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Komarekiella atlantica gen. et sp. nov. (Nostocaceae, Cyanobacteria): a new subaerial taxon from the Atlantic Rainforest and Kauai, Hawaii

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Abstract: Six strains of Cyanobacteria sampled in the Brazilian Atlantic rainforest and one strain from Kauai, Hawaii, were studied using morphological and molecular approaches, including 16S rRNA gene phylogenies and 16S–23S ITS secondary structures, and are herein described as *Komarekilla atlantica* gen. et sp. nov.. Morphologically they are similar to *Nostoc, Desmonostoc, Halotia,* and *Mojavia* and indistinguishable from *Chlorogloeopsis*. The parsimony and Bayesian phylogenies of the 16S rDNA show that these strains are close to nostocacean strains, in strongly supported clades and separated from all other genera. The secondary structures of the 16S–23S ITS were very consistent between strains of *K. atlantica*, but distinctly different from structures in other close taxa. Of special note, the Hawaiian strain of *K. atlantica* had 16S sequence identities of 99.5–100% to the Brazilian strains, and 16S–23S ITS sequence identities of 99.4–99.8% to the Brazilian strains, and consequently likely represents a very recent introduction of the species to Kauai from South America, the geographic source of many of the non–native plants in the Hawaiian Archipelago.

Key words: 16S rDNA, biodiversity, phylogeny, RNA secondary structures, taxonomy, tropical rainforest

Introduction

Carrying on the extensive study of cyanobacterial diversity of the Atlantic Rainforest, in which our research group has described many new taxa (Sant'Anna et al. 2013; Hentschke & Komárek 2014; Genuário et al. 2015; Hentschke et al. 2016), we describe another new terrestrial *Nostoc*–like genus using a combination of 16S rRNA phylogeny, 16S–23S ITS primary sequence and secondary structures, and morphological characters visible in the light microscope.

Nostoc Vaucher ex Bornet et Flahault is a widespread, commonly encountered genus of cyanobacteria, with 82 relatively well described species and many other poorly described species not in current usage (Komárek 2013). Phylogenetic studies based on 16S rRNA gene sequence have shown that strains assigned to *Nostoc* do not form a monophyletic group (Hrouzek et al. 2005; Řeháková et al. 2007; Kaštovský & Johansen 2008; Lukešová et al. 2009; Johansen et al. 2014), and recently three new *Nostoc*–like genera, phylogenetically closely related to *Nostoc sensu strictu*, have been described: *Mojavia* Řeháková et Johansen in Řeháková et al. (2007), *Desmonostoc* Hrouzek et Ventura in Hrouzek et al. (2013), and *Halotia* Genuário et al. (2015). Despite this progress, further revisionary work of the Nostocaceae is expected as new strains are isolated and characterized using both molecular and morphological characters.

One of the morphotypes of *Nostoc* commonly encountered from terrestrial habitats is *N. punctiforme* var. *populorum* Geitler, which has tightly compressed uniseriate trichomes in a common sheath, appearing to have division in multiple planes, such as *Mojavia* (Řeháková et al. 2007) and *Desmonostoc*. Genera with division in multiple planes similar to those forms

are *Chlorogloeopsis* MITRA et PANDEY and *Halotia*. Phylogenetic analysis of *Chlorogloeopsis* places this taxon sister to the clade containing true–branching genera such as *Hapalosiphon* BORNET et FLAHAULT, *Fischerella* BORNET et FLAHAULT, *Mastigocladus* BORNET et FLAHAULT, *Nostochopsis* BORNET et FLAHAULT, and *Westelliopsis* JANET (KAŠTOVSKÝ & JOHANSEN 2008), while the *N. punctiforme* var. *populorum*–like morphotypes, which are nearly indistinguishable from *Chlorogloeopsis* fall clearly into the *Nostoc sensu stricto* clade (see *N. indistinguendum* ŘEHÁKOVÁ et JOHANSEN and *N. desertorum* ŘEHÁKOVÁ et JOHANSEN in ŘEHÁKOVÁ et al. 2007). The true branched genus *Halotia*, unlike *Chlorogloeopsis* (also true branched), is phylogenetically closely related to nostocacean strains, as well as *Desmonostoc* (uniseriate trichomes) and *Mojavia* (uniseriate trichomes) (GENUÁRIO et al 2015). These incongruences reinforce the need of morphological and phylogenetic review of these genera.

We recently isolated six strains of a C*hlorogloeopsis/N. puntiforme* var. *popularum*–like taxon growing epiphytically on trees in subtropical and tropical regions of the Atlantic Rainforest (the coastal forest of Brazil) as well as one strain in rain puddles on cement in Hawaii. These isolates came up in culture from samples in which *Nostoc*–like species could not be detected, and thus are only known from culture. The strains produce multiseriate filaments in a common mucilage, and have cell divisions in more than one plane, like *Chlorogloeopsis*. They also have a life–cycle identical to that seen in *Chlorogloeopsis* (HINDÁK 2008). Some stages of their life cycle include filaments of microcolonies, like *Mojavia*, *Desmonostoc* and *Halotia* (ŘEHÁKOVÁ et al. 2007). Phylogenetic analyses show they likely belong to the Nostocaceae, but in a clade separate from *Nostoc, Desmonostoc, Halotia, Mojavia* and *Trichormus.* This paper will present evidence that establishes the uniqueness of these strains and will describe these isolates as *Komarekiella atlantica* gen. et sp. nov.

Material and Methods

Studied area, sampling and isolation of strains. Samples were collected from two tropical locations. First by scraping biofilms growing on the bark of trees, wood poles and concrete walls in the tropical and subtropical areas of the Brazilian Atlantic Rainforest, specifically in the State Parks of Santa Virginia (23º20'S and 45º09'W) and Ilha do Cardoso (25º04'12"S and 47º55'27"W) and in the Ecological Station Juréia–Itatins (24 26.481'S and 47 04.660'W). Material was kept dry in paper sacks, and subsequently inoculated in the laboratory onto BG–11 nitrogen–free (RIPPKA et al. 1979) agar media for enrichment and isolation of strains. Isolates were transferred into respective test tubes containing the same type of liquid media and kept under 14 h:10 h (light:dark) cycle with white fluorescent light (30 µmol photons.m^{-2}.s^{-1}), at a temperature of 23 (±2) °C, and held in the Culture Collection of the Institute of Botany, São Paulo, Brazil (CCIBt 3307, 3481, 3552, 3483, 3487, 3486 and 3485). Secondly, a sample was collected on a collecting trip in Hawaii. It was obtained from a mat found in a rain–fed puddle on a concrete pad near a house in Hawaii (21°54'56"N and 59°30'36"W). It was isolated from enrichment cultures on freshwater, agar–solidified Z–8 media (CARMICHAEL 1986) at John Carroll University, where it was kept on a 16 h:8 h (light:dark) cycle with white fluorescent light, at a temperature of 16 °C. Environmental samples and strain clones from Brazil were preserved in formaldehyde (4%) and deposited in the Herbarium of Institute of Botany (SP). The Hawaiian strain was preserved in formaldehyde as well as dried on filter paper and these materials were deposited in the Bishop Museum in Honolulu, Hawaii.

