmund *et al*. 2008, Strunecký *et al.* 2013) and its type species, *M. vaginatus* (Vaucher 1803: 200–201) Gomont ex Gomont (1892: 355) has been well characterized based both on morphology and DNA sequence (Garcia-Pichel *et al*. 2001, Komárek & Anagnostidis 2005, Starkenburg *et al*. 2011), *Trichocoleus* has received far less attention. This is likely due to two main reasons. First, *Trichocoleus* was created only recently, and it has not been widely adopted or reported since (Anagnostidis 2001, Komárek & Anagnostidis 2005). Second, *Trichocoleus* lacks precise definition, being identified essentially as "not *Microcoleus*". The type species *T. delicatulus* (West & West 1896: 164) Anagnostidis (2001: 369) was insufficiently described (lacking key details) and the original authors did not illustrate their material (West & West 1896). No species within *Trichocoleus* have been studied using molecular methods with the exception of a strain attributed to the genus (SAG 26.92) which actually belongs in the Phormidiaceae based on 16S rRNA phylogeny (Siegesmund *et al.* 2008, Garcia-Pichel & Wojciechowski 2009). With the increasing application of modern techniques, we can consider nearly any genus or species definition as insufficient and indefinite if lacking molecular data (i.e. 16S rRNA gene sequence).

With poor morphological characterization, no sequence, no herbarium specimen, and no reference culture of the type species available, it is hardly possible to establish the evolutionary position of *Trichocoleus* among other members of the Pseudanabaenales. In this paper we connect strains with known morphology and ecology consistent with the genus to 16S rRNA sequences, and thus create a firm phylogenetic reference point for *Trichocoleus*. We believe that it is necessary to create such reference points for existing taxa as part of the revision of the morphologically simple members of the Pseudanabaenales, which have been shown to have surprising molecular diversity (Bohunická *et al*. 2011, Johansen *et al*. 2011, Perkerson *et al*. 2011, Zammit *et al*. 2012). Without connecting existing taxa to sequenced strains it will be difficult in the future to decide whether newly sequenced taxa represent species or genera new to science, and with the increasing amount of sequences obtained from environmental DNA it will be even more difficult to move algal taxonomy forward without named reference points (*cf.* De Clerck *et al*. 2013).

We characterized 24 strains isolated from the Atacama, Colorado and Mojave Deserts using morphological and molecular approaches, and here describe *Trichocoleus desertorum* Mühlsteinová, Johansen et Pietrasiak. Additionally, based on the evaluation of available molecular data, we transfer *Leptolyngbya badia* Johansen et Lowe in Johansen *et al*. (2008: 26) to the genus *Trichocoleus* as well.

Materials and methods

Origin and morphological characterization of strains:—Strains used in this study were isolated from soils of three different arid regions: Atacama Desert, Mojave Desert, and Colorado Desert. Strains included in our analyses came from 17 different sites (Table 1). Soil samples were acquired by collecting the surface layer of soil including soil crusts where present. Upon returning to the laboratory, samples were either dilution plated on agar-solidified Z-8 medium (Carmichael 1986), or the Moistened Soil Method was applied (Johansen *et al.* 1984) to provide enrichment. In the dilution plating method, 0.5 g of dry soil was mixed with 50 ml of sterile liquid Z-8 medium, the mixture was shaken for 30 min and then 0.1 ml of this solution was plated on Z-8 agar plates. In the Moistened Soil Method three small petri dishes with 5 g of soil were used. Two of these soil samples were moistened with sterile DI water, the third one with sterile liquid Z-8. All strains were isolated into Z-8 medium and transferred on to agar slants after obtaining unialgal cultures. Cultures were maintained in a growth chamber with 16:8 h light:dark cycle at 15–18 °C.

TABLE 1. Sampling site information. Strain codes begin with site number in all isolates in the Johansen Culture Collection.

Site	Desert	GPS Coordinates	Elevation (m a.s.l.)	Collection	Site Desription
ATA1-4	Atacama, Chile	29° 49' 22" S, 71° 17' 28" W	24	11-May-09	Soil at bolder strewn site, near ocean, with cyanobacterial crust, plants.
ATA2-1	Atacama, Chile	29° 18' 59" S, 71° 14' 55" W	215	11-May-09	Fine sand with biological soil crusts, bryophytes, lichens, plants.
ATA3-4Q	Atacama, Chile	27° 50' 49" S, 71° 00' 34" W	190	13-May-09	Hypolithic under quartz rocks, lichens, plants very sparse.
ATA4-8	Atacama, Chile	27° 13' 18" S, 70° 20' 43" W	733	15-May-09	Soft sandy surface soil, well-developed crusts and lichens.
ATA12-5	Atacama, Chile	20° 07' 49" S, 69° 11' 34" W	2653	24-May-09	Fine sandy soil with rocks, algal crusts and very sparse plants.
ATA14-3	Atacama, Chile	18° 51' 55" S, 69° 07' 54" W	914	27-May-11	Sandy, gravelly soil with plants and lichens but no visible crusts, disturbed by animal tracks.
CMT-1BRIN	Mojave, USA	35° 30' 16" N, 115° 41' 55" W	1057	20-Mar-10	Clark Mountains, CA. Sandy soil in rock crevices, well-developed crusts, bryophytes, lichens, algae, plants.
CMT-1FDIN	Mojave, USA	35° 30' 19" N, 115° 42' 00" W	1058	20-Feb-10	Clark Mountains, CA. Sandy soil lacking well-developed crusts, with plants, disturbed by small burrowing mammals.
FB1	Chihuahuan, USA	32° 29' 29" N, 105° 47' 50" W	1462	15-Jul-98	Fort Bliss, NM. Loamy soil in Larrea community, well-developed algal crusts.
JO2	Chihuahuan, USA	32° 30' 56" N, 106° 44' 29" W	1338	17-Jul-98	Jornada Experimental Range, NM. Sandy loam with *Flourensia* and *Scleropogon*, mature algal crusts
CNP1	Colorado Plateau	38° 05' 36" N, 109° 50' 16" W	1646	11-May-99	Canyonlands Nat. Park, UT. Sandy loam in *Stipa* grassland, well-developed algal crusts.
TAA2	Mojave, USA	35º 09' 51" N, 116º 52' 23" W	884	6-Jun-98	Fort Irwin NTC, CA. Loamy sand in wash with *Ephedra* and *Larrea*, well-developed algal crusts
WJT8	Colorado, USA	33° 53' 35" N, 115° 47' 53" W	565	27-May-06	Joshua Tree Nat. Park, CA. Soil in lower alluvial fan with plants, and algal crusts (some darkened).
WJT16	Colorado, USA	33° 59' 45" N, 115° 33' 35" W	430	7-Jun-06	Joshua Tree Nat. Park, CA. Fine sandy to silty soil on desert pavement, without crusts, plants very sparse.
WJT40	Mojave, USA	33° 57' 35" N, 116° 07' 25" W	1355	17-Jul-06	Joshua Tree Nat. Park, CA. Sandy, gravelly soil from granitic pavement material, fragile algal crust.
WJT46	Colorado, USA	33° 49' 08" N, 115° 45' 50" W	796	18-Jul-06	Joshua Tree Nat. Park, CA. Sandy, gravelly soil, plants and well-developed algal crusts.
WJT55	Colorado, USA	33° 42' 56" N, 115° 35' 17" W	531	19-Jul-06	Joshua Tree Nat. Park, CA. Sandy, gravelly soil, plants and well-developed algal crusts.

The morphology of strains was investigated in exponential growth phase (1–3 weeks, depending on strain) and in senescent growth phase (17 weeks–24 months). The appearance of the culture on the agar slant was characterized and microphotographs were taken using a high resolution photomicroscope (Olympus BH-2) with Nomarski DIC optics equipped with a digital camera (Olympus DP25). Properties and dimensions of sheaths, filaments, trichomes, and cells were recorded. Photographic plates showing the main characteristics of the new species were prepared using Adobe Photoshop CS5.1.

Molecular characterization:—Genomic DNA of each strain was isolated using UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.; Carlsbad, CA, USA). A partial region of the 16S rRNA gene and the associated 16S–23S ITS region was amplified using polymerase chain reaction (PCR) with primers 1 and 2 *sensu* Boyer *et al.* (2001, 2002) yielding a DNA product of 1600 bp. The reactions were prepared in a total reaction volume of 25 µl as described in Lukešová *et al*. (2009). PCR was run in a S1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) set to 35 cycles of: 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 2 min 15 s, followed by 5 min extension at 72 °C and 4 °C hold for an indefinite amount of time. Amplified products were visualized and checked on a 1% agarose/ethidium bromide gel, and subsequently cloned into plasmids using the StrataClone PCR Cloning Kit (La Jolla, CA, USA). Plasmids were isolated from transformed cells using QIAprep Spin Kit (QIAGEN,

Carlsbad, CA, USA). The presence of the expected insert was confirmed by plasmid digestion using *Eco*R I. Purified plasmids with a confirmed insert of expected size (2 or 3 per each strain) were sequenced by Functional Biosciences, Inc. (Madison, WI, USA) using plasmid-anchored primers M13 forward, M13 reverse and internal primers 5, 7, and 8 *sensu* Boyer *et al.* (2001, 2002). Partial sequences were aligned into contigs using Sequencher (v. 4.9, Ann Arbor, MI, USA).

Phylogenetic analyses:—To resolve generic identity of our strains, consensus sequences of the 16S rRNA genes (a partial sequence of 1159–1163 nucleotides) from two or three ribosomal operons were manually aligned with other sequences available on GenBank (April 2013) using secondary structure to assist in alignment in regions with single-base insertions and deletions. These sequences were chosen using either Blast Search against our sequences, or by their identification with genera in the order Pseudanabaenales. In total this alignment of the 16S rRNA gene contained 326 sequences including *Gloeobacter violaceus* Rippka, Waterbury & Cohen-Bazire (1974: 435) FR798924 as an outgroup. The nexus file prepared from our alignment was run in PAUP v.4.02b (Swofford 1998) to construct the tree. The tree was found using parsimony as the optimality criterion in a heuristic search in which 'multree's = 'no', 'branch-swapping algorithm' = 'TBR', 'gapmode' = 'newstate', 'steepest descent' = 'yes' and 'nreps' = '1000'. Bootstrap support was based on running 1000 replicates. Bayesian analysis was run using the GTR-Γ model. Two runs of eight Markov chains were executed for 30 million generations with default parameters, sampling every 100 generations (the final average standard deviation of split frequencies was lower than 0.02), and the first 25% of sampled trees were discarded as burn-in. This analysis was conducted on the computer cluster MetaCentrum (www.metacentrum.cz), maintained in the Czech Republic.

