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Roholtiella volcanica sp. nov., a New Species of Cyanobacteria from Kamchatkan Volcanic Soils

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Abstract: During a study of biodiversity of cyanobacteria in Gorely volcano soils (Kamchatka Peninsula), a strain of heterocytous, a false branching cyanobacterium with gradually tapered filaments, was isolated. Prominent features of the strain were purplish-grey trichomes and firm, distinct multilayered sheaths. Based on the results obtained from the morphological, ecological, and phylogenetic analysis using the 16S rRNA and 16S–23S ITS region, 16S–23S ITS secondary structure analysis, comparison of flanking regions of BoxB and V3 helices, and the p-distance between the 16S–23S ITS region, we describe our strain K7 as a novel species of the genus *Roholtiella* with the name *Roholtiella volcanica* sp. nov., in accordance with the International Code of Nomenclature for algae, fungi, and plants. This work continues the rapid expansion of the description of new taxa of cyanobacteria, and particularly demonstrates a coming phase in cyanobacterial taxonomy in which the discovery of new species in recently described genera rapidly increases our understanding of the diversity in this phylum.

Keywords: 16S rRNA; 16S–23S ITS; 16S–23S ITS secondary structure; *Calothrix*; extreme habitats; Gorely volcano; mucilage; Nostocaceae

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1. Introduction

Despite of the long history of cyanobacterial research, we are still very far from the real estimation of the biodiversity within this group [1–7]. There are a number of reasons preventing the acquisition of knowledge about the real number of cyanobacterial species, one of which is the insufficient use of modern molecular genetic research methods. Many regions of the world are still unexplored. The study of cyanobacteria of volcanic substrates allows the detection of terrestrial cyanobacteria species resistant to a number of adverse environmental factors, such as high temperatures, humidity fluctuations, and high concentrations of toxic compounds.

Representatives of the family Nostocaceae occupy almost all habitats on our planet, including diverse terrestrial ecosystems [8–13]. These include, for example, representatives of the genera *Nostoc* Vaucher ex Bornet & Flahault, *Scytonema* Agardh ex Bornet & Flahault, *Hassallia* Berkeley ex Bornet & Flahault, and *Roholtiella* Bohunická, Pietrasiak et Johansen.

The genus *Roholtiella* was described in 2015 [14]. This genus of false branching cyanobacteria is characterized by gradually tapering trichomes and terminal and intercalary heterocytes. Originally, the genus included four species: *Roholtiella edaphica* Bohunická & Lukešová, *Roholtiella bashkiriorum* Gaysina et Bohunická, *Roholtiella fluviatilis* Johansen et Gaysina, and *Roholtiella mojaviensis* Pietrasiak et Johansen [14]. *Roholtiella edaphica* and *Roholtiella bashkiriorum* were found in temperate climate soils in the Czech Republic and the Republic of Bashkortostan in Russia, respectively. *Roholtiella fluviatilis* was detected in aquatic or hydro-terrestrial habitats in the Republic of Bashkortostan, and *Roholtiella mojaviensis,* in sandy, gravelly soil from granitic outcrops in Joshua Tree National Park, Wonderland of Rocks in USA [14].

Strain *Roholtiella* KZ-5-4-5 was isolated in 2016 from microbiotic crusts collected on Coquina beach on the coast of the Sea of Azov (Ukraine) [15]. Morphological and phylogenetic analysis using 16S rRNA and 16S–23S ITS markers revealed that *Roholtiella* KZ-5-4-5 belongs to *Roholtiella edaphica.*

In 2021 a new species of *Roholtiella*—*Roholtiella mixta*—was described from the entrance zone of a cave in the South Urals (Russia) [16]. Thus, this genus has a wide geographical distribution and ecological amplitude. It is necessary to note that representatives of *Roholtiella* are very similar with the species from the genera *Calothrix* and *Tolypothrix*, and in some floristic studies incorrect identification of this genus is quite possible. In this regard, the description of new species of the genus *Roholtiella*, including in extreme terrestrial habitats like volcanic soils, is expected.

During the study of the biodiversity of cyanobacteria of the volcanic soils of Gorely volcano in Kamchatka peninsula, a very interesting strain of false branching heterocytous cyanobacteria was isolated. Accordingly, the present study aims to clarify the taxonomic status of our isolated cyanobacterial strain based on a detailed morphological and molecular-genetic analysis based on the sequencing of 16S rRNA and 16S–23S ITS region and secondary structure of the 16S–23S ITS, allowing us to describe the strain as a new species of *Roholtiella*—*Roholtiella volcanica* sp. nov.

2. Materials and Methods

2.1. Study Site

The Kamchatka Peninsula is situated in eastern Russia in the north-west part of the Pacific Ocean [17]. It is one of the most volcanically active regions of the world. Under the influences of surrounding seas, the Kamchatka Peninsula is characterized by very variable weather, unstable climatic conditions, and diverse depositional environments and geographic reliefs [18]. Approximately one third of the area is covered by forests, another third by shrubs, and the remaining territory is occupied by alpine tundra, wetlands, and volcanic landscapes [19]. The main vegetation zones include maritime meadows, *Larix cajanderi* Mayr, *Picea ajanensis* Fisch. x Carrière, *Betula manii* Cham. forests, *Pinus pumila* (Pall.) Regeland, *Alnus kamtschatica* (Regel) Kudo ex Masam., shrublands, alpine meadows, mountain tundra, and cold deserts [20]. The conditions for soil formation on the Kamchatka Peninsula are related to the activity of the volcanic centers, which has produced eruption material of varying composition. The soil-forming rocks of most soils on the peninsula are volcanic ashes. A profile of volcanic soils progressively grows uphill due to the periodically supplied pyroclastic material falling on the surface (synlithogenic soil production) [21]. The soils of different regions of Kamchatka are characterized to varying degrees by relatively increased concentrations of elements mainly typomorphic to volcanic rocks of medium and basic compositions: Na, Ca, Mg, Cd, Mn, Co, Cu, and persistently low element contents, typical for acidic volcanites: La, Ce, Pr, Nd, Nb, Hf, Tl, Rb, and Th [22,23].

There are about 300 volcanoes in Kamchatka, more than 20 of which are active [17,24,25]. The Gorely volcano is a large long-lived volcanic center in southern Kamchatka with eruptive activity continuing to the present. This volcano consists of an ancient and modern edifice. The ancient edifice is shield-shaped with a caldera about 156 km^2 across at its center. The modern edifice is situated in the central part of the caldera and consists of three

merged cones [26]. The volcanites of the center of Gorely volcano are lime-alkaline, with moderate, only relative iron enrichment, significantly increased alkali content (especially K_2O), and the widespread development of hybridism. However, in the gabbro-dolerite inclusions related to its rocks, the basic clasts of ferro-andesite basalts containing 13.5% FeO are established [27].

