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Roholtiella, gen. nov. (Nostocales, Cyanobacteria)—a tapering and branching cyanobacteria of the family Nostocaceae

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Abstract

A total of 16 strains phylogenetically placed within the Nostocaceae were found to possess morphological features of the Rivulariaceae and Tolypothrichaceae (tapering trichomes and single false branching, respectively) in addition to their typical Nostocacean features (production of arthrospores in series). These strains formed a strongly supported clade separate from other strains that are phylogenetically and morphologically close. We describe four new species within the genus *Roholtiella gen. nov.* The four species include three distinguishable morphotypes. *Roholtiella mojaviensis* and *R. edaphica* are morphologically distinct from each other and from the other two species, *R. fluviatilis* and *R. bashkiriorum*. *Roholtiella fluviatilis* and *R. bashkiriorum* are cryptic species with respect to each other. All four species are easily distinguished based on the sequence of the 16S-23S ITS regions, in particular the flanking regions to the conserved Box-B and V3 helices. The species are further established by the elevated p-distance between species that is much reduced among strains within the same species. *Calochaete cimrmanii,* a recently described tapering species from tropical biomes, is the most likely sister taxon to *Roholtiella.*

Key words: 16S rRNA gene, 16S-23S ITS, cryptic species, morphology, new genus, Nostocophycideae, polyphasic approach, taxonomy

Introduction

With the advent of molecular sequencing and the polyphasic approach to cyanobacterial taxonomy, there has recently been considerable advance made in the systematics and taxonomy of cyanobacteria, including the description of numerous cyanobacterial genera and species. The first genera to be described with a combination of morphological and molecular methods appeared in 2002 (Abed *et al.* 2002, Flechtner *et al.* 2002, Suda *et al.* 2002). Since that time, over 40 genera have been described using a polyphasic approach that includes both morphology and molecular sequence data.

The heterocytous taxa have proven difficult to characterize and numerous morphologically well-defined genera have been found to be polyphyletic based on molecular phylogenetic analyses. Over the past century, characteristics such as tapering and false branching were thought to have evolved very seldom, and so most filaments that tapered were identified as *Calothrix* Agardh *ex* Bornet & Flahault (1886: 345) or *Rivularia* Agardh *ex* Bornet & Flahault (1887: 345), and false branching forms were commonly placed in either *Tolypothrix* Kützing ex Bornet & Flahault (1888a: 118) or *Scytonema* Agardh *ex* Bornet & Flahault (1888a: 85). When strains that were incorrectly assigned to these genera began to be sequenced (Rajaniemi *et al.* 2005), it created a good deal of taxonomic confusion, some of which persists to the present (Hauer *et al.* 2014).

The Nostocaceae have traditionally consisted of the heterocytous taxa that do not possess false branching or tapering (Geitler 1932, Komárek 2013). They form arthrospores that are solitary, or in series and are produced

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apoheterocytically (Komárek *et al.* 2014). Nostocaceaen taxa commonly have diffluent mucilage or a firm mucilage investment around the colony of trichomes, but rarely have individual sheaths around trichomes. Most taxa are isopolar, with only a few exceptions (e.g. *Cylindrospermum* Kützing *ex* Bornet & Flahault 1888b: 249). Furthermore, most have barrel-shaped or bead-like globose cells with strong constrictions at the crosswalls, a feature that further separates them from the heterocytous genera with cylindrical trichomes (e.g. *Tolypothrix, Scytonema, Brasilonema* Fiore *et al.* (2007: 794)*, Microchaete* Thuret ex Bornet & Flahault (1888a: 83)*, Fortiea* De Toni (1936: 3)). Recently, a number of strains assigned to *Tolypothrix, Calothrix,* and *Microchaete* have been found to form a clade within the Nostocaceae (Kaštovský & Johansen 2008, Kaštovský *et al.* 2014, Komárek *et al.* 2014). Strains in this clade have recurrently appeared in phylogenetic analyses without taxonomic correction, including *Nostoc* Fin 152, *Calothrix brevissima* West 1907: 180 (AB074504), *Tolypothrix* IAM M-259, and *Nostoc* PCC 7120 (Rajaniemi *et al.* 2005, Řeháková *et al.* 2007, Kaštovský & Johansen 2008, Lukešová *et al.* 2009, Johansen *et al.* 2014 (as "mixed Nostocaceae"), Kaštovský *et al.* 2014). We have long recognized that this group is problematic, but it is only recently (with the description of *Calochaete cimrmanii* Hauer *et al.* 2013: 38) that taxonomic revision has come to any members of this group.

While conducting a broader survey of terrestrial heterocytous cyanobacteria, we isolated 11 strains of morphologically distinct heterocytous, false branching cyanobacteria from various soil localities in Russia, USA and the Czech Republic. We characterized these strains using a polyphasic approach utilizing morphology, ultrastructure, habitat, and molecular markers. An additional five strains from more aquatic habitats (shallow waters near the banks of rivers) sequenced by others were found to fall in the same clade. All 16 strains bear morphological characteristics resembling both *Calothrix* (heteropolar tapering trichomes) and *Nostoc* Vaucher ex Bornet & Flahault (1888b: 181) (serial arthrospore formation, deeply constricted trichomes) in successive stages of their life cycle. They all form a highly supported compact clade within the Nostocaceae closely related to *Calochaete cimrmanii*. This clade is herein described as *Roholtiella*, with at least four species represented by our strains.

Materials and Methods

Isolation and maintenance of strains:—Cyanobacterial strains used in this study were isolated from soil samples collected in Russia, Czech Republic and USA (Table 1). Samples from Russia were collected in the territory of the Republic of Bashkortostan in different botanical-geographical zones (boreal forests, forest-steppe and steppes) following standard methods for soil phycology (Hollerbach & Shtina 1969). For isolation of pure cultures, the method of enrichment cultures on agar medium was used (Kostikov *et al.* 2001). In Petri plates with agar solidified BBM medium (Bischoff & Bold 1963), several drops of liquid BBM medium were transferred and evenly distributed with a glass spatula. A small amount of the soil sample was placed on the wet surface. Plates were placed into an incubator set at 4 °C with 12h:12h light:dark regime. Two strains were obtained from recultivated coal mining spoils in the Czech Republic and the USA (Table 1). Isolations were performed following the dilution plate method (Lukešová 1993, 2001) based on incubation of soil suspension (5 g of dried soil, 45 ml of sterile water) of different dilutions (dilution series from 10^{-1} to 10^{-4}) on agar-solidified BBM (1.75%) plate. The last two strains were obtained from composite soil samples collected in the Mojave Desert, USA (Table 1). Isolation of these strains was achieved by diluting 1 g of soil in 100 ml Z8 liquid medium (Carmichael 1986), shaken for 4 h at 150 rpm, and plated onto Z8 agar Petri plates with a 1/10 ml dilution. Plates were then incubated at 16:8 h light:dark cycle at 16 °C. In all cases, separate colonies of the target organisms were picked from the plates after several weeks of incubation and transferred onto agar slant tubes with a growth medium. For the duration of the project, all unialgal cyanobacterial isolates were transferred onto standard Z8 slant tubes as well as modified Z8 slant tubes with 1/10 of nitrogen content (both 1.5% agar solidified) and kept in 18 °C with a natural daylight regime.

All strains from Russia, Czech Republic and USA were deposited into CCALA Culture Collection of Autotrophic Organisms of the Institute of Botany of the ASCR, Třeboň, Czech Republic (accession numbers CCALA 1051, 1052 and 1055–1063) and their dried biomass to the CBFS Herbarium at the Department of Botany, University of South Bohemia, Czech Republic (accession numbers CBFS A-036 to A-046). Strain PCC 7415 was obtained from Pasteur Culture Collection of Cyanobacteria (Table 1) and was kept on BG11 slants (Stanier *et al.* 1971) under the same cultivation conditions as the rest of the tested strains. Other strains included in the study were previously characterized by Berrendero *et al.* (2011, Table 1) and the material (kept in UAM culture collection of Universidad Autónoma de Madrid) was not further examined during this project.

TABLE 1. Sampling sites and additional information of all strains belonging to the *Roholtiella* cluster. 1 = *R. fluviatilis* cluster, 2 = *R. bashkiriorum* cluster, 3 = *R. edaphica* cluster, 4 = *R. mojaviensis* cluster.

Cluster	Collection	Orig. no	Collected	Habitat	GPS	Locality
1	PCC 7415	PCC 7415	1972	soil	N/A	greenhouse, Stockholm, Sweden
1	CCALA 1058	RU7	Aug 2010	soil	55° 57’ 47” N, 58° 15’ 52” E	macroscopic growth of algae and cyanobacteria on riverside of river Ik on the edge of village Bolsheustikinskoye, Republic of Bashkortostan, Russia
1	UAM 332	CR1	2002	epilithon	40° 36’ 07” N, 3° 44’ 07” W	siliceous substrate submerged at the stream bank, Arroyo Cereal, Tres Cantos, Madrid, Spain
1	UAM 334	TJ6	2002	epilithon	40° 35’ 39” N, 3° 44’ 19” W	siliceous substrate submerged at the stream bank, Arroyo Tejada, Tres Cantos, Madrid, Spain
1	UAM 337	TJ10	2002	epilithon		
1	UAM 340	TJ13	2002	epilithon		
2	CCALA 1057	RU6	Aug 2010	soil	55° 57’ 47” N, 58° 15’ 53” E	macroscopic growth of algae and cyanobacteria on the path near river Ik on the edge village Bolsheustikinskoye, Republic of Bashkortostan, Russia
2	CCALA 1059	RU9	Aug 2010	soil	55° 57’ 29” N, 58° 15’ 50” E	meadow land near river Ik on the edge of the village Bolsheustikinskoye, Republic of Bashkortostan, Russia
3	CCALA 1055	RU1	Aug 2010	soil	55° 57’ 47” N, 58° 16’ 18” E	small ravine near forest near village Bolsheustikinskoye, Republic of Bashkortostan, Russia
3	CCALA 1056	RU2	Aug 2010	soil	55° 57’ 46” N, 58° 16’ 18” E	birch forest near village Bolsheustikinskoye, Republic of Bashkortostan, Russia
3	CCALA 1060	LG-S11	Jun 2010	soil	52° 38’ 04” N, 58° 43’ 42” E	forb-grass steppes near Sibay city, Republic of Bashkortostan, Russia
3	CCALA 1061	LG-P11	May 2010	soil	55° 25’ 29” N, 56° 34’ 31” E	hillside near the Red Rocks in village Pavlovka, Republic of Bashkortostan, Russia
3	CCALA 1062	JOH4	Sep 1996	soil	50° 14’ 31” N, 12° 42’ 07” E	dump after coal mining, tertiary cypris clay, recultivation area with sparsely planted pine seedlings, Vintířov, Czech Republic
3	CCALA 1063	JOH39	Nov 2009	soil	37° 42’ 21” N, 88° 40’ 30” W	dump after coal mining, recutivation area without addition of top soil, Sahara-Ashby Kolar Research Plots, Carbondale, Illinois, USA
4	CCALA 1051	WJT36-NPBG5B	Jun 2006	soil	34° 02’ 29” N, 116° 08’ 42” W	sandy, gravelly soil from granitic outcrops, plants and well-developed algal crusts, Joshua Tree National Park, Wonderland of Rocks, USA
4	CCALA 1052	WJT36-NPBG10	Jun 2006	soil		

Morphology and Ultrastructure:—The strains were studied in both light and transmission electron microscopes (TEM). General morphological observations and measurements were performed using an Olympus BX 51 microscope equipped with Nomarski DIC optics and Olympus DP71 digital camera. Cultures were repeatedly examined throughout all lifecycle stages, i.e. in cultures of different age since transfer. Growth medium with a lowered amount of nitrogen was used to induce heterocyte production.

