Winter 2016

CYANOMARGARITA GEN. NOV. (NOSTOCALES, CYANOBACTERIA): CONVERGENT EVOLUTION RESULTING IN A CRYPTIC GENUS

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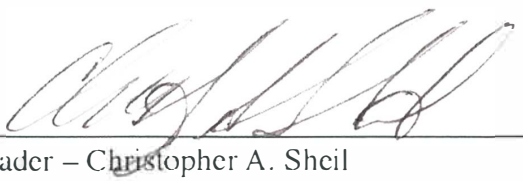
A Thesis Submitted to the
Office of Graduate Studies
College of Arts & Sciences of
John Carroll University
In Partial Fulfillment of the Requirements
For the Degree of
Master of Science

By
Sergei Shalygin
2016

The thesis of Sergei Shalygin is hereby accepted:



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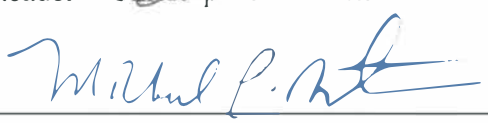

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ACKNOWLEDGEMENTS

I thank John Carroll University for salary support, supplies and use of laboratory facilities, and supportive faculty and staff in the Biology Department. Additionally, I am grateful to all lab mates from the Johansen laboratory for useful discussion of the work. This work was completed with support from the projects 31-15-11912S and 13-13368S of the Grant Agency of the Czech Republic and with a grant of the Faculty of Science, University of South Bohemia (GAJU 145-2013P)(KC)).

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ABSTRACT

Two populations of *Rivularia*-like cyanobacteria were isolated from ecologically diverse 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 *rbc*LX phylogenies. The new putative cryptic genus *Cyanomargarita*, with proposed type species *C. melechinii* sp. nov., and additional putative species *C. calcarea* are described herein. A new family for these taxa, the Cyanomargaritaceae, is proposed for this new genus.

INTRODUCTION

With the advent of molecular methods, many phycologists, 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, Patzelt et al. 2014, Mühlsteinová et al. 2014a, 2014b). However, though these papers suggest the existence of cryptic species, the species were not formally recognized taxonomically. Subsequently, cryptic species have been named in several algal groups, including euglenids (Marin et al. 2003, Karnkowska-Ishikawa et al. 2010, Kim et al. 2010, 2013, Linton et al. 2010), eustigmatophytes (Fawley et al. 2007, 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); however, very few cryptic genera have actually been described. *Pinocchia* Dvořák, Jahodárová et Hasler, which is morphologically identical to *Pseudanabaena* Lauterborn, has a phylogenetic position distant from *Pseudanabaena*, and was consequently described as a cryptic genus (Dvořák et al. 2015). *Kovacikia* Miscoe, Pietrasiak et Johansen, which is morphologically similar to *Phormidesmis* Turicchia et al., 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* Perkerson et. Casamatta, *Oculatella* Zammit, Billi et Albertano, *Limnolyngby*a X. Li et R. Li, *Pantanalinema* Vaz et al., and *Alkalinema* Vaz et al. All of the above 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* Roldán et al. is similar to *Gloeocapsa* Kützing, 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* Hasler et al. is the sole cryptic genus described (Hasler et al. 2014), being morphologically inseparable from *Phormidium* Kützing ex Gomont, as it is presently defined. Within the Nostocales, there is much greater morphological

complexity than the nonheterocytous orders. Some pseudocryptic genera have been described, including *Mojavia* Reháková et Johansen in Reháková et al. (2007), *Dapisostemon* Hentschke, Sant'Anna et Johansen in Hentschke et al. (2016), and *Petalocladus* Johansen et Vaccarino in Miscoe et al. (2016).

A population of tapering, heterocyte-bearing trichomes embedded in a hemispherical to spherical mucilage investment in a small, spring-fed, unpolluted stream was recently discovered near the town of Apatity in the Kola Peninsula, Russia. It was completely consistent with the description of *Rivularia*, 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 in a newly proposed genus, *Cyanomargarita*. This genus cannot be placed in any family-level grouping of taxa based on the phylogenetic analyses performed and will be placed in a family new to science, the Cyanomargaritaceae.

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. Living cultures were deposited into the Cyanobacterial Culture Collection at John Carroll University, Cleveland, 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 Scientific Centre of 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 species was deposited in the Herbarium for Nonvascular Cryptogams in the Monte L. Bean Museum, Provo, Utah, 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, Utah, 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 recover multiple rRNA operons (Siegesmund 2008). PCR amplicons and sequences of *rbc*LX and *rpo*C1 genes were obtained 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 (*rbc*LX, *rpo*C1, *nifD*) were directly sequenced, 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.

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 1 September, 2016. Sequences were aligned using Mega v. 6.06 (Tamura et al. 2013), and checked manually in Microsoft Word (Microsoft Corp., Redmond, Washington, USA) to ensure that alignments supported preservation of secondary structure (Lukešová et al. 2009, Řeháková et al. 2014). A full list of the OTU's used can be found in the uncollapsed phylogeny in supplemental materials (Fig. S1).

The public software jModeltest2 (Darriba et al. 2012) was used to determine the optimal Maximum Likelihood (ML) model, which was GTR+I+G, and Bayesian analysis was subsequently run using this model. Both analyses were run on CIPRES (Miller et al. 2012). Two runs of eight Markov chains were applied with 10 million generations with default settings, sampling every 100 generations. P-distance values for all sequences were calculated in PAUP v. 4.02b (Swofford 1998). Graphical representation of the ITS structures were created in Adobe Illustrator CS5.1 (Adobe Systems Inc., San Jose,

California, USA) based upon secondary structure configurations given by Mfold (Zuker 2003).

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). I treated protein coding gene sequences (*rbc*LX) as codons (Fawley et al. 2015), using the Ny98 evolutionary model with equal mutation rates (Miller et al. 2012). Baysian Analysis of the *rbc*LX alignment was conducted with two runs of eight Markov chains with 20 million generations, sampling every 100 generations, also using the GTR+I+G model (Miller et al. 2012, Darriba et al. 2012).

Line drawings. Drawings were made using stippling technique, completed digitally with Wacom Cintiq 24HD Pen Display utilizing the original photos as templates.

RESULTS

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

Cyanomargarita forms a cluster of two terminal OTUs corresponding to two new species: *C. melechinii* and *C. calcarea*, with high support (Fig 1; Fig S1(A,B,C,D)). *Cyanomargarita* is sister to a large clade containing Gloeotrichiaceae, Fortieaceae, Aphanizomenonaceae, Nostocaceae, and Tolypothrichaceae. Additionally, *Cyanomargarita* is also related to the *Scytonema crispum* group, which has an uncertain taxonomic position (*incertae familiae*), falling outside of the Scytonemataceae clade defined by the inclusion of the type species, *Scytonema hofmannii* (the basal clade in Fig. 1). *Cyanomargarita* is found outside of the Rivulariaceae, despite the convergent morphology between *Cyanomargarita* and *Rivularia*. Another piece of evidence supporting the independent origin of *Cyanomargarita* is that representatives of this new genus have ITS regions with only one tRNA gene ($tRNA^{Ile}$) across five different ribosomal operons (Table 1), which is different from both the Nostocaceae (with two or no tRNAs) 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.

Cyanomargarita has low similarity in 16S rRNA gene sequence with most other Nostocalaean taxa (Table 2). The highest similarity was with *Gloeotrichia pisum* Thuret

ex Bornet et 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 about 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 higher (<97-98%, see Flechtner et al. 2002, Patzelt et al. 2014, Berrendero Gómez et al. 2016). Therefore, the evidence is even stronger that *Cyanomargarita* is a new genus.

