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# Cyanomargarita gen. nov. (Nostocales, Cyanobacteria): convergent evolution resulting in

Jeffrey R. Johansen

*John Carroll University*, johansen@jcu.edu

Sergei Shalygin

Regina Shalygina

Nicole Pietrasiak

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CYANOMARGARITA GEN. NOV. (NOSTOCALES, CYANOBACTERIA): CONVERGENT EVOLUTION RESULTING IN A CRYPTIC GENUS<sup>1</sup>

*Sergei Shalygin*<sup>2</sup> 

Department of Biology, John Carroll University, University Heights, Ohio 44118, USA  
Polar-Alpine Botanical Garden-Institute, Kola Science Center, Russian Academy of Science, Kirovsk-6 184230, Russia

*Regina Shalygina*

Institute of Industrial Ecology Problems of the North, Kola Science Center, Russian Academy of Science, Akademgorodok 14a, Apatity 184209, Russia

*Jeffrey R. Johansen*

Department of Biology, John Carroll University, University Heights, Ohio 44118, USA  
Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 31, České Budějovice 370 05, Czech Republic

*Nicole Pietrasiak*

Department of Plant and Environmental Sciences, New Mexico State University, 945 College Drive, Las Cruces, New Mexico 88003, USA

*Esther Berrendero Gómez*

Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 31, České Budějovice 370 05, Czech Republic

*Markéta Bohunická*

Institute of Botany of the Academy of Sciences of the Czech Republic, Dukelská 135, Třeboň 379 82, Czech Republic  
Research and Breeding Institute of Pomology, Holovousy 129, Hořice 508 01, Czech Republic

*Jan Mareš*

Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 31, České Budějovice 370 05, Czech Republic

Institute of Botany of the Academy of Sciences of the Czech Republic, Dukelská 135, Třeboň 379 82, Czech Republic  
Biology Centre of the Academy of Sciences of the Czech Republic, Institute of Hydrobiology, Na Sádkách 702/7, České Budějovice 37005, Czech Republic

and *Christopher A. Sheil*

Department of Biology, John Carroll University, University Heights, Ohio 44118, USA

Two populations of *Rivularia*-like cyanobacteria were isolated from ecologically distinct and biogeographically distant sites. One population was from an unpolluted stream in the Kola Peninsula of Russia, whereas the other was from a wet wall in the Grand Staircase-Escalante National Monument, a desert park-land in Utah. Though both were virtually indistinguishable from *Rivularia* in field and cultured material, they were both phylogenetically distant from *Rivularia* and the Rivulariaceae based on both

16S rRNA and *rbclX* phylogenies. We here name the new cryptic genus *Cyanomargarita* gen. nov., with type species *C. melechini* sp. nov., and additional species *C. calcarea* sp. nov. We also name a new family for these taxa, the Cyanomargaritaceae.

*Key index words:* 16S rRNA gene phylogeny; 16S-23S ITS; cryptic genus; Cyanobacteria; *Cyanomargarita*; *rbclX* phylogeny; *Rivularia*

*Abbreviations:* BA, Bayesian Analysis; DIC, differential interference contrast; ITS, internal transcribed spacer; ML, maximum likelihood; MP, maximum parsimony; RAS, russian academy of sciences

With the advent of molecular methods, many phylogenists, including those who study cyanobacteria, began to recognize the existence of cryptic species (Boyer et al. 2002, Casamatta et al. 2003, Erwin and Thacker 2008, Joyner et al. 2008, Premanandh et al. 2009, Reñé et al. 2013, Johansen et al. 2014, Mühlsteinová et al. 2014a,b, Patzelt et al. 2014). However, while in these papers the existence of cryptic species was suggested, the species were not recognized taxonomically. Subsequently, cryptic species have been named in several algal groups, including euglenids (Marin et al. 2003, Kosmala et al. 2007, Kosmala et al. 2009, Karnkowska-Ishikawa et al. 2010, Kim et al. 2010, 2013, Linton et al. 2010), eustigmatophytes (Fawley and Fawley 2007, Fawley et al. 2015), chlorophytes (Fawley et al. 2005, 2011, Fučíková et al. 2014), and cyanobacteria (Osorio-Santos et al. 2014, Pietrasiak et al. 2014).

Some cyanobacterial systematists have suggested the existence of cryptic genera as well (Komárek et al. 2014, Dvořák et al. 2015a), although very few cryptic genera have actually been described. *Pinochia*, which is morphologically identical to *Pseudanabaena*, has a phylogenetic position distant from that genus, and therefore was described as a cryptic genus (Dvořák et al. 2015b). *Kovacikia*, which is morphologically similar to *Phormidesmis* but molecularly distinct, would also fit the definition of a cryptic genus, although the authors did not label it as such (Miscoe et al. 2016). There are also a number of pseudocryptic genera (genera defined by morphological traits that are minor or phenotypically plastic, and therefore not always expressed in the population) as well, such as *Nodosilinea*, *Oculatella*, *Limnolyngbya*, *Pantanalinema*, and *Alkalinema*. These genera belong to the Synechococcales, an order containing taxa with few morphological characteristics (simple filamentous forms, variations in trichome width and sheath characteristics).

Outside of the Synechococcales, few cryptic genera have been recognized. In the Chroococcales, *Chalicogloea* is similar to *Gloeocapsa* and could be considered a cryptic genus (Roldán et al. 2013). There are likely more cryptic genera in this order, but identifying them is more problematic because members of the genus are difficult to grow in culture, and consequently, fewer sequences are available. In the Oscillatoriales, *Ammassolinea* is the one of the described cryptic genera (Hašler et al. 2014), being morphologically inseparable from *Phormidium*, as it is presently defined, as well as *Moorea*, *Okeania* and *Microseira* (Engene et al. 2012, 2013, McGregor and Sendall 2015). Within the Nostocales, there is much greater morphological complexity than the nonheterocytous orders. Some pseudocryptic genera have been described, including *Mojavia* (Řeháková et al. 2007), *Dapisostemon* (Hentschke et al. 2016), and *Pelatocladus* (Miscoe et al. 2016).

We recently discovered a population of tapering, heterocyte-bearing trichomes embedded in a

hemispherical to spherical mucilage investment in a small, spring-fed, unpolluted stream near the town of Apatity in the Kola Peninsula, Russia. It was completely consistent with the description of *Rivularia* C. Agardh ex Bornet & Flahault, the type genus of Rivulariaceae, which contains tapering, heterocytous taxa. This taxon fit no established species in *Rivularia*, and upon sequencing was determined to be phylogenetically distant from all members of that family. A second species belonging to the same clade as the Russian material was found, and sequenced several years earlier from a wet wall in the Grand Staircase-Escalante National Monument in Utah, USA. These two populations differ morphologically and ecologically, and are described herein as two new species based on a modified phylogenetic species concept (Mishler and Theriot 2000, Johansen and Casamatta 2005) in a newly proposed genus, *Cyanomargarita* gen. nov. This genus cannot be placed in any family-level grouping of taxa based on the phylogenetic analyses performed – we place these taxa in a new family to science, the Cyanomargaritaceae fam. nov.

#### MATERIALS AND METHODS

*Isolation and strain characterization.* Both strains of *Cyanomargarita* were isolated from natural populations into unialgal cultures using standard microbiological methods, including enrichment plates and direct isolation from the original samples, in Z8 medium (Kotai 1972, Carmichael 1986). Cultures were observed under a Zeiss Axioskop photomicroscope with both bright field and DIC optics. All morphological measurements were obtained using AxioVision 4.8 software provided by Zeiss (Oberkochen, Germany). Living cultures were deposited into the Cyanobacterial Culture Collection at John Carroll University, Cleveland, OH, USA. Natural populations of material from which the strain *C. melechinii* APA-RS9 was derived were dried and deposited as an isotype in the Herbarium of the Polar-Alpine Botanical Garden-Institute, Kola Science Centre, RAS, Kirovsk-6, Murmansk Region, Russia, and information about habitat, coordinates and locality can be found in the online database Cyanopro (Melechin et al. 2013). The dried holotype material of this strain was deposited in the Herbarium for Nonvascular Cryptogams in the Monte L. Bean Museum, Provo, UT, USA. Liquid materials of both species fixed in 4% formaldehyde, as well as dried materials of *C. calcarea*, were also deposited in the Herbarium for Nonvascular Cryptogams in the Monte L. Bean Museum, Provo, UT, USA.