Morphological and life cycle studies. Morphological analysis was performed using a Zeiss Axioplan 2 microscope equipped with Zeiss Axiocam MRc digital camera and inverted microscope Zeiss Axiovert 25.

In order to report morphological plasticity and life cycle stages, all Brazilian strains were observed in the microscope every day for 7 days, then once a week for a month and finally after three months, after inoculation into new liquid BG11–nitrogen free media. Also, the strain CCIBt 3307 was inoculated onto agar with BG11 nitrogen–free media in an Üthermohl chamber and observed following the same frequency cited above, using an inverted microscope. Twenty vegetative cells, akinetes and heterocytes were measured for each studied strain in liquid media. The Hawaiian strain was identified subsequent to molecular work on all strains, and matched the morphology of the Brazilian strains, although the extent of characterization was not as extensive.

For the morphological comparisons between the macroscopic growing patterns of one strain in the new genus (CCIBt 3307) and a typical macroscopic *Nostoc commune* strain (CCIBt 3485), colonies of both strains were inoculated onto fresh Petri dishes with solid BG11 nitrogen–free media and observed for two months.

In a search for the natural populations, all dry environmental samples from the Atlantic Rainforest were rehydrated with distilled water for 20 hours before being analyzed using the microscope specified above.

DNA extraction, PCR amplification and sequencing. Total genomic DNA was isolated from liquid cultures of all Brazilian cyanobacterial strains using MOBIO Ultraclean DNA Isolation Kit. Nearly complete 16S rRNA gene fragments were amplified by PCR using the primers 27F1 (NEILAN et al. 1997) and 23S30R (TATON et al. 2003) in a Techne TC–412 thermocycler (BIBBY SCIENTIFIC). The reaction contained 10 ng of genomic DNA, 0.5 µM of each primer, 200 µM of dNTPs, 2.0 mM of $MgCl_2$, 1 × PCR buffer and 1.5 U Platinum Taq DNA polymerase (INVITROGEN, CARLSBAD, CA, USA), in a final volume of 25 µl. The PCR cycle had initial denaturation at 94 ºC for 5 min, followed by 10 cycles of 94 ºC for 45 s, 57 ºC for 45 s, 72 ºC for 2 min, another 25 cycles of 94 ºC for 45 s, 57 ºC for 45 s, 72 ºC for 2 min and a final extension step at 72 °C for 7 min. The resulting PCR product was cloned into a pGEM®–T Easy Vector System (PROMEGA, MADISON, WI, USA) according to the supplier's manual, cloned by heat–shock in E. coli DH5α cells and plated for blue–white selection (SAMBROOK & RUSSEL 2001). After growth, recombinant plasmids were extracted from white colonies by the alkaline lysis method (BIRNBOIM & DOLY 1979). The cloned

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gene fragment was sequenced using "Big Dye Terminator" version 3.0 (Applied Biosystems) with the plasmid primers T7 and M13 and the internal primers 357F/357R, 704F/704R and 1114F/1114R (Lane 1991). The cycle sequencing reaction was performed as follows: 25 cycles of 95 ºC for 20 s, 50 ºC for 15 s and 60 ºC for 1 m. the DNA was precipitated using 2 µL of sodium acetate buffer (1.5 M sodium acetate – pH 9.0 and 250 mM EDTA– pH 8.0) and 60 µl of 100% ethanol. The tubes were centrifuged at 4 ºC for 15 min at 12,000 × g and the supernatants were discarded. The DNA pellets were washed with 150 µl of 70% ethanol, centrifuged for 5 min and the supernatants removed. The pellets were air–dried overnight in the dark and at room temperature. The purified pellets were resuspended in HiDi formamide (Applied Biosystems), and the sample placed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequenced fragments were assembled into contigs using the software package Phred/Phrap/Consed (Philip Green, Univ. of Washington, Seattle, usa) and only bases with a quality >20 were considered.

A smaller partial 16S rRNA gene sequence fragment together with the full 16S–23S ITS region was isolated from the Hawaiian strain following protocols standard in the John Carroll University laboratory (Vaccarino & Johansen 2011, 2012; Pietrasiak et al. 2014; Miscoe et al. 2016).

Phylogenetic analyses. The 16S rRNA gene sequences obtained in this study (including *Nostoc commune* CCIBT 3485) and reference sequences retrieved from GenBank were manually aligned, and used to generate a similarity matrix and the phylogenetic trees.

The trees were constructed using the criterion of Maximum Parsimony (MP) using a heuristic search and 1000 replicates implemented by MEGA version 6 (Tamura et al. 2013). Gaps in the alignment were treated as missing data. Bayesian (BA) analysis were conducted using MrBayes 3.2 (Ronquist & Huelsenbeck 2003) in two independent runs, with four chains each, for 5×10^6 generations, sampling every 100 generations, and first 25% of sampled trees were discarded as burn–in.

The best–fitting evolutionary model GTR+G+I was selected for 16S rRNA gene sequences using ModelTest under the Akaike information criterion (Posada & Crandall 1998). The BA had an estimated sample size (ESS) exceeding 2000 for all parameters (ranging 2,025–26,923), well above the average of 200 typically accepted as sufficient by phylogeneticists (Drummond et al. 2006). The final average standard deviation of split frequencies was <0.0143. The potential scale reduction factor (PSRF) value for all the estimated parameters in the Bayesian analysis was 1.000, indicating that convergence of the MCMC chains was statistically achieved (Gelman & Rubin 1992).

To determine the relative position of our strains among orders of the Nostocophycidae subclass a first round of phylogenetic analysis, MP and BA trees were constructed with 466 OTU´s represented by Nostocophycidaean (Komárek & Anagnostidis 1989) sequences. Then, we chose closely related taxa within Nostocaceae with appropriate outgroups and constructed smaller trees based on 227 OTU´s. The outgroups used for all 16S rDNA phylogenetic analysis were represented by *Gloeobacter violaceus* PCC 8105, *Chroococcidiopsis* 9E–07, *Chroococcidiopsis cubana* SAG 39–79, *Chroococcidiopsis thermalis* PCC 7203 and *Chroococcidiopsis* BB 3 SAG 2024. A similarity matrix (p–distance) was also generated for related taxa.

The secondary structures of the helices D1–D1´, Box B, V2 and V3 were determined separately using Mfold version 2.3 (Zuker 2003), with folding temperature set at 20°C for sequences. For all ITS analyses, we used sequences containing both $tRNA^{Ile}$ and $tRNA^{Ala}$.

The 16S rDNA and 16S–23S ITS rDNA sequences of the cyanobacterial strains CCIBt 3307, 3481, 3552, 3483, 3487, 3486 and 3485, isolated in this study were deposited in the NCBI GenBank database under accession numbers KX638483, KX638484, KX638485, KX638486, KX638487, KX638488, KX638489, KX638490. The Hawaiian strain was deposited separately under accession numbers KX646831, KX646832.