For ultrastructural observations in TEM, a small amount of fresh biomass of a studied strain (CCALA 1061)

was first fixed with 6% glutaraldehyde and kept for several hours at room temperature. The sample was then washed with 0.05 M phosphate buffer (pH 7.2), postfixed with 2% osmium tetroxide in the same buffer at room temperature for 2 hours, and then repeatedly washed with 0.05 M phosphate buffer. Finally, cells were dehydrated with a graded isopropanol series and embedded in Spurr's resin (Spurr 1969) using propylene oxide as an intermediate stage. Thin sections were stained with uracil acetate and lead citrate and observed in a Jeol JEN 1010 (Tokyo, Japan) transmission electron microscope at 80 kV.

Molecular methods:—For strains from Russia and from the spoils in the Czech Republic and USA (see Table 1) genomic DNA from 60 mg of biomass previously dried for 48 hours in silicagel was extracted using the Invisorb™ Spin Plant Mini Kit (STRATEC Molecular GmbH, Berlin, Germany). An initial step was added for physically lysing cells by sonicator at 24 kHz for 8 min (Branson 5200; Branson Cleaning Equipment Company, Shelton, Conn.), with steel beads of 3 mm diameter and with the lysis buffer provided with the kit. DNA extraction of the desert strains from California was done with the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's protocol.

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) following the conditions described by Korelusová *et al.* (2009). 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) for the Czech and Russian strains and Stratagene cloning kit for the USA strains according to the manufacturer's instructions. Five colonies were chosen at random and PCR amplified using primers T7 and SP6 and following conditions described by Kaštovský *et al.* (2014). DNA sequencing was performed by the chain-termination method using Applied Biosystems BigDye™ Terminator Cycle Sequencing Kit v.3.1 (Life Technologies), according to the manufacturer's instructions. Sequences were deposited in GenBank under accession numbers KM268876-93.

Phylogenetic analyses:—A total of 142 OTUs, including sequences newly obtained by us together with representatives of main groups of heterocytous cyanobacteria available in GenBank, and two outgroup taxa (*Blennothrix* sp. AQS (EU586734) and *Chrococcidiopsis thermalis* Geitler 1933: 625 (AB039005)), were aligned using MAFFT v. 7 web server (http://mafft.cbrc.jp/alignment/server/, Katoh & Standley 2013) using default parameters. Phylogenetic calculations were run employing Bayesian inference in MrBayes 3.2.2 (Ronquist *et al.* 2012), maximum likelihood analysis in PhyML 3.0 (Guindon *et al.* 2010), and maximum parsimony analysis in SeaView 4.5.1 (Gouy *et al.* 2010). For the Bayesian analysis, two runs of eight Markov chains were executed for 5 million generations with default parameters, sampling every 100 generations (the final average standard deviation of split frequencies was lower than 0.01). The first 25% of sampled trees were discarded as burn-in. The ML tree was constructed applying the GTR+I+Γ model chosen according to Akaike Information Criterion provided by jModelTest 2 software (Darriba *et al.* 2012). A total of 1000 bootstrap replicate searches were conducted to evaluate the relative support of branches. A maximum parsimony analysis involved 1000 replicate searches to avoid local optima of most parsimonious trees, with starting trees obtained by random stepwise addition, using the tree bisection-reconnection (TBR) branch swapping algorithm. One thousand nonparametric bootstrap replications were run with the same settings to evaluate the relative branch support.

To resolve the relationships of our strains within the genus cluster, an alignment of the ITS regions containing no 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 12 non-identical sequences. This alignment was then concatenated to the associated 16S rRNA. Five additional strains in the ingroup were added with the ITS scored as missing data. A heuristic search in PAUP (Swofford 2004) utilizing parsimony as the optimality criterion, with multrees=yes, branch-swapping algorithm=TBR, gapmode=newstate, steepest descent=no, and nreps=10^4. Bootstrap support was based on running 10^4 replicates. The same alignment was used to run maximum likelihood analysis in PhyML 3.0 applying the GTR+I+Γ model. Finally, the same alignment was then modified to add a scoring of the indels in the ITS (1=base present, 0=base absent), and the mixed data alignment was then analyzed with MrBayes. Two runs of eight Markov chains were executed for 500 thousand generations with default parameters, sampling every 100 generations (the final average standard deviation of split frequencies was lower than 0.01). Posterior probabilities from the Bayesian analysis and bootstrap values from the first two analyses were mapped on to the Bayesian tree. The secondary structures of ITS regions were determined using a combination of comparative analysis and Mfold (Mfold web server, Zuker 2003).

The p-distances based on 16S rRNA sequence data between species and sister taxa outside of the genus were calculated in PAUP. P-distance based on the 16S-23S ITS sequence data was calculated just for the strains of *Roholtiella* for which operons lacking the tRNA genes were available.

Results

Taxonomic descriptions

Phylum **Cyanobacteria**
Class **Cyanophyceae**
Subclass **Nostocophycideae**
Order **Nostocales**

***Roholtiella* Bohunická, Pietrasiak *et* Johansen, *gen. nov.* (Figs. 1–4)**

Morphologically similar to *Calothrix*, from which it differs by having chains of arthrospores and not tapering to a hair and by living in freshwater and soil habitats rather than marine or brackish waters. Morphologically and phylogenetically close to *Calochaete*, from which it differs by not having cell division in more than one plane and in the markedly different structure of all conserved regions of the 16S-23S ITS region.

Type species:—*Roholtiella edaphica* Bohunická & Lukešová *spec. nov.*

Description:—Thallus flat, spreading, usually growing into the substrate. Filaments short (from 8 cells) to very long, single false-branched, less often also double false-branched. Young filaments heteropolar, often with terminal heterocyte or isopolar, tapered towards both ends, soon breaking in the middle between two heterocytes and becoming heteropolar. Mature filaments heteropolar, with basal heterocytes and distinctly tapered apices. Sheath thin, attached, colorless in rapidly growing cultures; dense, delimited, colorless to reddish in older filaments. Trichome constricted at the crosswalls, tapering when young, less evidently tapering or untapered when mature. Cells cylindrical or barrel-shaped, usually isodiametric, in some species shortened and compressed near the base or immediately after cell division, often widened in the basal part of trichome and narrower near the apices. End cells usually conical or conical rounded, slightly elongated. Heterocytes form in pairs in intercalary position, becoming terminal through breakage between paired heterocytes, sometimes solitary in intercalary position and giving rise to single false branching, hemispherical, barrel shaped, or almost spherical, with one or two pores, yellow, pale yellow or tan colored. Arthrospores with thick cell wall, typically forming in series and released from the ends of the filaments by disintegration of the sheath. Hormogonia with cells of smaller dimensions and typically 10 (8–16) cells long, likely germinating from arthrospores, observed in all stages of the culture.

Notes:—Macroscopically, the thallus looks like *Calothrix* when young and like *Nostoc* when old (rugged crumbly surface), which is also in accordance with the microscopic appearance. Heterocytes are not formed in standard media with supplied nitrogen. Microscopically most similar to *Calochaete cimrmanii*, which differs in that cell division in two planes was observed in the original material (Hauer *et al.* 2013, see fig. 1 L, M, O). In the description of *C. cimrmanii*, the authors stated that akinetes (called arthrospores in the Nostocaceae) were not present (Hauer *et al.* 2013). However, the authors show series of enlarged spherical cells at the trichome ends that bear some resemblance to the arthrospores of *Roholtiella* (Hauer *et al.* 2013, see fig. 1 D, E, L), although they consider these series of enlarged cells to be hormogonia as they become detached and maintain their filamentous integrity. The 16S-23S ITS sequence of *Calochaete* is not easily aligned with those in *Roholtiella,* and the secondary structures of the three conserved helices are notably different.

Etymology:—Named in honor of the champion for wilderness areas in California, Christopher Roholt.