Cyanomargarita is also outside of Rivulariaceae *sensu stricto*, according to our *rbc*LX phylogenetic analysis (Fig. 2). It is most closely related to the Tolypothrichaceae (containing the type species *Tolypothrix distorta* Kützing ex Bornet & Flahault) and diverse *Calothrix* strains. In contrast, *Rivularia* forms a well-supported clade with *Kyrtuthri*x, distant from *Cyanomargarita* (León-Tejera et al. 2016). The *rbc*LX phylogeny, with well supported separate clades of *Rivularia* and *Cyanomargariata,* is consistent with our conclusion based on the 16S rRNA gene phylogeny that *Cyanomargarita* is not congeneric with *Rivularia*, and, furthermore, is not in the Rivulariaceae.

ITS analysis. The 16S–23S ITS sequences of *C. calcarea* are about 50 nucleotides longer than the ITS sequences of *C. melechinii,* likely as a result of insertions flanking the $tRNA^{Ile}$ 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, I compare the secondary structures of

conserved ITS domains for homologous operons in the two species (e.g. operon 1, two additional operons were recovered in *C. melechinii* 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 5 base pairs (GACCU-AGGUC). Four substitutions across the two species in the upper part of the D1-D1′ helix were detected, with 3 of those located within the loop regions. Another 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. melechinii* has a short insertion (UAAU) within the terminal loop (Fig. 3). The Box B structure appears to be variable and informative, with a lager terminal loop in operon 1 of *C. melechinii*. The Box B of operon 1 in *C. calcarea* is actually more similar to the Box B of operon 2 of *C. melechinii*. It is not possible to determine whether this is a convergent mutation or gene conversion. In this particular case, differences in 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 2014, Pietrasiak 2014, Miscoe 2016).

Morphology and taxonomy. Based on morphology, ecology, distribution, 16S rRNA gene phylogeny, p-distance analyses of 16S rRNA gene, *rbc*LX phylogeny,

analysis of the secondary structure of the 16S–23S ITS region, and p-distance analysis of the 16S–23S ITS region, I 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 will be formally published in a peer-reviewed journal as required by the International Code of Nomenclature for Algae, Fungi and Plants (McNeill et al. 2012). The descriptions below are not valid under the code, but indicate the provisional taxonomy until the time of publication.

Cyanomargaritaceae Shalygin, Shalygina et Johansen **fam. prov.**

Diagnosis: Morphologically similar to the members of the Rivulariaceae, but phylogenetically distinct from that family. Phylogenetically closest to Gloeotrichaceae, with which it bears morphological similarity, but separated from that family by phylogeny and the absence of paraheterocytic, elongated akinetes. Molecularly similar to the "*Scytonema 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. prov.

Cyanomargarita Shalygin, Shalygina et Johansen **gen. prov.**

Diagnosis: Morphologically similar to *Rivularia*, but phylogenetically close to the clade containing Nostocaceae, Tolypothricaceae, and Aphanizomenonaceae, and

phylogenetically distant from all members of the Rivulariaceae, distinct from most other Nostocales by the occurrence of only one tRNA gene ($tRNA^{Ile}$) in the 16S–23S ITSregion.

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. prov.**

Description: Natural Populations (Figs 4, 5) − Macroscopic colonies slimy, spherical or hemispherical, with appearance of small blue-green pearls attached to mosses, less commonly irregularly shaped, greyish 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) µm wide near base, rarely with basal parts onion-like swollen. Sheaths thin to thick, 1−8 µm 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 base, rarely onion-like swollen, sometimes narrowing towards base, gradually tapering towards distal ends, unconstructed, slightly constricted to distinctly constricted at cross walls, typically constricted in basal part, becoming unconstructed in middle of long, mature trichomes, 7.5−12.5 µm wide near base, distally elongated into long, thin hairs, as narrow as 1 µm wide. 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 µm long, near the base shorter than wide to isodiametric, usually longer than wide in middle of long mature trichomes, up to 10 µm long, towards ends less intensely pigmented or colorless, 8−20 (27) µm long. Heterocytes often solitary, rarely in pairs or up to 3 in a row, olive-brown in color, usually with enlarged, single polar nodule, spherical, hemispherical, slightly conical, oval or cylindrical, elongated, flattened, within or outside of sheath, 10−15 (16) µm wide, 9−18 (20) µm long. Necridia and intercalary involution cells present.

Cultures (Fig. 6) – Macroscopic colonies dark-green to blue-green, spreading far from center, with several filaments upright from agar. Filaments entangled, long, in liquid Z8 medium forming huge, abundant nodules (20-60 µm wide), on solid medium, frequently having single- and double-false branching as well as geminate loops prior to branch formation, when young forming *Tapinothrix clintonii*-like stages 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*, (8.1) 10−16 µm wide. Sheaths always colorless, slightly lamellated, with 2−4 layers, usually straight, 1−6 µm wide. Trichomes in young stages taper, at basal part always clearly constricted, rarely forming long unconstricted hairs, 1−2 µm 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 zig-zag arrangement at the middle of the trichomes, also forming abruptly-conical apical cells on nitrogen-free medium, 3−10 µm wide. Cells often granulated, bright blue-green to olive-green, when actively dividing short, 2 µm long, in middle of long trichomes, 5−10 µm long, in the hair 3−15 (17) µm long, in nitrogen-free medium dividing parallel to filament axis to form a pair of cells (preheterocytes?) at the basal end of the trichome. Heterocytes forming only in nitrogen-free medium, 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 µm wide, 4−6 µm long. Necridia, intercalary involution cells, and dark-olive resting cells present.

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

Holotype to be designated: BRY37764, Monte L. Bean Museum, Provo, Utah.

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

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 young secondary 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.

Notes: According to morphology, most similar to the poorly known taxon, *Rivularia compacta* Collins in Collins et al. 1898, 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 Bohunicka **sp. prov.**

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 $tRNA^{Ile}$ region in the 16S-23S ITS, with percent similarity between ITS sequences of both species > 90.00%.

Description: Cultures (Figs 7, 8) − Macroscopic colonies dark-green to olive-green when old, radiating far from colony center, with several filaments erect from 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) µm 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 2 layers, 2 µm wide; in senescent cultures brown to slightly reddish, firm, as a rule covering only basal parts of trichomes, up to 5 µm wide, sometimes forming collars. Trichomes gradually attenuated, constricted at the cross walls when young, unconstricted when mature, 6−10 µm wide, tapering to a colorless hair many cells long, (2) 2.5−3 µm wide. Cells granulated, usually barrel-shaped or distinctly constricted, apical cells sometimes widened in comparison to 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 µm wide, 9−16 µm long. Heterocytes basal or intercalary, 2 or 3 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 µm wide, 9−12 µm long. Arthrospores variable in shape, spherical to barrel-shaped, also irregular and rhomboid, typically distinctly granulated, with thin walls, blue-green, 7−10 µm wide, 7−12 (17) µm long. Necridia present.

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

Holotype to be designated: BRY37768, Monte L. Bean Museum, Provo, Utah.

Isotype to be designated: BRY37769, Monte L. Bean Museum, Provo, Utah.

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.

DISCUSSION

Originally, tapering cyanobacteria capable of producing heterocytes were placed either in the Rivulariaceae (*Rivularia, Isactis, Brachytrichia* and *Gloeotrichia*) or the Mastichotricheae (*Calothrix, Dichothrix, Gardnerula* (as *Polythrix*), and *Sacconema*) (Bornet et Flahault 1886). In the early part of the 20th century, these taxa, as well as other tapering taxa, including non-heterocytous forms, such as *Leptochaete* and *Tapinothrix,* were all placed in a single family, Rivulariaceae (Fremy 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. (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* Agardh ex Bornet et Flahault, has not yet been sequenced, and is marine in origin. The accepted type species for *Rivularia, R. dura* Roth ex Bornet et Flahualt, 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, 2001b) work, more sequences in the tapering group were found (Sihvonen et al. 2007), yielding a phylogeny with four groups: 1) *Rivularia*, mostly from marine habitats, including the Bergey's Manual reference strain *Rivularia* PCC 7716 (Rippka et al. 2001b), 2) *Calothrix* marine clade I, 3) *Calothrix* marine clade II, 4) *Calothrix* freshwater clade, and 5) *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 (Fig. 1; Fig S1(A,B,C,D)).