Results

***Komarekiella* G.S. Hentschke, J.R. Johansen et C.L. Sant'Anna gen. nov. (Fig. 1)**

Description: In nature, growing on the bark of trees, wood, or concrete. Thallus microscopic in nature and in culture. In culture primarily growing attached to the glass walls of the test tubes in liquid media, and released as tufts which detach and settle to the bottom of the test tube as they grow, on agar growing radially from the center via hormogonia release, forming a dense creeping mat. Colonies spherical when developing from akinetes, elongate when developing from hormogonia, eventually aggregated loosely into macroscopic thalli through release of diffluent mucilage. Akinetes apoheterocytic, spherical or subspherical, granulated, developing from vegetative cells in trichomes, germinating into two equal vegetative cells or alternatively into a large vegetative cell and a smaller heterocyte, either of which develops into spherical colonies with a firm mucilage layer, which eventually become larger colonies enclosed within a diffluent mucilage. Hormogonia developing from older more diffuse colonies; when first formed motile, short, lacking firm mucilage, later immotile with heterocytes and akinetes, developing into multiseriate filaments, which eventually become elongate colonies enclosed in a firm mucilage. Cells quadratic in young hormogonia, becoming spherical or subspherical as filament matures, dividing perpendicular or longitudinal to the main axis of filament. Heterocytes spherical, compressed subspherical or oval.

Type species: *Komarekiella atlantica*.
Etymology: Named in honor of Professor Jiří Komárek.

***Komarekiella atlantica* G.S. Hentschke, J.R. Johansen et C.L. Sant'Anna sp. nov.**
(Fig. 1)

Description: Thallus at first bright blue–green, becoming a darker duller blue–green with age, and consisting of many subspherical colonies or aggregations of filaments enclosed in a common mucilage, up to 1 cm long. Colonies with compact aggregations of cells, at first with filamentous arrangement not visible, later

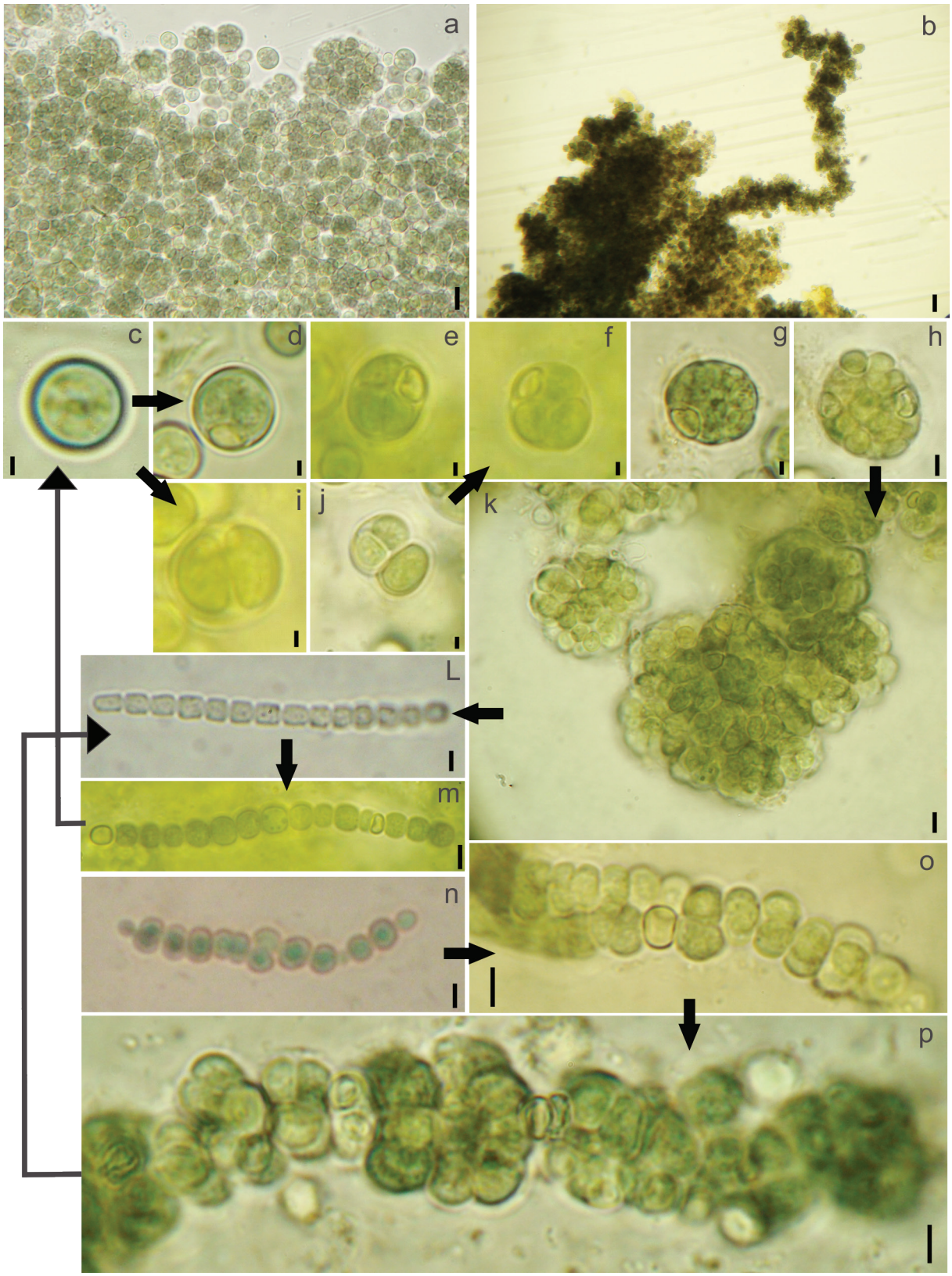

Fig. 1. Life cycle of *Komarekiella*; arrows indicate the developmental directons: (a, b) General view of macrocolonies; (c–h, k) unequal division of an akinete into a bigger vegetative cell and a smaller heterocyte and subsequent divisions; (i, j) equal division of an akinete into two vegetative cell and subsequent division; (l–p) developmental stages of hormogonia; longitudinal divisions of cells are shown in (n–p). Scales a 10 µm; b 50 µm; c, d, e–g, i 1 µm; h, l 3 µm; k, m, n–p 5 µm.

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more diffuse with trichomes apparent. Hormogonia released within a few days of transfer to fresh media, motile, 3 µm wide. Filaments at first uniseriate, later multiseriate, up to 10 µm wide. Vegetative cells in hormogonia quadratic, isodiametric to longer than wide, 3–5 µm long, becoming spherical or compressed subspherical in mature filaments, dividing in more than one plane, 3.4–5.5µm in diameter. Heterocytes developing in germinating akinetes 2–3 µm in diameter, later 3–5.5 µm in diameter. Akinetes 3.5–6 µm in diameter.