2.2. Sampling, Strain Isolation, and Cultivation

Samples were collected following traditional methods of soil phycology [28] from the soils of Gorely volcano (52°33′31.0″ N 158°02′16.0″ E) in August 2010 during a period of eruptive activity. Pure cultures of cyanobacteria were isolated using the method of enrichment cultures on agar medium [29]. According to this method, on the surface of agar-solidified Z8 medium [30] in Petri plates, a small amount of liquid Z8 medium was transferred and carefully spread with a glass spatula. A pinch of carefully crushed soil was scattered on a moistened surface. Plates were incubated in the refrigerator with a transparent door at a temperature of +4 °C and light intensity 700 lx at 12 h:12 h light:dark regime.

The strain K7 was cultured in liquid nutrient media according to the standard methods [31], and agar slants with agar concentration 1.5% in tubes. In some agar slants, 1–2 mL of liquid media was added. As a result, at the boundary of the solid and liquid media favorable conditions for the growth of cyanobacteria were created. For observation of the life cycle, liquid Waris-H medium [32] was used. To obtain heterocytes, media with one tenth nitrogen content was used. The purity of the strain from other cyanobacteria was confirmed by microscopic observation.

2.3. Phenotypic Analysis

Morphological observation was conducted by an Axio Imager A2 (Carl Zeiss, Oberkochen, Germany) and an Olympus BX 53 (Olympus, Tokyo, Japan) with Nomarski DIC optics. Images were made by Axio Cam MRC (Carl Zeiss, Germany) and Olympus DP27 (Olympus, Tokyo, Japan) cameras at magnification ×1000.

Observation of the life cycle of the strain was conducted over an extended period beginning in August 2011 and lasting until April 2022. The morphology of apical cells, the shape and size of vegetative cells and heterocytes, the position of heterocytes, the shape of trichomes, the presence of false branching, arthrospore formation and release, sheath peculiarities, and other features were analyzed.

2.4. DNA Extraction, PCR, and Sequencing

Genomic DNA was extracted from 10–12 days-old log phase culture using Qiagen DNeasy® Power Soil® Pro Kit (Qiagen, Hilden, Germany). Subsequently, the partial 16S rRNA gene (~1160 nt long) and the 16S–23S ITS region (complete ITS and short partial 23S) was amplified using the primers P1 (5′-CTCTGTGTGCCTAGGTATCC-3′) [33] and P2 (5′-GGGGAATTTTCCGCAATGGG-3′) [34]. The PCR was performed in a 25 µL reaction consisting of 12.5 µL of LongAmp® Taq 2X Master Mix (NEB), 0.5 µM each of the primers and 2µL of DNA on a Bio–Rad C1000TM Thermal Cycler. The amplified product was thereafter cloned using StrataClone PCR Cloning Kit (Agilent Technologies, Santa Clara, CA, USA) followed by restriction digestion using EcoR1, and the selected cloned product was sent to Functional Biosciences Inc. (Madison, WI, USA) for sequencing. The sequences obtained were assembled into contigs and proofread using the Sequencher program (version 4.8, Ann Arbor, MI, USA).

2.5. Phylogenetic Analysis

The 16S rRNA gene-based phylogenetic trees were constructed using 240 nucleotide sequences (each about 1160–1400 nt in length). The sequences were aligned using MAFFT [35], and the alignment was manually curated in order to retain the secondary structures. In addition, an alignment of the 16S–23S ITS region was also prepared in order to differentiate strain K7 from the closely related species. Orthologous ITS operons without tRNA were

used for the phylogenetic analysis, and the assembled alignment for this analysis was 346 nt long. All the phylogenetic trees reconstructed in this study were inferred using the Bayesian Inference (BI), Maximum Likelihood (ML), and Neighbor Joining (NJ) methods. The BI tree was reconstructed using Mr. Bayes 3.2.6 [36] and the tree was set to run for 80 million generations or until the standard deviation of split frequency reached below 0.01 (for the 16S tree) or 0.001 (for the 16S–23S ITS tree). In both cases it reached that stop point before the 80 million generations were complete. The average ESS values for all the parameters were greater than 100 (for the 16S tree) or greater than 8000 (for the 16S–23S tree), whereas the PSRF values were near to or at 1 for both analyses. Further, the diagnostic frequency, sampling frequency, and print frequency were set to 1000, and 25% of the samples from the beginning of the chain were discarded as burnin. The ML tree was reconstructed on the IQ-TREE web server [37] using the ultrafast bootstrap method with 1000 replications [38]. The BI and ML analysis was executed using the suitable model predicted by jModelTest [39], which led to the selection of GTR + G + I. The NJ tree was reconstructed in Mega X [40] and the reliability of the tree was assessed using the bootstrap method with 1000 replications [41]. An additional Maximum Parsimony (MP) analysis was run on the 16S–23S ITS alignment, counting indels as a fifth base, and conducting 10,000 bootstrap replicates. This analysis was run in PAUP v. 4b10.

2.6. Analysis of 16S–23S ITS Region

The full length 16S–23S ITS operon without tRNA was recovered from strain K7, and the folded secondary structures were obtained for the regions D1-D1′, BoxB, and V3 helices using Mfold on the UNAFold web server [42]. Further, the folded secondary structures of the other members of the genus *Roholtiella* were obtained, and comparative secondary structure analysis of the above-mentioned region was performed. All the secondary structures were redrawn in Adobe Illustrator (ver. 24.1.1). Furthermore, the sequence flanking the BoxB and V3 helices of strain K7 were compared with the corresponding region of the phylogenetically related taxa.

2.7. Calculation of Percentage Similarity and p-Distance

The *p*-distance values corresponding to the 16S rRNA gene and the 16S–23S ITS region were determined for strain K7 along with the phylogenetically related strains. P-distance was determined by running the sequence alignments in PAUP, using distance as the criterion, with distance = *p* and the SHOWDIST command. Percentage similarity for the 16S rRNA gene was calculated using the formula [(1–*p*-distance) × 100] whereas the percentage dissimilarity for the 16S–23S ITS region was calculated using the formula (*p*-distance × 100).

3. Results

3.1. Morphological Characterization

Roholtiella volcanica K7 was isolated in September 2011 from the east slope of the Gorely volcano, from tundra volcanic illuvial–humus soils at pH 4.65, soil moisture 55%, and soil temperature 12 °C [43]. No alga or cyanobacterium apart from *R. volcanica* was isolated from this sample, and the low algal diversity of the Gorely volcano soils in general has been documented by others as well [44].

All *Roholtiella* species described so far are from soil habitats, although the soils vary in availability of moisture (riverbanks to desert soils) and light (caves to full sun). *R. volcanica* is ecologically distinguished from these species by its occurrence in soil that is depauperate in most minerals and plant nutrients but has elevated toxic metals.

Roholtiella volcanica possesses all the morphological synapomorphies of other species in the genus. Over a period of observation lasting more than a decade, utilizing both liquid and agar-solidified medium, all stages of its life cycle were seen (Figure 1A–K). Hormogonia are isopolar and untapered (Figure 1B), becoming tapered and heteropolar quickly with the production of a basal heterocyte and apical growth (Figure 1A,C). Trichomes are deeply

constricted at the crosswalls, with cells barrel-shaped, usually isodiametric to shorter than broad, even in apical portions of the trichomes (Figure 1C,D). Single false branching is evident (Figure 1E–G). Rounded arthrospores are produced in long series (Figure 1I). Heterocytes are all terminal and mostly hemispherical (Figure 1C,D), and are rare in replete media but common in media with reduced nitrogen content.