To resolve the relationships of our strains within the genus cluster, alignment of the ITS regions containing both tRNA genes was made using a combination of ClustalW (EMBL-EBI 2013, Cambridgeshire, UK) and manual alignment utilizing secondary structure of conserved domains. In total this alignment contained 24 sequences. A heuristic search was run in PAUP utilizing parsimony as the optimality criterion, with 'multrees' = 'yes', 'branch-swapping algorithm' = 'TBR', 'gapmode' = 'newstate', 'steepest descent' = 'no' and 'nreps' = '10000'. Three different analyses were run using different outgroups: *Nodosilinea nodulosa* (Li & Brand 2007: 397–399) Perkerson & Casamatta in Perkerson *et al.* (2011: 1405) UTEX2910 (KF307598), *Leptolyngbya* Zehnder 1965/U140 (HM018692), and no outgroup. Bootstrap support was based on running 10000 replicates.

All trees were viewed using FigTree (Rambaut 2007) and subsequently re-drawn using Adobe Illustrator CS5.1. P-distance of 16S sequences was determined in PAUP to reveal similarity among our strains of interest. The ITS secondary structures of helices D1-D1', Box-B, V2, and V3 were derived using M-fold (Zuker 2003) and re-drawn in Adobe Illustrator CS5.1 for easy comparison. *Leptolyngbya* Zehnder 1965/U140 and *N. nodulosa* were included as outgroups for secondary structure analysis. All sequences available were used to postulate the structural variability among operons with varying numbers of tRNA genes (two or none).

Herbarium specimens and accession numbers:—Herbarium specimens of each strain, as well as fresh biomass preserved in 4% formaldehyde, were prepared by the authors and placed in the Herbarium for Nonvascular Cryptogams, Monte L. Bean Museum, Provo, Utah, USA with accession numbers BRY 37724–40. Cultures of each isolate are kept in Dr. Johansen's algal collection at John Carroll University, Cleveland, Ohio, USA, and selected strains were deposited in the Culture Collection of Autotrophic Organisms (CCALA) at the Institute of Botany, Academy of Sciences of the Czech Republic, Třeboň, CZ (strains CCALA 1032–1036). Sequences were submitted to GenBank database with accession numbers KF307600–620.

Results

Class **Cyanophyceae**
Subclass **Synechococcophycideae**
Order **Pseudanabaenales**
Family **Schizothrichaceae**

Trichocoleus desertorum Mühlsteinová, Johansen et Pietrasiak, *sp. nov.* (Figs. 1–2)

Most similar to *Trichocoleus sociatus* (West & West 1897: 272) Anagnostidis (2001: 369), from which it differs by having slightly attenuated trichomes, involution cells, and more highly variable cell lengths, as well as terrestrial habitat.

Type:—CHILE. Atacama Desert, Copiapó, 27° 13' 18" S, 70° 20' 43" W, 733 m a.s.l., *K. Osorio-Santos, L. Baldarelli, J. Johansen, S. Warren, L. Aguilera, K. Godoy, 15-5-2009* (holotype BRY! 37728, Herbarium for Nonvascular Cryptogams, Provo, Utah, USA. Reference strain ATA4-8-CV2, CCALA 1032, Algal Collection at John Carroll University, Cleveland, USA, and Culture Collection of Autotrophic Organisms at the Institute of Botany, Třeboň, CZ. Isotypes BRY! 37729, 37730, Herbarium for Nonvascular Cryptogams, Provo, Utah, USA. Additional reference strains ATA4-8-CV3, ATA4-8-CV12, CCALA 1033, CCALA 1034, Algal Collection at John Carroll University, Cleveland, USA, and Culture Collection of Autotrophic Organisms at the Institute of Botany, Třeboň, CZ).

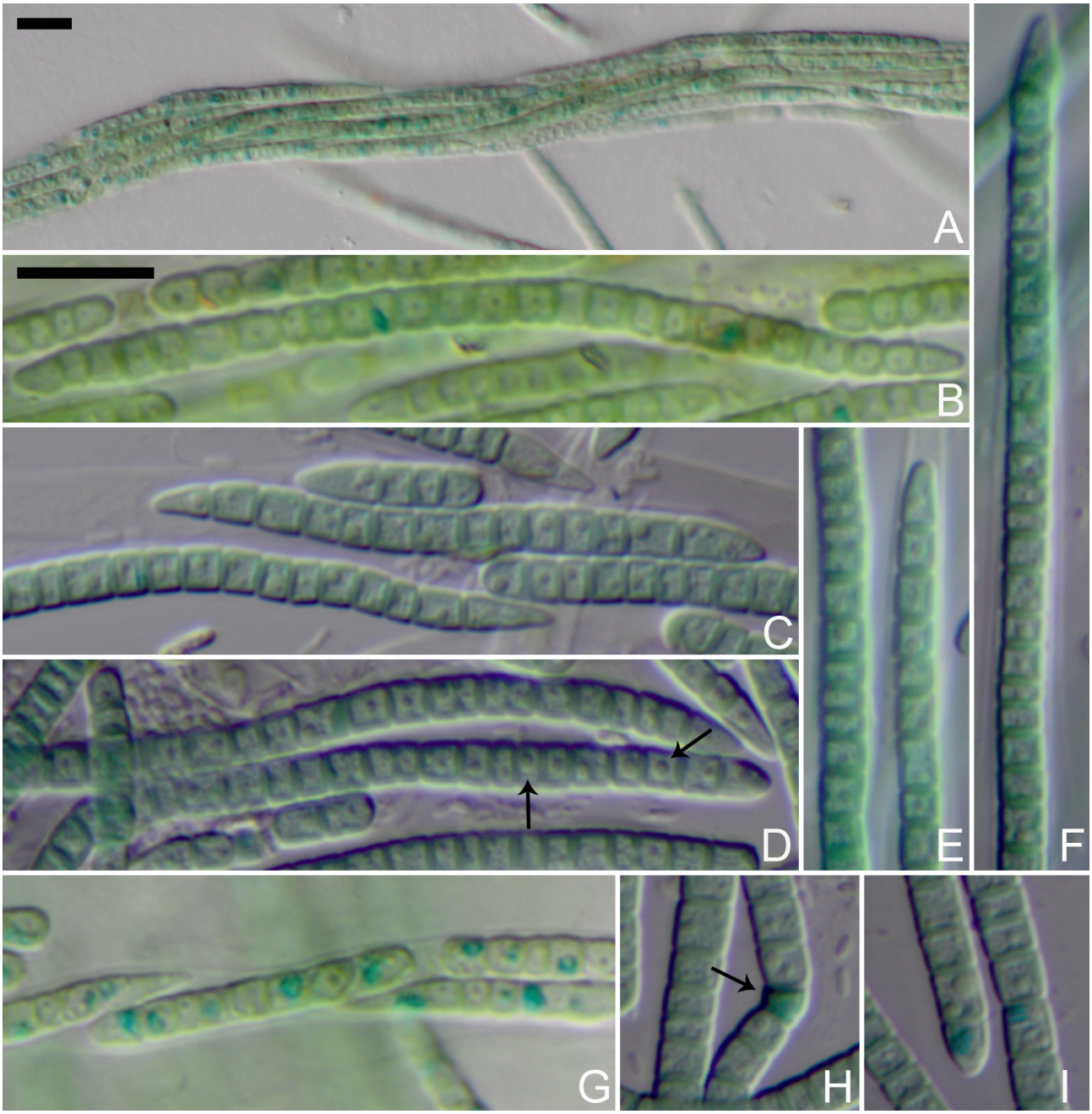

FIGURE 1. Morphological variability of the type population of *T. desertorum* (ATA4-8). A. Multiple trichomes in a sheath. B. Short trichome with conical apical cells. C. Different shapes of apical cells and tapering of trichome. D. Granules in cells (arrows). E–F. Variable cell lengths and sheath properties. G. Short trichomes with pointed apical cells and vivid cyan spots in cells, senescent culture. H. Necridium (arrow). I. Cyan spots in young trichome. Scale = 10 µm (A at lower magnification, B–I share the scale bar from B).

Description:—Thallus consisting of blue-green filaments creeping on agar, sometimes forming dark flat mats. Filaments containing multiple trichomes up to 33 µm wide, when containing single trichomes only 2.3–6.3 µm wide. Sheath colorless, in older cultures sometimes refracting light in DIC to look gray or blackish, lamellated, thick or thin, containing one to several trichomes entangled together. Trichomes motile, mostly pale blue-green or blue-green, rarely slightly yellowish or greenish, occasionally with vivid cyan inclusions in some cells, constricted

at cross-walls, not or slightly attenuated at the ends, rarely attenuated to a narrow point, short (e.g. 19–40 cells) or long, with swollen involution cells sometimes present, 1.5–4.3 (5.3) µm wide. Cells quadratic as well as shorter or much longer than wide, sometimes dividing asymmetrically, occasionally with one to a few small or large granules in cell center, 0.8–14.0 (15.8) µm long. Thylakoids parietal, sometimes (young cultures mostly) forming simple fascicles in the centroplasm. Apical cells longer than wide, conical to sharply pointed, 1.0–3.5 (3.8) µm wide in middle part and 2.0–11.5 (19.5) µm long. Necridia present.