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 one of the marine *Calothrix* clades (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 Gloeotrichaceae (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,* and *Brachytrichia,* in the Rivulariaceae; *Calothrix, Dichothrix* and *Macrochaete* in the Mastichotricheae (which will need renaming), *Roholtiella* and *Calochaete* in the Fortiaceae, *Gloeotrichia* in the Gloeotrichaceae, *Goleter* in the Nostocaceae, and *Cyanomargarita* in the Cyanomargaritaceae, indicating that tapering likely arose independently in the Nostocales at least six times.

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 Stigonematales. Based on molecular data, we now know that true branching occurs in the Scytonemataceae (*Symphyonemopsis* and *Iphinoe*), Stigonemataceae (*Stigonema*), Tolypothricaceae (*Rexia*), and Hapalosiphonaceae (*Hapalosiphon, Fischerella, Westiellopsis* and *Nostochopsis*, etc.), indicating this character arose at least four times. 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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Table 1. Nucleotide lengths of the 16S–23S ITS region of various genera representing diverse families in the Nostocales. Both species of *Cyanomargarita* lacked $tRNA^{Ala}$ gene, consequently the segment after the $tRNA^{Ile}$ 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	$tRNA^{Ile}$ gene	Spacer+V2+ spacer	$tRNA^{Ala}$ gene	Spacer+ Box-B+spacer	BoxA	D4+spacer	V3+ending of ITS	Total length
Cyanomargarita melechinii OP1	7	64	34	73	74	-	-	139	11	25	86	513
Cyanomargarita melechinii OP2	7	64	34	66	74	-	-	138	11	25	81	500
Cyanomargarita melechinii OP3	7	63	34	71	74	-	-	139	11	25	80	504
Cyanomargarita calcarea OP1	8	64	33	77	74	-	-	176	11	26	90	559
Cyanomargarita calcarea OP1	8	64	33	79	74	-	-	176	11	26	90	561
Tolypothrix distorta ACOI 731	8	66	36	15	74	-	-	224	11	22	87	542
Rivularia sp. PCI185B PUNA NP3	9	64	39	19	74	56	73	80	11	38	88	551
Roholtiella edaphica LG-S11	8	67	32	14	74	82	73	170	11	27	115	672
Spirirestis rafaelensis WJT-71-NPBG6	8	66	33	16	74	60	73	75	11	26	60	501
Cylindrospermopsis raciborskii KLL07	8	64	34	11	74	3	73	55	11	23	38	394
Gloeotrichia pisum SL6-1-1	8	64	31	44	-	-	-	45	11	25	49	277
Calothrix parietina 102-2B	7	66	30	45	-	-	-	53	11	25	92	329
Scytonema cf. *crispum* 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. *Cyanomargarita melechinii* APA-RS9									
2. *Cyanomargarita calcarea* GSE-NOS12-04C	99.05								
3. *Rivularia* sp. PCI185B PUNA NP3	92.84	92.75							
4. *Scytonematopsis contorta* HA4292-MV4	92.32	92.28	91.81						
5. *Macrochaete santannae* KT336441	93.89	94.38	93.26	91.16					
6. *Gloeotrichia pisum* SL6-1-1	95.44	95.31	93.34	92.16	92.34				
7. *Roholtiella edaphica* LG-S11	95.35	95.08	93.59	93.58	94.03	97.45			
8. *Calothrix* sp. PCC6303 (Marine)	92.11	92.39	91.64	91.07	91.73	91.88	92.73		
9. *Calothrix* sp. PCC7715 (Freshwater)	91.94	92.44	91.28	91.44	92.72	91.78	92.44	90.95	
10. *Scytonema* cf. *crispum* UCFS21	95.34	95.43	92.90	93.46	95.14	95.70	95.15	93.66	93.40

FIGURE LEGENDS

Fig. 1. Bayesian phylogeny for *Cyanomararita* spp. within Nostocales based on a maximum of 1495 nucleotides from the 16S rRNA gene (254 OTUs). Branch support values are shown as Bayesian posterior probability. Two species of *Cyanomargarita* are highlighted in bold, the Rivulariaceae and Cyanomargaritaceae clades are highlighted in dark gray boxes; remaining family-level clades are highlighted with light grey boxes. Drawings of the spherical colonies in the right part of the boxes indicates tapering filaments showing similar morphology between *Cyanomargarita* and *Rivularia*.

Fig. 2. Bayesian phylogeny for *Cyanomararita* spp. within Nostocales based on a maximum of 600 nucleotides from the *rbc*LX region (86 OTUs). Branch support values are shown as Bayesian posterior probability. Branch support values are shown as Bayesian posterior probability. 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*.

Fig. 3. Secondary structures of the 16S–23S ITSregion from both species. OP stands for different operons, with three operons recovered from *C. melechinii* and one operon from *C. calcarea*. Arrows on *C. calcarea* structures indicate base changes from the homologous operon 1 for *C. melechinii*.

Fig. 4. Photographs and light micrographs of *C. 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.

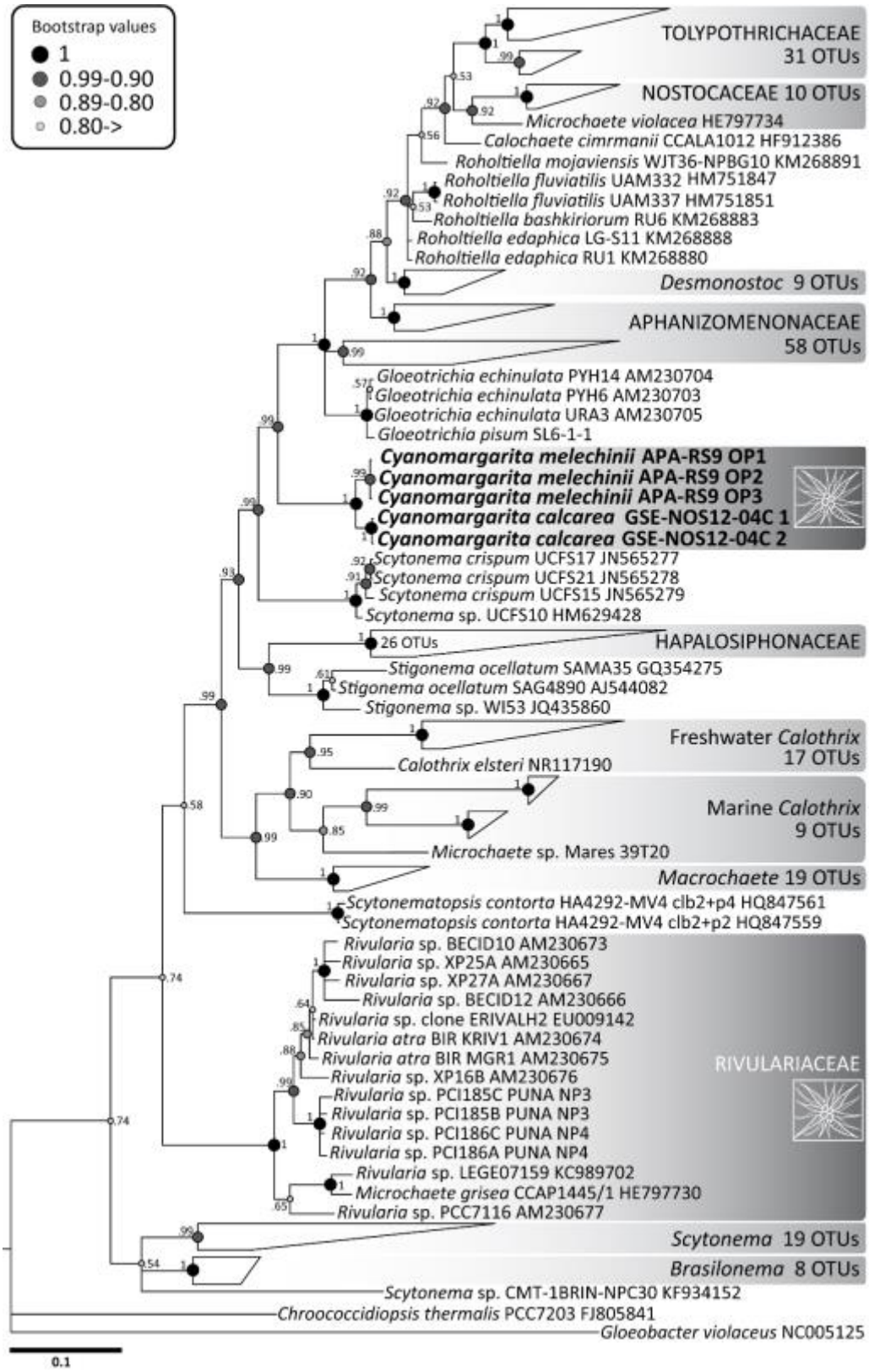
Fig.5. Line drawings of *C. melechinii* from natural populations. (A) Underwater colonies on stones and mosses. (B) Spherical macrocolonies on mosses leaf. (C and D) Filaments forming tufts within colony. (E) Single filaments with false branching, firm sheath, and constrictions at crosswalls. (F) Variably-shaped 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.