*Molecular methods.* Genomic DNA was extracted following techniques described in Pietrasiak et al. (2014). PCR amplification of the 16S rRNA gene was accomplished following Osorio-Santos et al. (2014), with the exception that forward primer 8F was used instead of forward primer VRF2, for amplification of a longer sequence, starting near the beginning of the 16S rRNA gene (Perkerson et al. 2011). The 16S rRNA amplicons were cloned to avoid problems in sequencing of multiple operons (Siegesmund et al. 2008). PCR amplicons and sequences of *rbcLX* and *rpoC1* genes were obtained exactly according to Rudi et al. (1998) and Seo and Yokota (2003), respectively. The *nifD* gene amplification was completed using a protocol described in Roeselers et al. (2007). All three protein-encoding genes (*rbcLX*, *rpoC1*, *nifD*) were sequenced after purification procedure using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA); rather than cloned, because they are single-copy genes

in the cyanobacterial genome. All sequences obtained in this study were deposited in the NCBI Nucleotide database, under accession numbers – for 16S rRNA gene: KY296602, KY296603, KY296604, KY296605, KY296606, KY296607, KY296608; *rbdLX*: KY296611, KY296612; *nifD*: KY296609, KY296610; *rpoC1*: KY296613, KY296614.

**Phylogenetic analyses.** All sequences chosen for alignment and phylogenetic analyses were obtained from our internal set of sequences and relevant sequences (chosen based on both BLAST searches and named taxon searches) from the NCBI Nucleotide database before September 1, 2016. Ribosomal (16S) sequences were aligned using MUSCLE in Mega v. 6.06 (Tamura et al. 2013), and checked manually in Microsoft Word (Microsoft Corp., Redmond, WA, USA) to ensure that alignments supported preservation of secondary structure (Lukešová et al. 2009, Řeháková et al. 2014). Protein coding genes were aligned as codons using MUSCLE in Mega v. 6.06 (Tamura et al. 2013).

The public software jModeltest2 (Darriba et al. 2012) was used to determine the optimal Maximum Likelihood (ML) model, which was GTR+I+G for 16S rRNA genes, and both ML and Bayesian Analysis (BA) were subsequently run using this type of model. The exact parameters of the substitution models were individually estimated from the data during analysis by MrBayes v. 3.2.6 (Ronquist et al. 2012) and RaxML v. 7.2.8 (Stamatakis et al. 2008) software. The jModeltest2, ML, and BA calculations were all run on CIPRES (Miller et al. 2012). In the BA, two runs of eight Markov chains were applied with 10 million generations, sampling every 100 generations, with 25% burn-in. The sump command in Mr. Bayes was used. The ML analysis was conducted in RAXML v.7.2.8 with 1,000 bootstrap replicates, using GTR+I+G substitution model. Parameters for the models, with rate matrix, base frequencies, and invariant and gamma settings are given in Table S1 in the Supporting Information. The Maximum-parsimony (MP) analysis was performed using PAUP v. 4.02b (Swofford 2002) with steepest descent, the tree bisection and reconnection branch swapping, and 1,000 bootstrap replicates.

Phylogenies utilizing 16S rRNA gene sequences can yield ambiguous or unsupported trees, and in such cases a multiple loci approach is recommended (da Silva Malone et al. 2015, Song et al. 2015). We treated the *rbdLX* alignment as codons in the BA calculation (Fawley et al. 2015), using the Ny98 equal rate ratio model available in MrBayes for analysis of protein-coding genes (Ronquist et al. 2012). In the ML analysis the GTR+I+G model chosen by jModelTest2 was applied. Parameters for the models are given in Table S1. We conducted BA calculation of the *rbdLX* alignment with two runs of eight Markov chains with 20 million generations, sampling every 100 generations, with 25% burn-in. Additionally, MP and ML analyses were performed for *rbdLX* gene using the same settings as in the 16S rRNA phylogeny. The tree topology from BA was chosen for the resulting visualization for both 16S rRNA gene and *rbdLX* gene phylogenies, as well as for the mapping of support values from MP and ML analyses.

For 16S rRNA gene the BA had an estimated sample size (ESS) exceeding 2,000 for most of the parameters (790–6,877, average = 2,621 from 13 parameters), 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 <0.016. The potential scale reduction factor (PSRF) value for all the estimated parameters in the BA was 1.000, indicating that convergence of the MCMC chains was statistically achieved (Gelman and Rubin 1992).

For *rbdLX* gene the BA had an ESS exceeding 2,000 for some of the parameters (79–2,193, average = 345 from 67 parameters), 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 <0.010. The PSRF value for all the estimated parameters in the BA was 1.000, indicating that convergence of the MCMC chains was statistically achieved (Gelman and Rubin 1992).

P-distance values for all sequences were calculated in PAUP v. 4.02b (Swofford 2002). Graphical representations of the ITS structures were created in Adobe Illustrator CS5.1 (Adobe Systems Inc., San Jose, CA, USA) based upon secondary structure configurations given by Mfold (Zuker 2003).

**Line drawings.** Drawings were made by the senior author using stippling technique, completed digitally with Wacom Cintiq 24HD Pen Display (Wacom Technology Corporation, Portland, OR, USA) utilizing the original photos as templates.

## RESULTS

**Phylogenetic analyses.** The 16S rRNA gene phylogeny has posterior probability support on all nodes in the backbone of the BA, with the exception of four nodes at the base of the tree marked with small light gray circles (Fig. 1). Overall topology of the tree is consistent with recent studies of Nostocales (Berrendero et al. 2011, Hauer et al. 2014, Kaštovský et al. 2014, Berrendero Gómez et al. 2016, León-Tejera et al. 2016).

*Cyanomargarita* gen. nov. forms a cluster of two terminal OTUs (Operational Taxonomic Units) corresponding to two new species: *C. melechinii* and *C. calcarea*, with high support (Fig 1). *Cyanomargarita* is sister to a large clade, containing the well-defined monophyletic Gloeotrichiaceae, as well as the Fortieaceae, Aphani-zomenonaceae, Nostocaceae, and Tolypothrichaceae. Additionally, *Cyanomargarita* is also related to the “*Scytonema* cf. *crispum*” group, which requires revision (i.e., it is not *Scytonema* C.Agardh ex É.Bornet & C.Flahaault) and has an uncertain taxonomic position (incertae familiae), falling outside of the Scytonemataceae clade defined by the inclusion of the type species, *Scytonema hofmannii* C.Agardh ex Bornet & Flahaault (the basal clade in Fig. 1). *Cyanomargarita* is found outside of the Rivulariaceae sensu stricto, despite the similar morphology between *Cyanomargarita* and *Rivularia*.

The BA in this study and the MP and ML analyses had highly similar topology, although the latter two analyses had poorer support. The MP analysis differed slightly in the placement of *Roholtiella* Bohunická, Pietrasiak & J.R.Johansen and *Gloeotrichia* J.Agardh ex Bornet & Flahaault, but these taxa were still associated with the Nostocaceae and Aphani-zomenonaceae. In the parsimony analysis, *Macrochaete* Berrendero Gomez, J.R.Johansen & Kaštovský moved from a position proximate to *Calothrix* C.Agardh ex Bornet & Flahaault to a position sister to *Cyanomargarita*, but *Cyanomargarita* remained sister to the same polyfamily clade as in the BA (Fig. 1). The ML analysis generally had the poorest node support, but had the same topology as the BA except for a slight change in the position of “*S. cf. crispum*.” In all three analyses, *Cyanomargarita* was always a supported clade outside of the Rivulariaceae and outside of any other family-level clade.