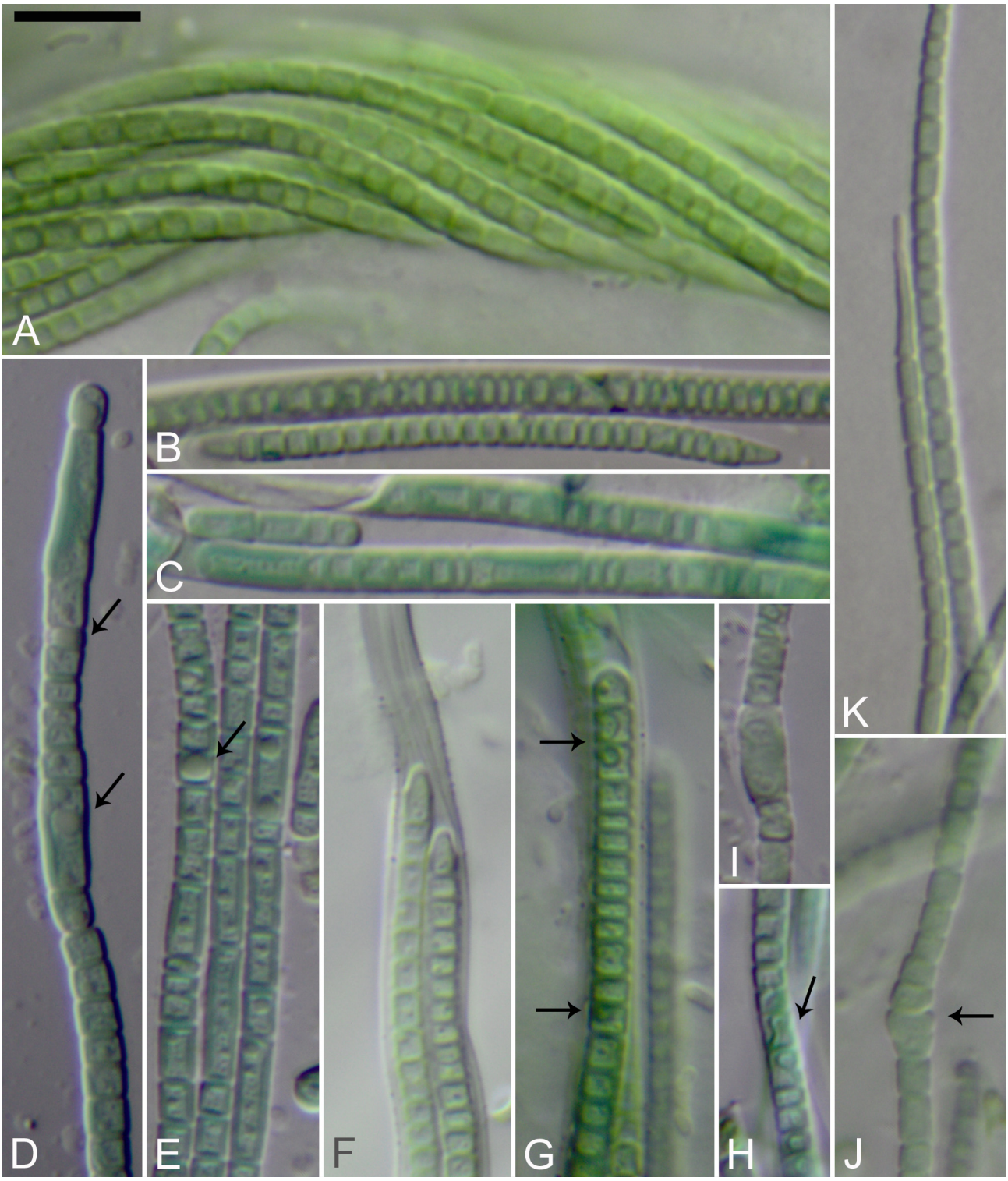

FIGURE 2. Morphological variability of various *T. desertorum* populations. A. Multiple trichomes in a common sheath (ATA3-4Q-KO9). B. Short trichome with short, strongly constricted cells (ATA1-4-KO5). C. Asymmetrical cell division (WJT40-NPBG1). D–E. Asymmetrical cell division and granules (arrows) in cells (CMT-1BRIN-NPC4B). F. Multiple trichomes in a colorless lamellated sheath with greyish light refraction (ATA14-3-RM36). G–H. Thylakoids in fascicles (arrows) (G. ATA14-3-RM36, H. ATA2-1-CV2). I–J. Involution cells (arrow) (I. WJT40-NPBG1, J. WJT55-NPBG8). K. Tapering trichome with pointed apical cell (ATA1-4-CV13). Scale = 10 µm.

Etymology:—*desertorum* = of the deserts.

Habitat:—Desert soils.

Observations:—Our strains comprise a well-supported clade in the Pseudanabaenales separate from other similar taxa, such as *Leptolyngbya* Anagnostidis & Komárek (1988: 390), *Tapinothrix* Sauvageau (1892: CXXIII), *Pseudophormidium* (Hansgirg ex Forti 1907: 493) Anagnostidis & Komárek (1988: 409), *Nodosilinea* Perkerson & Casamatta in Perkerson *et al.* (2011: 1404), and *Oculatella* Zammit, Billi & Albertano (2012: 351) (Fig. 3), and are morphologically distinguished from most of the other Pseudanabaenales by the common occurrence of multiple trichomes in a single sheath (Table 2; Figs. 1A; 2A, F). While sheath characteristics are often variable in culture (Pearson & Kingsbury 1966, Boyer *et al.* 2002), this feature was always evident in healthy cultures of *Trichocoleus*. The character of multiple trichomes in a common sheath is thought to have evolved several times independently in phylogenetically separate lineages (Garcia-Pichel & Wojciechowski 2009).

TABLE 2. Morphological characteristics of investigated *Trichocoleus* strains. ND = not determined. Reference strains used for designation of type materials are in bold font.

Strain Name	Trichomes in Sheath	Necridia	Fascicul. Thylakoids	Involution Cells	Motility	Filament Width [µm]	Trichome Width [µm]	Cell Length [µm]	Length of apical cells [µm]	Width of apical cells[µm]
ATA1-4-CV13	1	-	-	-	-	2.5–3.5	1.5–2.3	1.5–4.3	2.5–6.3 (11.5)	(1) 1.3–1.8
ATA1-4-KO5	1	+	+	-	+	2.5–5.0	1.8–3.3	0.8–8.8	4.0–10	1.0–2.3
ATA2-1-CV2	1	+	+	-	+	3.0–6.3	2.3–3.3	1.3–6.3	3.3–6.5	1.5–2.5
ATA3-4Q-KO9	several	-	-	-	+	3.3–4.0; up to 33	1.8–3.0	1.8–4.0	3.8–6.5	1.5–3.0
ATA4-8-CV2	several	+	-	-	+	4.0–5.8; up to 17	2.5–3.8	1.5–5.5	3.5–9.3	1.3–2.8
ATA4-8-CV3	1	-	-	-	-	4.5	2.5–3.5	1.5–3.5	4.5–7.3	1.5–2.3
ATA4-8-CV12	1	-	-	-	-	3.8–4.0	2.8–3.5	1.5–3.5 (6.3)	3.5–6.8 (8.3)	1.5–2.5
ATA12-5-KO5	several	+	+	+	-	up to 23	2.8–4.3 (5.3)	1.8–7.3	2.0–6.5	1.8–3.5
ATA14-3-RM35	1	+	+	-	+	3.8–6.3	2.3–4.0	1.8–7.5	3.5–7.3	1.8–2.5
ATA14-3-RM36	several	+	-	-	-	4.3–12.8	2.8–4.0	1.5–3.8	3.0–5.0 (5.8)	1.8–2.5
CMT-1BRIN-NPC4B	1	+	+	+	+	±	2.0–4.0	1.3–11.3 (15.8)	2.3–6.8	1.5–2.8
CMT-1FDIN-NPC12A	1	-	+	+	+	3.0–3.5	2.3–4.3	1.5–7.5	3.8–6.8	1.8–2.3
WJT16-NPBG1*	1	+	-	-	-	3.0–3.8	2.3–3.3	1.8–3.8	2.3–4.3	1.5–2.3
WJT40-NPBG1	1	+	+	+	+	3.0–3.8	2.3–3.8	1.3–14	2.5–8.8 (19.5)	1.5–2.8
WJT40-NPBG2	1	-	-	-	+	2.3–3.0	1.8–2.8	1.3–3.3	2.5–4.3	1.3–1.8
WJT46-NPBG1	1	-	-	-	-	2.5–3.3	1.8–3.0	1.5–4.0	2.5–6.5	1.3–2.3
WJT55-NPBG8	1	-	-	+	-	±	2.5–3.0 (3.8)	1.5–4.0	2.8–6.3	1.5–2.3
Trichocoleus badius	1–2	-	-	-	-	up to 2.5	1.0–1.3	2	ND	ND

* not in ITS tree; ** Johansen *et al.* 2008

The most similar taxon is *T. sociatus*. Multiple trichomes in a sheath, conical to pointed-end cells, and constriction of trichomes are features shared by *T. sociatus* and *T. desertorum*. However, attenuation of trichomes (attenuation over several cells, not just the apical cell), presence of involution cells and highly variable lengths of cells are characteristics unique for *T. desertorum* (Komárek & Anagnostidis 2005). We consider these differences, together with the fact that *T. sociatus* was originally described from a freshwater habitat in Africa (West & West 1897), to be too significant to be shared by a single species. We have consequently described *T. desertorum* as a new species from desert soil habitats.

Trichocoleus desertorum cultures investigated in this study demonstrated marked variability both among and within strains (Table 2). However, morphology and molecular data were not congruent and did not yield clear criteria for recognizing strains or groups of strains as separate species. It is likely that with further study, particularly additional sequence data and phylogenetic analysis, more species will be described in this genus in the future. In anticipation of this possibility, the morphology of the reference strains is shown independently (Fig. 1) from the rest of the investigated cultures (Fig. 2).

Trichocoleus badius (Johansen & Lowe) Mühlsteinová, Johansen et Pietrasiak, *comb. nov.*
Basionym: *Leptolyngbya badia* Johansen & Lowe in Johansen *et al.* (2008: 26; figs. 1D, 2C–D, 4A–B, E–F, H–K).

Phylogenetic analyses, 16S rRNA:—Analyses of the 16S rRNA sequences showed clear separation of the genus *Trichocoleus* from the other 298 taxa (Fig. 3). The node for the *Trichocoleus* clade received the highest possible support from both Bayesian (1.00) and parsimony analyses (100). The tree obtained with Bayesian analyses contained more supported nodes than the parsimony analysis, and is consequently the tree reported here. All *Trichocoleus* strains were isolated and sequenced at John Carroll University, and with the exception of *T. badius*, all were isolated from desert soils. Some strains reported in GenBank as *Leptolyngbya* have since been lost, and consequently they are simply listed as *Trichocoleus* sp. (Fig. 4). The eight basal OTUs (including *T. badius*) have long branches, and it is likely that these represent strains that do not belong in *T. desertorum* (Fig. 4).