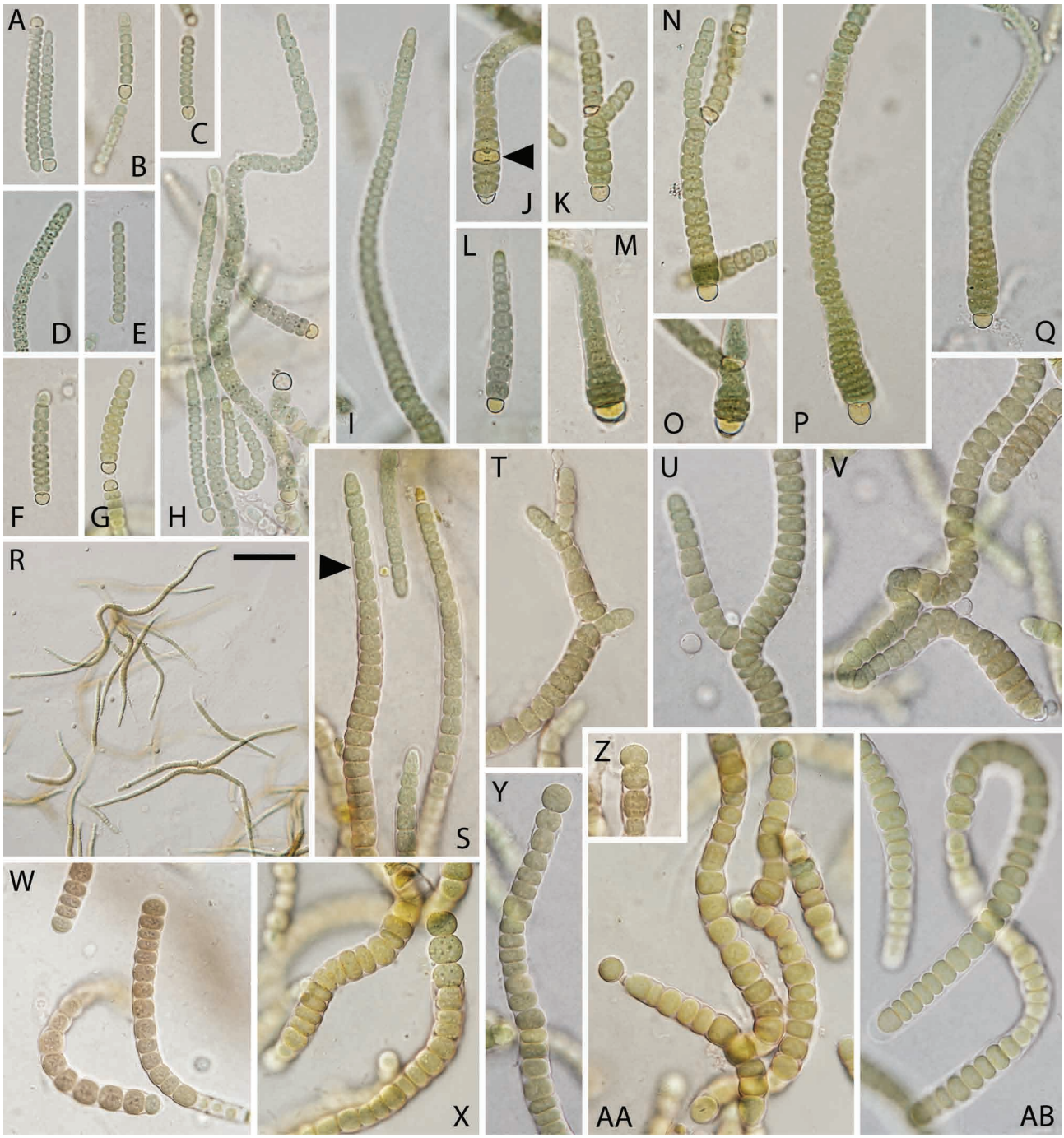

FIGURE 1. A–AB. *Roholtiella edaphica*, sp. nov. A–G. Hormogonia. H–I. Young tapered filaments with conical end cell. J–Q. *Calothrix*-like filaments with swollen base and basal heterocyte. J. Filament with intercalary heterocyte (marked with arrow). K, N–O. Filaments false branched at the heterocyte. R. Young filaments with isopolar growth. S–W. Mature filaments. S. Tapered mature filaments with brownish colored sheath, diffluent at the ends (marked with arrow). T–U. Single false branching. X–AB. Formation and releasing of arthrospores. Strains used in this figure: CCALA 1055 = I–J, Q, AB; CCALA 1056 = D–E, U–V, Y; CCALA 1060 = L, W; CCALA 1061 = A, H, R–T, Z; CCALA 1062 = B–C, F, M, O, X; CCALA 1063 = G, K, N, P, AA. Scale bar applies to all figures, in A–Q and S–AB = 20 µm, in R = 100 µm.

***Roholtiella edaphica* Bohunická *et* Lukešová, *spec. nov.* (Figs. 1A–AB, 4A–F)**

Differing from all other species in the genus through the evident swelling of the trichome near basal heterocytes in mature filaments. Further differing from *R. mojaviensis* in occurring in temperate, mesic soils and having olive coloration rather than deep blue-green coloration. Differing from all other species in the sequence of the flanking regions of the Box-B and V3 helices of the 16S-23S ITS region (Fig. 5).

Type:—USA. Sahara Number 6 Mine, Ashby-Kolar Research Plots, recultivated spoil after coal mining, without addition of top soil, west south-west of Harrisburg, IL. Dried specimen from culture of sample, *A. Lukešová, November 2009* (holotype: CBFS! A-041-1, Herbarium at the Department of Botany, Faculty of Science, University of South Bohemia, Czech Republic). Reference strain: CCALA 1063 (Culture Collection of Autotrophic Organisms at the Institute of Botany of the ASCR, Třeboň, Czech Republic).

Description:—Thallus flat spreading, sometimes with bundles of filaments visible on the substrate surface or *Nostoc*-like rugged surface when old, growing into the substrate, green, olive-green, brown-olive, brown-green, red-brown or black-green, sometimes releasing brown or brownish-red pigment into the substrate. Filaments short to long, single or rarely double false branched (Figs. 1K, N, T, U), isopolar (Fig. 1R) or heteropolar (Figs. 1J–Q). Sheath thin, firm (Figs. 1S–V), sometimes widened, closed at the ends (Fig. 1AB) or becoming diffluent towards the ends (Fig. 1S), or open when releasing arthrospores (Fig. 1Y), colorless, slightly pinkish, orange, red to red-brown. Trichomes constricted at crosswalls, not tapered or distinctly gradually tapered, slightly (Figs. 1J–K, N) or distinctly (Figs. 1M, O–Q) swollen at the base, widest cells basal or intercalary, 6.2–12.3 µm wide. Cells typically shorter than wide, sometimes isodiametric (Figs. 1H, AA) or slightly longer than wide, barrel-shaped to almost spherical or spherical compressed (Figs. 1M–Q), with smooth to finely granulated content, typically olive-green but also blue-green, grey-green, brown, green, orange-green or orange, 1.6–8.2(9.0) µm long. End cells conical (Figs. 1H–I), conical rounded or rounded (Figs. 1W–AB), 3.3–5.7(6.5) µm wide, 3.2–6.8 µm long. Heterocytes both intercalary (Fig. 1J) and terminal (Figs. 1K–Q), with smooth pale yellow content, when intercalary barrel shaped, shorter than wide, when terminal spherical, hemispherical or elongated rounded, pale yellow or yellow, 4.0–10.7 µm wide, 2.8–9.0 µm long. Hormogonia short (Figs. 1A–G), with cells 2.9–5.2(5.7) µm wide, 1.6–4.9 µm long. Arthrospores released from the end of the filament by dissociation (Figs. 1X–AA), typically 6.1–11.1 µm wide, 4.6–9.3 µm long. Necridic cells rarely present.

Habitat:—temperate climate soil.

Etymology:—From the Latin *edaphica* (= soil-inhabiting), referring to the habitat of origin of the taxon.

Other strains:—CCALA 1055 (CBFS! A-046-1), CCALA 1056 (CBFS! A-042-1), CCALA 1061 (CBFS! A-044-1), CCALA 1060 (CBFS! A-043-1), CCALA 1062 (CBFS! A-045-1)

Roholtiella bashkiriorum **Gaysina *et* Bohunická, *spec. nov.* (Figs. 2A–O)**

Morphologically most similar to *R. fluviatilis*, from which it differs by living on damp to dry soil rather than in rivers or water's edge of rivers. Differing from *R. mojaviensis* in occurring in temperate, mesic soils and having olive coloration rather than deep blue-green coloration. Differing from all other species in the sequence of the flanking regions of the Box-B and V3 helices of the 16S-23S ITS region (Fig. 5).

Type:—Russia. Macroscopic growth of algae and cyanobacteria on the path near river Ik on the edge of the village Bolsheustikinskoye, Republic of Bashkortostan, *L.A. Gaysina, August 2010* (holotype: CBFS! A-036-1). Reference strain: CCALA 1057.

Description:—Thallus flat spreading, slightly growing into the substrate, olive-green to brown, with rugged surface (*Nostoc*-like) when old. Filaments short to long, single (Figs. 2F, J) or more rarely double (Fig. 2I) false branched. Sheath thin, attached (Figs. 2J, M, N) or diffluent, colorless, trichomes sometimes curled inside the sheath. Trichomes constricted at crosswalls, not tapered to distinctly gradually tapered without swollen base (Figs. 2A, B, C, E, F), 6.6–9.8 µm wide in the widest portions. Cells shorter than wide to isodiametric, barrel-shaped to rounded, with smooth or finely granulated content, olive-green, grey-green or orange-green, 2.1–8.5 µm long. End cells conical rounded, 3.3–6.9 µm wide, 3.6–7.2 µm long (Figs. 2B, D–F). Heterocytes terminal hemispherical (Figs. 2 B, F) or intercalary cylindrical and often hemispherical in pairs before the filament breakage (Fig. 2K), yellow, 4.1–8.2 µm wide, 2.5–7.4 µm long. Arthrospores or short rows of arthrospores released from the ends of the filaments (Figs. 2G, H, M–O), spherical compressed to almost spherical, 6.2–10.5 µm wide and 4.9–9.0 µm long. Hormogonia 2.5–5.2 µm wide, 1.6–3.8 µm long (Fig. 2A).

Habitat:—soil.

Etymology:—*bashkiriorum* = of the Bashkirs, named for the Bashkir people of the Republic of Bashkortostan, Russia.

Other strain:—CCALA 1059 (CBFS! A-037-1)