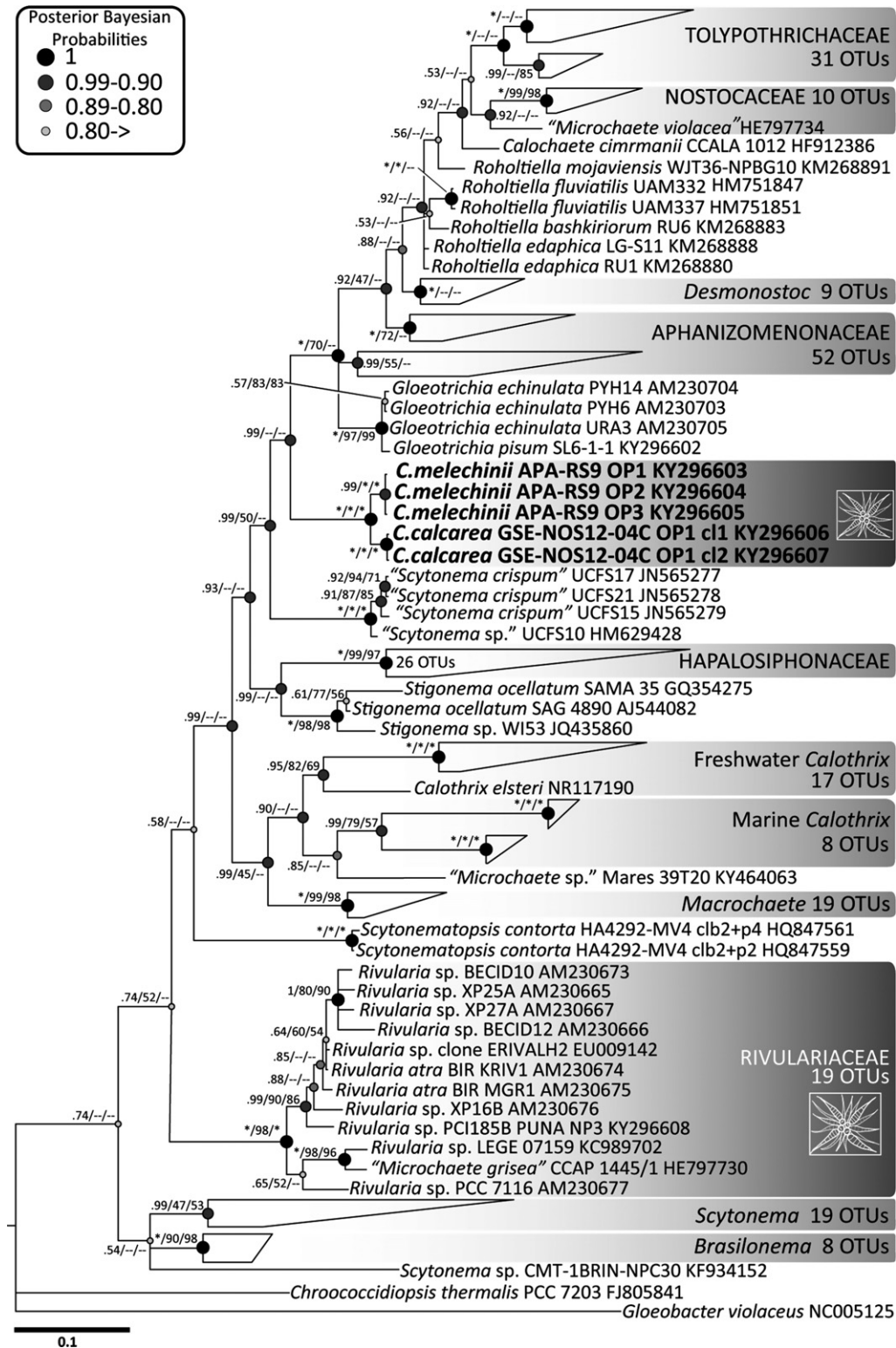


FIG. 1. Phylogeny for *Cyanomargarita* spp. inferred by Bayesian analysis within Nostocales based on a maximum of 1,495 nucleotides from the 16S rRNA gene (240 OTUs). Branch support values are shown as BA/ML/MP. Support values of 100 in ML and MP, or 1.00 (BA), is displayed as "\*", respectively, nodes with no support are shown as "-". Two species of *Cyanomargarita* are highlighted in bold, with sequences representing different operons and clones indicated (OP1, OP2, OP3, etc.), the Rivulariaceae and Cyanomargaritaceae clades are highlighted in dark gray boxes; remaining family-level clades are highlighted with light gray boxes. Drawings of the spherical colonies in the right part of the boxes indicates tapering filaments showing similar morphology between *Cyanomargarita* and *Rivularia*. OTUs in quotation marks are strains we consider to either be incorrectly identified or in need of revision.

Another piece of evidence supporting the placement of *Cyanomargarita* outside the Rivulariaceae is that representatives of this new genus have ITS regions with only one tRNA gene (tRNA<sup>Ile</sup>) across four different ribosomal operons (Table 1), which is different from both the Nostocaceae (with two or no tRNAs; see Boyer et al. 2001, Řeháková et al. 2007, Johansen et al. 2014) and the Rivulariaceae (with two tRNAs only). We conclude that, based on current phylogenetic evidence, *Cyanomargarita* requires its own family-level rank, and propose the family Cyanomargaritaceae fam. nov.

*Cyanomargarita* has low similarity in 16S rRNA gene sequence with most other Nostocales taxa (Table 2). The highest similarity was with *Gloeotrichia pisum* Thuret ex Bornet & Flahault from an alkaline wetland in Ohio, USA (95.4%). However, our new taxon differs from members of *Gloeotrichia* based on the absence of paraheterocytic akinetes with well-developed exospore. Moreover, 16S rRNA gene similarity between our taxon and a *Rivularia* strain from Argentina is only ~92.3%. Historically, less than 95% similarity among 16S rRNA gene sequences was considered good evidence for separation of prokaryotic genera (Stackebrandt and Goebel 1994), but within the heterocytous genera the cutoff is likely much higher (Flechtner et al. 2002, Patzelt et al. 2014, Berrendero Gómez et al. 2016).

*Cyanomargarita* is also outside of Rivulariaceae sensu stricto, according to our *rbclX* phylogenetic analysis (Fig. 2). According to this analysis, it is most closely related to the Tolypothrichaceae (containing the type species *Tolypothrix distorta* Kützing ex Bornet & Flahault) and diverse strains of *Calothrix*. In contrast, *Rivularia* forms a well-supported clade with *Kyrtuthrix* Ercegovic, distant from *Cyanomargarita* (León-Tejera et al. 2016). The *rbclX* phylogeny, with posterior probability support in the BA showing separated clades of *Rivularia* and *Cyanomargaritata*, is consistent with our conclusion based on the 16S rRNA gene phylogeny that *Cyanomargarita* is not congeneric with *Rivularia*, and, furthermore, is not even in the Rivulariaceae. The MP and ML phylogenies had much poorer support but similar topologies. *Cyanomargarita* was sister to the Tolypothrichaceae in MP and in a polytomy with Tolypothrichaceae and *Calothrix/Macrochaete* as in the BA in the ML analysis (data not shown).

**ITS analysis.** The 16S-23S ITS sequences of *Cyanomargarita calcarea* are ~50 nucleotides longer than the ITS sequences of *C. melechinii*, likely as a result of insertions flanking the tRNA<sup>Ile</sup> gene on the 3' side of the gene (Table 1). In general, secondary structures of D1-D1', V3, and Box B helices show similar structures across both species with minor base substitutions in all three domains. Below, we compare the secondary structures of conserved ITS domains for homologous operons in the two species (e.g., operon 1, we recovered two additional

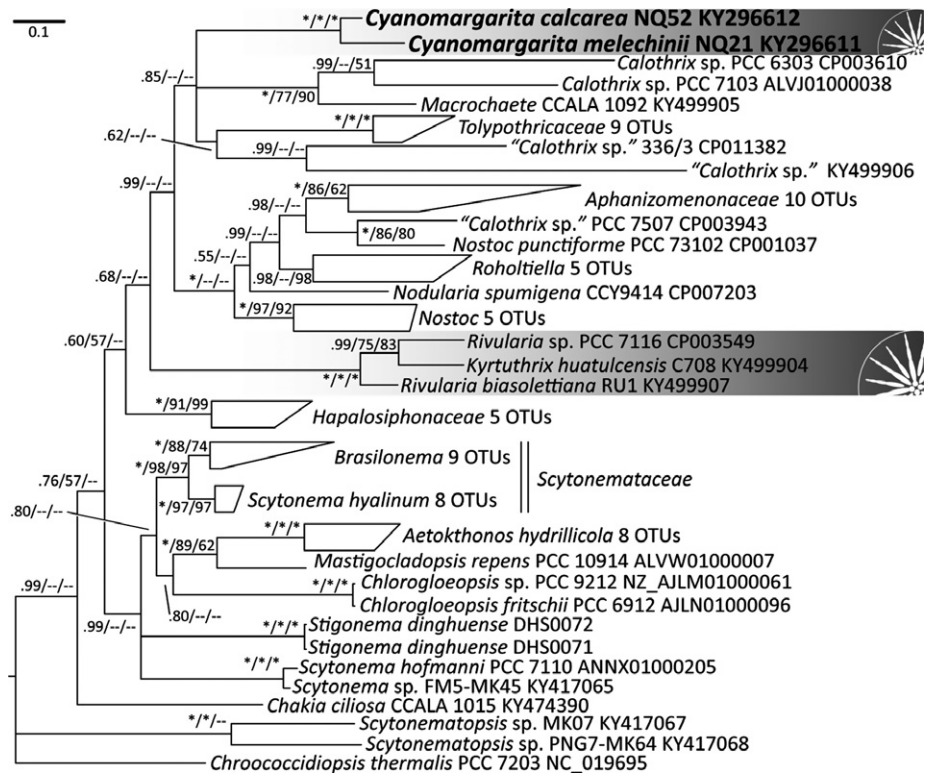
TABLE 1. Nucleotide lengths of the 16S-23S ITS region of various genera representing diverse families in the Nostocales. Both species of *Cyanomargarita* lacked tRNA<sup>Ala</sup> gene, consequently the segment after the tRNA<sup>Ile</sup> is Spacer+Box-B+Spacer. In taxa lacking both tRNA genes (last three in list), Spacer+D3 + spacer went all the way to the Box-B helix, so there was no spacer preceding the Box-B helix in Spacer+Box-B+Spacer for those taxa.