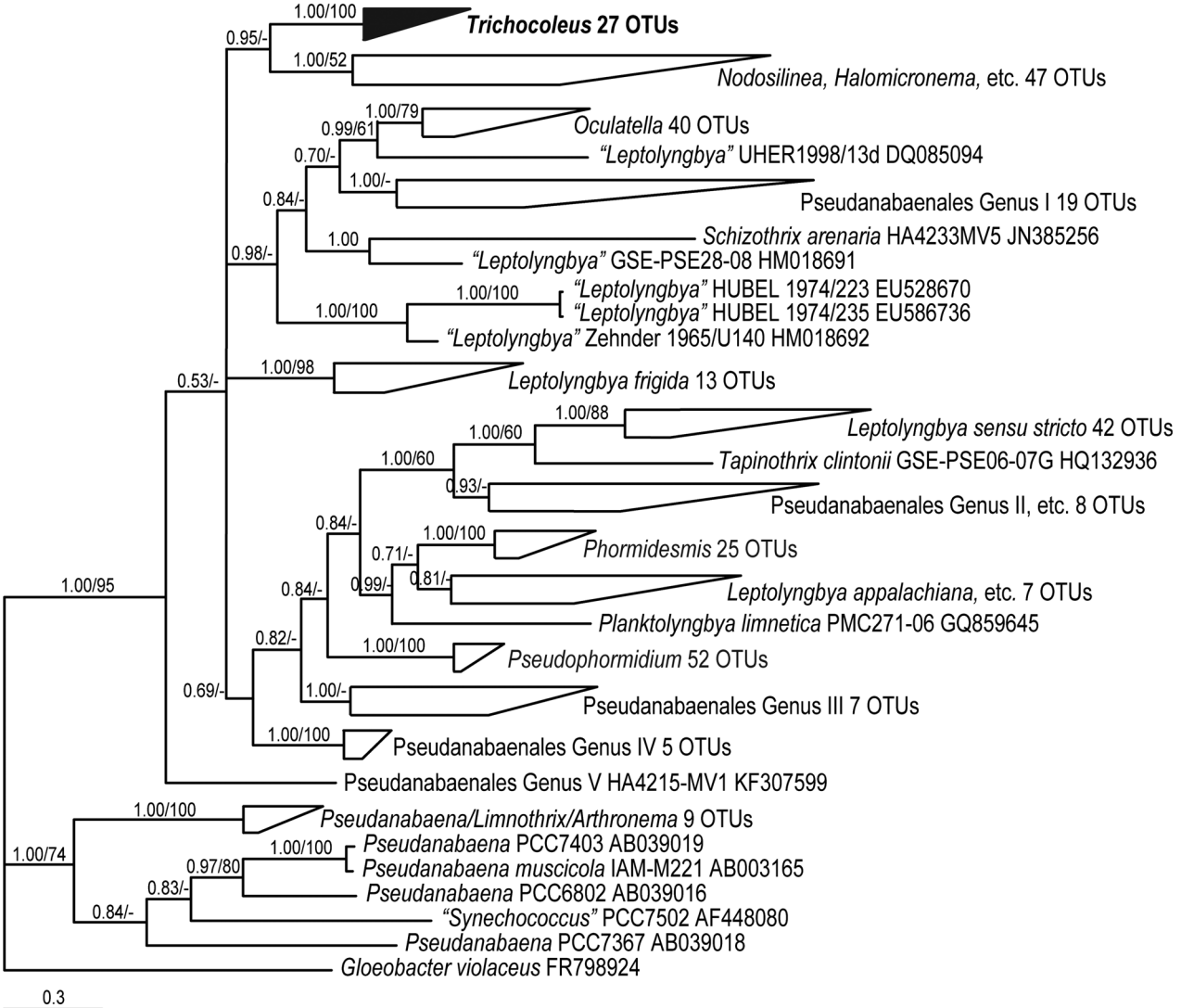

FIGURE 3. Phylogenetic position of the genus *Trichocoleus* within the Pseudanabaenales based on Bayesian analysis with 16S rRNA gene sequence data. Posterior probabilities/bootstrap support from parsimony analysis reported above node. Taxa in quotation marks are outside of correct genus clade but reflect unrevised names given in GenBank.

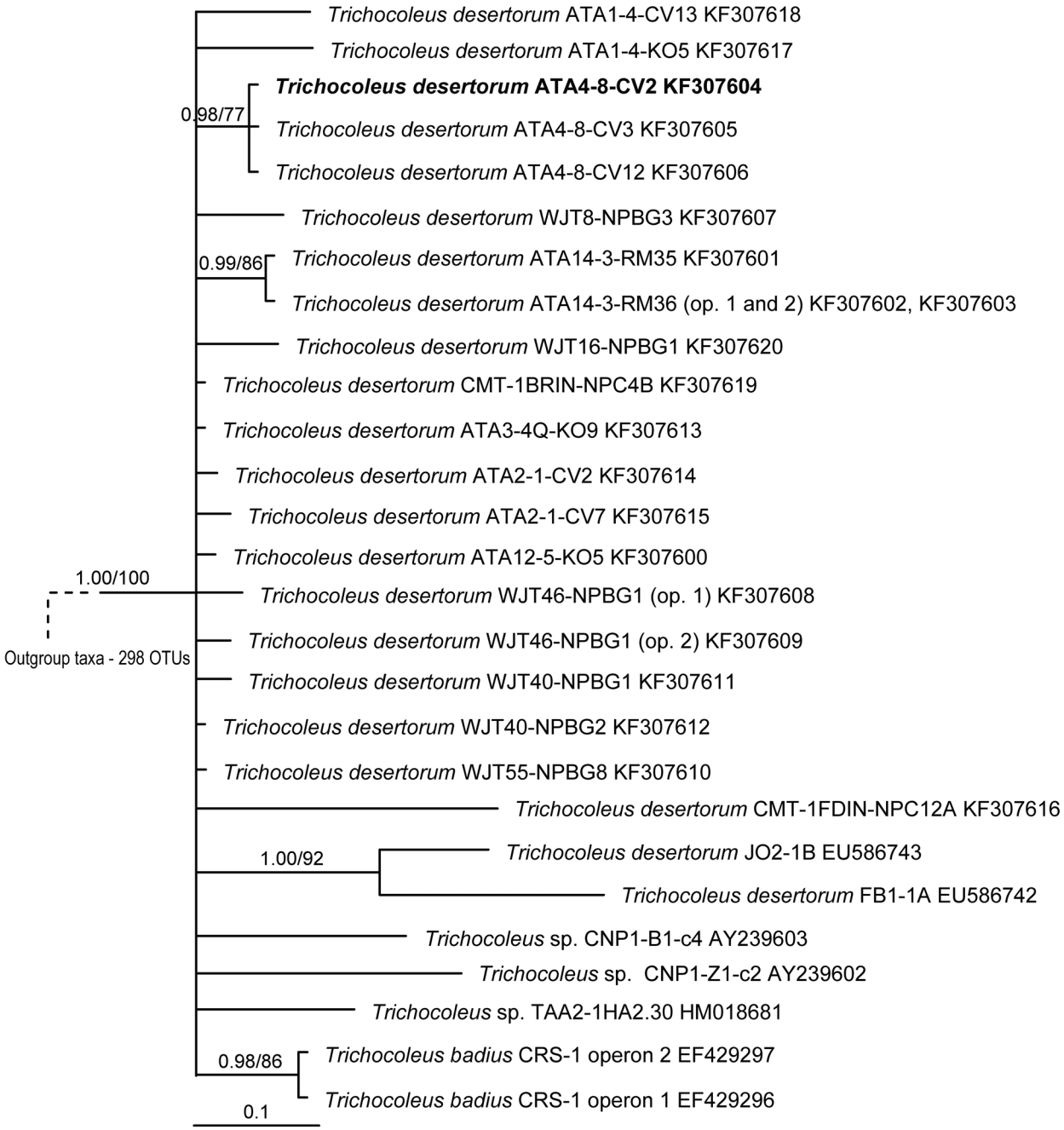

FIGURE 4. Detail of the *Trichocoleus* cluster based on Bayesian analysis with 16S rRNA gene sequence data. Posterior probabilities/ bootstrap support from parsimony analysis reported above node. op. = operon. Holotype is based on strain in bold font.

For comparison of the 16S rRNA gene sequence similarity, two outgroups were used—*N. nodulosa* UTEX2910 and *Leptolyngbya* sp. Zehnder 1965/U140. *Nodosilinea* was a supported sister group in Bayesian analysis (Fig. 3), whereas parsimony analysis showed *Leptolyngbya* Zehnder 1965/U140 as a sister taxon (data not shown). Both of them were significantly less than 95% similar to strains representing *Trichocoleus* (Table 3), which further justified our perception that the *Trichocoleus* cluster is a distinct genus. However, the 16S rRNA gene similarities in *T. desertorum* were highly variable (Table 4). Whereas most of the 22 compared taxa were >98% similar to each other, there were three strains (*T. desertorum* FB1-1A, JO2-1B, CMT-1FDIN-NPC12A) whose similarities among each other were significantly below this value (96.6%–97.9%). Also the similarity of these strains to the other taxa was often lower than 98%. In case of strain FB1-1A it dropped to 97.2% (Table 4). Based on their phylogenetic placement (Fig. 4) and the similarity analysis, these strains do not belong to *T. desertorum.*

TABLE 3. Comparison of the 16S rRNA gene sequence similarity among supported *Trichocoleus* clusters representing different taxonomic units and three outgroups (*"T. sociatus"* SAG 26.92, *N. nodulosa* UTEX2910*, Leptolyngbya* Zehnder 1965/U140). Values are in percent similarity calculated from P-distance as reported in PAUP. Taxa in quotation marks are incorrectly identified according to phylogenetic evidence.

Strain		1	2	3	4	5	6	7
1	*Trichocoleus desertorum*	–	–	–	–	–	–	–
2	*Trichocoleus badius* CRS1 (op. 1, 2)	97.4–99.5	–	–	–	–	–	–
3	*Trichocoleus* sp. CNP1-Z1-c2	97.0–98.5	98.6	–	–	–	–	–
4	*Trichocoleus* sp. CNP1-B1-c4	97.1–98.9	98.8	98.2	–	–	–	–
5	*Trichocoleus* sp. TAA2-1HA2.30	97.3–99.2	99.1	98.3	98.2	–	–	–
6	*"Leptolyngbya"* ZEHNDER 1965/U140	92.9–94.2	93.9	93.3	93.7	93.4	–	–
7	*Nodosilinea nodulosa* UTEX2910	91.1–92.8	92.8	91.1	91.3	91.9	92.1	–
8	*"Trichocoleus sociatus"* SAG 26.92	90.2–91.7	91.4	91.0	91.2	91.2	91.9	89.7

TABLE 4. Comparison of the 16S sequence similarity in *T. desertorum*. Selected strains are represented**.**