Fig. 6. Light micrographs of *C. 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.

Fig. 7. Line drawings of *C. calcarea* from cultures. (A) Initial stages with hormogonium (arrow) and single filaments without sheaths. (B) An isopolar filament divided by intercalary heterocyte formation 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.

Fig. 8. Light micrographs of *C. 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.

Fig. S1 (In 4 parts :A, B, C, D). Uncollapsed Bayesian phylogeny for *Cyanomararita* spp. within Nostocales based on maximum of 1495 nucleotides from the 16S rRNA gene (254 OTUs). Level of support (Bayesian posterior probabilities) indicated with different colors.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Roholtiella bashkiriorum RU6 KM268883
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Desmonostoc 9 OTUs
APHANIZOMENONACEAE
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Gloeotrichia echinulata PYH6 AM230703
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Cyanomargarita melechinii APA-RS9 OP2
Cyanomargarita melechinii APA-RS9 OP3
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Cyanomargarita calcarea GSE-NOS12-04C 2
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Scytonema crispum UCFS21 JN565278
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Scytonema sp. UCFS10 HM629428
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Stigonema ocellatum SAG4890 AJ544082
Stigonema sp. WI53 JQ435860
Freshwater Calothrix
17 OTUs
Calothrix elsteri NR117190
Marine Calothrix
9 OTUs
Microchaete sp. Mares 39T20
Macrochaete 19 OTUs
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Scytonematopsis contorta HA4292-MV4 clb2+p2 HQ847559
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Rivularia sp. XP25A AM230665
Rivularia sp. XP27A AM230667
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Brasilonema 8 OTUs
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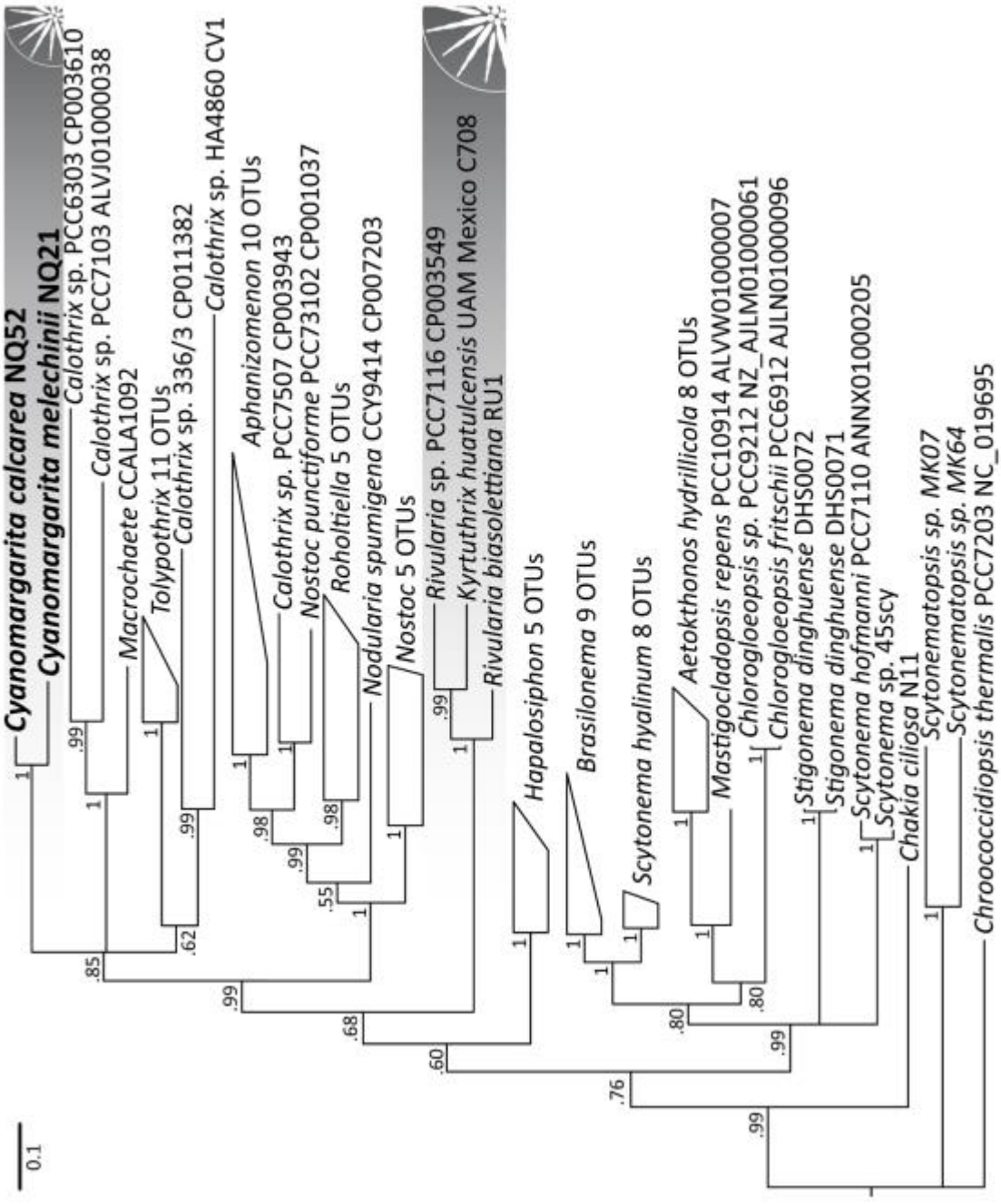
Fig.1.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Hapalosiphon 5 OTUs
Brasilonema 9 OTUs
Scytonema hyalinum 8 OTUs
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Chlorogloeopsis sp. PCC9212 NZ_AJLM01000061
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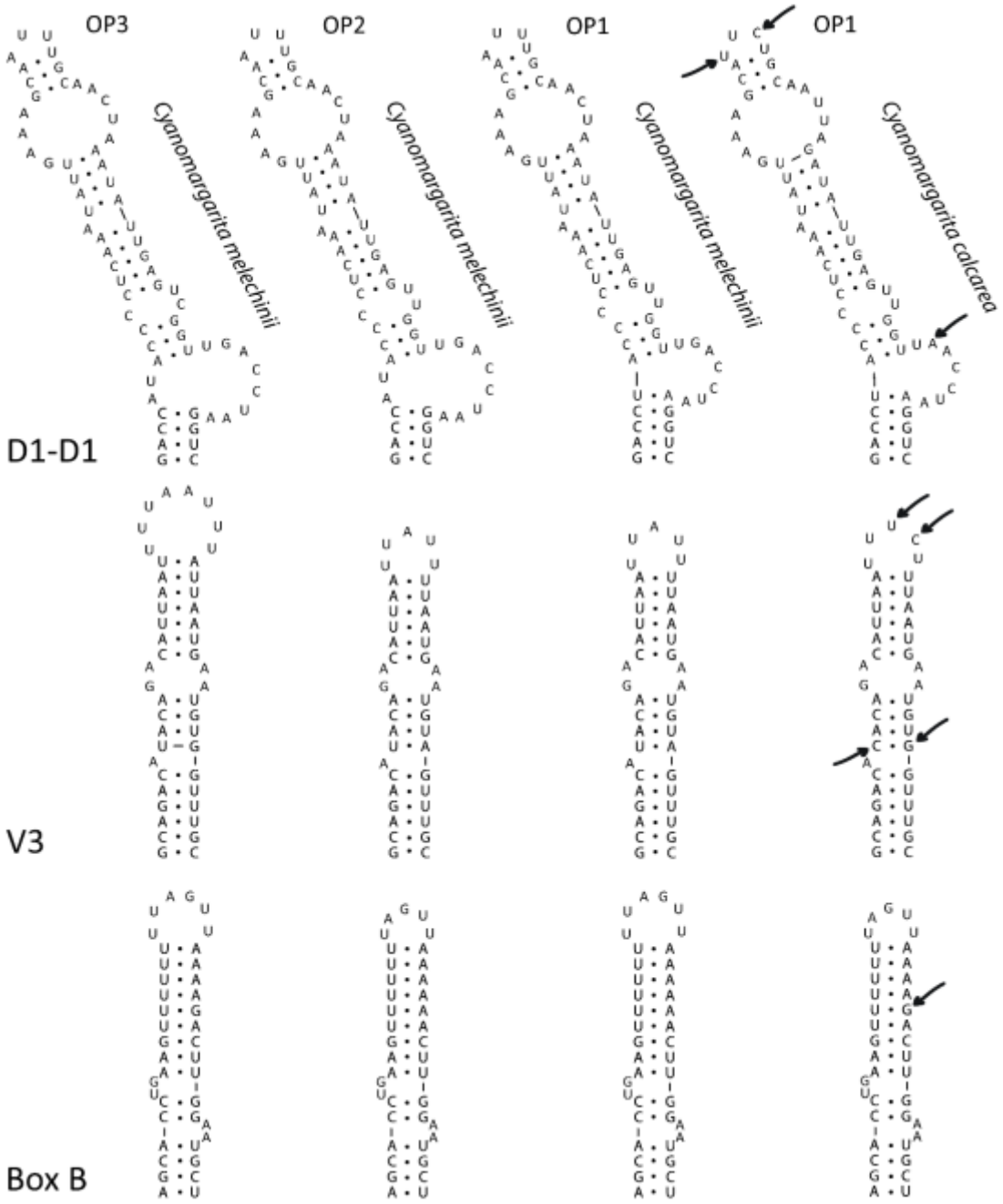
Fig.2.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OP1
OP1
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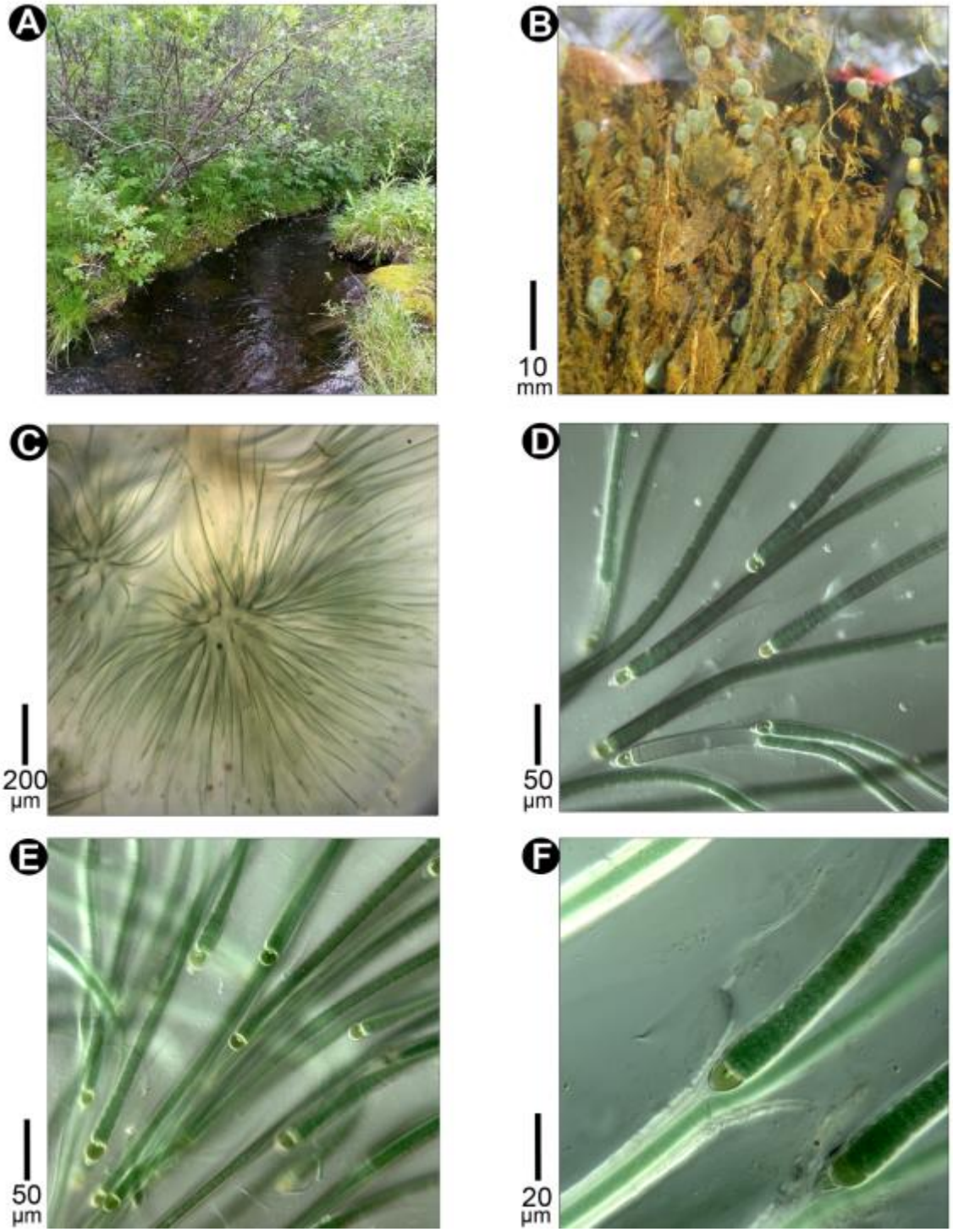
Cyanomargarita melechinii
Cyanomargarita calcarea
D1-D1
V3
Box B

Fig.3.