Holotype here designated: SP469725 (unialgal population preserved in 4% formaldehyde), Herbarium of Institute of Botany (SP), São Paulo, Brazil.
Isotypes: materials are preserved also in 4% formaldehyde (SP469723, SP469724, SP469726, SP469728, SP469729, SP469730, SP729431).
Type locality: State Park of Santa Virginia (23º20'S and 45º09'W).
Habitat: growing on a concrete wall.
Etymology: Named after the Atlantic Rainforest, where the species was first found.
Reference strain: CCIBt 3483.

Komarekiella could not be found in the environmental samples which served as the source material for strains CCIBt 3307, 3481, 3552, 3483, 3487 and 3486, but the taxon grew vigorously when inoculated into culture media (nitrogen–free BG11) and was easily isolated from enrichment cultures.

Morphologically *Komarekiella* is close to *Mojavia*, *Desmonostoc*, *Halotia* and microscopic species of *Nostoc*, although they differ from each other in some aspects. The new genus has the aggregated filaments of microcolonies like *Mojavia* and *Halotia;* the absence of a firm outer layer of the colonial mucilage (in some stages) like *Desmonostoc*; and stages in its life cycle which resemble some species of microscopic *Nostoc* (e.g. *N. punctiforme* var. *populorum*). If only isolated stages of the life cycle of *Komarekiella* were observed, it would be possible to incorrectly assume that those stages were one or more of these other taxa. Comparing the whole life cycle of *Komarekiella* with other genera, we observed that this taxon is indistinguishable from *Chlorogloeopsis*, especially with reference to the unequal germination of the akinetes, which, until this paper, served as the diagnostic feature of the last. A full summary (Table 1) of the comparison of these genera illustrates the difficulty of using morphology alone to define them.

Evaluation of the macroscopic growth form showed that the strains of *Komarekiella* grow in a sub-

Table 1. Morphological comparisons among *Komarekiella* and related genera.

Genera/characters	Thallus	Number of division planes in trichomes	Sheaths	Germination of akinetes (originating)	Width of vegetative cells in trichomes (µm)	Habitat
Nostoc Bornet & Flahault	Macroscopic	One	Firm	Two vegetative cells	4.5–6	Cosmopolitan mainly on unshaded and temporary humid soils
Desmonostoc Hrouzek & Ventura	Microscopic				3.5 or wider in avarage	Cosmopolitan, mainly on meadow soils.
Mojavia Řeháková & Johansen					4–10	Soils of Mojave desert, USA
Halotia Genuário et al.		Two	Firm or sometimes diffluent		2.3–4.6	Brazilian mangroves and maritime Antarctica
Chlorogloeopsis Mitra & Pandey				Commonly a larger vegetative cell and a small heterocyte	(2)3–6(8)	Cultivated soil, India
Komarekiella Hentschke et al.					3.4–5.5	Wood and concrete, Atlantic Rainforest Brazil.

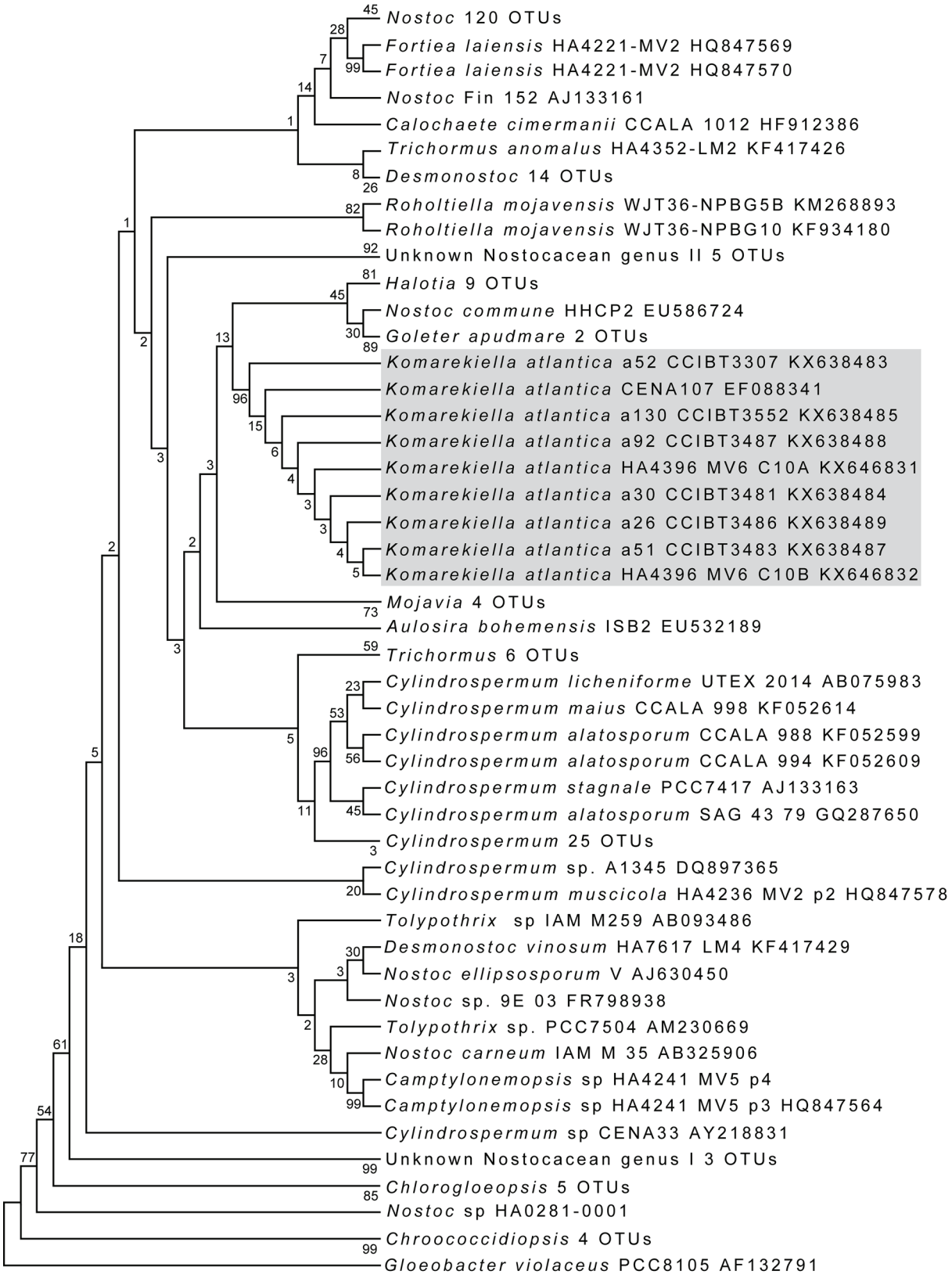

Figure 2. MP tree based on the 16S rRNA gene sequences of 227 strains of nostocacean cyanobacteria. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. A total of 816 characters were analyzed.