| Strain   | Leader | D1-D1' helix | Spacer+D2 | Spacer+D3 + spacer | tRNA <sup>Ile</sup> gene | Spacer+V2 + spacer | tRNA <sup>Ala</sup> gene | Spacer+Box-B+spacer | BoxA | D4 + spacer | V3 + ending of ITS | Total length |
|--|--------|--------------|-----------|--------------------|--------------------------|--------------------|--------------------------|---------------------|------|-------------|--------------------|--------------|
| <i>Cyanomargarita melechinii</i> OP1             | 7      | 64           | 34        | 73                 | 74                       | -                  | -                        | 139                 | 11   | 25          | 86                 | 513          |
| <i>Cyanomargarita melechinii</i> OP2             | 7      | 64           | 34        | 66                 | 74                       | -                  | -                        | 138                 | 11   | 25          | 81                 | 500          |
| <i>Cyanomargarita melechinii</i> OP3             | 7      | 63           | 34        | 71                 | 74                       | -                  | -                        | 139                 | 11   | 25          | 80                 | 504          |
| <i>Cyanomargarita calcarea</i> OP1, cl1          | 8      | 64           | 33        | 77                 | 74                       | -                  | -                        | 176                 | 11   | 26          | 90                 | 559          |
| <i>Cyanomargarita calcarea</i> OP1, cl2          | 8      | 64           | 33        | 79                 | 74                       | -                  | -                        | 176                 | 11   | 26          | 90                 | 561          |
| <i>Tolypothrix distorta</i> ACOI 731             | 8      | 66           | 36        | 15                 | 74                       | -                  | -                        | 224                 | 11   | 22          | 87                 | 542          |
| <i>Rivularia</i> sp. PCI185B PUNA NP3            | 9      | 64           | 39        | 19                 | 74                       | 56                 | 73                       | 80                  | 11   | 38          | 88                 | 551          |
| <i>Rohollia edaphica</i> LG-S11                  | 8      | 67           | 32        | 14                 | 74                       | 82                 | 73                       | 170                 | 11   | 27          | 115                | 672          |
| <i>Spirirestis rajadensis</i> WJT-71-NPBG6       | 8      | 66           | 33        | 16                 | 74                       | 60                 | 73                       | 75                  | 11   | 26          | 60                 | 501          |
| <i>Cylindrospermopsis raciborskii</i> KLL07      | 8      | 64           | 34        | 11                 | 74                       | 3                  | 73                       | 55                  | 11   | 23          | 38                 | 394          |
| <i>Gloeotrichia pisum</i> SL6-1-1                | 8      | 64           | 31        | 44                 | -                        | -                  | -                        | 45                  | 11   | 25          | 49                 | 277          |
| <i>Calothrix parviflora</i> 102-2B               | 7      | 66           | 30        | 45                 | -                        | -                  | -                        | 53                  | 11   | 25          | 92                 | 329          |
| " <i>Scytonema</i> cf. <i>crispum</i> " U55-MK38 | 9      | 82           | 33        | 45                 | -                        | -                  | -                        | 50                  | 11   | 28          | 71                 | 329          |

TABLE 2. Percent 16S rRNA similarity (based on p-distance) of some tapering representatives on Nostocales, including *Cyanomargarita* spp.

| Strains  | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     |
|--|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1. <i>Cyanomargarita melechirii</i> APA-RS9        |       |       |       |       |       |       |       |       |       |
| 2. <i>Cyanomargarita calcarea</i> GSE-NOS12-04C    | 99.05 |       |       |       |       |       |       |       |       |
| 3. <i>Rivularia</i> sp. PCI185B PUNA NP3           | 92.84 | 92.75 |       |       |       |       |       |       |       |
| 4. <i>Scytonematopsis contorta</i> HA4292-MV4      | 92.32 | 92.28 | 91.81 |       |       |       |       |       |       |
| 5. <i>Macrochaete santannae</i> KT336441           | 93.89 | 94.38 | 93.26 | 91.16 |       |       |       |       |       |
| 6. <i>Gloetrichia pisum</i> SL6-1-1                | 95.44 | 95.31 | 93.34 | 92.16 | 92.34 |       |       |       |       |
| 7. <i>Roholtiella edaphica</i> LG-S11              | 95.35 | 95.08 | 93.59 | 93.58 | 94.03 | 97.45 |       |       |       |
| 8. <i>Calothrix</i> sp. PCC6303 (Marine)           | 92.11 | 92.39 | 91.64 | 91.07 | 91.73 | 91.88 | 92.73 |       |       |
| 9. <i>Calothrix</i> sp. PCC7715 (Freshwater)       | 91.94 | 92.44 | 91.28 | 91.44 | 92.72 | 91.78 | 92.44 | 90.95 |       |
| 10. " <i>Scytonema</i> cf. <i>crispum</i> " UCFS21 | 95.34 | 95.43 | 92.90 | 93.46 | 95.14 | 95.70 | 95.15 | 93.66 | 93.40 |

FIG. 2. Phylogeny for *Cyanomargarita* spp. inferred by Bayesian analysis within Nostocales based on a maximum of 600 nucleotides from the *rbcLX* region (83 OTUs). Branch support values are shown as BA/ML/MP. Support values of 100 in ML and MP, or 1.00 (BA), is displayed as "\*", nodes with no support are shown as "-". Two species of *Cyanomargarita* are highlighted in bold, the Rivulariaceae and Cyanomargaritaceae clades are highlighted with dark gray boxes. Drawings of the spherical colonies in the right part of the boxes indicates tapering filaments showing similar morphology between *Cyanomargarita* and *Rivularia*. OTUs in quotation marks are strains we consider to be incorrectly identified.



operons in *Cyanomargarita melechirii* that were not obtained from *C. calcarea*). The configuration of D1-D1' helices for both species share features seen in most members of the Nostocales: a small terminal loop; a sub-terminal bilateral bulge; and a basal unilateral bulge on the 3' side of the helix, with a highly conserved basal clamp of five base pairs (GACCU-AGGUC). We detected four substitutions across the two species in the upper part of the D1-D1' helix, with three of those located within the loop regions. The last substitution on D1-D1' helix occurs within the basal 3' unilateral bulge, a transition mutation from G to A (Fig. 3). The V3 helix was very similar in both species, but with some minor differences such as two substitutions in the apical loop and a compensatory change in a single base pair in the middle part of the stem (indicated

by arrows). The V3 helix from operon 3 of *C. melechirii* has a short insertion (UAAU) within the terminus of the helix (Fig. 3). The Box B structure appears to be variable and informative, with a notably different terminal loop in operon 1 of each species. The Box B of operon 1 in *C. calcarea* is actually more similar to the Box B of operon 2 of *C. melechirii*. We do not know if this is a convergent mutation or gene conversion. In our particular case, differences of ITS structures across different operons inside one lineage can be more significant than differences detected between homologous operons of different species. The overall differences between the ITS sequences from homologous operons of the two species exceeds the differences used in the past to justify species separation (Osorio-Santos et al. 2014, Pietrasiak et al. 2014, Miscoe et al. 2016).