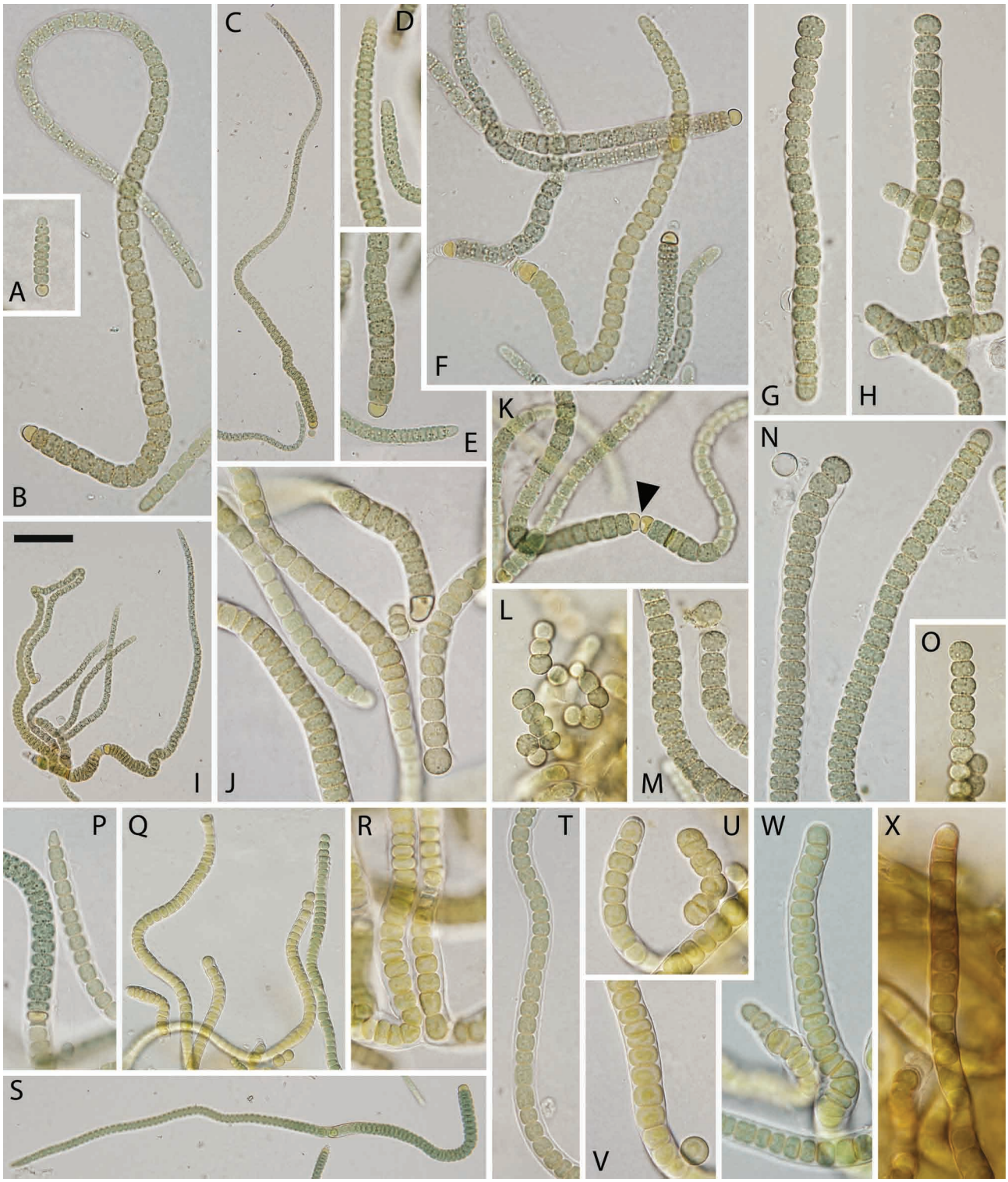

FIGURE 2. A–O. *Roholtiella bashkiriorum*, sp. nov. A. Hormogonium. B–E. Young tapered, *Calothrix*-like filaments. F. Branching at the heterocyte. I, K. Isopolar filaments with intercalary heterocyte(s), arrow marks a point of subsequent breakage. G–H, J, M–N. Mature filaments becoming arthrospores and being released from the opened ends. L, O. Rows of arthrospores. P–X. *Roholtiella fluviatilis*, sp. nov. P, S. Young tapered filaments. Q–X. Mature filaments and formation of arthrospores. R. Double false branching. U. Row of arthrospores. X. Filaments with reddish colored sheath. Strains used in this figure: CCALA 1057 = B–C, F, I–J, L, O, CCALA 1059 = A, D–E, G–H, K, M–N. CCALA 1058 = P–X. Scale bar applies to all figures, in A, B, D–H, J–P, R, T–X = 20 μm, in C, I, Q, S = 50 μm.

Roholtiella fluviatilis Johansen *et* Gaysina, *spec. nov.* (Figs. 2P–X)

Morphologically most similar to *R. bashkiriorum*, from which it differs by living on rocks in rivers or on sediment at the water's edge of rivers. Differing from all other species by being aquatic or hydroterrestrial. Differing from all other species in the sequence of the flanking regions of the Box-B and V3 helices of the 16S-23S ITS region (Fig. 5).

Type:—Russia. Macroscopic growth of algae and cyanobacteria on riverside of river Ik on the edge of the village Bolsheustikinskoye, Republic of Bashkortostan, *L.A. Gaysina, August 2010* (holotype: CBFS! A-038-1). Reference strain: CCALA 1058.

Description:—Thallus flat or unevenly spreading, olive-green, blackish-green to orange green. Filaments short to long, single (Fig. 2W) or sometimes double (Fig. 2R) false branched. Sheath thin, attached, sometimes widened, colorless, orange to reddish. Trichomes constricted at crosswalls, not tapered to clearly tapered *Calothrix*-like with basal heterocyte (Fig. 2S), not distinctly swollen at the base, 7.9–9.8 µm wide in the widest portions. Cells shorter than wide up to longer than wide, barrel-shaped, with smooth or finely granulated content, olive-green, blue-green, grey-green, orange-green to orange, 2.8–10.8 µm long. End cells rounded (Figs. 2U, W, X) or conical (Fig. 2P), 3.9–6.9 µm wide, 4.1–6.4 µm long. Heterocytes terminal hemispherical (Fig. 2S), or intercalary rounded cylindrical, yellowish, 4.3–7.4 µm wide, 3.2–6.8 µm long. Arthrospores or short rows of arthrospores released from the ends of the filaments (Fig. 2Q), with smooth, unevenly distributed cell content, spherical compressed to almost spherical. Hormogonia observed only rarely, 5.2–6.0µm wide, 2.8–4.0 µm long.

Habitat:—In mid-order streams or in hydroterrestrial communities at the water's edge of such streams.

Etymology:—From the Latin *fluviatilis* (= river-inhabiting), referring to the habitat of origin of the taxon.

Other strains:—UAM 332, UAM 334, UAM 337, UAM 340

Roholtiella mojaviensis Pietrasiak *et* Johansen, *spec. nov.* (Figs. 3A–S)

Differing from all other species by having shorter, narrower, more consistently tapered trichomes (Table 2) and bright blue-green pigmentation and desert soil habitat. Also differing from all other species in the sequence of the flanking regions of the Box-B and V3 helices of the 16S-23S ITS region (Fig. 5).

Type:—USA. Microscopic in sandy, gravelly soil from granitic outcrops, with plants and well-developed algal crusts, Joshua Tree National Park, Wonderland of Rocks, CA, *N. Pietrasiak, June 2006* (holotype: CBFS! A-039-1). Refence strain: CCALA 1051.

TABLE 2. Cell dimensions of *Roholtiella* strains investigated in detail. Reference strains for the type material are marked in bold. [x] row of arthrospores within the sheath, not yet released, (x) observed exceptionally.

Species	CCALA	Max cell	End cell		Cell	Heterocyte		Hormogonia		Arthrospores	
	Strain ID	width	width	length	length	width	length	width	length	width	length
R. edaphica	1055	6.4–10.3	4.1–4.8	3.2–6.4	2.0–7.5(9.0)	4.0–9.0	2.8–9.0	3.4–5.2	1.6–4.1	[7.0–10.2]	[3.3–8.5]
R. edaphica	1056	6.9–10.0	3.8–5.7(6.5)	3.6–6.4	2.0–7.2(9.0)	4.6–7.5	3.0–7.5	3.0–4.0	2.0–4.0	8.2–9.5	6.6–8.7
R. edaphica	1060	6.6–8.2	3.3–5.4	3.2–5.9	2.1–5.7	4.6–6.9	3.3–6.4(8.5)	3.3–4.4	1.7–4.1	6.1–8.2	[2.9]–8.2
R. edaphica	1061	6.2–10.1	3.3–5.4	3.2–6.8	3.1–8.2	4.1–8.0	2.5–7.0	2.9–4.2	1.6–4.9	7.8–9.8	[3.3]5.6–9.3
R. edaphica	1062	7.7–12.3	3.6–4.9	3.6–6.4	2.3–6.4	4.1–10.7	3.0–7.2	3.6–5.0	2.0–3.3	6.2–11.1	[4.1]–8.5
R. edaphica	**1063**	7.9–11.0	4.1–4.9	3.2–5.5	1.6–5.7	4.8–8.0(10.1)	2.8–7.5	(5.2–5.7)	(2.1–3.3)	6.9–9.0	4.6–9.3
R. bashkiriorum	**1057**	6.6–9.8	3.3–6.6	4.1–7.2	3.0–8.2	4.6–8.2	2.5–7.4	2.5–4.0	1.6–3.0	6.2–10.2	5.0–9.0
R. bashkiriorum	1059	6.6–9.5	3.9–6.9	3.6–6.4	2.1–8.5	4.1–7.2	3.3–4.9	3.6–5.2	1.6–3.8	8.2–10.5	4.9–7.9
R. fluviatilis	**1058**	7.9–9.8	3.9–6.9	4.1–6.4	2.8–10.8	5.9–8.4	3.9–7.2	(5.2–6)	(2.8–4.0)	6.1–10.4	3.9–10.0
R. mojaviensis	**1051**	5.7–9.6	3.3–4.6	3.2–5.5	1.6–6.6	4.3–7.4	3.2–6.8	2.8–3.6	2.0–3.6	5.6–7.9	4.1–7.5
R. mojaviensis	1052	5.7–8.4	2.8–5.2	3.2–6.4	2.1–7.3	N/A	N/A	2.9–4.2	1.6–4.1	4.5–4.9	4.9–9.0

Description:—Thallus evenly flat, spreading, appressed to the agar surface, growing into the substrate, blue-green to olive-green, rarely turning brown. Filaments short, sometimes single (Figs. 3E, K) or more rarely double (Fig. 3D) false branched. Sheath thin, attached, rarely widened and lamellated (Figs. 3L, S), firm, colorless to slightly pinkish (Fig. 3S). Trichomes constricted at crosswalls, slightly to distinctly tapered towards ends, commonly *Calothrix*-like with basal heterocyte at the slightly widened base, 5.7–9.6 µm wide. Cells shorter than wide, rarely longer than wide, cylindrical, barrel-shaped or compressed spherical, with smooth content, blue-green to olive-green, 1.6–7.3 µm long. End cells conical-rounded (Figs. 3B–C, I) or conical (Figs. 3A, D, S), 2.8–5.2 µm wide, 3.2–6.4 µm long. Heterocytes terminal, hemispherical or slightly conical, rarely intercalary in pairs, yellow or tan, 4.3–7.4 µm wide, 3.2–6.8 µm long. Arthrospores or rows of arthrospores released from the ends of the filament, with thick cell wall, almost spherical, olive-green, 5.6–7.9 µm wide, 4.1–9.0 µm long. Hormogonia constricted at crosswalls, typically 8–12 celled (Fig. 3A), without sheath or with sheath thin, attached, with cells 2.8–4.2 µm wide, 1.6–41 µm long.

Habitat:—desert soil.

Etymology:—From the Latin *mojaviensis* (= from Mojave Desert, USA), referring to the geographical origin of the taxon.