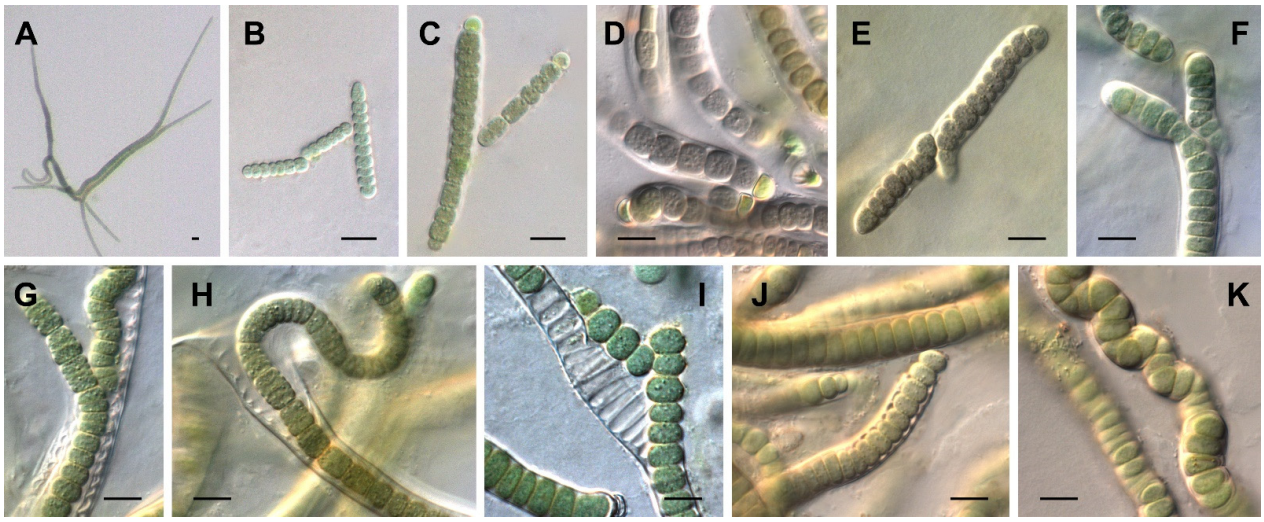

Figure 1. (**A**) Young trichomes, tapered to the ends, in liquid media; (**B**) hormogonia in liquid media; (**C**) young filaments with terminal heterocytes; (**D**) trichomes immediately after first isolation in pure cultures on agar solidified media; (**E**) the beginning of the false branching on agar solidified media; (**F**) the false branching in old culture on agar tubes with the liquid media; (**G**) false branching filaments, partly surrounded by the thick multilayered sheath in old culture on agar tubes with liquid media; (**H**) releasing of the filament from the thick multilayered sheath in old culture on agar tubes with liquid media; (**I**) release of arthrospores after the destruction of the sheath from the one side of the filament in old culture on agar tubes with liquid media; (**J**) release of arthrospores from the end of filament in old agar solidified cultures; (**K**) tightly coiled filaments in old agar-solidified media. Scale bar: 10 μm.

Morphological differences from other species are few. The dimensions of the trichomes, (2.9–9.1 μm wide), cells (3.1–10.6 long), heterocytes (5.0–7.4 μm wide, 3.3–5.3 μm long), and arthrospores (6.7–8.9 μm wide, 3–7.1 μm long) are similar to the previously described species of *Roholtiella* [14–16]. Immediately after the isolation in pure culture the strain grew on the agar surface in the form of small blue-green, olive-green, or brown upright tufts.

The primary difference from other species was in the color of the cells and the characteristics of the sheath. Purplish-grey trichomes and cells were often present as a distinct feature, particularly on agar-solidified media (Figure 1D,E), although olive green and blue-green trichomes were also evident. In the lower part of the tube in the interface of solid and liquid media, the trichomes were blue-green (Figure 1F–I), whereas in the upper part on agar they were purplish to olive brown (Figure 1D,E,J,K). In 9 month-old cultures, cells became pale olive and lost granulation (Figure 1J,K). In addition, variously curved and coiled trichomes were observed in old cultures (Figure 1J). The occurrence of tightly coiled and bent trichomes was present in older cultures as well, and were also a distinct characteristic of the species (Figure 1K).

Except for hormogonia and young filaments, *R. volcanica* consistently had firm, distinct multilayered sheaths (Figure 1D–K). The sheath was often structured, taking on the form of the trichomes and showing the impression of the cells even after coming free of the trichomes or cells (Figure 1G–I). In addition to the release of arthrospores from an open end of the sheath (Figure 1J), the sheath often split open and released the arthrospores from the side of the filament, a characteristic unobserved in all other species. The sheath could also split open to release growing trichomes (Figure 1H). In old cultures (6–24 months after

transferring to fresh media) the multilayered structure of the sheath became especially evident. The layers of the sheath were fused together firmly, forming a single structure (Figure 1G).

3.2. Phylogenetic Analysis

In the phylogenetic trees inferred using the 16S rRNA gene, *R. volcanica* clustered within the *Roholtiella* clade, confirming its genus-level identification. Whereas *R. fluviatilis* and *R. bashkiriorum* had distinct and supported nodes, the other species were united into a node with poor support and were not resolved (Figure 2). *R. volcanica* and *R. edaphica* KZ-5-4-5 clustered together with some support (Figure 2), but the branch was embedded in a paraphyletic collection of *R. edaphica* strains that also included *R. mojaviensis* and *R. mixta*, in addition to the *R. volcanica*/*Roholtiella* KZ-5-4-5 branch.