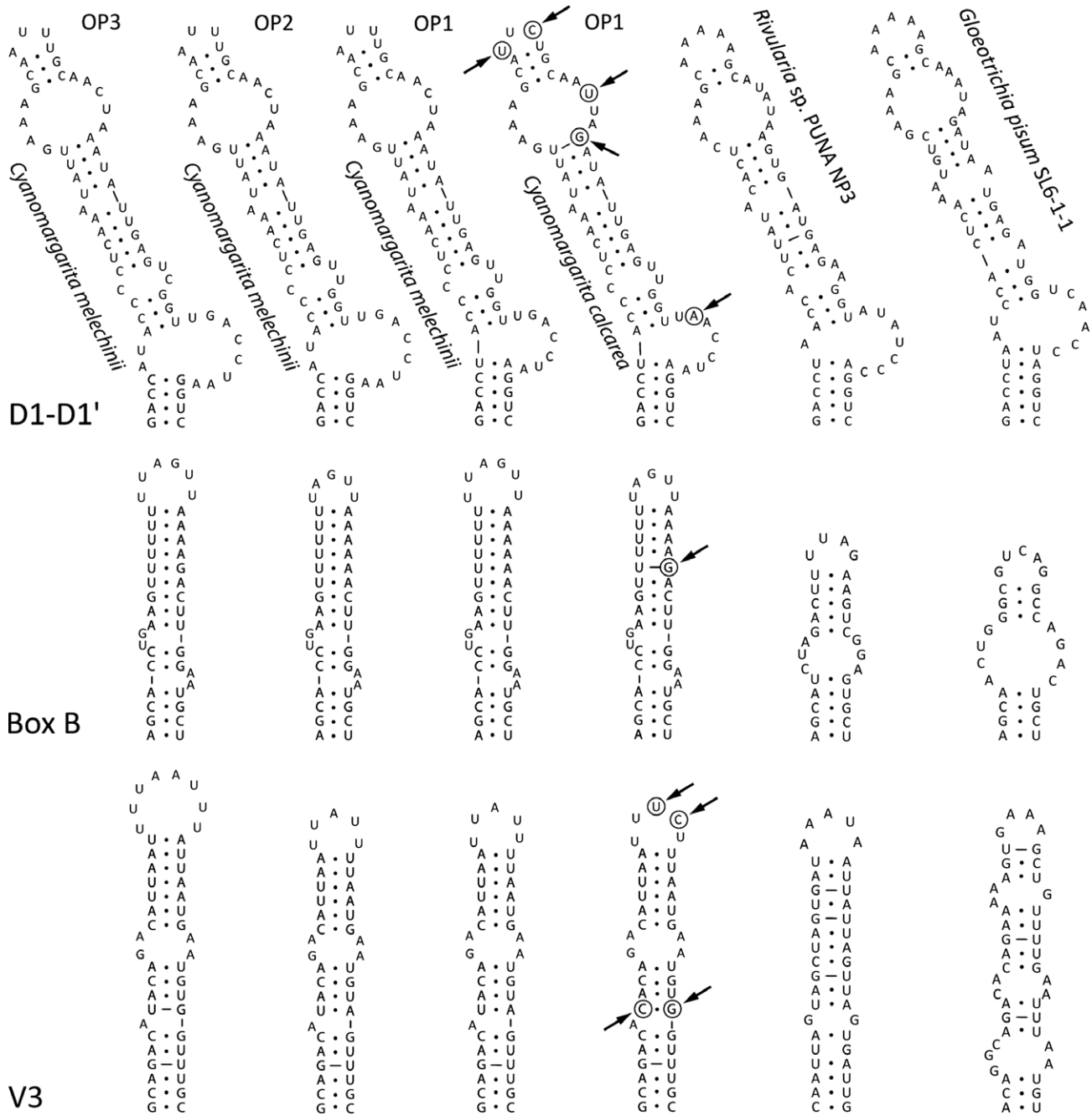


FIG. 3. Secondary structures of the 16S-23S ITS region from both species. OP stands for different operons, with three operons recovered from *Cyanomargarita melechinii* and one operon (with two clones with identical sequence and structure) from *Cyanomargarita calcarea*. Arrows on *C. calcarea* structures indicate base changes from the homologous operon 1 for *C. melechinii*.

Based on morphology, ecology, distribution, 16S rRNA gene phylogeny, p-distance analyses of 16S rRNA gene, *rbcLX* phylogeny, analysis of the secondary structure of the 16S-23S ITS region, and p-distance analysis of the 16S-23S ITS region, we conclude that the two strains of the *Cyanomargarita* clade appear to be evolutionarily independent lineages distant from representatives of Rivulariaceae, with the genus *Cyanomargarita* gen. nov. belonging

to a monogeneric family, Cyanomargaritaceae. These taxa are described here.

*Morphology and taxonomy.* **Cyanomargaritaceae** Shalygin, Shalygina & J.R.Johansen **fam. nov.**

*Diagnosis:* Morphologically similar to the members of the Rivulariaceae, but phylogenetically distinct from that family. Phylogenetically closely related to clade containing Gloeotrichiaceae, with which it bears morphological similarity, but separated from that family by phylogeny and the absence of



paraheterocytic, elongated akinetes. Also related to the “*Scytonema cf. crispum*” clade, which is phylogenetically distant from *Scytonema sensu stricto*, but differing from that group by tapering, copious mucilage formation, and hemispherical to spherical colony formation.

*Etymology*: named for the single genus in the family, *Cyanomargarita*.

*Type genus*: *Cyanomargarita* Shalygin, Shalygina et Johansen gen. et sp. nov.

*Cyanomargarita* Shalygin, Shalygina & J.R.Johansen **gen. nov.**

*Diagnosis*: Morphologically similar to *Rivularia*, but phylogenetically close to the clade containing Nostocaceae, Tolypothricaceae, and Aphani-zomenonaceae, and phylogenetically distant from all members of the Rivulariaceae, distinct from most other Nostocales by the occurrence of only one tRNA gene (tRNA<sup>le</sup>) in the 16S–23S ITS region.

*Description*: Macroscopic colonies in nature hemispherical to spherical to irregularly globular, with tapering trichomes embedded in the colonial mucilage but extending outside of the mucilage to impart a fuzzy appearance to the colony. Filaments with distinct lamellated sheath, which is often funnel- or collar-like at the distal ends. Trichomes typically largest at the base and tapering to a thin hair distally, arranged in parallel, singly, or doubly false branched, sometimes forming concentric layers in large colonies. Heterocytes basal or rarely intercalary. Akinetes absent, but large swollen arthrospores present in some species.

*Etymology*: named for the pearl-like appearance of blue-green colonies growing on mosses; *cyaneus* (L) = greenish-blue; *margarita* (L) = pearl.

*Type species*: *Cyanomargarita melechinii*

*Cyanomargarita melechinii* Shalygin, Shalygina et Johansen **sp. nov.**

*Diagnosis*: Akin to *M. calcarea*, but differing in possession of broad, colorless to slightly blackish sheaths and shorter hairs, with shorter spacer regions flanking the tRNA<sup>le</sup> region in the 16S–23S ITS, with percent identity between ITS sequences of both species >90.00%.

*Description*: Natural Populations (Figs. 4 and 5) – Macroscopic colonies slimy, spherical, or hemispherical, with appearance of small blue-green pearls attached to mosses, less commonly irregularly shaped, grayish blue-green to blue-green, attached to the substrate (in type locality on the submersed moss *Fontinalis* sp. and on stones), growing up to 5 mm in diameter. Filaments more or less radially arranged, sometimes arranged in concentric layers in the colony, attenuated towards the ends, densely arranged in parallel orientation, abundantly single false branched, with young, short filaments having geminate branching, 12.5–18 (21)  $\mu\text{m}$  wide near base, rarely with basal parts onion-like swollen. Sheaths thin to thick, 1–8  $\mu\text{m}$  wide, often strongly lamellated with 3–5 distinct layers, colorless to

slightly blackish in old filaments, funnel-like widened at the distal ends and near site of branching, rarely firm, compacted to give wavy or transverse striations. Trichomes usually gradually widened at the base, rarely onion-like swollen, sometimes narrowing towards the base, gradually tapering towards the distal ends, unconstricted, slightly constricted to distinctly constricted at the cross walls, typically constricted in the basal part, becoming unconstricted in the middle of long, mature trichomes, 7.5–12.5  $\mu\text{m}$  wide near the base, distally elongated into long, thin hairs, as narrow as 1  $\mu\text{m}$ . Cells usually granulated, rarely with large, spherical clear vesicular spaces devoid of thylakoids, bright blue-green to blue-green, when actively dividing as short as 2  $\mu\text{m}$  long, near the base shorter than wide to isodiametric, usually longer than wide in the middle of long mature trichomes, up to 10  $\mu\text{m}$  long, towards the ends less intensely pigmented or colorless, 8–20 (27)  $\mu\text{m}$  long. Heterocytes often solitary, rarely in pairs or up to three in a row, spherical, hemispherical, slightly conical, oval, or cylindrical, elongated, flattened, within, or outside of sheath, olive-brown in color, usually with an enlarged, single polar nodule, 10–15 (16)  $\mu\text{m}$  wide, 9–18 (20)  $\mu\text{m}$  long. Necridia and intercalary involution cells present.

Cultures (Fig. S1 in the Supporting Information) – Macroscopic colonies dark-green to blue-green, spreading far from the center, with several filaments upright from agar. Filaments very entangled, long, in liquid Z8 medium forming huge, abundant nodules (20–60  $\mu\text{m}$  wide), on the solid medium, frequently having single-, and double-false branching as well as geminate loops prior to branch formation, when young forming stages similar to *Tapinothrix clintonii* Bohunická et Johansen with one isopolar filament tapered at both ends fragmenting to produce two heteropolar filaments with widened base and tapered ends, rarely on nitrogen-free medium arranged in parallel like representatives of *Coleodesmium* Borzi ex Geitler, (8.1) 10–16  $\mu\text{m}$  wide. Sheaths are always colorless, slightly lamellated, with 2–4 layers, usually straight, 1–6  $\mu\text{m}$  wide. Trichomes in young stages taper, at the basal part always clearly constricted, rarely forming long unconstricted hairs, 1–2  $\mu\text{m}$  wide, in mature stages also distinctly constricted, often slightly tapering or untapered but forming conical apical cells, usually long and entangled, releasing small tapered hormogonia, or with pairs of cells with zigzag arrangement at the middle of the trichomes, also forming abruptly conical apical cells on nitrogen-free medium, 3–10  $\mu\text{m}$  wide. Cells often granulated, bright blue-green to olive-green, when actively dividing short, 2  $\mu\text{m}$  long, in middle of long trichomes, 5–10  $\mu\text{m}$  long, in the hair 3–15 (17)  $\mu\text{m}$  long, in nitrogen-free medium dividing parallel to filament axis to form a pair of cells (proheterocytes?) at the basal end of the trichome. Heterocytes forming only in nitrogen-free medium,