	Strain	1	2	3	4	5	6	7	8	9	10	11
1	*T. desertorum* FB1-1A	–	–	–	–	–	–	–	–	–	–	–
2	*T. desertorum* JO2-1B	97.9	–	–	–	–	–	–	–	–	–	–
3	*T. desertorum* CMT-1FDIN-NPC12A	96.6	97.7	–	–	–	–	–	–	–	–	–
4	*T. desertorum* CMT-1BRIN-NPC4B	97.4	98.0	98.4	–	–	–	–	–	–	–	–
5	*T. desertorum* WJT40-NPBG1	97.2	97.8	98.2	99.8	–	–	–	–	–	–	–
6	*T. desertorum* WJT55-NPBG8	97.3	97.9	98.3	99.9	99.7	–	–	–	–	–	–
7	*T. desertorum* ATA1-4-CV13	97.5	98.2	98.4	99.3	99.1	99.2	–	–	–	–	–
8	*T. desertorum* ATA12-5-KO5	97.2	97.8	98.2	99.7	99.6	99.8	99.1	–	–	–	–
9	*T. desertorum* ATA14-3-RM35	97.4	97.7	97.8	99.5	99.3	99.6	98.8	99.7	–	–	–
10	*T. desertorum* ATA4-8-CV2	97.6	97.9	98.1	99.6	99.4	99.5	98.9	99.4	99.7	–	–
11	*T. desertorum* ATA3-4Q-KO9	97.4	98.0	98.2	99.8	99.7	99.7	99.1	99.7	99.7	99.7	–
12	*T. desertorum* ATA2-1-CV7	97.2	97.8	98.0	99.7	99.5	99.6	99.0	99.5	99.5	99.6	99.8

Phylogenetic analyses, 16S–23S ITS:—To gain better insight into the intrageneric identities of investigated strains, analyses of the 16S–23S ITS region were performed. For these analyses we tried to use the same outgroups which we used for the similarity table above. However, the ITS region of *N. nodulosa* was so different from our strains both in nucleotide sequence and lengths of some regions (Table 5) that it was not possible to accurately align them. The ITS region of *Leptolyngbya* Zehnder 1965/U140 was a little less different, but still too different to allow determination of species level relationships (data not shown). Therefore, we present a tree which was created with no outgroup by aligning only strains belonging to *Trichocoleus* (Fig. 5). Only operons containing both tRNAs (alanine and isoleucine) were used for the ITS phylogenetic analyses. Consensus sequences were made for strains where more than one operon with both tRNAs were sequenced. However, for two strains we were not able to make consensus sequences (*T. desertorum* ATA14-3-RM36 operons 1, 2; *T. badius* CRS-1 operons 1, 2), so we included both of them in the analyses. Unlike the other strains, where consensus can be assumed, in the case of these two organisms we indicate here whether we talk about the consensus or not. These operons did not vary in conserved regions (Table 5).

TABLE 5. Lengths of 16S–23S ITS regions in the analyzed strains. In the two instances where operons lacking tRNA genes were recovered, the V2 does not form, but the region is reported in place of D2, D3, and Pre-Box-B and associated spacers simply as the V2 spacer.

Strain	Leader	D1-D1' helix	D2 with spacer	D3 with spacer	tRNAIle gene	V2 spacer	tRNAAla gene	Pre-Box-B spacer	Box-B helix	Post-Box-B spacer	Box-A	D4	V3	D5
Nodosilinea nodulosa UTEX2910	8	62	31	60	74	6	73	24	40	18	11	38	27	34
Leptolyngbya Zehnder 1965/U140	8	65	30	16	74	84	73	46	38	17	11	13	?	?
Trichocoleus sp. TAA2-1HA2.30	10	62	38	17	74	63	73	33	36	16	11	14	65	21
T. badius CRS-1 operon 1	8	62	37	17	74	51	73	33	36	16	11	14	65	21
T. badius CRS-1 operon 2	8	62	37	17	74	37	73	33	36	16	11	14	65	21
T. desertorum ATA1-4-CV13	8	62	35	16	74	24	73	14	36	17	11	14	63	21
T. desertorum ATA1-4-KO5	8	62	35	16	74	24	73	14	36	17	11	14	63	21
T. desertorum ATA2-1-CV2	8	62	37	17	74	53	73	14	37	17	11	14	65	21
T. desertorum ATA2-1-CV7	8	62	37	17	74	53	73	14	37	17	11	14	65	21
T. desertorum ATA3-4Q-KO9	8	62	37	17	74	24	73	14	37	17	11	14	65	21
T. desertorum ATA4-8-CV2	8	62	37	17	74	24	73	14	37	17	11	14	63	21
T. desertorum ATA4-8-CV3	8	62	37	17	74	24	73	14	37	17	11	14	63	21
T. desertorum ATA4-8-CV12	8	62	37	17	74	24	73	14	37	17	11	14	63	21
T. desertorum ATA12-5-KO5	8	62	36	17	74	24	73	14	37	17	11	14	65	21
T. desertorum ATA14-3-RM35	8	62	37	17	74	24	73	14	36	17	11	14	63	21
T. desertorum ATA14-3-RM36 op. 1	8	62	37	17	74	24	73	14	36	17	11	14	63	21
T. desertorum ATA14-3-RM36 op. 2	8	62	35	17	74	24	73	14	36	17	11	14	66	21
T. desertorum CMT-1BRIN-NPC4B	8	62	38	16	74	24	73	14	37	17	11	14	63	21
T. desertorum CMT-1FDIN-NPC12A	8	62	38	17	74	9	73	14	37	17	11	14	65	20
T. desertorum FB1-1A	8	62	35	17	74	9	73	15	36	17	11	14	66	21
T. desertorum JO2-1B	8	62	35	17	74	9	73	15	36	17	11	14	63	21
T. desertorum WJT8-NPBG3	8	62	38	16	74	24	73	13	37	17	11	14	63	21
T. desertorum WJT16-NPBG1*	8	62	31			390			35	19	11	21	48	17
T. desertorum WJT40-NPBG1	8	62	38	16	74	24	73	13	37	17	11	14	63	21
T. desertorum WJT40-NPBG2	8	62	38	16	74	24	73	13	37	17	11	14	63	21
T. desertorum WJT46-NPBG1 op. 1	8	62	38	16	74	24	73	13	37	17	11	14	63	21
T. desertorum WJT46-NPBG1 op. 2*	8	63	31			301			35	19	11	21	48	17
T. desertorum WJT55-NPBG8	8	62	38	16	74	24	73	13	37	17	11	14	64	21

* operons with no tRNAs not included in the ITS tree

The ITS phylogeny provided much finer resolution of intrageneric relationships of the strains than the 16S rRNA phylogeny. Whereas the Atacama strain clusters seemed to represent only populations from certain sites, all WJT strains together with CMT-1BRIN-NPC4B grouped in single well supported clade accompanied by strains from site ATA1-4 as a sister clade (Fig. 5). Sequences of *T. badius* and *Trichocoleus* sp. TAA2-1HA2.30 were placed on the edge of the tree, outside of the well supported cluster including all desert strains. These results indicate that *T. desertorum* and *T. badius* are different species, and that the strain TAA2-1HA2.30 is an undescribed third species. Unfortunately, the culture of this strain has been lost, and we do not have enough information to

describe it as a new species. The three taxa exhibiting 16S rRNA similarity <98% to the other *T. desertorum* strains (FB1-1A, JO2-1B, CMT-1FDIN-NPC12A) created a well-supported group in the center of the *T. desertorum* cluster (Fig. 5). This represents a disagreement with the 16S rRNA phylogeny (Fig. 4), in which the three dissimilar strains are outside of the main cluster of *T. desertorum*. Consequently, this phylogeny suggests there is more taxonomic diversity in this clade of *Trichocoleus* than we are able to diagnose at this time.

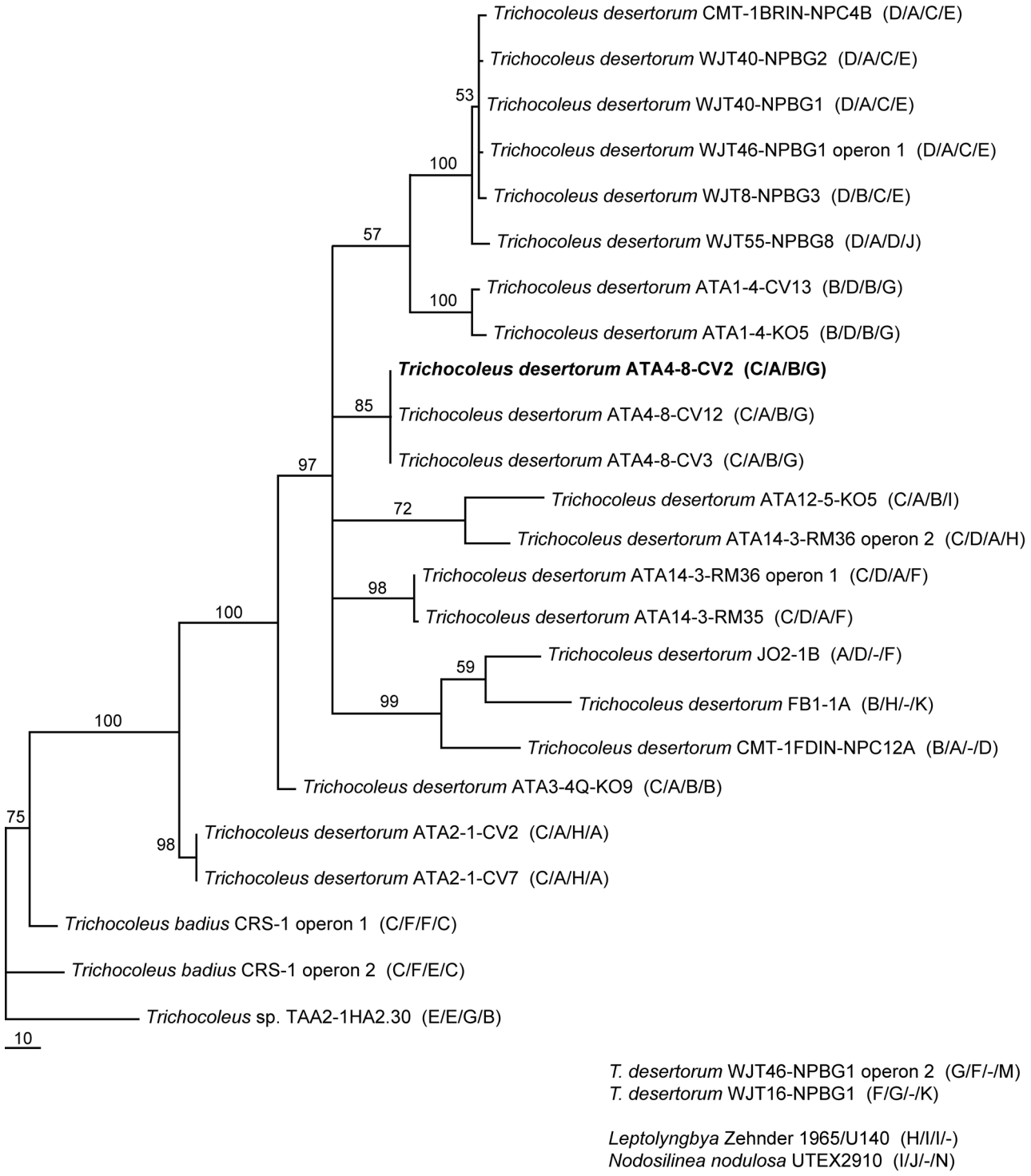

FIGURE 5. Phylogenetic relationships among *Trichocoleus* strains based on parsimony analysis with 16S–23S ITS region sequence. Values above branches represent bootstrap values from this analysis. Holotype is based on strain in bold font. Letters in parentheses represent ITS helices shown in Figs. 6–9 in order: D1–D1'/Box-B/V2/V3. Helix pattern for operons not included in the ITS tree are shown in lower right corner.