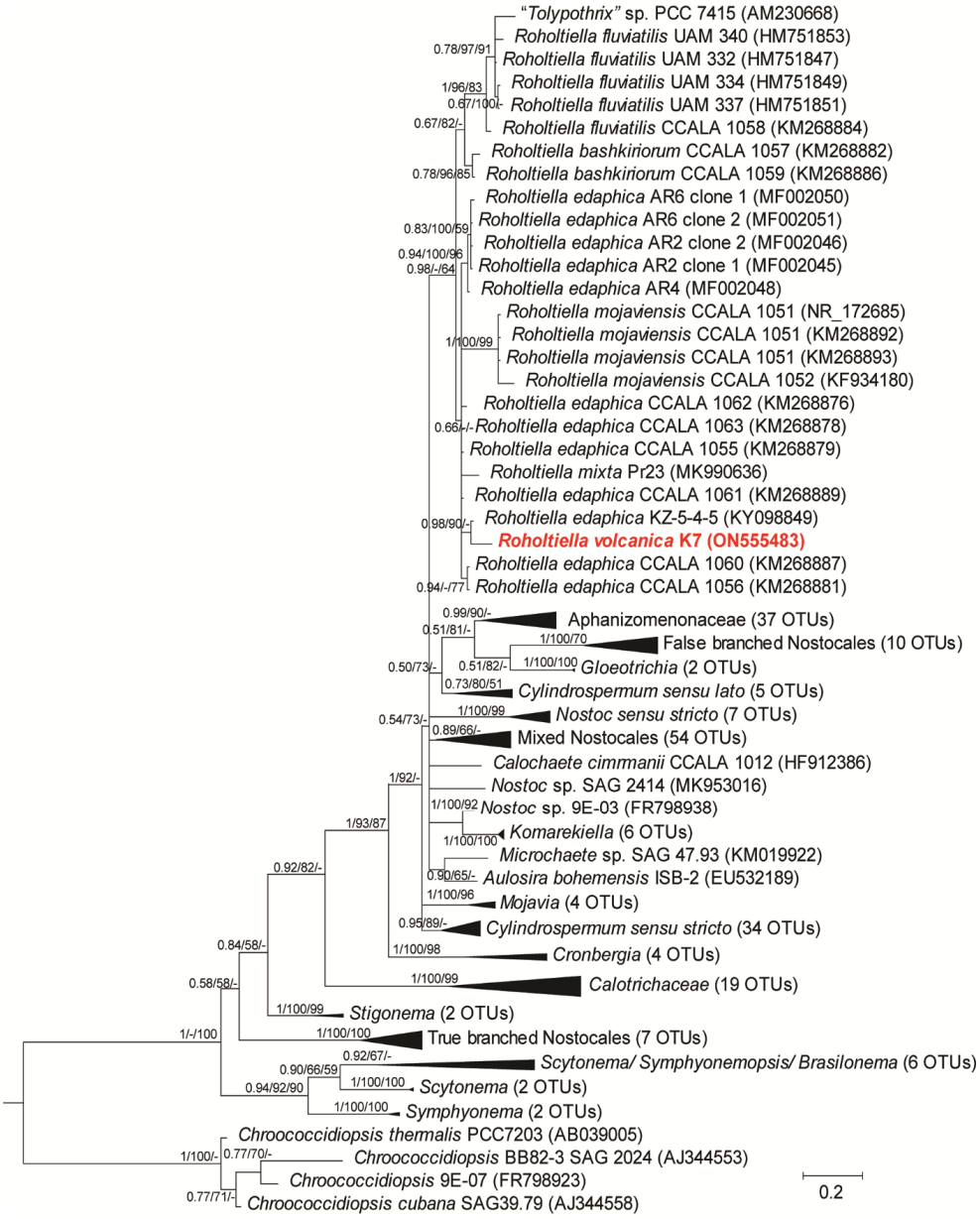

Figure 2. Phylogenetic positioning of *Roholtiella volcanica* K7 (in red font) based on the 16S rRNA gene sequence of 240 cyanobacterial taxa in the Nostocales, rooted with *Chroococcidiopsis*, with the posterior probability/bootstrap values representing BI/ML/NJ mapped on to the nodes in BI analysis. Bar, 0.2 changes per nucleotide position.

3.3. Analysis of the 16S–23S ITS Region

The phylogenetic analysis of the strains of *Roholtiella* for which an ITS gene sequence is available clearly separated *R. volcanica* from all other species (Figure 3). Furthermore, all species were monophyletic in this analysis, including *R. edaphica* which has been the most widespread and commonly observed species. *R. volcanica* was phylogenetically closest to *R. bashkiriorum* (Figure 3). The set of 11 strains used in the alignment for this analysis had very few indels (9 nucleotides only over a length of 346 nucleotides). This is an unusually close group and may reflect a recent diversification. As part of the analysis, we discovered that at least two different ribosomal operons are present in *R. edaphica*, with the less commonly recovered operon having a much shorter V3 helix of 35 nucleotides in comparison to a V3 of 91 nucleotides found in all other strains. We removed these two sequences (KZ-5-4-5 KY098849 and CCALA 1063 KM268878) from the alignment for the ITS analysis so that we were determining phylogeny based on orthologous operons. When the sequences were left in the alignment, the two *R. edaphica* strains with different operons still clustered with *R. edaphica* but formed very long branches; the topology of the rest of the tree was the same as in Figure 3.

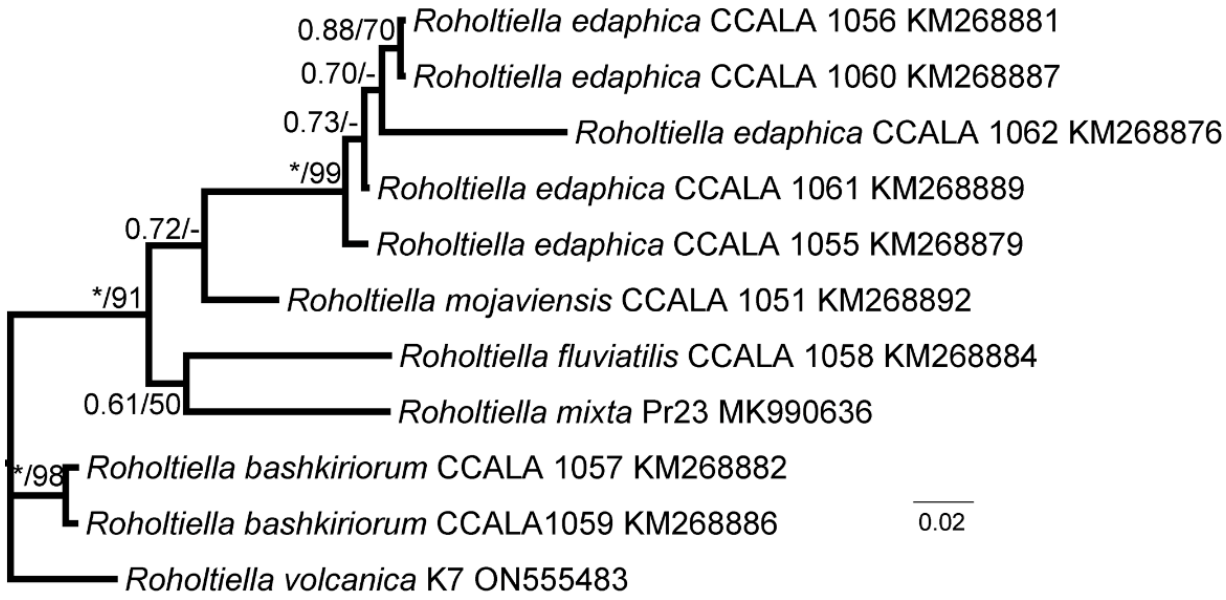

Figure 3. Phylogenetic positioning of *Roholtiella volcanica* K7 based on the 16S–23S ITS region of 11 cyanobacterial strains belonging to the genus *Roholtiella*, with the posterior probability/bootstrap values representing BI/MP mapped on to the nodes of the BI analysis. Posterior probabilities of 1.00 are indicated by an asterisk (*).