Other strain:—CCALA 1052 (CBFS! A–040-1)

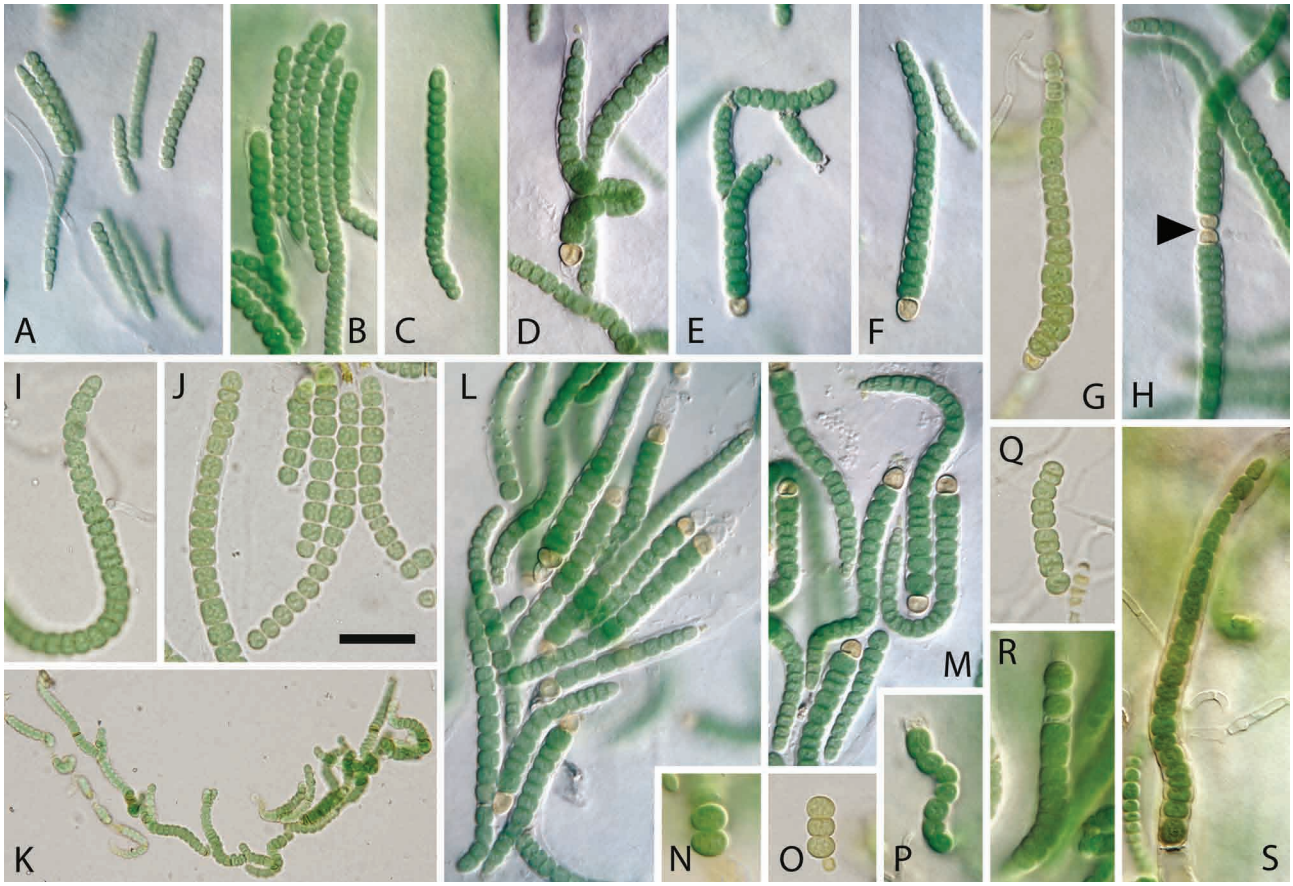

FIGURE 3. A–S. *Roholtiella mojaviensis*, sp. nov. A. Hormogonia. B–C. Maturing hormogonia. D–H, L–M. Tapered *Calothrix*-like filaments. E. False branching. H. Isopolar filament with two adjacent intercalary heterocytes, arrow marks a point of subsequent breakage. I–J. Mature filaments with near-spherical cells. K. False branching. N–Q. Rows of arthrospores. R. Releasing of arthrospores from the opened end of the filament. S. Mature tapered filament with thick structured colored sheath. Strains used in this figure: CCALA 1051 = A, D–I, K–O, Q–R, CCALA 1052 = B–C, J, P, S. Scale bar applies to all figures, in A–J and L–S = 20 µm, in K = 50 µm.

Ultrastructure

The ultrastructure of a selected *Roholtiella* strain (CCALA 1061) was examined in TEM (Fig. 4). The most conspicuous characteristic of the thin sections is the thylakoid arrangement in which the thylakoid membranes form dense entangled curves and spirals filling almost all of the cell content, leaving only a small interthylakoidal space in the central part of the cell (Figs. 4A–F). The nature of the hyaline mucilaginous sheath is evident (Figs. 4A–C), as is the formation

of crosswalls and position of carboxyzomes (Fig. 4C). While the thylakoid structure is fairly unique in appearance, in general it is congruent with the irregular thylakoid structure of other heterocytous cyanobacteria (compare e.g. Brito *et al.* 2012).

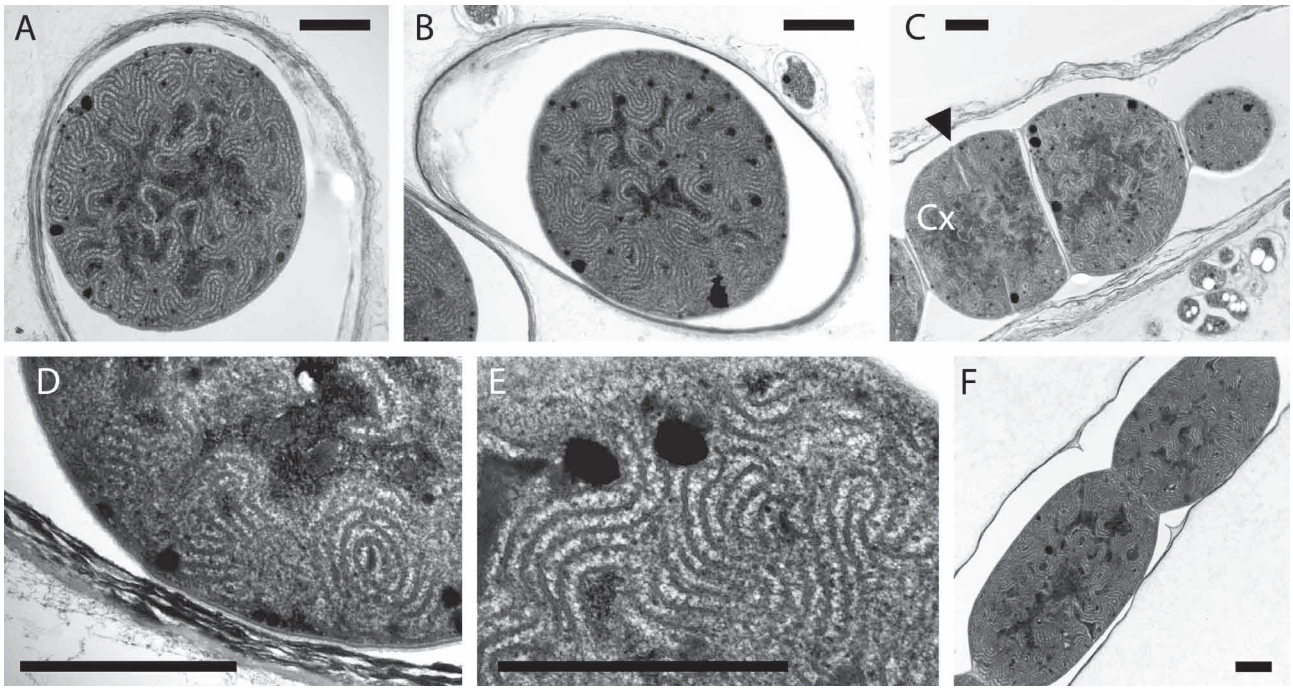

FIGURE 4. Ultrastructure of *Roholtiella*, strain CCALA 1061 *R. edaphica* in TEM. A–B. Cross-section of the vegetative cell. C. Longitudinal section of the filament surrounded by mucilaginous sheath, new cross-wall formation marked with arrow. D–E. Detail of the cell content and arrangement of thylakoids. F. Elongated cells in a young filament, longitudinal section. Cx = carboxyzome. Scale bars = 1 μm.

Operons with no tRNA genes

```
                                bp128                          bp154       bp186       bp199
                                *                              *           *           *
R. fluviatilis CCALA 1058       ATAATCATTACAAGTATTGGAAATAGTAA...BoxB...TTAT-TCCAGCCA...
R. bashkiriorum CCALA 1057, 1059 AGAATCATCAAAAGTACAGGGAATAGTAC...BoxB...AGAA-TCCAGCCA...
R. mojaviensis CCALA 1055, 1056 ACAATCATARAAACTACTGGGAATAGTG-...BoxB...TAATRTCCAGCCA...
R. edaphica CCALA 1055-1060     AAAATCATAAAAACTACTGGGAATAGTG-...BoxB...TATTGTCCAGCCA...
R. edaphica CCALA 1062          AAAATGATAAAAACTACTGGGAATAGTG-...BoxB...TATTGTCCAGCCA...

                                          bp226     bp237    bp330              bp354
                                          *         *        *                  *
R. fluviatilis CCALA 1058       BoxA-D4...GA-AAAAAGCAG...V3...AGTGGTGAA-ACCAATTGTTTAAGT
R. bashkiriorum CCALA 1057, 1059 BoxA-D4...GAAAAAAAGCAG...V3...AGTGGTGAA-ACCAA--TGAATTGT
R. mojaviensis CCALA 1055, 1056 BoxA-D4...GA-AAAAAGCAG...V3...AGTGGTGAA-ACCAAT-TGTTTAGT
R. edaphica CCALA 1055-1060     BoxA-D4...GATTTAAAGCAG...V3...AGTGGTGAACACCAAA-TGTATWGT
R. edaphica CCALA 1062          BoxA-D4...GATTTAAAGCAG...V3...AGTGGTGAACACCAAA-TGTATTGT
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Operons with both tRNA genes

```
                                bp414                                                  bp472       bp510
                                *                                                      *           *
R. fluviatilis CCALA 1058       ACTTTTTGTGGCGGAGCAGACAGTGTGATACAATTAAAGATTGTGGATAAA...BoxB...TAATA
R. bashkiriorum CCALA 1057      ACTTTTTGTGGCGGAGCAGACAGTGTGATAAAATTAAAGATTGTGGATAAA...BoxB...-AGAA
R. mojaviensis CCALA 1056       GTTTTTTGTGGCGAAACAACCAGTGTGATAAAATCAAAGATTGTGGATAAA...BoxB...TAATA
R. edaphica CCALA 1055, 1060    AATTTTTGTGGCGAAACAAATAGTGTGATAYAGTTARAGATYGTCGATAAA...BoxB...TATTG
R. edaphica CCALA 1062          AATTTTTGTGGCGAAACAAACAGTGTGATATAGTTAAAGATTGTCGATAAA...BoxB...TATTG