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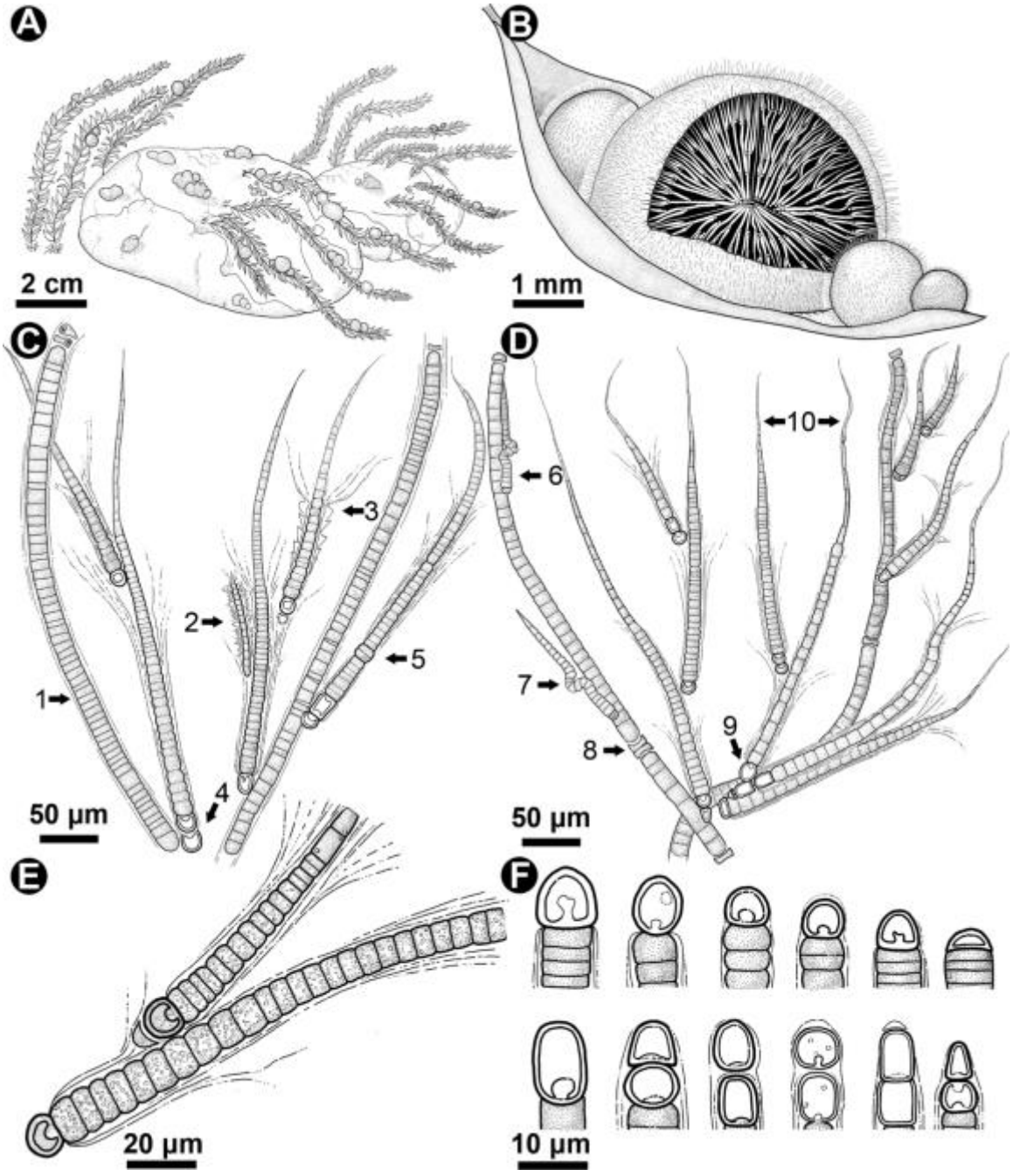

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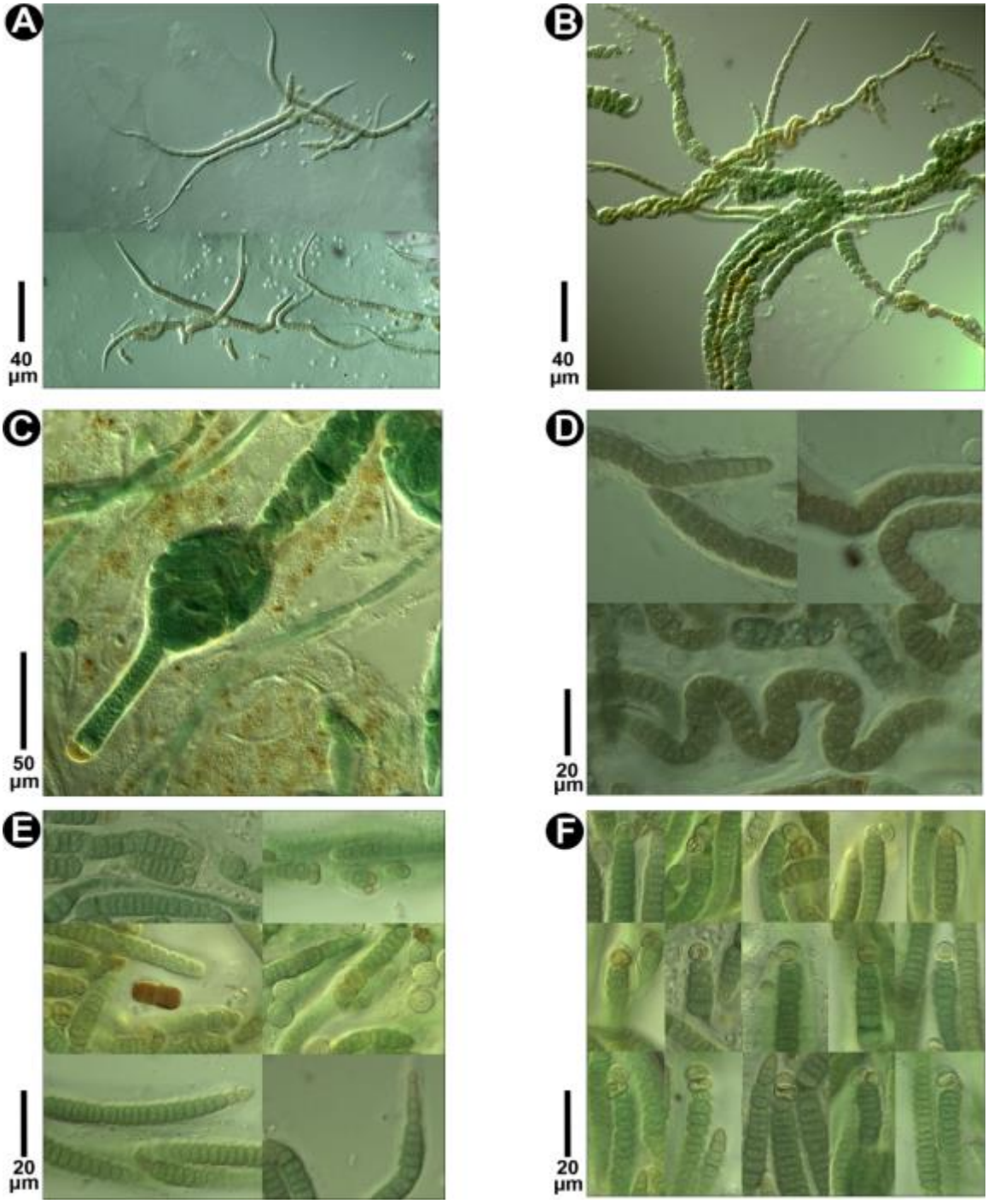