Secondary structure of the 16S–23S ITS:—The D1–D1' helix was relatively conserved among all the strains (Fig. 6). The base of the stem always consisted of 5 bp, followed by a side loop of 7 bases, with the only exception being *N. nodulosa* whose side loop consisted of only 6 unpaired bases. The terminal loop was also fairly conserved. In most cases it contained 16 bases (Figs. 6B–F, I); exceptionally 15 (Fig. 6A), or 17 and 18 bound partially together (Figs. 6G–H). The terminal loop with 18 bases was observed only in the outgroup represented by *Leptolyngbya* Zehnder 1965/U140. The middle part of the D1–D1' helix was minimally variable with several minor side loops or single unpaired bases. *Trichocoleus badius* shared the shape of this structure with various strains of *T. desertorum* from the Atacama Desert (Fig. 6C). The structure of TAA2-1HA2.30 was unique, with a distinct 3:3 base bilateral bulge following the conserved basal part of the stem (Fig. 6E).

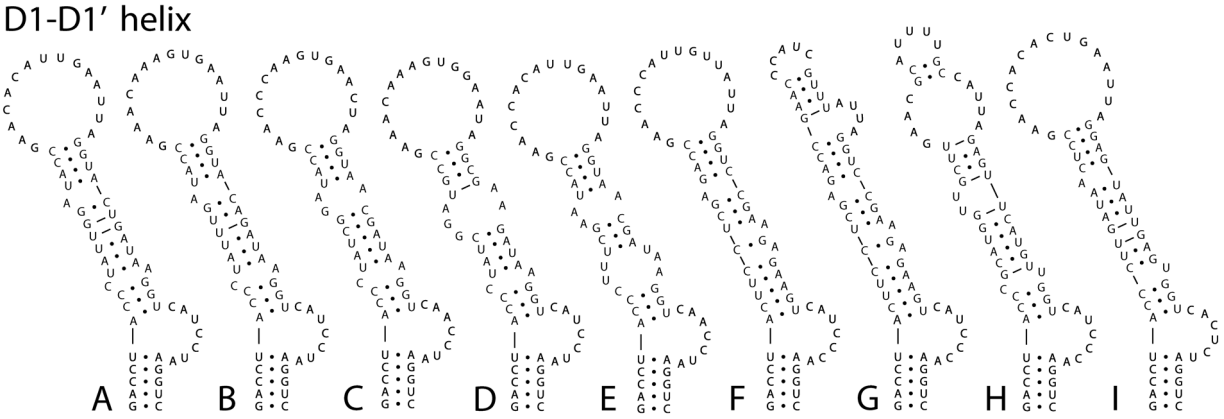

FIGURE 6. D1–D1' helix. A. *Trichocoleus desertorum* JO2-1B. B. *Trichocoleus desertorum* ATA1-4-KO5/CV13, CMT-1FDIN-NPC12A, FB1-1A. C. ATA2-1-CV2/CV7, ATA4-8-CV2/CV3/CV12, ATA3-4Q-KO9, ATA12-5-KO5, ATA14-3-RM35/RM36 op. 1 and 2; *Trichocoleus badius* op. 1 and 2. D. *Trichocoleus desertorum* CMT-1BRIN-NPC4B, WJT8-NPBG3, WJT40-NPBG1/NPBG2, WJT46-NPBG1 op. 1, WJT55-NPBG8. E. *Trichocoleus* sp. TAA2-1HA2.30. F. *Trichocoleus desertorum* WJT16-NPBG1. G. *Trichocoleus desertorum* WJT46-NPBG1 op. 2. H. *Leptolyngbya* Zehnder 1965/U140. I. *Nodosilinea nodulosa.*

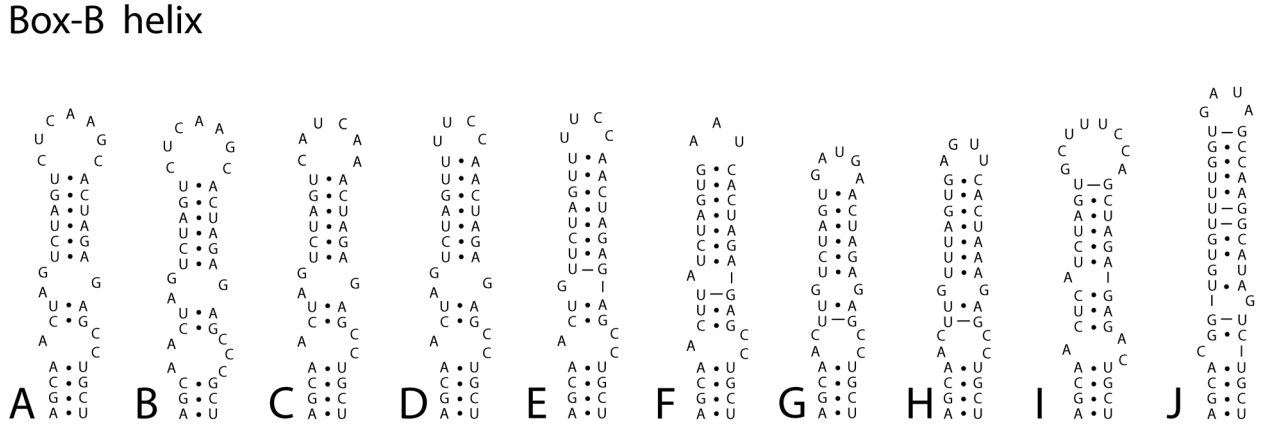

FIGURE 7. Box-B helix. A. *Trichocoleus desertorum* ATA2-1-CV2/CV7, ATA3-4Q-KO9, ATA4-8- CV2/CV3/CV12, ATA12-5-KO5, CMT-1BRIN-NPC4B, CMT-1FDIN-NPC12A, WJT40-NPBG1/NPBG2, WJT46-NPBG1 op. 1, WJT55-NPBG8. B. *Trichocoleus desertorum* WJT8-NPBG3. C. *Trichocoleus desertorum* ATA14-3-RM35/RM36 op. 1 and 2. D. *Trichocoleus desertorum* ATA1-4-KO5/CV13, JO2-1B. E. *Trichocoleus badius* op. 1 and 2, *Trichocoleus* sp. TAA2-1HA2.30. F. *Trichocoleus desertorum* WJT46-NPBG1 op. 2, G. *Trichocoleus desertorum* WJT16-NPBG1. H. *Trichocoleus desertorum* FB1-1A. I. *Leptolyngbya* Zehnder 1965/U140. J. *Nodosilinea nodulosa.*

The Box-B helix presented a more variable structure (Fig. 7). The basal part of the helix was conserved in all investigated strains. It consisted of a 4 bp helix followed by a bilateral bulge (usually 3 bp in total). The only exception was in strain WJT8-NPBG3, where the substitution of cytosine by uracil on the 3' side of the helix disabled binding with adenine on the corresponding side, and thus, the basal stem consisted only of 3 bp followed by a larger bilateral bulge (Fig. 7B). The helix length varied among the strains as well as the size of the terminal

loop. The most divergent structure was in *N. nodulosa*—it lacked all loops in the stem, only 2 unpaired bases were present (Fig 7J). The largest terminal loop was observed in *Leptolyngbya* Zehnder 1965/U140, with 8 bases (Fig 7I), whereas in the rest of the structures the terminal loop ranged from 3–7 bases (Figs. 7A–H, J). *Trichocoleus badius* together with TAA2-1HA2.30 shared their own unique structure (Fig. 7E).

The V2 region represented the most variable helix (Fig. 8). It was either rather long (Figs. 8F–H), or completely missing as in case of strains JO2-1B, FB1-1A, and CMT-1FDIN-NPC12A (Table 5). TAA2-1HA2.30 was represented by its own structure (Fig. 8G) and unique structures were present also in each operon of *T. badius* (Figs. 8E–F).

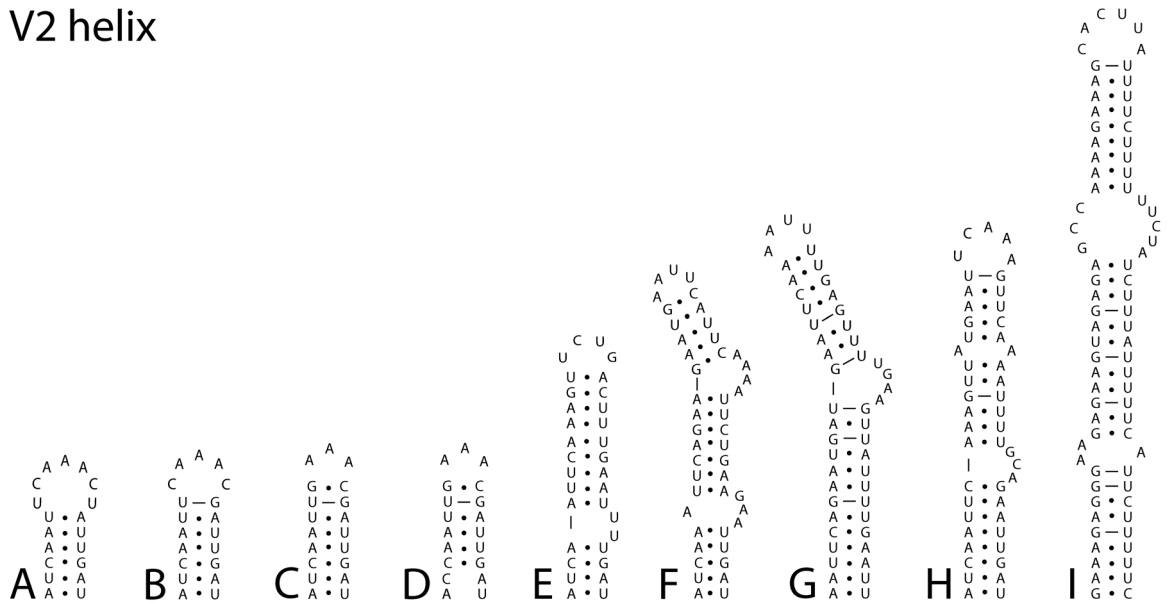

FIGURE 8. V2 helix. A. *Trichocoleus desertorum* ATA14-3-RM35/RM36 op. 1 and 2. B. *Trichocoleus desertorum* ATA1-4-KO5/CV13, ATA3-4Q-KO9, ATA4-8-CV2/CV3/CV12, ATA12-5-KO5. C. *Trichocoleus desertorum* WJT8-NPBG3, WJT40-NPBG1/NPBG2, WJT46-NPBG1 op. 1, CMT-1BRIN-NPC4B. D. *Trichocoleus desertorum* WJT55-NPBG8. E. *Trichocoleus badius* op. 2. F. *Trichocoleus badius* op. 1. G. *Trichocoleus* sp. TAA2-1HA2.30. H. *Trichocoleus desertorum* ATA2-1-CV2/CV7. I. *Leptolyngbya* Zehnder 1965/U140.