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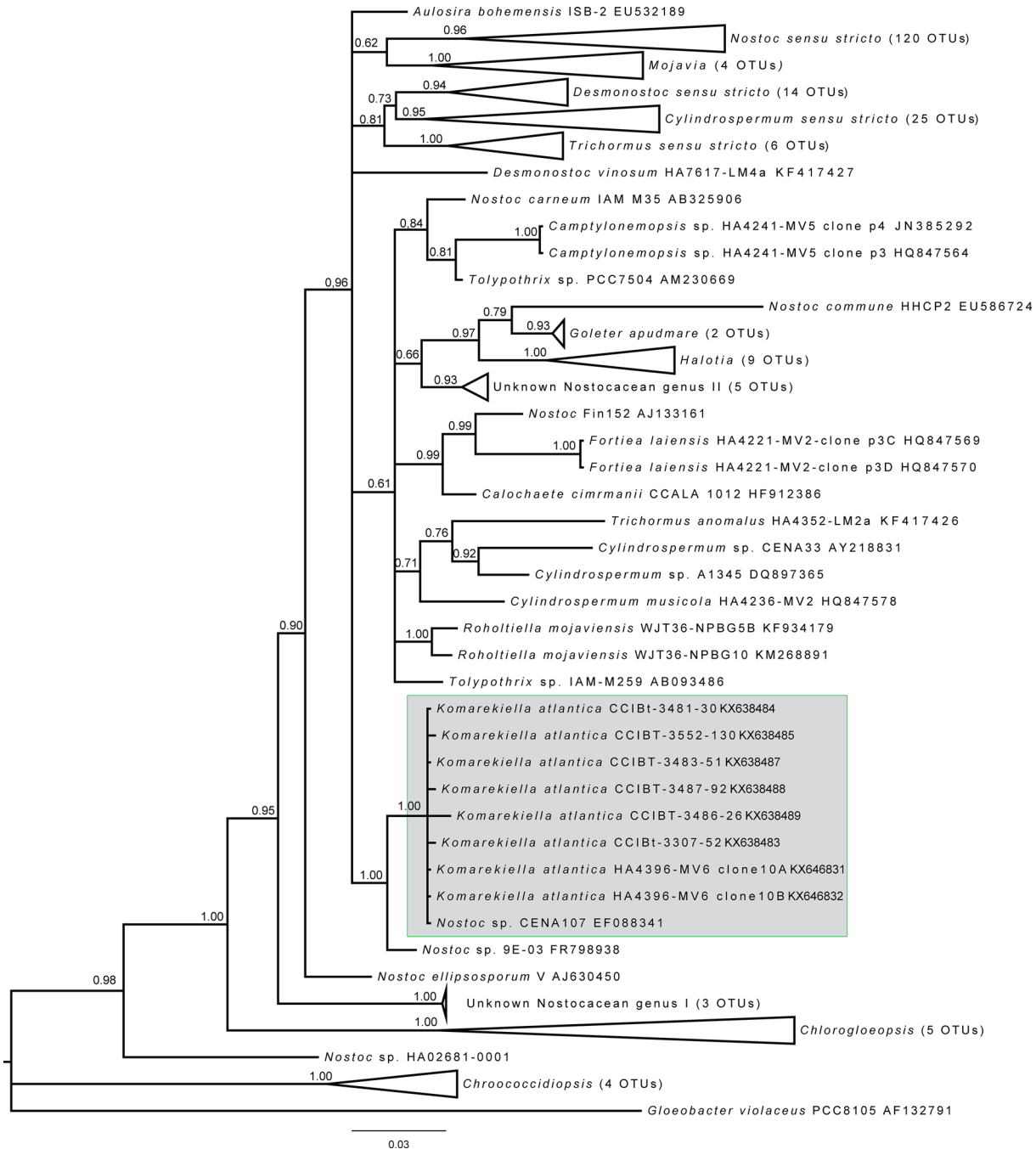

Figure 3. Bayesian tree based on the 16S rRNA gene sequences of 227 strains of nostocacean cyanobacteria. The probabilities obtained are displayed at the nodes. A total of 816 characters were analyzed.

stantially different way from our *N. commune* strain (CCIBt 3485) isolated from soils in the Atlantic Rainforest. In nature *N. commune* was macroscopic and amorphous, and in our laboratory trials, it typically formed well–defined spherical macroscopic mucilaginous colonies on solid media (nitrogen–free BG–11). *Komarekiella* grew radially creeping under the same conditions.

Molecular Characterization

Maximum Parsimony and Bayesian analysis based on 16S rDNA sequence data from 227 OTU's were largely in agreement and show *Komarekiella* as highly supported clades (100 and 1.0 bootstrap support and posterior probability, respectively), although the backbones of both trees were weakly supported. In the parsimony tree, *Komarekiella* is in a clade with *Halotia, Goleter apudmare, Mojavia pulchra* and *Aulosira bohemensis* (Fig. 2). In the Bayesian tree, *Komarekiella* is clearly supported as a separate genus–level clade, but is in a large polytomy with *Nostoc, Mojavia, Halotia, Goleter, Aulosira bohemensis, Cylindrospermum, Trichormus, Desmonostoc, Calochaete* and *Fortiea* (Fig. 3).