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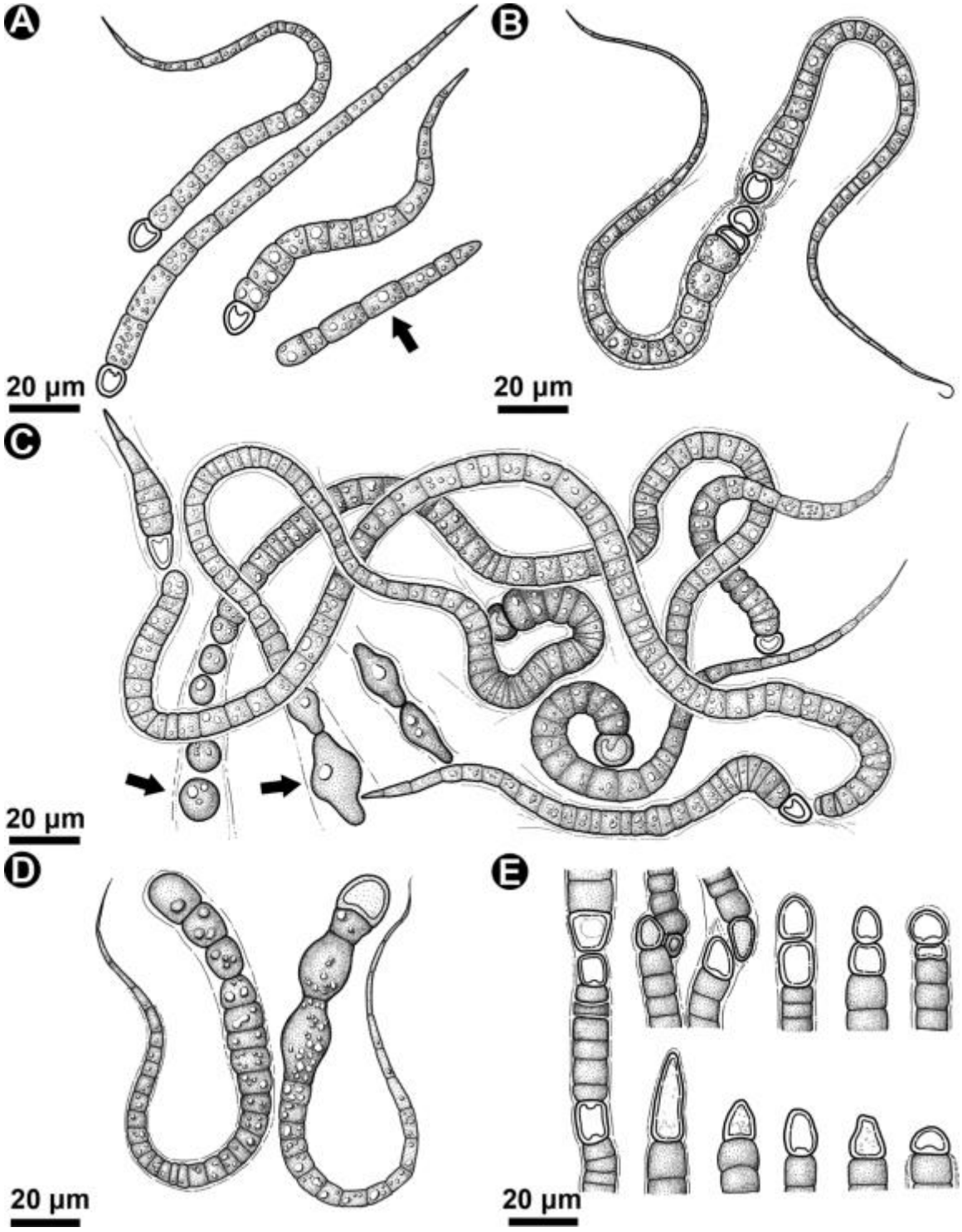

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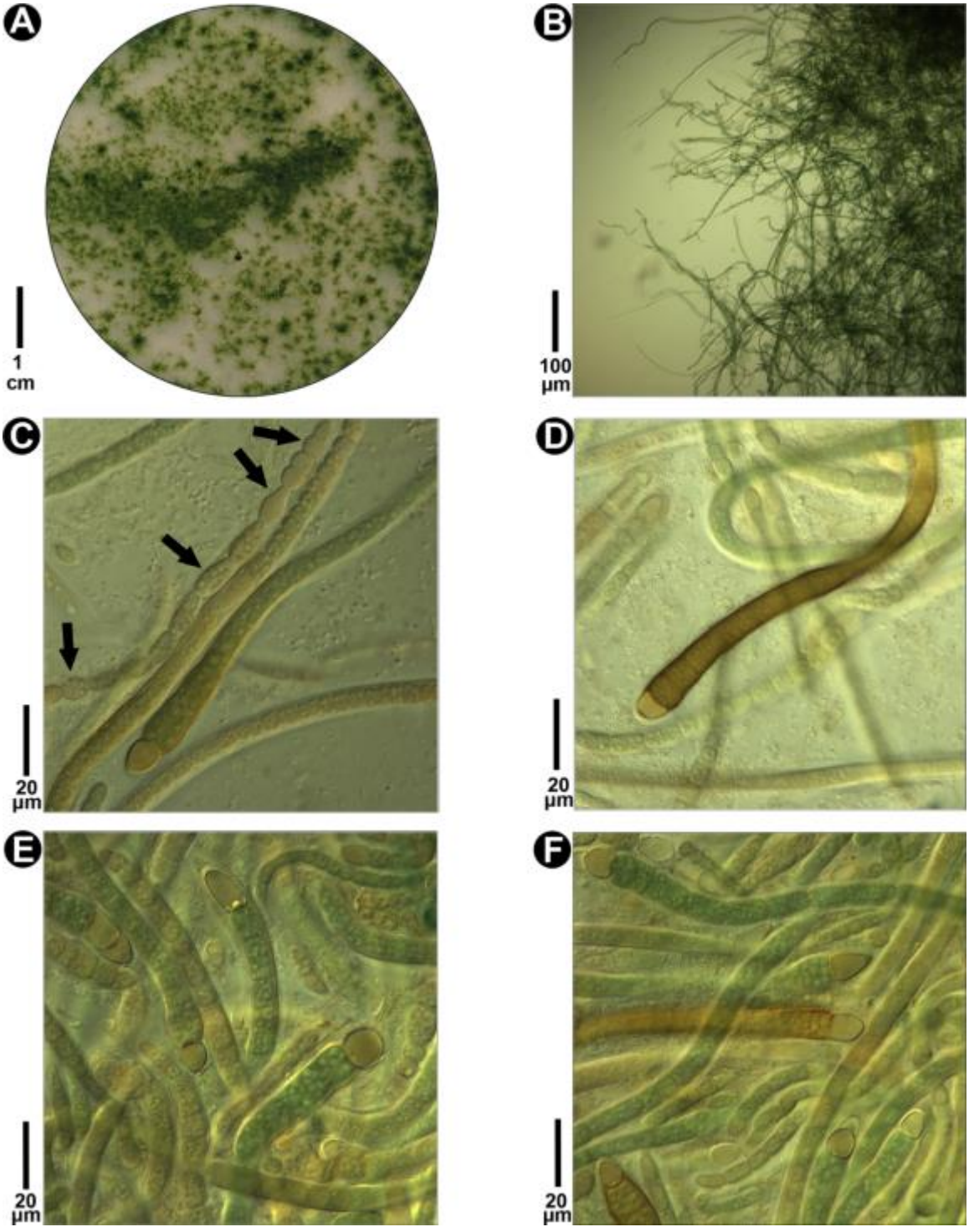