The secondary structures of the conserved helical domains of the ITS region were not very informative. The D1-D1′ helix was unusually similar among species, with the basal 3′ unilateral bulge opposite a 5′-AU-3′ mispairing on the 5′ side (Figure 4). The only exception to this was *R. mixta* with a solitary C residue opposite the 3′ unilateral bulge. The D1′D1′ helix of *R. volcanica* had an identical structure to those of *R. bashkiriorum* and *R. fluviatilis*. All strains and species had a terminal loop of five nucleotides, subtended by a helix of 3 nucleotide pairs, and a bilateral bulge below the short helix (Figure 4). Variations in structure and sequence were evident in some of the mid-helix regions between the basal unilateral bulge and the subterminal bilateral bulge.

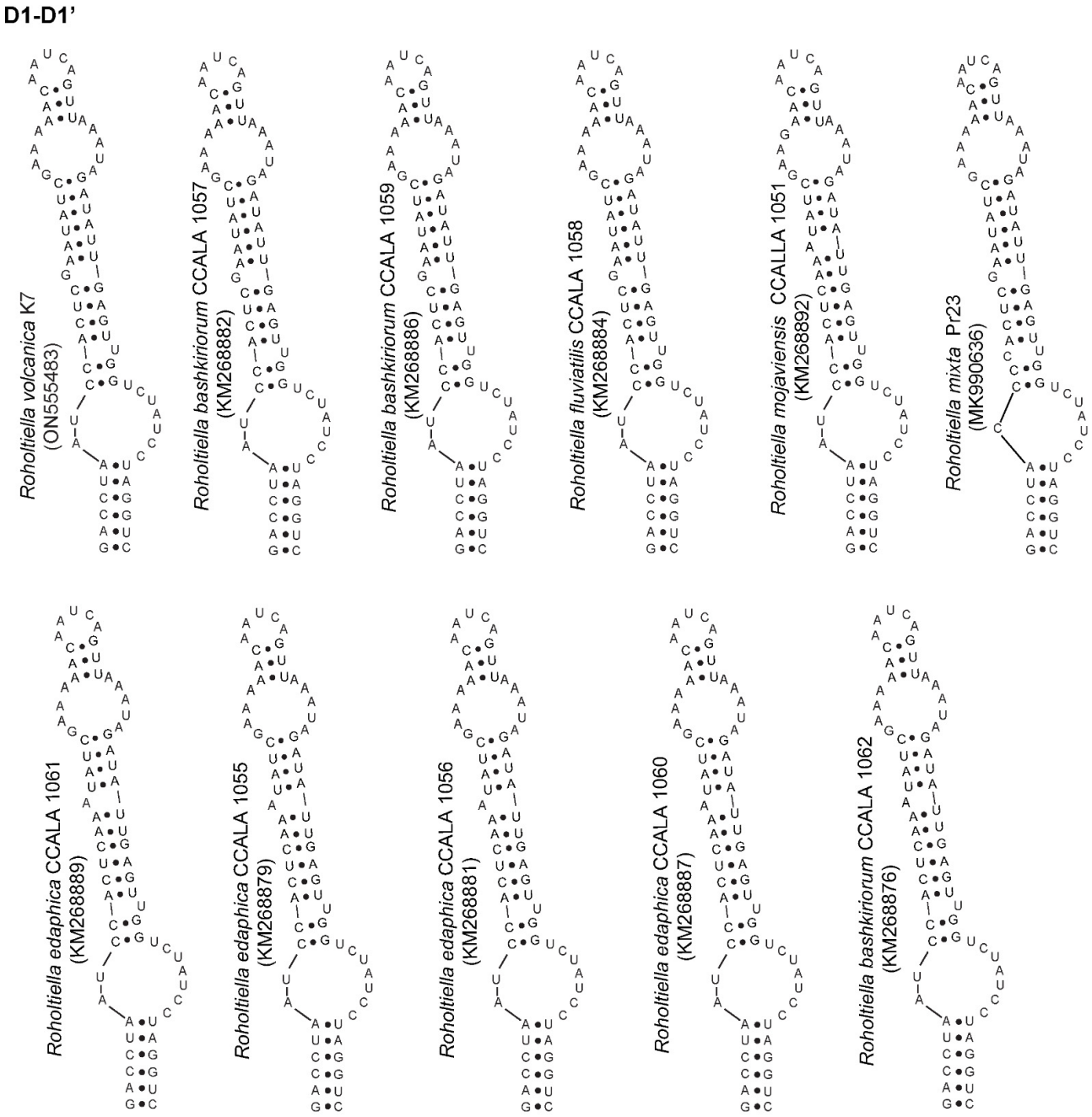

Figure 4. Comparative secondary structure analysis of the D1-D1′ helix region of *Roholtiella volcanica* K7 with the other members of the genus *Roholtiella*.

The Box-B helix was also almost invariant in the secondary structure among species, with only a few differences in the size of the central bilateral bulge (Figure 5). Differences in the primary sequence of these helices were noted in the terminal loop. *R. volcanica* had 5′-UUAC-3′ as its terminal sequence, as did *R. bashkiriorum*, and possibly *R. mixta*, which had one ambiguous nucleotide (Figure 5). The rest had 5′-AAUU-3′ or 5′-AAUC-3′.