                                bp515  bp522          bp551     bp560     bp653              bp676
                                *      *              *         *         *                  *
R. fluviatilis CCALA 1058       TCCAGCCA...BoxA-D4...-AAAAAGCAG...V3...AGTGGTGAAACCAATTGTTTAAGT
R. bashkiriorum CCALA 1057      TCCAGCCA...BoxA-D4...AAAAAAGCAG...V3...AGTGGTGAAACCAATGGATT--GT
R. mojaviensis CCALA 1056       TCCAGCCA...BoxA-D4...-AAAAAGCAG...V3...AGTGGTGAAACCAATTGTTTA-GT
R. edaphica CCALA 1055, 1060    TCCAGCCA...BoxA-D4...TTTAAAGCAG...V3...AGTGGTGAACACCAAATGTATWGT
R. edaphica CCALA 1062          TCCAGCCA...BoxA-D4...TTTAAAGCAG...V3...AGTGGTGAACACCAAATGTATTGT
```

FIGURE 5. Sequence of 16S-23S ITS flanking regions of the BoxB and V3 helices for *Roholtiella* species. Base pair position shown above sequences, with sequences of BoxB, BoxA-D4, and V3 not shown. Variable bases shaded in gray.

Molecular sequence analyses

Roholtiella forms a well-supported (0.98 posterior probability) monophyletic clade (Fig. 6A, top cluster) within the Nostocaceae (Fig. 6A, c3). The *Roholtiella* clade contains a total of 17 OTUs, including 11 new isolates from several localities (one strain is represented by two sequences), four strains characterized previously (Berrendero *et al.* 2011), and one other sequence obtained from the GenBank sequence database (see also Table 1). *Roholtiella* is part of a subcluster of strains in the Nostocaceae that have been a source of taxonomic confusion in the literature (Fig. 6A, c1). This subcluster includes forms that taper (*Calochaete*, "*Calothrix*", *Microchaete diplosiphon* Gomont *ex* Bornet & Flahault 1888a: 84) as well as forms that possess false branching ("*Tolypothrix*"). It is clearly more affiliated with the Nostocaceae based on phylogenetics, but is distant from the Tolypotrichaceae clade that contains *Tolypothrix sensu stricto* (*T. distorta* Kützing *ex* Bornet & Flahault 1888a: 119 is the type, see Fig. 6A, c5) and from the Rivulariaceae clade that contains *Calothrix sensu stricto* (Fig. 6A, c6). The Nostocaceae in our analysis is clade c3 (Fig. 6A, c3), sister to the Aphanizomenonaceae (Fig. 6A, c4) as defined in Komárek *et al.* (2014). Clade c2 contains the type species for *Nostoc, N. commune* Vaucher *ex* Bornet & Flahault (1888b: 181) (Fig. 6A, c2). Clade c1, to which *Roholtiella* belongs, may eventually be recognized at the family level if more consistent morphological and phylogenetic separation is established.

An independent analysis was performed using an alignment of the 16S rRNA gene concatenated with the 16S-23S ITS region (Fig. 6B). Four separate supported clusters were evident within the genus. The first cluster contains the strain *Roholtiella fluviatilis* CCALA 1058 isolated from a hydroterrestrial habitat on the bank of the River Ik in Bashkortostan, Russia, together with four strains isolated from rocks within streams in Spain (the UAM strains) and one strain from a greenhouse in Sweden (PCC 7415). We consider the UAM strains to be conspecific with *R. fluviatilis,* although in GenBank they are listed as *Tolypothrix* species. *Tolypothrix* PCC 7415 had a long branch within the clade and was morphologically distinct from *R. fluviatilis,* and so at this time the evidence is less convincing that PCC 7415 belongs in *R. fluviatilis.* The second cluster is formed by two strains of *R. bashkiriorum* (CCALA 1057, 1059) isolated from soils in the Ik River watershed in Bashkortostan, Russia. The largest cluster includes six newly isolated strains of *Roholtiella edaphica* from dry soils in Russia, Czech Republic, and USA (strains CCALA 1055, 1056, 1060–1063). The last cluster consists of two strains of *Roholtiella mojaviensis* (CCALA 1051, 1052) from Mojave Desert soil, of which one is represented by two varying operons. All four of the clusters are highly supported in all three analyses (BI/ML/MP).

Sequence similarity of the 16S rRNA gene was ambiguous in resolving species relationships in *Roholtiella* as well as in resolving genera in the Nostocales (Table 3). All *Roholtiella* species were above 97.9% similar, meaning they were above the similarity cut-off which is considered clear evidence of speciation (97.5%). The two representative sister taxa were 96.9–98.8% similar to *Roholtiella* species, which is above the similarity cut-off which is considered clear evidence for recognition of genera (95%). However, the distantly related *Tolypothrix distorta* in a separate family, the Tolypothrichaceae, was also above this cut-off (95.2–96.1% similar to *Roholtiella*). Only *Calothrix sensu stricto* was below 95% similar (Table 3), and it was very distant from all Nostocaceae (Fig. 6A).

The p-distance based on an alignment of the 16S-23S ITS region within and between species of *Roholtiella* was consistent with the phylogenetic analysis (Fig. 6B) and with past studies in which cryptic species were identified using this method (Erwin & Thacker 2008, Osorio-Santos *et al.* 2014, Pietrasiak *et al.* 2014). P-distance within species in past studies has always been less than 4.00, with mean values below 2.00. P-distance between species is typically over 7.00. This provides a much clearer separation of taxa as the discontinuity between species is fairly clear. *Roholtiella edaphica* CCALA 1062 was fairly different from other strains in that species cluster, and when this species was excluded, the p-distance within the species was much reduced (Table 4). However, even with this strain of *R. edaphica* left in, the discontinuity between species was clear and unambiguous.

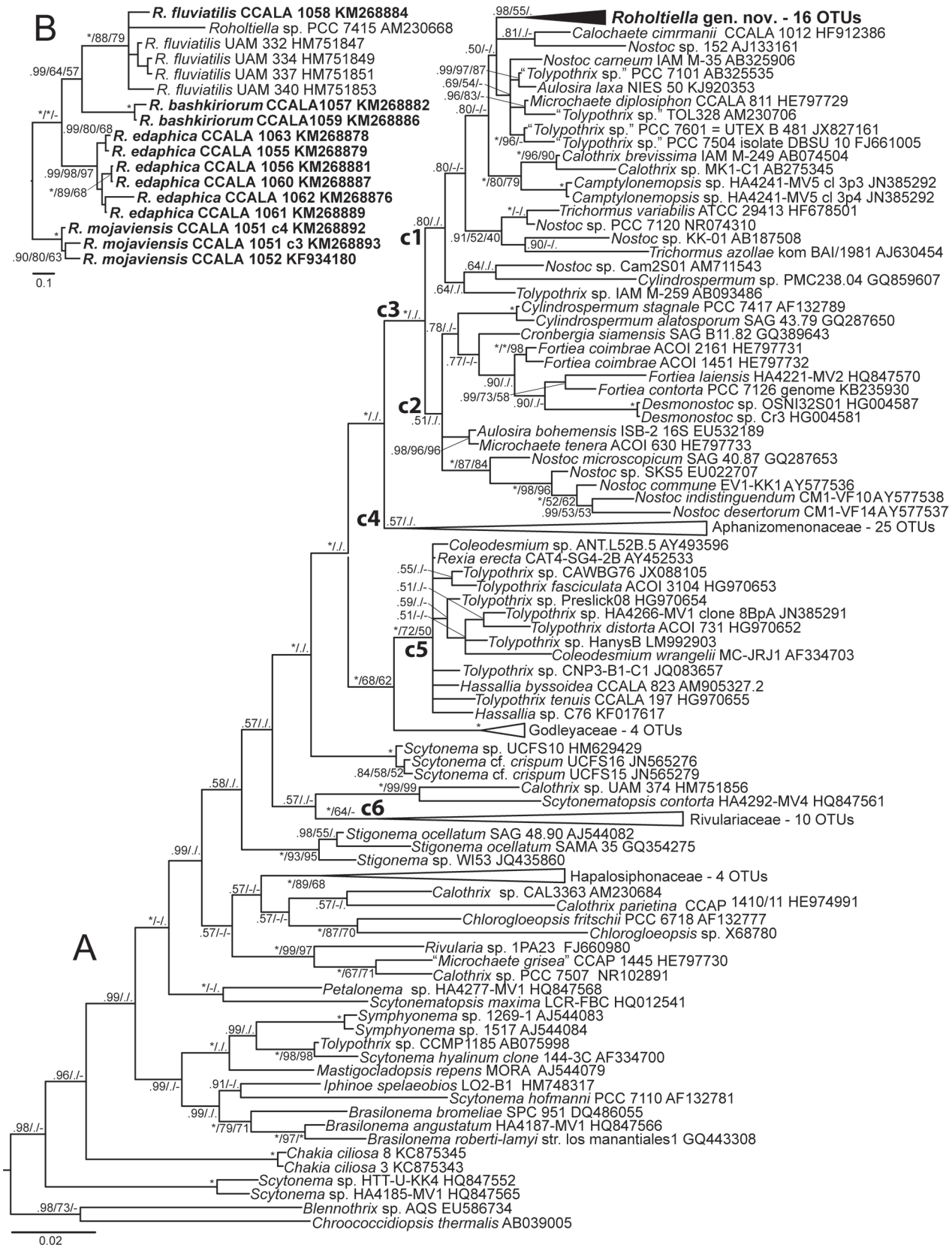

FIGURE 6. A. Phylogenetic analysis based on 16S rDNA sequences of 142 OTUs demonstrating position of *Roholtiella*, *gen. nov.* B. Phylogenetic analysis based on concatenated 16s rDNA and 16S-ITS rDNA (operon lacking sequence for $tRNA^{Ile}$ and $tRNA^{Ala}$) sequences including all members of the *Roholtiella* clade shown in Fig. 6A. The trees are both based on Bayesian topology and the support values are given for Bayesian posterior probabilities, maximum likelihood, and maximum parsimony (BI/ML/MP). The cut-off values for bootstrap and probability are 50 and 0.5, respectively. Clades c1-c5 represent different taxonomic groups at the family or subfamily level (see text).

TABLE 3. Percent similarity of the 16S rRNA gene of all known strains of *Roholtiella* with outgroup taxa *Calochaete cimrmanii* CCALA 1012 and *Aulosira laxa* NIES 50. Strains sequenced specifically for this study are in bold font. Additional strains 100% identical to first strain listed are indicated in parentheses.

	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13
1	***R. edaphica* CCALA 1056 (CCALA 1060)**													
2	***R. edaphica* CCALA 1055 (CCALA 1063)**	99.8												
3	***R. edaphica* CCALA 1061**	99.9	99.9											
4	***R. edaphica* CCALA 1062**	99.8	99.8	99.9										
5	***R. bashkiriorum* CCALA 1057**	99.5	99.7	99.6	99.5									
6	***R. bashkiriorum* CCALA 1059**	99.8	100	99.9	99.8	99.7								
7	***R. fluviatilis* CCALA 1058 (**UAM334, UAM337, UAM332**)**	99.5	99.5	99.6	99.5	99.2	99.5							
8	*R. fluviatilis* UAM340	99.2	99.2	99.3	99.2	98.8	99.2	99.7						
9	***R. mojaviensis* CCALA 1051 (CCALA 1052)**	99.2	99.2	99.3	99.2	98.8	99.2	98.8	98.7					
10	*"Tolypothrix"* sp. PCC 7415 (= *Roholtiella* sp.)	98.3	98.3	98.4	98.3	98.0	98.3	98.8	98.5	97.9				