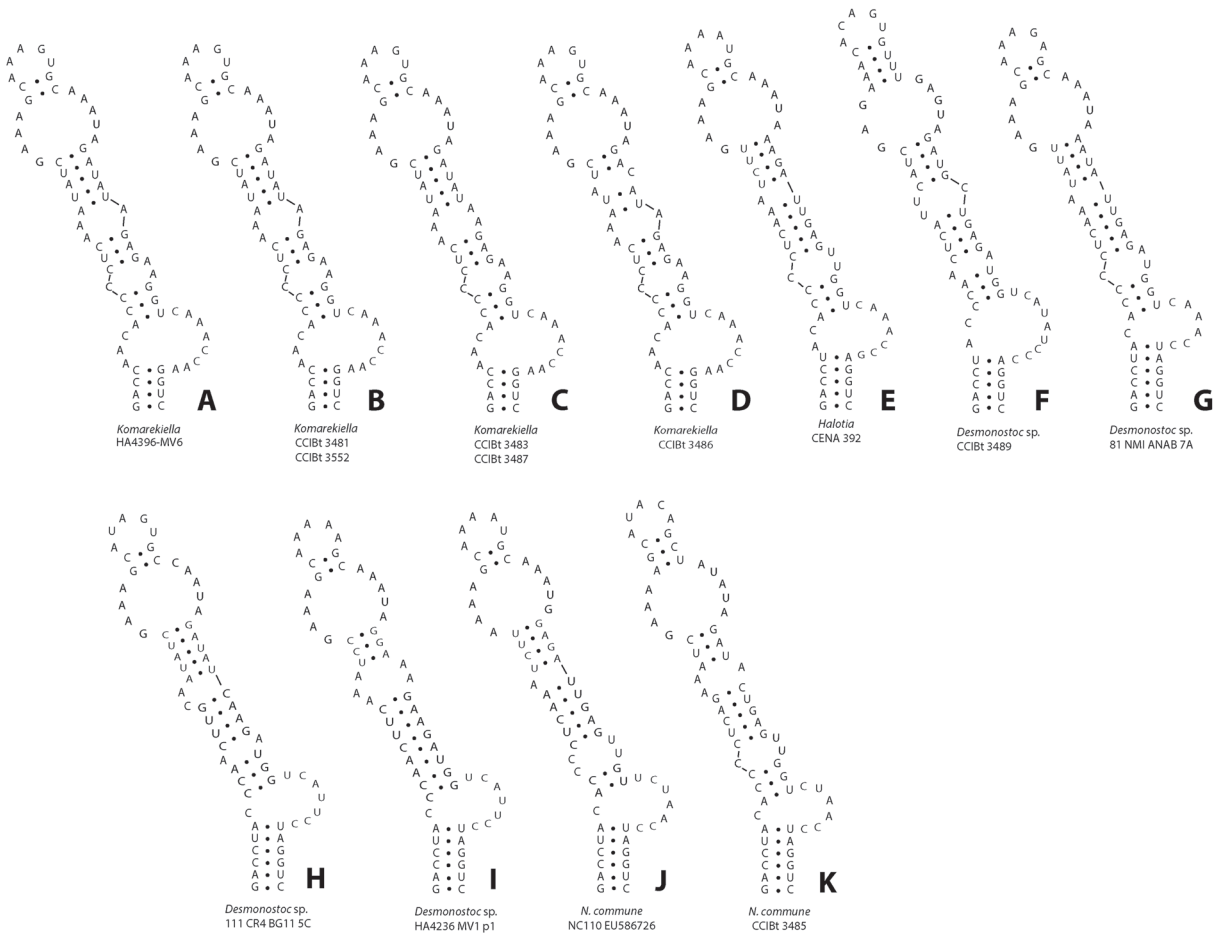

Figure 4. Secondary structures of D1–D1` helices (16–23S ITS) in related Nostocaceae genera. *Komarekiella* differs from other genera by featuring the basal unilateral bulge opposed by three unpaired nucleotides on the 5' side of the helices.

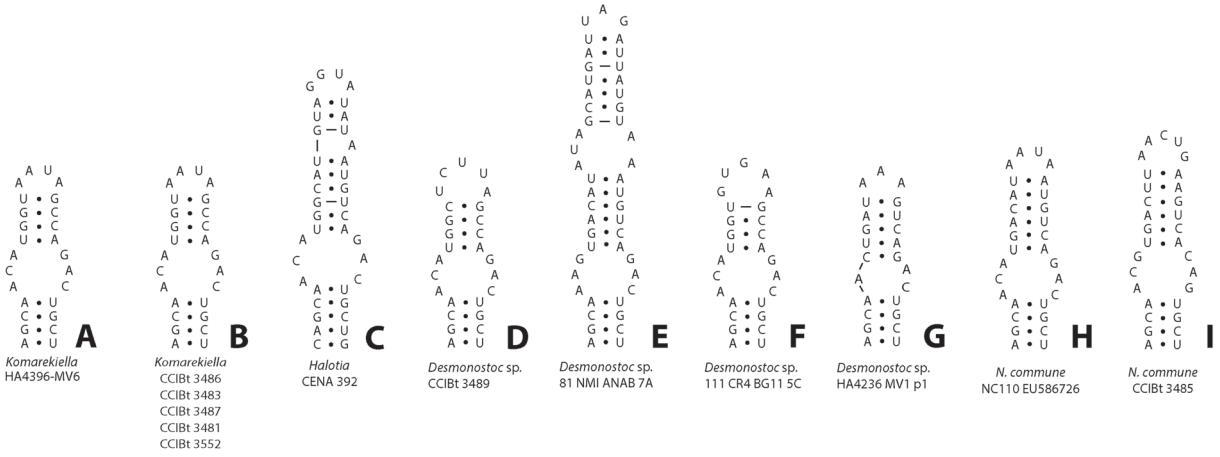

Figure 5. Secondary structures of Box B helices (16–23S ITS) in related Nostocaceae genera. The helices are unique and conserved among *Komarekiella* strains.

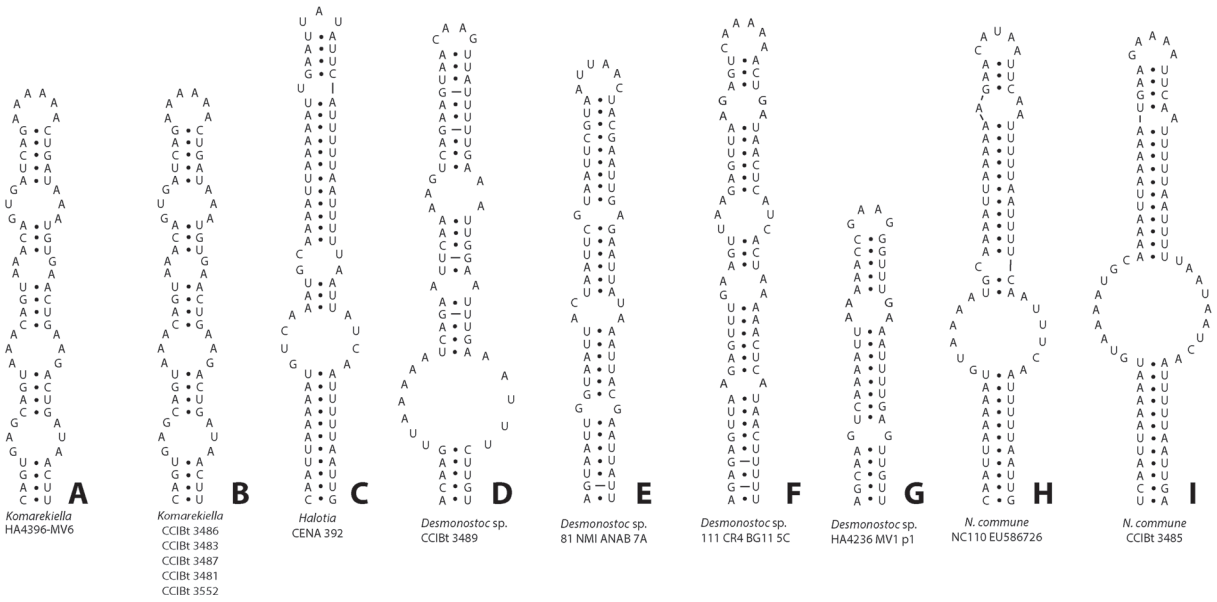

Figure 6. Secondary structures of V2 helices (16–23S ITS) in related Nostocaceae genera. The helices are identical among *Komarekiella* strains, but highly variable among other genera.