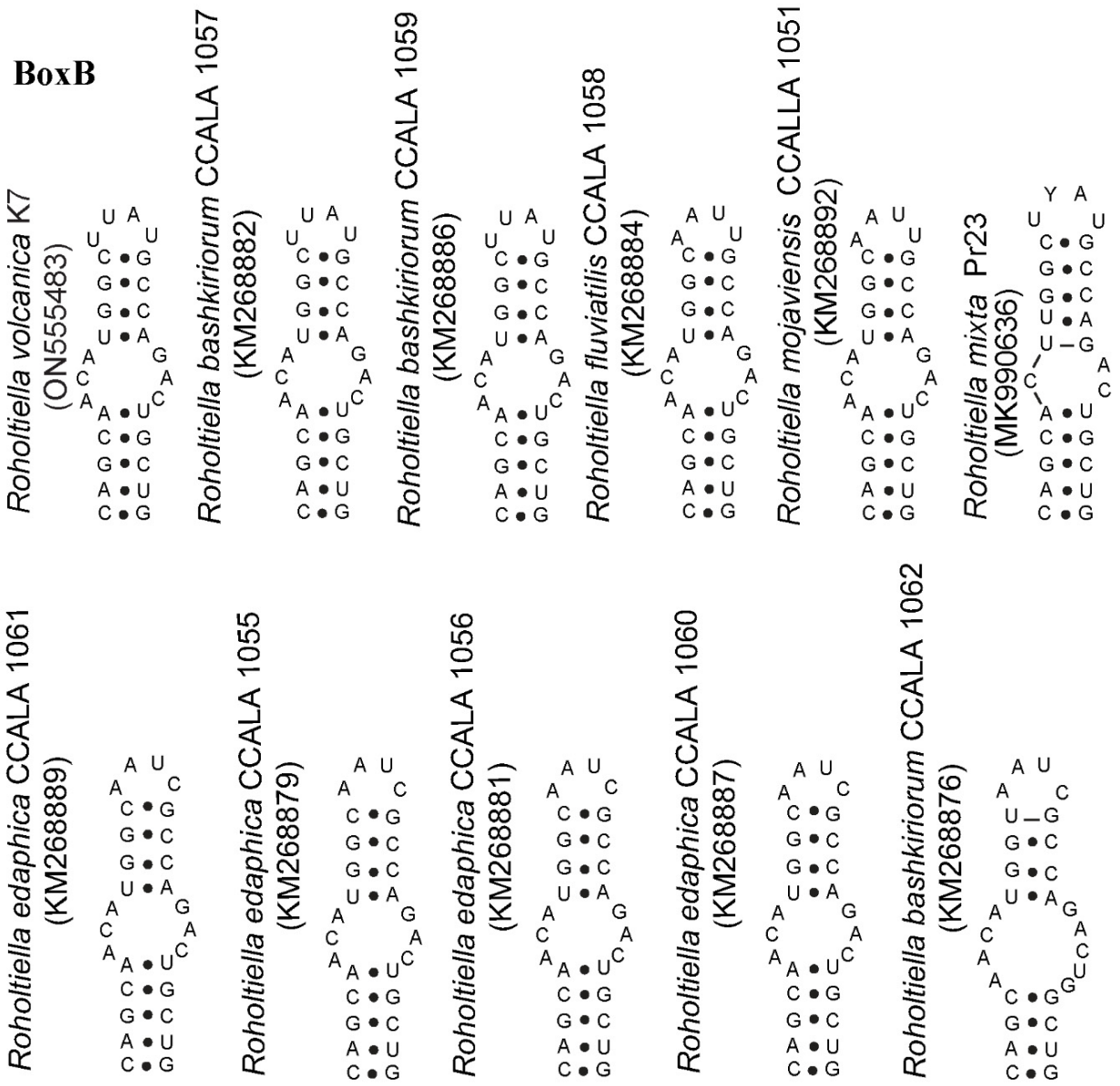

Figure 5. Comparative secondary structure analysis of the BoxB region of *Roholtiella volcanica* K7 with the other members of the genus *Roholtiella*.

The V3 helix was the most variable among species, and *R. volcanica* had a secondary structure similar to many, but not identical to any other V3 helix (Figure 6). All strains shared the same basal clamp, but above the bilateral bulge ending the clamp region, the primary sequence and pairing of nucleotides varied up to the end.

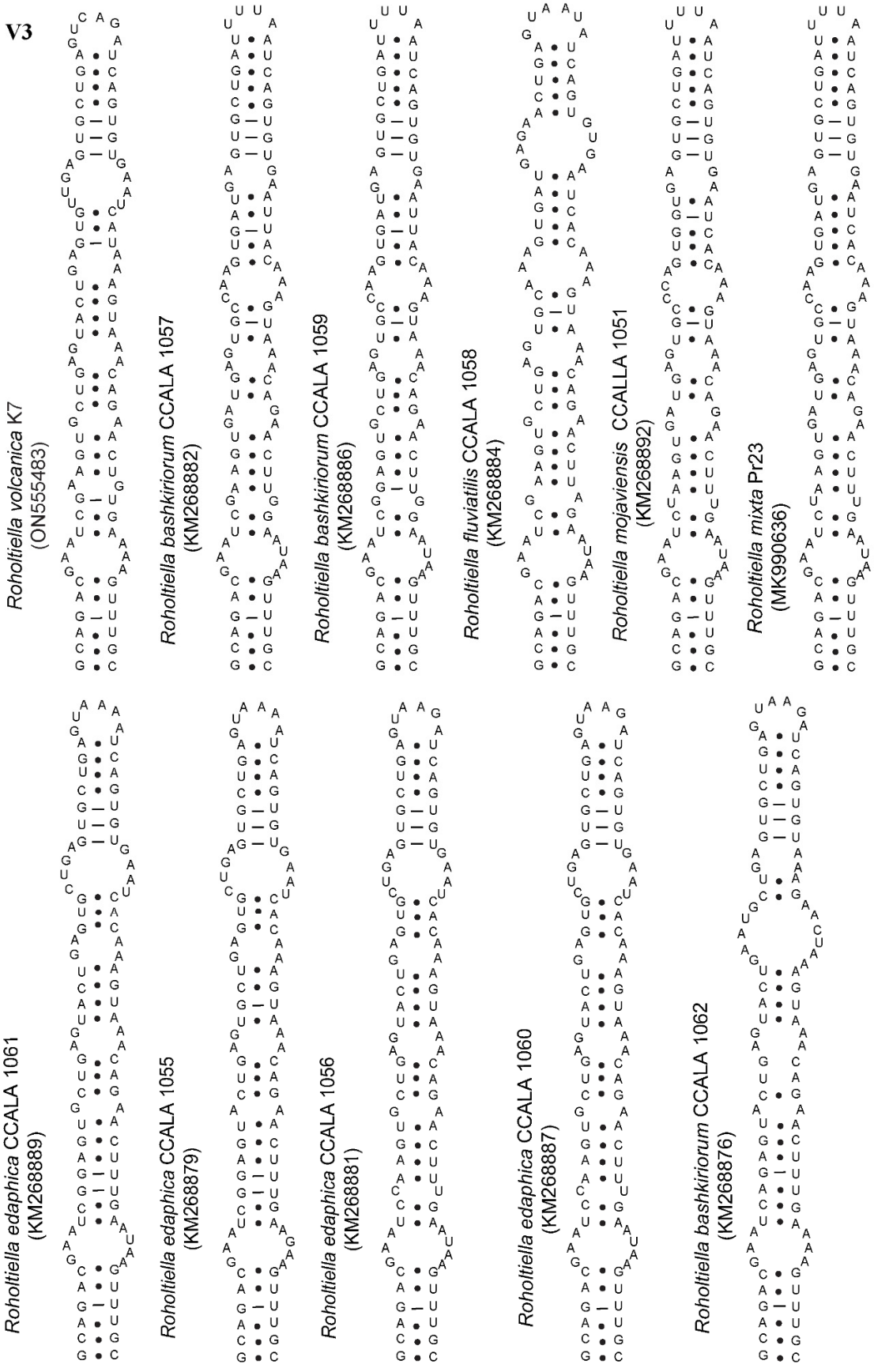

Figure 6. Comparative secondary structure analysis of the V3 helix region of *Roholtiella volcanica* K7 with the other members of the genus *Roholtiella*.

Further, we also compared the flanking regions of the BoxB (Figure 5) and V3 helices (Figure 6) of *R. volcanica* with all strains of *Roholtiella* (Table 1). *R. bashkiriorum* was found to be the closest species to *R. volcanica* based on this comparison. The flanking regions of *R. volcanica* corresponding to the BoxB region differed at a single position, whereas the flanking region corresponding to the V3 helix differed at only two positions (Table 1). These flanking regions were more divergent from *R. volcanica* in other strains.