The V3 helix was quite variable in structure (Fig. 9), although some parts were still conserved. The basal part of the stem consisting of a 4 bp helix followed by a bilateral bulge with a single unpaired adenine in the 5' portion of the helix and 3 unpaired bases 5'–ACA–3'on the opposite side of the bulge, further followed by 4 bp long helix was the same for all the *Trichocoleus* operons containing both tRNAs (Figs. 9A–K). Even the following loop consisting of 4:3 unpaired bases was well conserved among *Trichocoleus* strains (Figs. 9A–J) with the only exception being in *Trichocoleus* FB1-1A where this loop was smaller (Fig. 9K). The V3 helices of operons with no tRNA genes were much smaller than the V3 helices in operons with both tRNAs (Figs. 9L–M). However, the smallest helix was present in *N. nodulosa*, even though this operon contained both tRNAs (Fig. 9N). Both operons of *T. badius* possessed structures different in the upper portion of the helix from all those in the *T. desertorum* strains (Fig. 9C). We can conclude that the conserved part of V3 helix is consistent for the genus *Trichocoleus*, when operons with both tRNAs are investigated.

Discussion

Based on the 16S similarity values, *Trichocoleus* is a distinct genus, with maximum similarity of 94.2% to its possible sister taxa (Table 3). However, the similarity among 22 strains representing *T. desertorum* was unusually variable (Table 4). Other studies which have used multiple strains to describe new taxa showed that the 16S rRNA gene sequence similarity can be as low as 98% within one species (*cf.* Siegesmund *et al.* 2008, Strunecký *et al.* 2011). Most of the *T. desertorum* strains had similarity values well above this number; however, there were three exceptions with values close to or considerably below 98%. These exceptions were represented by strains FB1-1A,

JO2-1B, and CMT-1FDIN-NPC12A with similarity as low as 97.2% to the other *T. desertorum* strains and 96.6% among themselves (Table 4). This is certainly not common within single species. Even though comparison of 16S rRNA gene sequence similarity was shown to be unreliable for confirmation of cyanobacterial species when used as the only characteristic (Johansen & Casamatta 2005), the large range of similarities observed in *T. desertorum* further suggests the presence of cryptic species.

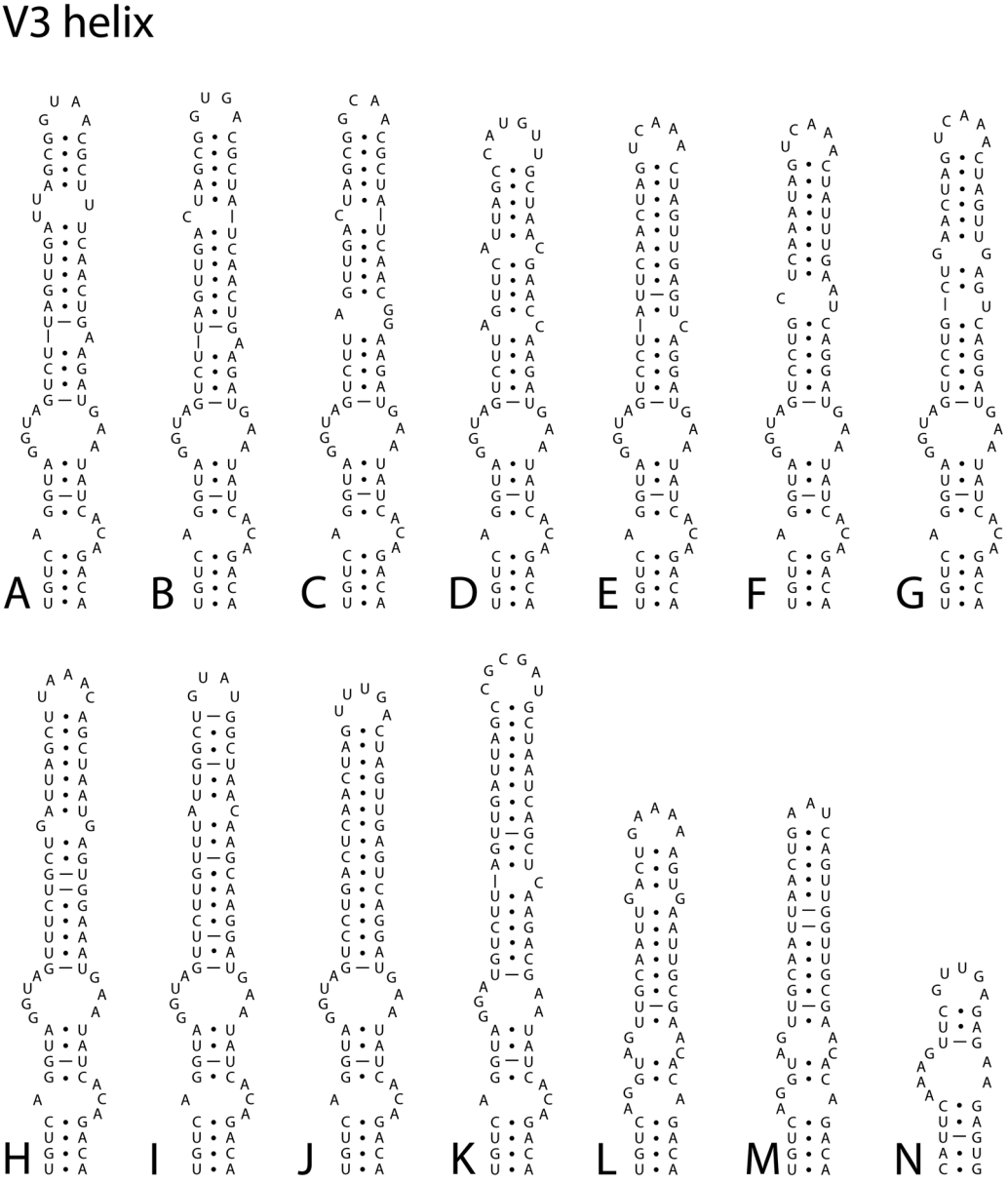

FIGURE 9. V3 helix. A. *Trichocoleus desertorum* ATA2-1-CV2/CV7. B. *Trichocoleus desertorum* ATA3-4Q-KO9, *Trichocoleus* sp. TAA2-1HA2.30. C. *Trichocoleus badius* op. 1 and 2. D. *Trichocoleus desertorum* CMT-1FDIN-NPC12A. E. *Trichocoleus desertorum* CMT-1BRIN-NPC4B, WJT8-NPBG3, WJT40-NPBG1/NPBG2, WJT46-NPBG1 op. 1. F. *Trichocoleus desertorum* ATA14-3-RM35/RM36 op. 1, JO2-1B. G. *Trichocoleus desertorum* ATA1-4-KO5/CV13, ATA4-8-CV2/CV3/CV12. H. *Trichocoleus desertorum* ATA14-3-RM36 op. 2. I. *Trichocoleus desertorum* ATA12-5-KO5. J. *Trichocoleus desertorum* WJT55-NPBG8. K. *Trichocoleus desertorum* FB1-1A. L. *Trichocoleus desertorum* WJT16-NPBG1. M. *Trichocoleus desertorum* WJT46-NPBG1 op. 2. N. *Nodosilinea nodulosa*.

As can be seen from the phylogenetic comparison of the ITS regions, strains grouped into several supported clusters (Fig. 5). The cluster containing WJT strains represented a well-supported group of cyanobacteria from five different sites in two different deserts (Colorado, Mojave), whereas strains from the Atacama Desert (ATA) created multiple small groups, each usually representing only a single site. Rarely did ATA strains form groups representing more than one site. Remarkably, two different operons with the same tRNA pattern from the strain

ATA14-3-RM36 fell into two different supported groups, thus making the strain "polyphyletic". Similarly, *T. badius,* as shown on our ITS tree, is a "paraphyletic" organism. Therefore, we can conclude that the operon variability can obscure the signal from geographic origin, particularly if only single operons are recovered for a given strain.

Whereas presence of multiple rRNA operons in cyanobacteria is well known and three different patterns according to presence or absence of tRNAs have been revealed and described (e.g. Boyer *et al.* 2001), variability among operons with the same number of tRNAs present in the ITS region remains rather unknown. Boyer *et al.* (2001) warned against using operons with different patterns for the phylogenetic analyses, but it seems that even differences among operons with the same number of tRNA genes can negatively affect the accuracy of phylogenetic analysis. The three strains exhibiting low 16S similarity to each other and to the other *T. desertorum* strains (FB1-1A, JO2-1B, CMT-1FDIN-NPC12A) grouped together into a well-supported cluster in the center of the *T. desertorum* group. All three of these problematic strains lacked the V2 region, which probably played a role in their clustering together. The absence of the V2 region may be strain-specific or only operon specific, which would again suggest that if different operons were used, inaccurate conclusions about phylogenetic placement could be made. Currently, we have not observed differences in the 16S rRNA gene of operons expressing different sequences in the ITS region.

The variability among operons can be also demonstrated in the ITS secondary structures. The operons including no tRNA genes had very different and unique secondary structures when compared to the ones containing both tRNA genes (Figs. 6F–G, 7F–G, 9L–M). Therefore, it is important to always compare operons with the same number of tRNA genes, even though in general the same type of structure is present in the conserved helices of all of them. However, sometimes there are differences in same-pattern operons of the same strain: ATA14-3-RM36 significantly differed in the variable part of the V3 helices (Figs. 9F–H), *T. badius* differed in both length and sequence of their V2 regions (Figs. 8E–F).