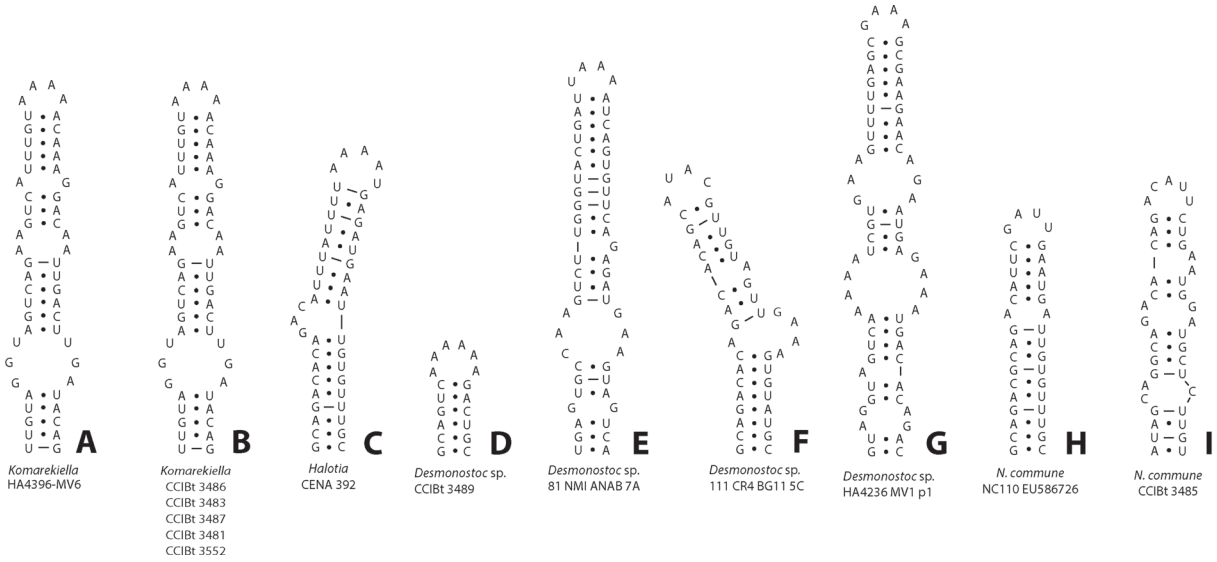

Figure 7. Secondary structures of V3 helices (16–23S ITS) in related Nostocaceae genera. The helices are identical among *Komarekiella* strains, but highly variable among other genera.

Both trees showed *Komarekiella* distant from *Chlorogloeopsis fritschii*, which forms basal clades in each of the trees. Phylogenetic analyses with greater taxon sampling (466 OTU's including the set of 227 OTU's as well as taxa from other genera and families of heterocytous cyanobacteria) had less bootstrap support and poorer resolution, although the placement of *Komarekiella* was still in the Nostocaceae, distant from all true branching taxa (tree not shown). Generally considering the clades of our trees, the Bayesian tree presented better support for genera, specially for *Nostoc* sensu stricto clade, in which our strain CCIBT 3485 is included, as well as *Desmonostoc*.

Examination of percent identity among taxa phylogenetically close to *Komarekiella* showed high similarity among genera. The *Komarekiella* strains were all >99.4% similar to each other, and were >95% similar to other taxa in the *Halotia* clade: *Goleter apudmare* 97.7–98.0%, *Aulosira bohemensis* 97.5–97.8%, *Mojavia* 96.2–96.6%, *Halotia branconii* 95.4–96.3%. The use of percent identities below 95% to establish genera has been recommended by some (Stackebrandt & Goebel 1994; Yarza et al. 2014), but these authors also recognize that genera that have higher levels of similarity can still be recognized if phylogenetic and phenotypic evidence exists for such recognition. *Komarekie-*

lla is >96% similar to representatives of the following morphologically distinct and well established genera: *Anabaena, Aulosira*, *Calochaete*, *Calothrix*, *Cylindrospermum, Desmonostoc, Dolichospermum, Fortiea, Goleter, Gloeotrichia, Hassallia*, *Halotia*, *Microchaete*, *Mojavia*, *Nostoc*, *Rexia*, *Roholtiella*, *Tolypothrix*, *Trichormus* and *Scytonema.*

An alignment of the 16S–23S ITS region could be constructed among the taxa most closely related to *Komarekiella*. The lengths of *Komarekiella* ITS regions were perfectly conserved among the strains in the genus, but distinctly different from all other genera (Table 2). This basic analysis is in agreement with the 16S rRNA gene phylogeny: *Komarekiella* is a distinct, supported clade separate from its closest phylogenetic neighbors.

The analysis of the secondary structures of the 16–23S ITS region showed that the regions D2, D3, Box A and D4 were conserved among *Komarekiella, Halotia, Desmonostoc* and *N. commune* strains (Figs. 4–7). Among the *Komarekiella* strains, the Box B, V2 and V3 helices were identical (Figs. 5–7), but the D1–D1' helix presented three slightly different patterns in the central helix, specifically on the 3' side of the helix (Fig. 4). CCIBt 3486 D1–D1' helix differs from the others owing to a mismatch in the stem below the upper bulge caused by a change of the 42nd nucleotide (A for C). The folded D1–D1' helices for the strains CCIBt 3483 and 3487 differs from the ones for CCIBt 3481 and 3552, by the insertion of an adenine after nucleotide 45, forming a bulge instead of the mismatch presented in the same position for the latter strains. However, the D1–D1' helix of all *Komarekiella* strains showed a very distinct synapomorphy: the basal unilateral bulge was opposed by three unpaired nucleotides on the 5' side of the helix, a feature not observed in any other cyanobacterial taxon to date. *Halotia, Desmonostoc* and *Nostoc* present two or one bases in this position.

Comparing the ITS structures between *Komarekiella*, *Desmonostoc, Halotia* and *N. commune*, the V2, V3, and Box B helices were highly variable. No consistent patterns were found for these helices among the strains studied, although other studies have shown high consistency of these structures within *Nostoc sensu stricto* (LUKEŠOVÁ et al. 2009). *Komarekiella* was distinctly different in the structure of the V2 and V3 helices from all other taxa (Figs. 6, 7), while the Box B helix shared relatively high similarity in the basal portions of the helix to the Box B helices of the other taxa (Fig. 5).

DISCUSSION

The five populations are morphologically very similar, all of them presenting polyseriate trichomes and unequal division of akinetes. Despite some color variations in cell content, it is not possible to separate them as

Table 2. Nucleotide lengths of the regions of the 16S–23S ITS of several studied strains.