Table 1. Sequences of 16S–23S ITS flanking region of the BoxB and V3 helices for *Roholtiella* species. Variable bases are in bold and the differences between *R. volcanica* and all other strains are highlighted in grey for each of those strains. Note that the fewest differences are between *R. volcanica* and *R. bashkiriorum*.

Roholtiella volcanica K7	A**G**AAT**C**AT**CAA**AA**C**TA**CA**GG**G**AATAGT**AC**TT–**BoxB**–**AGAA**–TCCAGCCA
Roholtiella bashkiriorum CCALA 1057	A**G**AAT**C**AT**CAA**AA **G** TA**CA**GG**G**AATAGT**AC**TT–**BoxB**–**AGAA**–TCCAGCCA
Roholtiella bashkiriorum CCALA 1059	A**G**AAT**C**AT**CAA**AA **G** TA**CA**GG**G**AATAGT**AC**TT–**BoxB**–**AGAA**–TCCAGCCA
Roholtiella edaphica CCALA 1063	A **A** AAT**C**AT **A** **AA**AA**C**TA**C** **T** GG**G**AATAGT **G** –TT–**BoxB**– **TATTG** TCCAGCCA
Roholtiella edaphica KZ_5_4_5	A **C** AAT**C**AT **A** **AA**AA**C**TA **TT** GA**G**AATAGT **G** –TT–**BoxB**– **TATTT** TCCAGCCA
Roholtiella edaphica CCALA 1062	A **A** AAT**G**AT **A** **AA**AA**C**TA**C** **T** GG**G**AATAGT **G** –TT–**BoxB**– **TATTG** TCCAGCCA
Roholtiella edaphica CCALA 1055-56; 1060-61	A **A** AAT**C**AT **A** **AA**AA**C**TA**C** **T** GG**G**AATAGT **G** –TT–**BoxB**– **TATTG** TCCAGCCA
Roholtiella fluviatilis CCALA 1058	A **T** AAT**C**AT **T** **A** **C** AA **G** TA **TT** GG **A** AATAGT**A** **A** TT–**BoxB**– **TT** **A** **T** –TCCAGCCA
Roholtiella mojaviensis CCALA 1051-52	A **C** AAT**C**AT **AR** **AA**A**C**TA**C** **T** GG**G**AATAGT **G** –TT–**BoxB**– **TA** **A** **TA** TCCAGCCA
Roholtiella mixta Pr23	A **C** AA**C**AT **TG** **AA**A**C**TA **TT** GG **A** AATATA**A** **A** TT–**BoxB**– **TT** **A** **T** –TCCAGCCA
Roholtiella volcanica K7	GA**AAA**AAAGCAG–**V3**–AGTGGTGAA**C**ACCAA**A**–**TGTATT**GT
Roholtiella bashkiriorum CCALA 1057	GA**AAA**AAAGCAG–**V3**–AGTGGTGAA–ACCAA—**TG** **A** **ATT**GT
Roholtiella bashkiriorum CCALA 1059	GA**AAA**AAAGCAG–**V3**–AGTGGTGAA–ACCAA—**TG** **A** **ATT**GT
Roholtiella edaphica CCALA 1063	GA **TTT** AAAGCAG–**V3**–AGTGGTGAA**C**ACCAA**A**–**TGTATT**GT
Roholtiella edaphica KZ_5_4_5	GA–**AA**AAAGCAG–**V3**–AGTGGTGAA**C**ACCAA**A**–**TGTAT** **A** GT
Roholtiella edaphica CCALA 1062	GA **TTT** AAAGCAG–**V3**–AGTGGTGAA**C**ACCAA**A**–**TGTATT**GT
Roholtiella edaphica CCALA 1055-56; 1060-61	GA **TTT** AAAGCAG–**V3**–AGTGGTGAA**C**ACCAA**A**–**TGTATT**GT
Roholtiella fluviatilis CCALA 1058	GA–**AA**AAAGCAG–**V3**–AGTGGTGAA–ACCAA **TTGT** **T** **TAA** GT
Roholtiella mojaviensis CCALA 1051-52	GA–**AA**AAAGCAG–**V3**–AGTGGTGAA–ACCAA **T** –**TGT** **T** **T** **A** GT
Roholtiella mixta Pr23	GA–**AA**AAAGCAG–**V3**–AGTGGTGAA**C**ACCAA–**TGTA**– **A** GT

Lastly, the percent dissimilarity among ITS sequences was indicative that *R. volcanica* was a distinct species (Table 2). Among described species, *R. volcanica* was least dissimilar to *R. bashkiriorum* (PD = 5.0%). All other species were considerably more dissimilar (7.6–12%). In some instances, PD $\geq$ 4.0 has been taken as evidence that the strains are different species [45], so PD = 5.0 supports the conclusion that *R. volcanica* has sufficient lineage separation from *R. bashkiriorum* to be considered a different species. Based on the fact that ecological and morphological differences also exist between the species in addition to phylogenetic separation, we describe our strain K7 as *Roholtiella volcanica*, in accordance with the International Code of Nomenclature for algae, fungi, and plants [46].

Table 2. Percent dissimilarity of the 16S–23S ITS region based on orthologous operons found among strains of *Roholtiella*.

	***Roholtiella volcanica* K7**	***Roholtiella bashkiriorum* CCALA 1057**	***Roholtiella bashkiriorum* CCALA 1059**	***Roholtiella fluviatilis* CCALA 1058**	***Roholtiella mojaviensis* CCALA 1051**	***Roholtiella mixta* Pr23**	***Roholtiella edaphica* CCALA 1061**	***Roholtiella edaphica* CCALA 1055**	***Roholtiella edaphica* CCALA 1056**
Roholtiella volcanica K7									
Roholtiella bashkiriorum CCALA 1057	5.0								
Roholtiella bashkiriorum CCALA 1059	5.0	0.6							
Roholtiella fluviatilis CCALA 1058	12.0	10.2	10.2						
Roholtiella mojaviensis CCALA 1051	10.0	7.0	7.6	8.5					
Roholtiella mixta Pr23	12.1	9.	9.8	10.3	7.4				
Roholtiella edaphica CCALA 1061	7.9	9.3	8.7	10.5	6.7	10.6			
Roholtiella edaphica CCALA 1055	8.7	9.9	9.3	10.5	6.7	10.6	1.29		
Roholtiella edaphica CCALA 1056	7.6	9.6	9.6	10.5	6.7	10.6	0.9	2.0	
Roholtiella edaphica CCALA 1060	7.6	9.6	9.6	10.5	6.7	10.6	0.9	2.0	0

3.4. Taxonomic Description

Roholtiella volcanica Gaysina and Singh, Johansen, Saraf, Pal, Allaguvatova sp. nov. (Figure 1A–J).