11	*Calochaete cimrmanii* CCALA 1012	98.1	98.1	98.2	98.1	98.0	98.1	97.8	97.7	98.4	96.9			
12	*Aulosira laxa* NIES50	98.2	98.2	98.3	98.2	97.9	98.2	97.9	98.0	98.4	97.0	97.8		
13	*Tolypothrix distorta* ACOI 731	95.9	96.0	96.1	96.0	95.9	96.1	95.9	95.6	95.9	95.2	96.0	95.3	
14	*Calothrix* sp. RM201	93.7	93.8	93.9	93.8	93.7	94.0	93.6	93.8	94.3	92.3	93.3	93.0	92.8

TABLE 4. P-distance (expressed as percent dissimilarity) among *Roholtiella* species using aligned 16S-23S ITS sequences. Mean is given with range in parentheses. Numbers in bold along the diagonal reflect P-distance within the species when multiple strains are available. Note: internal similarity of *R. edaphica* is 0.82 (0.00–2.02) when CCALA 1062 is excluded.

	edaphica	*bashkiriorum*	*fluviatilis*	*mojaviensis*
R. edaphica	**1.42 (0.00–3.20)**			
R. bahskiriorum	9.33 (8.19–11.69)	**0.58 (0.58)**		
R. fluviatilis	9.44 (7.31–12.00)	9.68 (9.68)	**NA**	
R. mojaviensis	6.67 (3.82–9.61)	7.31 (6.73–7.90)	8.17 (7.87–8.46)	**0.58 (0.29–0.87)**

16S-23S ITS Secondary Structure

The secondary structure of the D1–D1' helix of the ITS region was very similar among all *Roholtiella* species, but substantially different from representative sister taxa (Fig. 7). Two equally stable structures were possible for most species (see Figs. 7A–B). One operon of *R. mojaviensis* CCALA 1055 had a slightly different structure in the basal portion of the helix, while *R. edaphica* CCALA 1063 had an additional mismatching base pair in the center of the helix (Figs. 7A–B). Apart from these minor differences, the secondary structures of the D1–D1' helices were identical in *Roholtiella*. The structure of the BoxB helix was consistent within operons in *Roholtiella*. In the operons containing $tRNA^{Ile}$ and $tRNA^{Ala}$, the stem of the helix had a mismatch between A and AC (or CC) which caused a small bilateral bulge, and had a larger terminal loop (Figs. 8A–F). In operons not containing tRNA genes, the stem of the helix had a more prominent bilateral bulge and smaller terminal loop (Figs. 8G–K). The BoxB structures recovered for *Calochaete cimrmanii* and *Nostoc indistinguendum* Řeháková & Johansen in Řeháková *et al.* (2007: 485) were noticeably different (Figs. 8F, L, M). The V3 helices for *R. fluviatilis* and *R. bashkiriorum* were identical in structure (Figs. 8Q, R), and

these helices were very similar to the V3 helices in *R. mojaviensis* (Fig. 8P). *Roholtiella edaphica* was the most different in terms of sequence and structure, especially strain CCALA 1062 (Figs. 8N, O). The *Roholtiella* V3 helices were substantially different from those in *C. cimrmanii* and *N. indistinguendum* (Figs. 8S–T).

The four species of *Roholtiella* were best distinguished by the sequences flanking the BoxB and V3 helices (Fig. 5). These regions have received scant attention in other studies, but they were significant in our taxa. These flanking regions are likely what led to the phylogenetic analysis being as clear as it was (Fig. 6B). We recommend use of these sequences to diagnose the species of *Roholtiella* discovered thus far.

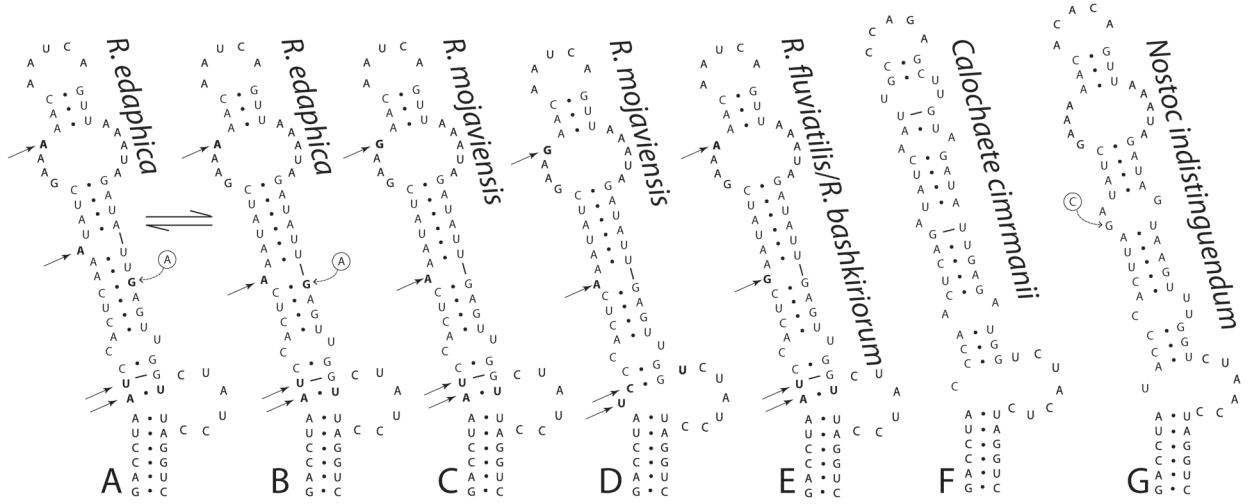

FIGURE 7. Secondary structures for the D1–D1' helix in the ITS regions for *Roholtiella* spp. and representative outgroup taxa, *Calochaete cimrmanii* and *Nostoc indistinguendum*. Arrows and a bold font style show bases variable among species, while the circled adenine residue is an alternate base in *R. edaphica* CCALA 1063 and circled cytosine is an alternate base in *N. indistinguendum*. A–B. Equally thermodynamically stable structures in *R. edaphica* are shown for the following strains: CCALA 1061 for the operon with no tRNA genes; CCALA 1055-56, 1060, 1062 for the no tRNA operon as well as the operon with both tRNA genes. C–D. Differences in operons are shown for *R. mojaviensis*. C. Operon with no tRNA for CCALA 1051 and 1052 as well as the operon with both tRNA for CCALA 1052. D. Variation of the operon with no tRNA of CCALA 1051. E. No sequence differences existed between operons with no or with both tRNAs for *R. fluviatilis* (CCALA 1058), and *R. bashkiriorum* (CCALA 1057, 1059). F. D1–D1' helix for *C. cimrmanii* (strain CCALA 1012) showing the operon with no tRNA. G. D1–D1' helix for recovered operons with and without both tRNAs for *N. indistinguendum* (strain CM1-VF10).

Discussion

In a broader study of the cyanobacterial types morphologically belonging to the traditional family Microchaetaceae (Hauer *et al.* 2014), we encountered a group of genetically closely related strains characterized by morphological features not fully corresponding with any genus described thus far. The morphology of these strains varied throughout their life cycle. Typical small-celled hormogonia grew into clearly tapered, heteropolar filaments with terminal heterocytes, strongly resembling *Calothrix*. In this early phase, strains were often also false-branched. During aging, the tapering became less evident and a series of arthrospores, similar to those known in *Nostoc,* were formed. Arthrospores were later released from the mucilaginous sheath. Subsequent germination of arthrospores into small-celled hormogonia was anticipated, although never directly observed. The phylogenetic analysis revealed that these strains form a well-supported monophyletic cluster within the family Nostocaceae, and therefore we described these new types as genus *Roholtiella*.

The four species within *Roholtiella* were well supported in the analysis based on 16S rRNA gene sequence concatenated with the 16S-23S ITS region. The species separation was evident also in the analysis of 16S rRNA gene only, although the individual branches were very short and not well supported (not shown in this study). The failure of the 16S rRNA gene to resolve species was not surprising considering that the members of the *Roholtiella* cluster were at least 98.7% similar (97.9% if strain PCC 7415 is included) to each other in their 16S rRNA sequence (Table 3). This result is in agreement with other studies suggesting that the artificial limits set for species and genera (97.5% and 95% rRNA sequence similarity, respectively) proposed for prokaryotic taxa by Stackebrandt and Goebel (1994) are too low

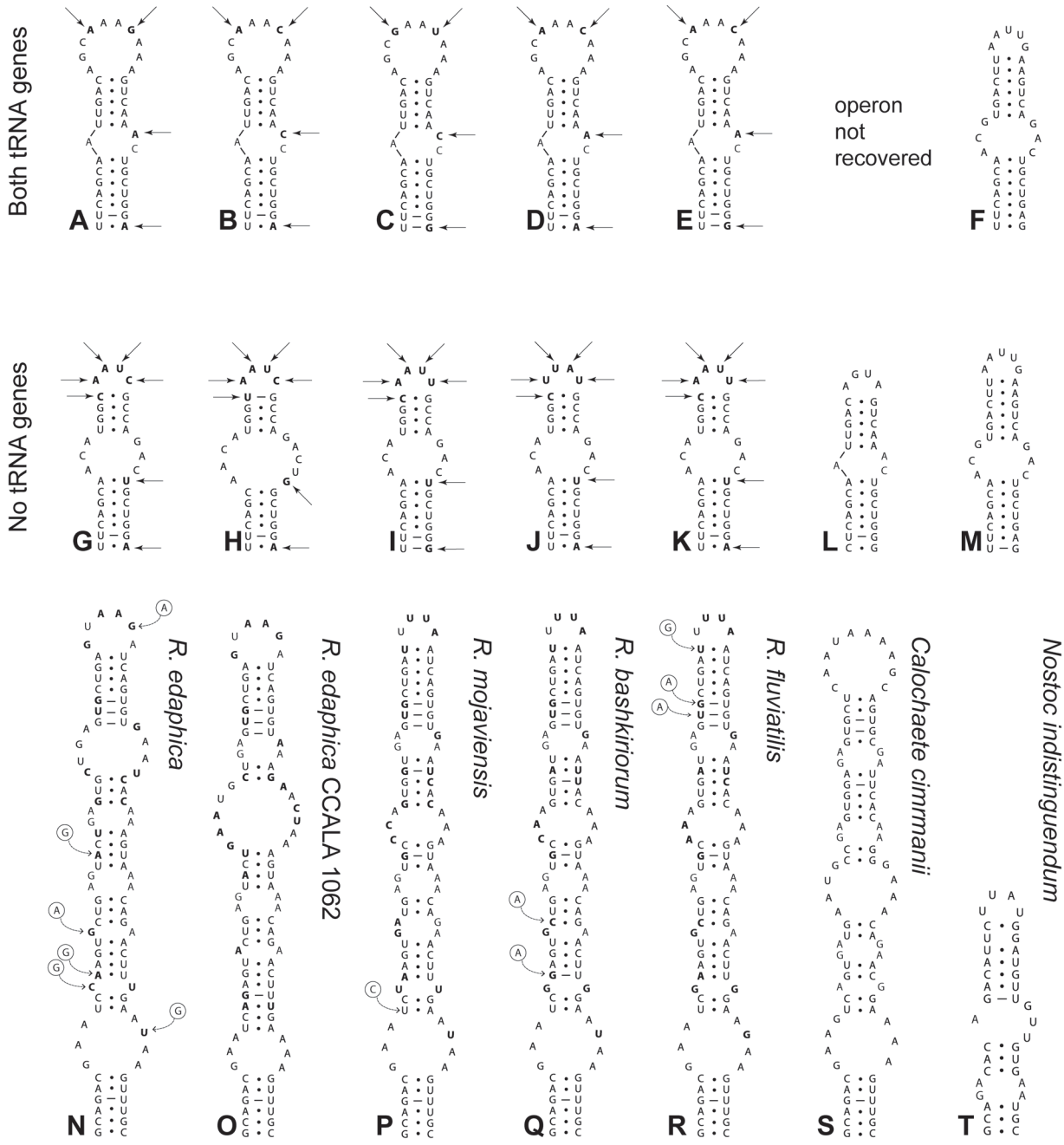