Strain	Leader	D1–D1' helix	Spacer+D2	Spacer+D3+spacer	tRNA Ile gene	Space+V2+spacer	rRNAAla gene	Spacer+BoxB+spacer	Box A	D4	Spacer+V3
K. atlantica CCIBt 3481	9	65	35	16	73	82	72	84	11	12	62
K. atlantica CCIBt 3486	9	65	35	16	73	82	72	84	11	12	62
K. atlantica CCIBt 3483	9	66	35	16	73	82	72	84	11	12	62
K. atlantica HA4396–MV6	9	65	35	16	73	82	72	84	11	12	62
Desmonostoc sp. CCIBt 3489	8	69	35	14	73	85	72	83	11	11	33
Desmonostoc sp. 81 NMI ANAB 7A	9	65	36	15	74	79	73	103	11	11	87
N. commune CCIBt 3485	8	67	42	16	73	89	72	92	11	11	48
Halotia branconii CENA 392	9	65	40	16	74	88	73	75	12	11	59
Halotia longispora CENA 184	9	66	40	14	74	88	73	74	11	11	77
Mojavia JT2–VF2	8	64	32	11*	NA	NA	NA	54**	11	11	118

*Spacer+D3, **Box B+Spacer, NA = not available

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different species based on morphology or ecology. The same holds true for the molecular data as we observed very high similarities among the 16S rDNA sequence data (99.4–100%) of *Komarekiella* OTUs as well as among sequences of the 16S–23S ITS gene region (99.4–99.8%).

Morphological analysis also showed that the genus *Komarekiella* is morphologically similar to *Chlorogloeopsis, Mojavia*, *Desmonostoc*, *Halotia* and microscopic *Nostoc*, because of their overlapping features and the almost total lack of derived characters in all these genera. However, *Komarekiella* clearly differs from typical *Nostoc*, because it does not form the macroscopic mucilaginous colonies, found in the latter genus. *Komarekiella* differs from *Desmonostoc, Halotia, Mojavia*, and microscopic *Nostoc* by the special type of germination of akinetes, originating a bigger vegetative cell and a smaller heterocyte as the first division. It is molecularly most similar to *Goleter apudmare*, but this taxon demonstrates heteromorphic uniseriate trichomes with clear tapering and basal heterocytes, akinete production in series, and a complete absence of *Chlorogloeopsis*–like globular colonies.

When compared to *Chlorogloeopsis*, we noticed that both genera share the same type of cell division in trichomes, colonies wrapped by mucilaginous firm and diffluent sheaths, apoheterocytic akinetes, and the same type of akinete germination. Before this paper, this special type of akinete germination, originating a larger vegetative cell and a smaller heterocyte, was the main diacritical character for *Chlorogloeopsis*. However, the phylogenetic analysis of the 16S rDNA and the 16S–23S ITS, show that *Komarekiella* and *Chlorogloeopsis* are genetically not closely related and this is probably the only way to reliably differentiate these genera. The fact that *Chlorogloeopsis, Mojavia*, and *Komarekiella* are still known only from culture material makes the comparisons among them even more difficult, since it is known that culturing can impact morphology (Hentschke & Sant'Anna 2014).

Ecologically, these genera differ from each other (Table 1), although *Mojavia*, *Komarekiella, Halotia, Desmonostoc* and *Chlorogloeopsis* were never found in environmental samples due to their symplesiomorphic microscopic morphology (all are likely confused with microscopic *Nostoc* in environmental samples). Hypothetically, they could be opportunist populations from elsewhere, which grow from propagules in culture. However, the fact that all of the *Mojavia* and *Komarekiella* strains were found strictly in the habitats where they were originally described is evidence of environmental specificity. We found five populations of *Komarekiella* from similar environments in Mata Atlantica and Hawaii (tropical forests), and *Mojavia* has been found repeatedly in desert soils. The probability that these strains are growing as opportunists in culture is likely very low.

In the case of the polyphyletic *Chlorogloeopsis*, the type *C. fritschii* was originally described from wheat fields in India and reported later from many different environments such as thermal springs in Greenland (DQ430996) and Slovakia (Hindák 2008) and in a dry salt lake in Tunisia (Sahara desert) (Hindák 2008). Our phylogenetic analysis shows that the Indian type *C. fritschii* PCC 6912 is in the same clade as the Greenland strains with 100% of bootstrap support, at the base of Nostocaceae clade. Unfortunately, there is no molecular data for the Tunisian and Slovakian populations, but probably those two clades can be different species of *Chlorogloeopsis*, from very different environments, an example of a genus with relaxed environmental dependence. According to this, we treat *Komarekiella* and *Mojavia* as environmentally restricted genera, considering that their habitats are important for their taxonomy for now, but we conclude that further studies must be done to confirm this, since they are recently–described genera and it is still unknown whether or not they will be found in other habitats.

In the ITS secondary structures comparisons, *Komarekiella* is separated from all other cyanobacterial genera by presenting three bases opposing the basal unilateral bulge in the D1–D1' helix. This is highly unusual. In all other cyanobacteria for which secondary structure of the D1–D1' helix has been determined, there are 0, 1, or rarely 2 bases opposite the bulge. In a single taxon, *Pelatocladus maninholoensis,* there are 4 bases opposite the bulge (Miscoe et al. 2016). The significance of this structure is not at present known, but the basal sequence and secondary structure are highly conserved in all D1–D1' helices thus far studied in the cyanobacteria.

The genus *Komarekiella* is likely only one of the many *Nostoc*–like genera that eventually will be described. *Nostoc* is known to be a polyphyletic, morphology–poor genus (Hrouzek et al 2005), and there are a number of strains identified as *Nostoc* in sequence databases that fall outside of *Nostoc sensu stricto*. We still do not know if these clades or singletons are really morphologically indistinguishable from *Nostoc*, or if these morphological incongruences are due to the lack of life cycle studies and natural material observations (Hentschke & Sant'Anna 2014). *Trichormus, Halotia, Desmonostoc,* and *Mojavia* are also very morphologically similar to *Nostoc*, but careful study of strains of these taxa provided morphological means for their separation.

Finally, we were very surprised to find *K. atlantica* in Kauai. We isolated the Mata Atlantica populations in Brazil, in 2010 at the Institute of Botany in São Paulo, Brazil. We isolated the Kauai strain in 2009 at John Carroll University. All populations were originally identified as *Chlorogloeopsis* or *Nostoc* sp., but early sequencing of the Mata Atlantica material demonstrated they could not be placed phylogenetically in those or other existing genera, and the more intensive life–cycle work commenced. It is highly

unusual to have multiple populations of a new genus and species in the protologue, but to have populations 13,000 km apart is practically unique. Numerous plant species have been introduced to the Hawaiian Archipelago from South America, and we suspect that *K. atlantica* in Kauai was introduced from South America in one of these introductions. The very high sequence similarities we observed among all strains, particularly among the ITS regions, which often show differences at the intraspecies population–level, suggest that they share a very recent common ancestor. While not proven at this time, this unusually disjunct distribution of near–identical genotypes could well serve as a clear example of unintentional long–distance introduction of a microalga. To give a perverse twist on the Baas–Becking hypothesis (Baas–Becking 1934), we might say "Everything is *not* everywhere, *but* humans will soon fix that."

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