Description: Thallus flat, in the form of upright tufts with visible filaments on the substrate, blue-green, olive-green, or brown-green. Filaments short to long, single or rarely false branched, heteropolar. Sheath thick, firm, clearly delimited, in old cultures widened, distinctly layered, closed at the ends or becoming diffluent towards the ends, colorless. Trichomes constricted at crosswalls, not tapered or distinctly gradually tapered, slightly or distinctly swollen at the base, 2.9–9.1 μm wide. Cells shorter than wide, isodiametric or sometimes longer than wide, barrel-shaped, cylindrical, to almost spherical or spherical compressed, with smooth to finely granulated content, olive-green, brown, purplish-grey, or blue-green, 3.1–10.6 long. End cells conical, conical rounded, or rounded. Heterocytes terminal, with smooth pale-yellow content, shorter than wide, hemispherical or spherical, pale green, 5.0–7.4 μm wide, 3.3–5.3 μm long. Hormogonia short, with cells 3.5–6.5 μm wide, 1.4–6.5 μm long. Arthrospores forming in chains, released from one side or the end of filament by destruction of the sheath, 6.7–8.9 μm wide, 3–7.1 μm long. In old cultures some filaments tightly coiled and variously curved.

Etymology: From the Latin *volcanica* (=from volcano), referring to the habitat of origin of the taxon.

Type locality: Gorely volcano, Kamchatka peninsula, Russia, 52°33′10.8′′ N 158°02′06.0′′ E.

Ecology of type locality: tundra volcanic illuvial–humus soils down the east slope of Gorely volcano, pH 4.65, soil moisture 55%, soil temperature 12 °C.

Reference strain: *Roholtiella volcanica* was deposited in the Bashkortostan Collection of Algae and Cyanobacteria (BCAC) as BCAC 516 *Roholtiella volcanica* K7.

Holotype here designated: dried biomass of the strain *Roholtiella volcanica* K7, deposited in the Herbarium of BCAC under accession number 1.

4. Discussion

Roholtiella was recognized almost simultaneously among culture collections maintained by researchers in Russia (Gaysina), the Czech Republic (Lukešová, Bohunická, and Hauer), Spain (Berrendero), and the USA (Pietrasiak and Johansen) and through fortunate and effective communication between these workers it was published collaboratively with the description of four species from very diverse locales: *R. edaphica*, *R. bashkiriorum*, *R. fluviatilis*, and *R. mojaviensis* [14]. *R. edaphica* is the most commonly encountered species, both in the protologue for the genus and since [15]. Most recently, *R. mixta* was described from soil in a cave entrance in the south Ural mountain region of Russia [16]. These five species are morphologically consistent and recognizable as belonging to *Roholtiella* even without the detailed study, including molecular sequencing, that has gone into the naming of each species. The slightly tapered filaments that evidence false branching, barrel-shaped cells deeply constricted at the crosswalls of the trichome, and rows of round arthrospores set the genus apart from all other Nostocales. A re-examination of the numerous *Calothrix* species chronicled in Komárek [10] did not reveal any taxa with the rows of round arthrospores. The closest taxon to the morphology of *Roholtiella* is *Calothrix elenkenii* Kosinskaja. That taxon was not as deeply constricted, but if it were available in culture, sequencing might reveal it to be a *Roholtiella* species. Like *R. fluviatilis*, it was first seen in a river in Russia. Most *Roholtiella* taxa were isolated from soils (*R. fluviatilis* was the only exception, and it was from the sediment near the bank). The capacity to live in sediment and soil matrices is an ecological characteristic that may be universal in the genus.

R. volcanica was closest phylogenetically to *R. bashkiriorum*, and the two could be considered sister taxa. For the closest species, *R. bashkiriorum*, the dissimilarity was 5.0%. Percent dissimilarity in the 16S–23S ITS sequence was the lowest of any recognized species pair, and so this criterion might be considered ambiguous in isolation. As has been noted in numerous papers, there appears to be a large discontinuity in dissimilarity between members of the same species and members of different species. Percent dissimilarity (PD) $\geq$ 7.0% is considered strong evidence for separate species, and PD $\leq$ 3.0% is considered evidence that the populations (strains) belong to the same species, with intermediate values (3.0 $\leq$ PD $\leq$ 7.0) being ambiguous [47–51].

However, the ecological and morphological differences are significant. *R. bashkiriorum* was collected in meadowland soil near the River Ik in Bashkortostan [14]. This habitat was more fertile and much less extreme than the nutrient-deficient soil in Gorely volcano. These volcanic soils are harsh and nutrient-poor. They are low density, have high hydrophilicity, and have high filtration capacity, and their low content of organic material and high content of heavy metals and acidity are limiting to photosynthetic organisms [52]. Furthermore, the purplish coloration common in trichomes growing on agar, and the very firm, multilayered sheath, serve to separate the species from all others in the genus. *R. edaphica*, *R. bashkiriorum*, and *R. fluviatilis* were mostly pale brown in cell content, *R. mixta* was grey, to greyish blue-green, and *R. mojaviensis* was bright blue-green [14,16]; none produced purplish cells. *R. volcanica* produced blue-green cells in liquid culture, and the color delineation was especially noticeable on agar slants with the addition of liquid medium. All other species of *Roholtiella* have thin, soft to diffluent, colorless, and unstructured sheaths [14,15]. Trichomes often appear to have no sheath. These morphological features of *R. volcanica* K7 may be a mechanism for its survival in the adverse conditions of volcanic soils, tying the morphology of the species to its ecophysiology.

The essence of the polyphasic approach to cyanobacterial taxonomy is to weigh all evidence from multiple character sets. The degree of difference in any one of these character sets might not be enough to justify the recognition of this species, but the combination of the differences in all character sets indicates that these lineages have been separate for a long period of time, and thus can certainly be considered different evolutionary species.

They also fit the criteria given for species recognition using the monophyletic species concept [46,53].

Although several algal isolation methods with varying media were attempted, only *R. volcanica* was isolated from the sample (K9) in which it occurred. This indicates that even for a volcanic soil, this sample was particularly harsh. It was collected from the east slope in a tundra illuvial–humus soil, but had recently received an input of pyroclastic ash containing As, Ba, Bi, Cd, Cu, Fe, Mn, V, and Zn [54]. During the study of 27 different soil samples from Gorely and neighboring Mutnovsky volcanoes, 48 species of algae and cyanobacteria were found [43]. *R. volcanica* was recognized morphologically as belonging to the genus *Roholtiella*, and sequencing confirmed this placement. However, although considerable sequencing of algal strains was conducted, Allaguvatova et al. [43] did not name any new species, and reserved that effort for this work.

Volcanic soils have been given some attention with regard to algal colonization and succession. It has long been suggested that cyanobacteria were the first to colonize ash [55–59]. However, other studies have demonstrated algal communities in which green algae are equally or more diverse on ash [43,60–62]. Unfortunately, the work on these floras of volcanic regolith and soil has usually been based only on morphology, and molecular studies are just emerging [43]. Given that these soils are often very early in their genesis on volcanoes, they are unique, and worthy of further diversity studies. We anticipate that in future, volcanic soils will receive the study they deserve.

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