FIGURE 8. Secondary structures for the BoxB and V3 helices in the ITS regions for *Roholtiella* spp. and representative outgroup taxa, *C. cimrmanii* and *N. indistinguendum*. Arrows and a bold font style show bases variable among species. Helices are arranged by taxon in vertical columns, with taxon label next to the V3 helix. A–F. BoxB helices from operons with both tRNA genes. A. *Roholtiella edaphica* strains CCALA 1055-56, CCALA 1060-61. B. *Roholtiella edaphica* strain CCALA 1062. C. *Roholtiella mojaviensis* strains CCALA 1051-52. D. *Roholtiella bashkiriorum* strains CCALA 1057 and 1059. E. *Roholtiella fluviatilis* strain CCALA 1058. F. *Nostoc indistinguendum* strain CM1-VF10. G–M. BoxB helices from operons with no tRNA genes. G. *Roholtiella edaphica* strains CCALA 1055 and 1060. H. *Roholtiella edaphica* strain CCALA 1062. I. *Roholtiella mojaviensis* strain CCALA 1052. J. *Roholtiella bashkiriorum* strain CCALA 1057. K. *Roholtiella fluviatilis* strain CCALA 1058. L. *C. cimrmanii* strain CCALA 1012. M. *Nostoc indistinguendum* strain CM1-VF10. N–T. V3 helices. Circled residues represent alternate bases in different strains, or in the case of *R. fluviatilis,* between different operons in the same strain. N. The structure without the circled bases was obtained for *R. edaphica* strains CCALA 1060 (no and both tRNA operons) and CCALA 1056 (no tRNA operon).The alternative structure with the circled bases was obtained for strains CCALA 1061 (no tRNA operon) and CCALA 1055 (no and both tRNA operons). O. *Roholtiella edaphica* strain CCALA 1062. P. *Roholtiella mojaviensis* strains CCALA 1051-52. Q. The structure without the circled bases was obtained for *R. bashkiriorum* strains CCALA 1057 (both tRNA operons) and CCALA 1059 (no tRNA operon). The alternative structure with the circled bases was obtained for strain CCALA 1057 (no tRNA operon). R. The structure without the circled bases was obtained for *R. fluviatilis* strain CCALA 1058 for the operon with both tRNA. The alternative structure with the circled bases was obtained for the operon with no tRNA. S. *C. cimrmanii* strain CCALA 1012 showing the operon with no tRNA T. *Nostoc indistinguendum* strain CM1-VF10 for recovered operons with and without both tRNAs.

for Nostocaceae (Flechtner *et al*. 2002, Casamatta *et al.* 2006, Johansen *et al.* 2014, Kaštovský *et al*. 2014, Řeháková *et al.* 2014). Due to the insufficient amount of informative sites in the 16S rRNA gene in this group, we believe that the use of ITS sequence together with the 16S is a good tool for the recognition of species (Erwin & Thacker 2008, Perkerson *et al.* 2011, Osorio-Santos *et al.* 2014, Pietrasiak *et al.* 2014).

The sister taxon of *Roholtiella* was not unambiguously resolved, but close relatives include *Calochaete*, *Aulosira laxa* Kirchner *ex* Bornet & Flahault (1888b: 256), and taxa incorrectly assigned to *Microchaete*, *Nostoc,* and *Tolypothrix*. Further removed were *Camptylonemopsis* Desikachary (1948: 46), *Trichormus* (Ralfs *ex* Bornet & Flahault 1888b: 226) Komárek & Anagnostidis (1989: 303), and taxa incorrectly assigned to *Calothrix* and *Tolypothrix.* The Nostocaceae has in the past been considered to lack forms that possess either tapering or false-branching and this historical morphological assumption is likely what has led to the misidentification of several of the strains in this subcluster. As recently shown by Hauer *et al.* (2014) and also following from our phylogenetic analyses, the family Tolypothrichaceae and the typical genus *Tolypothrix* as well as the family Rivulariaceae and the genus *Calothrix* are genetically quite distant. Also *M. diplosiphon* CCALA 811 was recently found to not belong to *Microchaete sensu stricto* (Hauer *et al.* 2014), although it was not placed in a different genus. One of the "*Tolypothrix*" strains, strain PCC 7415, which was deposited into the Pasteur Culture Collection as "*Calothrix*" was according to our results a species within *Roholtiella*. Although it belongs to a supported clade for *R. fluviatilis*, we have decided to not assign it to this species, as it was genetically relatively dissimilar based on 16S rRNA gene sequence (up to 1.5% distant, compared to other members of the species which were maximally 0.3% distant, see Table 3). Moreover, the morphology of this strain was not typical, e.g. the sheath was lacking (not shown here), which is likely a result of long-term cultivation in the PCC collection.

Most of the above mentioned close relative strains are inadequately characterized and ambiguously identified. The exceptions are *Aulosira laxa,* which was examined by us and found to fit the original description both morphologically and ecologically (not published), and *Calochaete cimrmanii,* which was fully characterized and recently described by Hauer *et al.* (2013). Of these two genera, *Calochaete* is very similar to *Roholtiella* in the morphological features of the life cycle stages (tapering, releasing arthrospores in series from the trichome end, heteropolar filaments) and habitat from which it was described (soil). However, *Roholtiella* and *Calochaete* are here recognized as separate genera based on one morphological feature (*Calochaete* has cell division in two planes), molecular distance, and secondary structure of the 16S-23S ITS region. In the phylogenetic analyses, *Calocheate* was always close to the cluster of *Roholtiella*, but these two genera never formed a single supported clade. Also the analysis of 16S-23S ITS secondary structures revealed significant differences between these two genera in all three studied helices (Figs. 7, 8).

This is the fourth study to use the p-distance within the 16S-23S ITS region among cyanobacterial strains within a genus to recognize species boundaries in morphologically cryptic species (Erwin & Thacker 2008, Osorio-Santos *et al.* 2014, Pietrasiak *et al.* 2014). This method is proving to be very effective. Erwin and Thacker (2008) first used p-distance of the 16S-23S ITS region to recognize cryptic species within invalidly described *Synechococcus spongiarum* Usher *et al.* (2004: 190), but they did not recognize the 12 distinct clades within the species taxonomically. Both Osorio-Santos *et al.* (2014) and Pietrasiak *et al.* (2014) did recognize the cryptic species they identified using Erwin and Thacker's metric. Even among very different clades within the cyanobacteria (Synechococcales, Pseudanabaenales, Nostocales), this metric has been consistent and effective, and shows great promise for recognizing species boundaries in the cyanobacteria. The clear discontinuity between strains assigned to the same species and strains assigned to different species has been maintained in all four studies, and the p-distances have had strikingly similar ranges between species and among strains of the same species. This method certainly needs further validation, but its promise as a diagnostic tool appears bright at this point in time.

The differing structures of the BoxB helix in the two different operons of *Roholtiella* is very intriguing (Figs. 8 A–E, G–K). The consistency within operons suggests that the operons containing both tRNA genes and the operons containing no tRNA genes shared a common ancestry that preceded speciation within the genus, and possibly preceded the phylogenetic separation among what are now considered separate genera (Figs. 8 A–M). The uniformity of the BoxB secondary structure within ribosomal operons is striking as it suggests that these structures are very conservative, and apparently persist even through speciation events. The conservative nature of ITS secondary structures within homologous operons has been observed in a few other cases, such as in the structure of the D1–D1' helix in *Leptolyngbya* Anagnostidis & Komárek (1988: 390) (Johansen *et al.* 2011). This difference among nonhomologous operons within strains and species needs further examination, and may reveal critical information on the evolution within the ribosomal genes of the cyanobacteria.

The family Nostocaceae, as presently defined (Komárek 2013), requires taxonomic revision and definition, as well

as more extensive taxon sampling to resolve the genera that likely exist within the clade. By describing *Roholtiella* using a polyphasic approach, we hope to have made progress towards this end.

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