Since the phylogenetic analyses revealed considerable molecular diversity in our strains, we were interested to see if their morphology reflected this diversity as well. However, from our observations it seemed that most of the diversity, such as presence of multiple trichomes in a sheath (Fig. 1A, Figs. 2A–F), creation of involution cells (Figs. 2I–J), asymmetrical division (Figs. 2C–E), or thylakoids creating fascicles (Figs. 2G–H), were variably present in a way not consistent with either phylogeny or biogeography. Even though all strains were grown in the same light and temperature conditions, using the same media (Z-8), some of them appear to differ in expression of the above features (Table 2). Observation of rare thylakoid fascicles in the centroplasm in *T. desertorum*, a cyanobacterium clearly belonging to the order Pseudanabaenales, is very unique and unusual. The whole subclass Synechococcophycidae, to which the Pseudanabaenales belongs, is defined by the presence of parietal thylakoids. This separates it from subclass Oscillatoriophycideae (also containing simple filamentous cyanobacteria) characterized by radial/fasciculate thylakoids (Hoffmann *et al.* 2005). *Trichocoleus* clearly exhibits parietal thylakoids in all trichomes, but the few fascicles visible rarely in the centroplasm further explains the easy confusion of this genus with *Microcoleus* in the Oscillatoriophycideae.

Based on all the characteristics listed above, we did not have sufficient evidence to separate our strains into more than one species. The ITS clusters were not congruent with morphology and ecological conditions, and the operon variability seemed significant enough that we decided to treat the whole supported cluster containing all of the desert soil strains as single taxon (Fig. 5). However, with regard to all the evidence, cryptic species are likely present. To separate them, additional data such as sequences from other genes or physiological/biochemical characterizations are probably needed.

The results of our phylogenetic analyses led us to transferring *L. badia* to *Trichocoleus* and creating the new combination—*T. badius*. This cyanobacterium was originally described from a seep wall in Great Smoky Mountains National Park and its morphology was quite different from *T. desertorum*. The maximum number of trichomes in a sheath was two, trichomes had untapered, bluntly rounded end cells and were only up to 1.3 µm wide, with sheaths thick, rigid, and brown (Johansen *et al.* 2008). Such distinctive morphological divergence from *T. desertorum* is slightly surprising with regard to the high sequence similarity with some of the strains (up to 99.5% similarity, see Table 3). One potential explanation is that even though closely related, the strikingly different habitats selected for very different morphological traits in these two taxa. Unfortunately, Johansen *et al.* (2008) noted that the original culture died after the second transfer, and thus, it is difficult to add more information on this organism's morphology.

The phylogenetic position of our strains in the Pseudanabaenales group, presence of multiple trichomes in a sheath, and morphological similarity to *T. sociatus* (as shown in the Results section) lead us to the conclusion that our strains represent the genus *Trichocoleus*. Strains attributed to *T. sociatus* have been recorded from several deserts all over the world and have been used to study how cyanobacteria can withstand extreme physiological conditions represented by desert soils (e.g. Lange *et al.* 1992, 1994, Karsten & Garcia-Pichel 1996, Maya *et al.* 2002, Büdel *et al.* 2009). However, *T. sociatus* was described originally from fresh waters in Angola (West & West 1897), a habitat posing quite different challenges than the desert ecosystem. We believe that ecology and biogeography are important parts of a polyphasic approach. For example, as shown in a study of true-branched heterocytous cyanobacteria, different evolutionary lineages were represented by hot-spring inhabitants than were represented in morphologically similar soil morphs (Kaštovský & Johansen 2008).

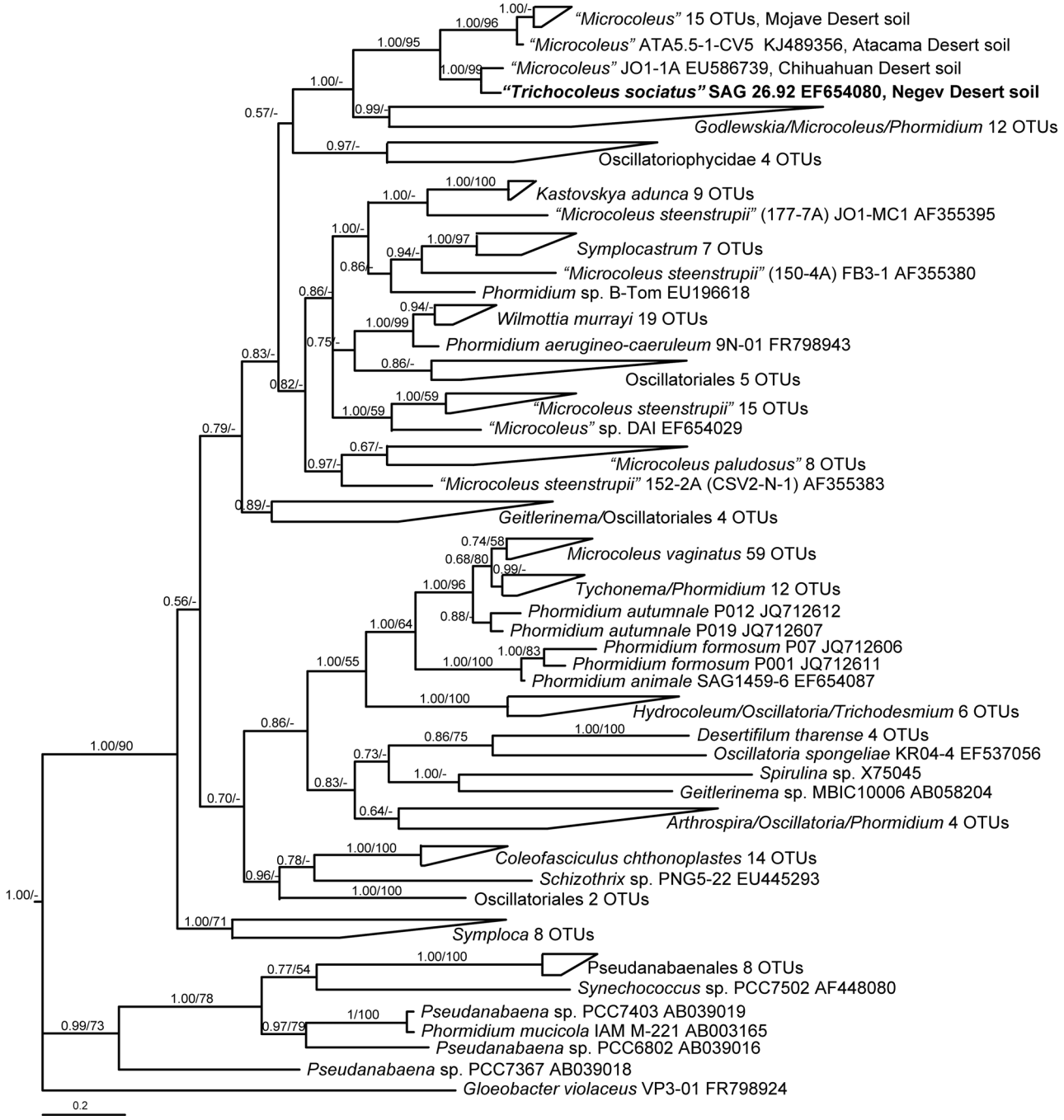

FIGURE 10. Bayesian analysis of 225 strains within the Oscillatoriales showing the phylogenetic position of *Trichocoleus* (*Microcoleus*) *sociatus* SAG 26.92 in a clade of an unnamed desert soil genus outside of the *Microcoleus sensu stricto.* Taxonomic epithets applied to GenBank sequences that we consider to be incorrect are indicated by enclosure in quotation marks.

So far, there is only one sequence available in GenBank with the name *Trichocoleus,* and it is called *T. sociatus* (SAG 26.92, EF654080). However, this sequence groups with *Microcoleus*-like strains in the Oscillatoriales (Siegesmund *et al.* 2008, Mühlsteinová *et al.* 2014), and has very low similarity with all *T. desertorum* strains (Table 3). Furthermore, examination of the strain in LM revealed it does not have parietal thylakoids. It consequently cannot represent the genus *Trichocoleus*. The original culture (BB 92.2) was isolated from the Negev Desert, Israel as *Microcoleus sociatus* West & West (1897: 272), and deposited in a culture collection in 1992 as SAG 26.92 (SAG Culture Collection Database). The collectors of the original culture noticed differences in morphology between natural samples and culture—while in nature trichomes were about 2.8 µm wide, in culture they measured about 3.5 µm (Lange *et al.* 1994). We ran a phylogenetic analysis based on parsimony of 711 taxa in both the Oscillatoriales and Pseudanabaenales to determine the placement of SAG 26.92 and it clearly places with the Oscillatoriales (data not shown). In a phylogeny based upon 16S rRNA sequences (225 Oscillatoriales plus 14 outgroup taxa) the position of this strain is shown (Fig. 10). It is in a supported clade with 17 other desert soil strains which appear to show some geographical separation and which have all been previously reported as *Microcoleus*. This lineage falls well outside of the *M. vaginatus* and *Trichocoleus* clades, and we consider *T. sociatus* SAG 26.92 to be neither conspecific with *T. sociatus* nor to have any relationship with the genus *Trichocoleus*, a conclusion also reached nine years ago by Komárek & Anagnostidis (2005, see p. 318).

Thus, we present here the first clearly defined phylogenetic reference point for the genus *Trichocoleus* based on the 16S rRNA gene sequence and associated 16S–23S ITS sequence. Of course, unless the sequence of the type species is available, there is no 100% guarantee that our reference point truly represents *Trichocoleus* as defined based on *T. delicatulus.* In contrast to the morphological definition of this genus in Komárek & Anagnostidis (2005), *T. desertorum* does not have cells always longer than wide, and its trichomes can be wider than 3 µm. However, as discussed above, morphology of simple filamentous cyanobacterial forms can be very variable and can depend on whether they are described from natural samples or from cultures. Probably neither of the other *Trichocoleus* species currently known was studied in culture. Because *T. delicatulus* lacks any drawing, herbarium reference, or strain culture, the confirmation or refutation of its actual position in *Trichocoleus* as defined in this study will likely never occur. Presumably the only way of doing that would be visiting the original type locality, isolating a morphotype corresponding with the initial, rather poor, description, and sequencing it. Meanwhile, it is highly important for the current progress in the field of cyanobacterial taxonomy to decrease the amount of dark taxa (Page 2012 as cited in De Clerck *et al.* 2013) in sequence databases, and connect as many well defined reference points in phylogenetic trees (i.e. based on molecular data) with already existing taxa as possible. This of course has to take into careful consideration morphological and ecological descriptions, because often that is all that is available for the taxa defined in the past. Even though there is risk that eventually sequences of type species in classic genera will show that some strains have been improperly assigned to these genera, we think it is worth taking a chance and moving forward with naming new taxa and re-defining old taxa using the polyphasic approach.

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