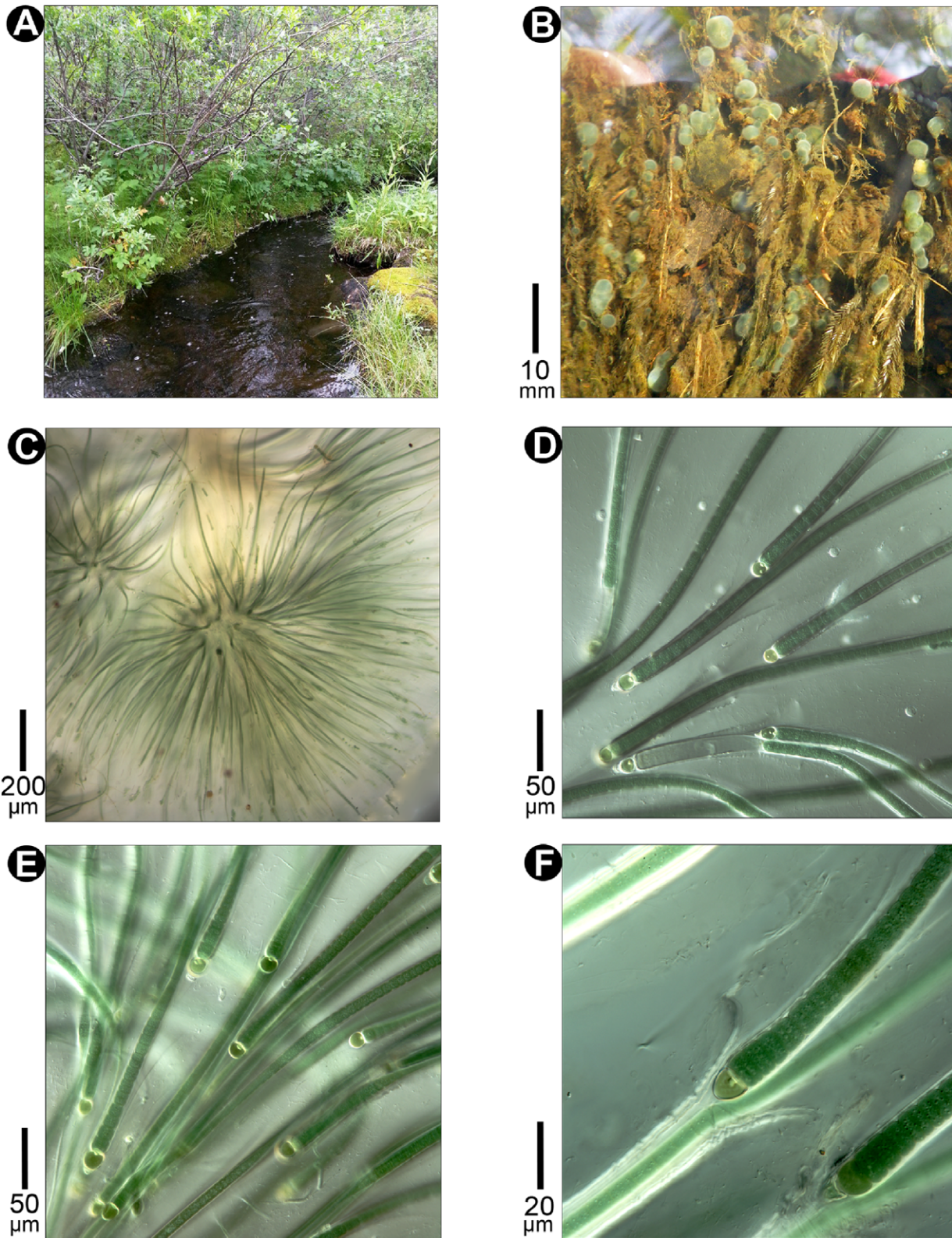


FIG. 4. Photographs and light micrographs of *Cyanomargarita melechinii* from natural populations (A) Habitat. (B) Underwater spherical and hemi-spherical macrocolonies on the *Fontinalis* sp. stems. (C) Colonial growth of radially arranged filaments. (D and E) Multiple filaments with funnel-like widened sheaths and variably shaped heterocytes. (F) Distinctly lamellated sheath and clear constrictions at branching trichome.

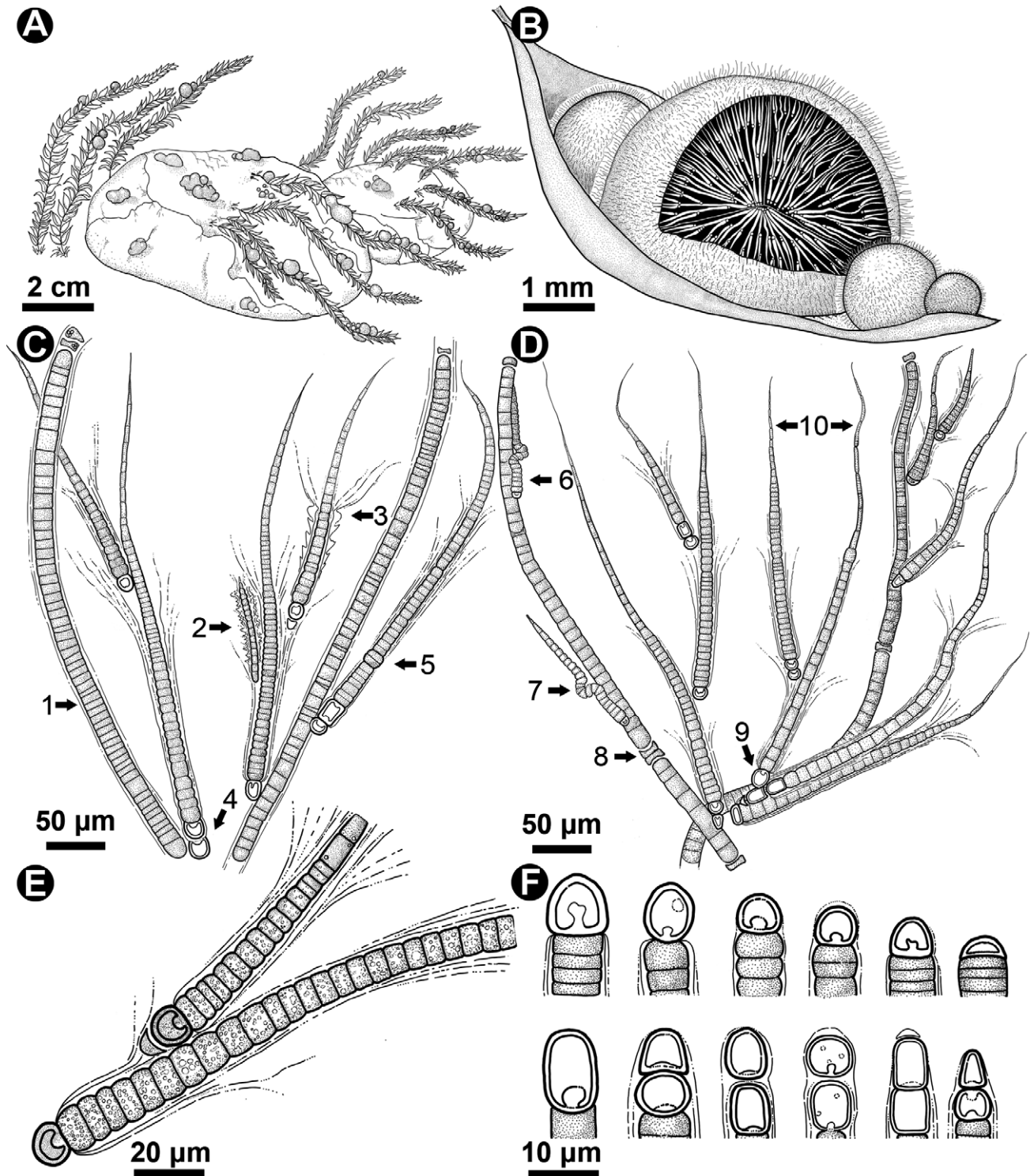


FIG. 5. Line drawings of *Cyanomargarita melechinii* from natural populations. (A) Underwater colonies on stones and mosses. (B) Spherical macrocolonies on moss leaf. (C and D) Filaments forming tufts within colony. (E) Single filaments with false branching, firm sheath, and constrictions at crosswalls. (F) Variably shaped heterocytes. Numerals indicate diagnostic characteristics used in species description: 1, Filament without constrictions; 2, sheath with wavy striations; 3, funnel-like widened sheaths; 4, two heterocytes in the row; 5, intercalary involution cells; 6, juvenile single trichome without individual sheath; 7, geminate branching on juvenile single trichome; 8, two necridia in a row; 9, different shaped heterocytes; 10, thin apical hairs.

basal, slightly brownish or colorless, of different shapes, from oval or spherical to hemispherical, flattened or irregular, often solitary, rarely two in a row or two side by side, within or outside of sheath, 5–7  $\mu\text{m}$  wide, 4–6  $\mu\text{m}$  long. Necridia, intercalary involution cells, and dark-olive resting cells present.

*Etymology*: Named in honor of Alexey Melechkin, the lichenologist who originally found *Cyanomargarita* in its type locality and informed the author of its existence.

*Holotype here designated*: BRY37764, Monte L. Bean Museum, Provo, Utah, USA.

*Isotypes here designated*: KPABG(C):3804, Herbarium of the PABGI under *Rivularia* sp., Kirovsk-6, Russia; BRY37765, BRY37766, BRY37767, Monte L. Bean Museum, Provo, Utah, USA.

*Type locality*: Russia, Kola Peninsula, Murmansk province, Apatity District, vicinity of the Apatity town, 67°32' 38.4" N, 33°30' 14" E, from cold, small, spring-fed, unpolluted, flowing stream in secondary, young forest with coniferous and deciduous trees, below the water surface on the mosses and stones (–10 cm), pH 8.4.

*Reference Strain*: *Cyanomargarita melechinii* APA-RS9, deposited in the Cyanobacterial Culture Collection at John Carroll University.

*NCBI GenBank Accession numbers*: KY296603, KY296604, KY296605, KY296609, KY296611, KY296613.

*Notes*: According to morphology, most similar to the poorly known taxon, *Rivularia compacta* Collins, described from Northern America, from which it differs by larger size of the filaments and trichomes, as well as geminate branching and character of the sheath (Komárek 2013).

*Cyanomargarita calcarea* Shalygin, Shalygina et Bohunická **sp. nov.**

*Diagnosis*: Akin to *C. melechinii*, but differing by possession of brownish sheaths closely attached to the trichomes, with longer hairs, with arthrospores, and with longer spacer regions flanking the tRNA<sup>Leu</sup> region in the 16S-23S ITS, with percent identity between ITS sequences of both species >90.00%.

*Description*: Cultures (Figs. 6, S2 in the Supporting Information) – Macroscopic colonies dark-green to olive-green when old, radiating far from the colony center, with several filaments erect from the agar, in liquid medium forming hemispherical colonies with parallel and radial arranged filaments. Filaments relatively long, entangled, sometimes irregularly coiled or screw-like coiled, frequently with single-, and double-false branching as well as with geminate loops prior to branch formation, gradually tapering from the base, 7–12 (16)  $\mu\text{m}$  wide, rarely with basal parts of filaments onion-like swollen. Sheath in the juvenile stages usually colorless, soft, thin, always attached to trichomes, maximally with two layers, 2  $\mu\text{m}$  wide; in senescent cultures brown to slightly reddish, firm, covering only basal parts of trichomes, up to 5  $\mu\text{m}$  wide, sometimes forming

collars. Trichomes gradually attenuated, constricted at the cross walls when young, unconstricted when mature, 6–10  $\mu\text{m}$  wide, tapering to a colorless hair many cells long, (2) 2.5–3  $\mu\text{m}$  wide. Cells granulated, usually barrel-shaped or distinctly constricted, apical cells sometimes widened in comparison to the adjacent subterminal cells but abruptly narrowing to a conical end, blue-green, bright blue-green to dark olive-green, longer than wide, isodiametric, or shorter than wide, longer than wide towards the ends, 2–3.5  $\mu\text{m}$  wide, 9–16  $\mu\text{m}$  long. Heterocytes basal or intercalary, two or three in a row, flattened, quadratic, or elongated oval, with shape spherical, hemispherical, conical, or irregular, rarely with two heterocytes side by side, within or outside sheath, bright brown to olive in color, 6–12  $\mu\text{m}$  wide, 9–12  $\mu\text{m}$  long. Arthrospores variable in shape, spherical to barrel-shaped, also irregular and rhomboid, typically distinctly granulated, with thin walls, blue-green, 7–10  $\mu\text{m}$  wide, 7–12 (17)  $\mu\text{m}$  long. Necridia present.

*Etymology*: Named for its occurrence on limestone; *calcarius* (L) = calcareous.

*Holotype here designated*: BRY37768, Monte L. Bean Museum, Provo, Utah, USA.

*Isotype here designated*: BRY37769, Monte L. Bean Museum, Provo, Utah, USA.

*Type locality*: Wet limestone wall in the Sheep Creek Drainage, in the Carmel Formation, pH 7.9, Grand Staircase-Escalante National Monument, Utah, USA, 37°29' 06.30" N, 112°03' 47.36" W.

*Reference Strain*: *Cyanomargarita calcarea* GSE-NOS12-04C, deposited in the Cyanobacterial Culture Collection at John Carroll University.

*NCBI GenBank Accession numbers*: KY296606, KY296607, KY296610, KY296612, KY296614.

*Notes*: Natural material could not be obtained as populations were microscopic embedded in a mixture of other algae.

## DISCUSSION

Originally, tapering cyanobacteria capable of producing heterocytes were placed either in the Rivulariaceae (*Rivularia*, *Isactis* Thuret ex Bornet et Flahault, *Brachytrichia* Bornet et Flahault and *Gloeotrichia*) or the Mastichotricheae (*Calothrix*, *Dichothrix* Zanardini ex Bornet et Flahault, *Gardnerula* De Toni (as *Polythrix* Grunow ex Bornet et Flahault), and *Saccinema* Borzi ex Bornet et Flahault; Bornet and Flahault 1886–1888). In the early part of the 20th century, these taxa, as well as other tapering taxa, including non-heterocytous forms, such as *Lep-tochaete* Borzi ex Bornet et Flahault and *Tapinothrix* Sauvageau, were all placed in a single family, Rivulariaceae (Frémy 1929, Geitler 1932). The non-heterocytous forms were removed from the family in the revision of the Nostocales completed by Komárek and Anagnostidis (1989) – this system continued in both Komárek (2013) and Komárek et al.

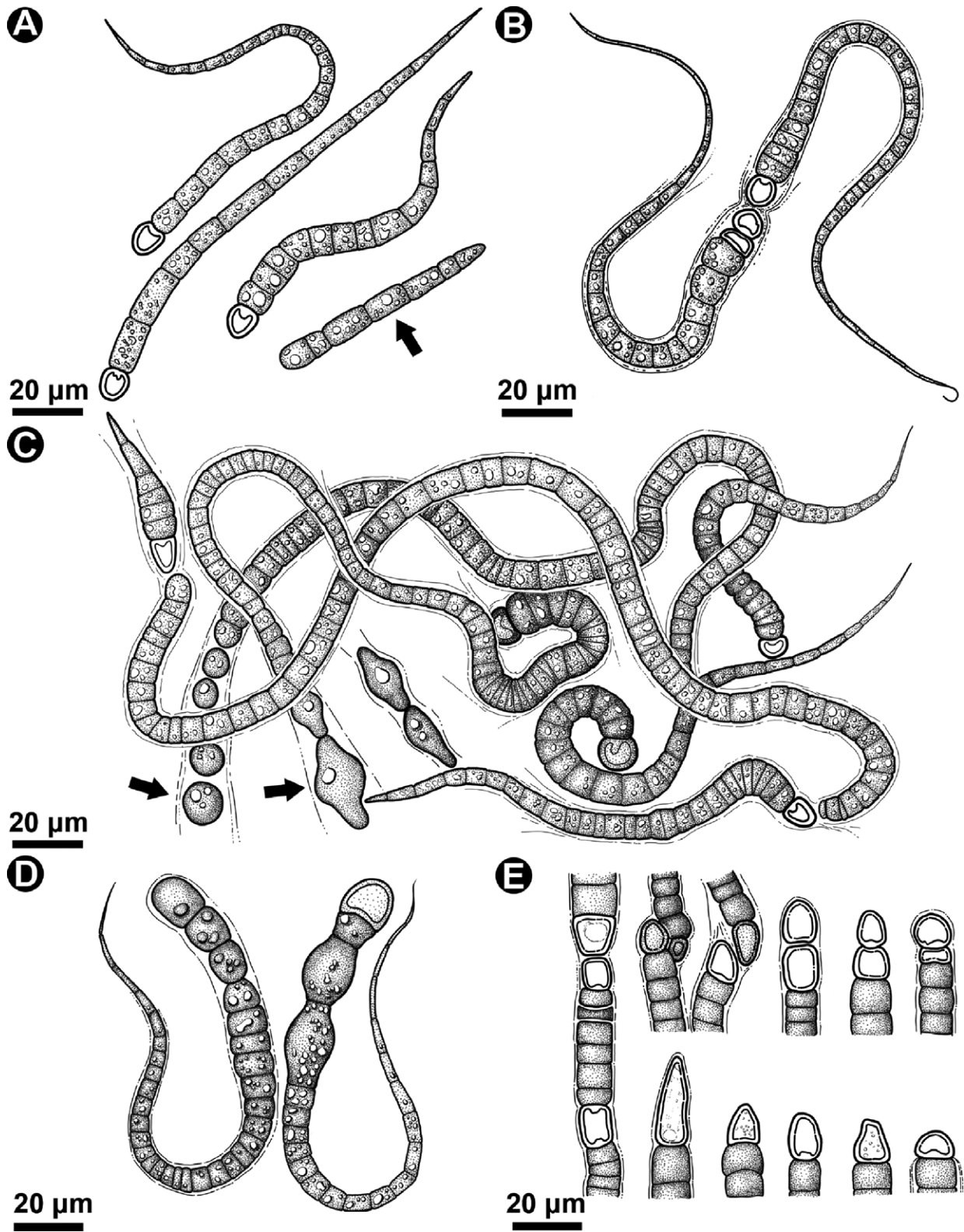


FIG. 6. Line drawings of *Cyanomargarita calcaria* from cultures. (A) Initial stages with hormogonium (arrow) and single filaments without sheaths. (B) An isopolar filament divided by intercalary heterocytes, disintegrating into two heteropolar filaments within a common sheath. (C) Entangled filaments in stationary phase, with separation of arthrospores indicated by arrows (that will grow into new filaments, D). (D) Arthrospores, germinating to form juvenile filaments. (E) Variably shaped heterocytes.

(2014). Morphologically, these taxa are well-defined, although the colonial morphology and production of hairs is typically lost in culture. The type species for *Calothrix*, *C. confervicola* C.Agardh ex Bornet & Flahault, has not yet been sequenced, and is marine in origin. The accepted type species for *Rivularia*, *R. dura* Roth ex Bornet & Flahault, has also not been sequenced, and is freshwater in origin.

Confusion regarding the diagnosis of *Calothrix* from *Rivularia* clearly exists in the modern literature. In Bergey's Manual of Systematic Bacteriology (Second Edition), the reference strains for *Calothrix* are all freshwater in origin (Rippka et al. 2001a), whereas the three reference strains for *Rivularia* are all from saline habitats (Rippka et al. 2001b). This ecological niche is the opposite of what one would expect based on the type ecology of the species. Subsequent to Rippka et al.'s (2001a,b) work, more sequences in the tapering group were found (Sihvonen et al. 2007), yielding a phylogeny with five groups: (i) *Rivularia*, mostly from marine habitats, including the Bergey's Manual reference strain *Rivularia* PCC 7716 (Rippka et al. 2001b), (ii) *Calothrix* marine clade I, (iii) *Calothrix* marine clade II, (iv) *Calothrix* freshwater clade, and (v) *Gloeotrichia* clade. Berrendero et al. (2008) confirmed this result (although *Gloeotrichia* was not in their phylogeny), but showed that all three marine clades had at least some strains assigned to *Calothrix* and some strains assigned to *Rivularia*. In subsequent papers (Berrendero Gómez et al. 2016, León-Tejera et al. 2016), the five clades noted by Sihvonen et al. (2007) persisted in the phylogenetic analyses based on larger taxon sets. Our 16S rRNA phylogeny has the most taxa, and these five clades persist in our phylogeny as well (Figs. 1, S3 in the Supporting Information).

Although some confusion persists in the names assigned to strains in culture collections, the identity of these five clades is fairly stable. We suspect that the type for *Calothrix*, when it is isolated and sequenced, will fall within either marine *Calothrix* Clade I or Clade II; *Rivularia dura*, when sequenced, will fall in the *Rivularia* clade defined in Berrendero Gómez et al. (2016) and León-Tejera et al. (2016). *Gloeotrichia* has already been moved to another family, the Gloeotrichiaceae (Komárek et al. 2014). We anticipate that *Calothrix*-like taxa (Freshwater, Marine I, Marine II) likely will be revised and separated into three genera and placed in their own families, separate from the Rivulariaceae (Fig. 1). Based on either morphology or phylogeny, *Cyanomargarita* does not fall into any previously described families, and will be placed in the Cyanomargaritaceae.

Much of the confusion in cyanobacterial taxonomy today is the result of the assumptions by earlier authors that a number of morphological features evolved within the phylum only once, or, at best, only a few times. Tapering trichomes inhabiting soft mucilage to form adherent colonies, false branching, and true branching were all characteristics that

were thought to be significant and sufficient to group taxa into relatively few higher level taxa. We now know that these derived characters have arisen multiple times through the process of convergent evolution. Tapering trichomes occur in very phylogenetically distant and diverse groups: *Rivularia*, *Isactis*, *Kyrtuthrix*, *Scytonematopsis* Kiseleva, and *Brachytrichia*, in the Rivulariaceae; *Calothrix*, *Dichothrix* and *Macrochaete* in the Mastichotricheae (which will need renaming), *Roholtiella* Bohunická, Pietrasiak et Johansen and *Calochaete* Hauer, Bohunická et Mühlsteinová in the Fortiaceae, *Gloeotrichia* in the Gloeotrichiaceae, *Goleter* Miscoe et Johansen in the Nostocaceae, and *Cyanomargarita* in the Cyanomargaritaceae, indicating that tapering likely arose independently in the Nostocales at least six times (Komárek et al. 2014, Berrendero Gómez et al. 2016, León-Tejera et al. 2016, Miscoe et al. 2016).

True-branching was similarly considered to have been a unique feature that arose only once in the heterocytous cyanobacteria, and all true-branching forms were at one time in the Stigonemataceae. Based on molecular data, we now know that true branching occurs in the Scytonemataceae (*Symphyonemopsis* Tiwari et Mitra and *Iphinoe* Lamprinou et Pantazidou), Stigonemataceae (*Stigonema* Agardh ex Bornet et Flahault), Tolypothricaceae (*Rexia* Casamatta, Gomez et Johansen), Hapalosiphonaceae (*Hapalosiphon* Kirchner ex Bornet et Flahault, *Fischerella* Gomont, *Westiellopsis* Janet, and *Nostochopsis* Wood ex Bornet et Flahault) Chlorogloeopsidaceae (*Chlorogloeopsis* Mitra et Pandey), and *Aetokthonos* Wilde et Johansen, indicating this character arose at least six times (Gugger and Hoffmann 2004, Wilde et al. 2014, Mareš et al. 2015). Indeed, in the Cyanomargaritaceae, cell division in two planes is present in both species, and this is a prerequisite character to true-branching, although at present we have only seen the phenomenon in the basal cells of the trichomes in culture material.

Polyphyly in cyanobacterial genera should not be a surprise. Given that relatively few characters were given inordinate weight by early taxonomists, thinking that these characters could arise independently did not seem parsimonious or likely. However, with a molecular understanding, we realize that many supposed synapomorphies in cyanobacteria are actually not homologous characters. It seems apparent that they are useful in the definition of genera, where they appear to be consistent across the entire group, but they fail in the definition of higher-level taxa. The exception appears to be the formation of heterocytes and akinetes, which are restricted to the Nostocales and therefore likely arose only once.

Given the convergence of morphological traits in evolutionarily distant lineages, the use of molecular sequence data to define family- and order-level taxa is likely going to increase. The morphological definition of families will likely be replaced by a phylogenetic definition (a monophyletic cluster of

genera). This is already happening in other algal groups, such as the Sphaeropleales (Fučíková et al. 2014). We anticipate that as more molecular sequence data become available for more genera, the difficulty in using existing family-level taxonomy will increase in many algal groups, including cyanobacteria, and more families will be described and recognized in order to maintain monophyly and to stabilize taxonomy. These families will, unfortunately, often be difficult to characterize morphologically, and so will lose their meaning and value to the taxonomic novice. However, a taxonomic system consistent with evolutionary history has long been the goal of taxonomists.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

**Figure S1.** Light micrographs of *Cyanomargarita melechinii* from cultures. (A) *Tapinothrix clintonii*-like stages. (B) Spiraled and very entangled filaments. (C) Huge nodule from liquid medium. (D) Single, double and geminate branching types. (E) Unusual cell division in the perpendicular plane, dark-olive resting cells and strange endings of trichomes. (F) Variably shaped heterocytes.

**Figure S2.** Light micrographs of *Cyanomargarita calcarea* from cultures. (A) Macrocolonies on agar surface. (B) Entangled filaments with single and double false branching. (C) Individual filaments with variably shaped arthospores (arrows). (D) Mature filaments with intensely brown sheath. (E and F) Variably shaped heterocytes on well granulated trichomes.

**Figure S3.** Uncollapsed phylogeny for *Cyanomargarita* spp. inferred by Bayesian analysis within Nostocales based on a maximum of 1,495 nucleotides from the 16S rRNA gene (240 OTUs). Branch support values are shown as BA/ML/MP. Support values of 100 in ML and MP, or 1.00 (BA), is displayed as “\*,” respectively, nodes with no support are shown as “–.”

**Table S1.** Summary of likelihood substitution model parameters for the GTR+G+I nucleotide models and Ny98 codon model.