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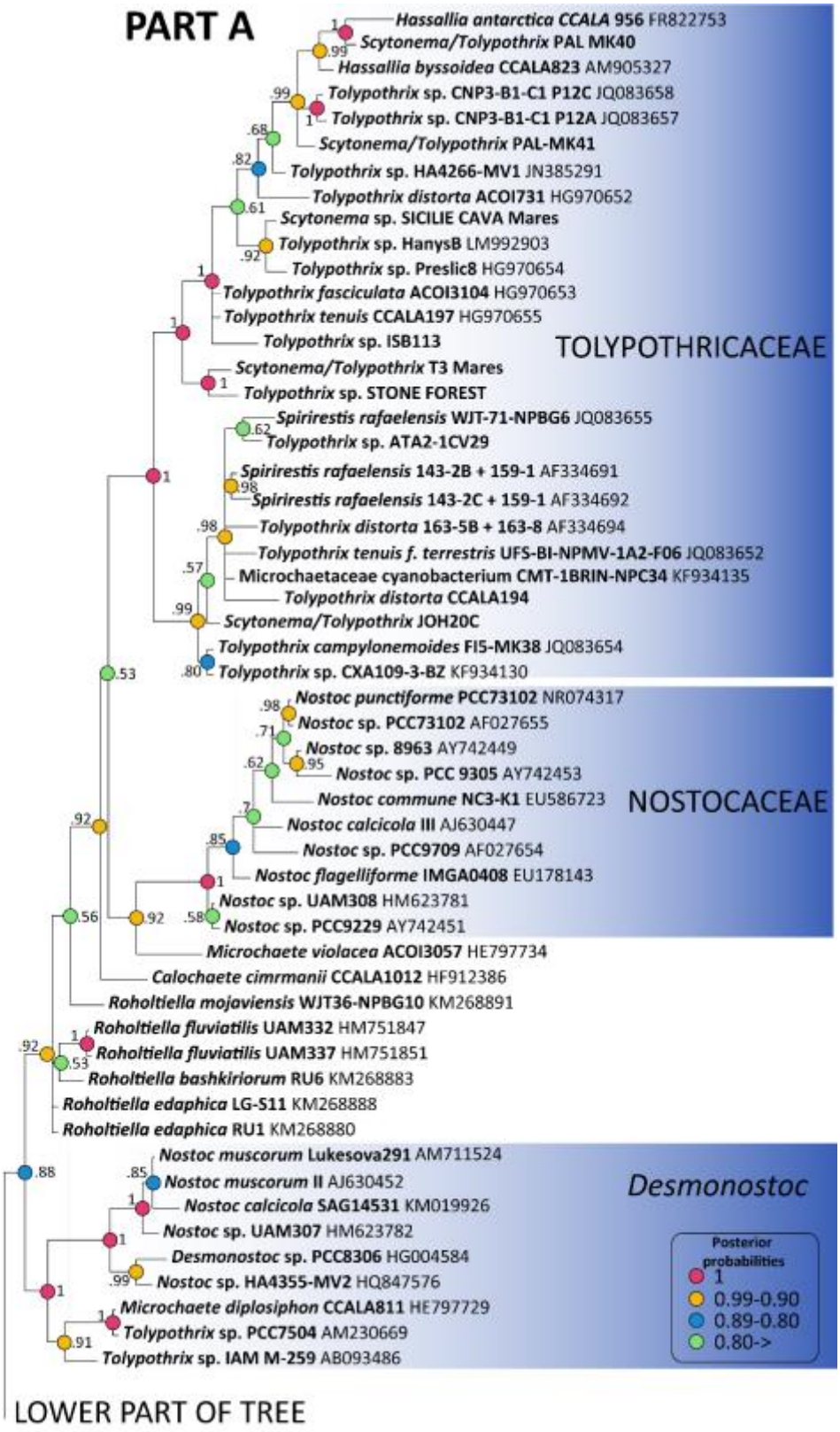
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Scytonema sp. SICILIE CAVA Mares
Tolypothrix sp. HanysB LM992903
Tolypothrix sp. Preslic8 HG970654
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Tolypothrix sp. ISB113
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Tolypothrix sp. STONE FOREST
TOLYPOTHRICACEAE
Spirirestis rafaelensis WJT-71-NPBG6 JQ083655
Tolypothrix sp. ATA2-1CV29
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Calochaete cimrmanii CCALA1012 HF912386
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Roholtiella bashkiriorum RU6 KM268883
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0.80->
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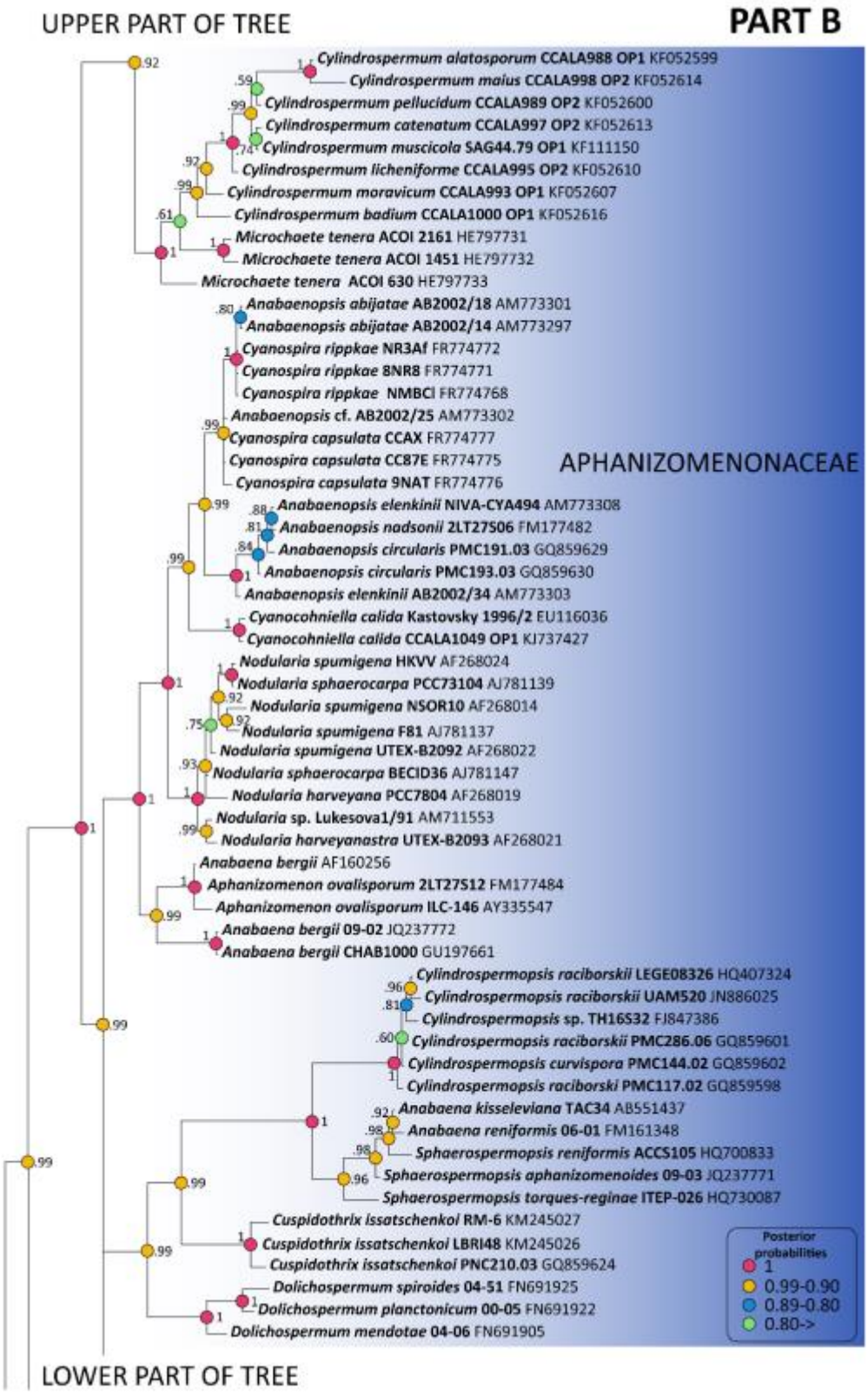
Fig. S1A.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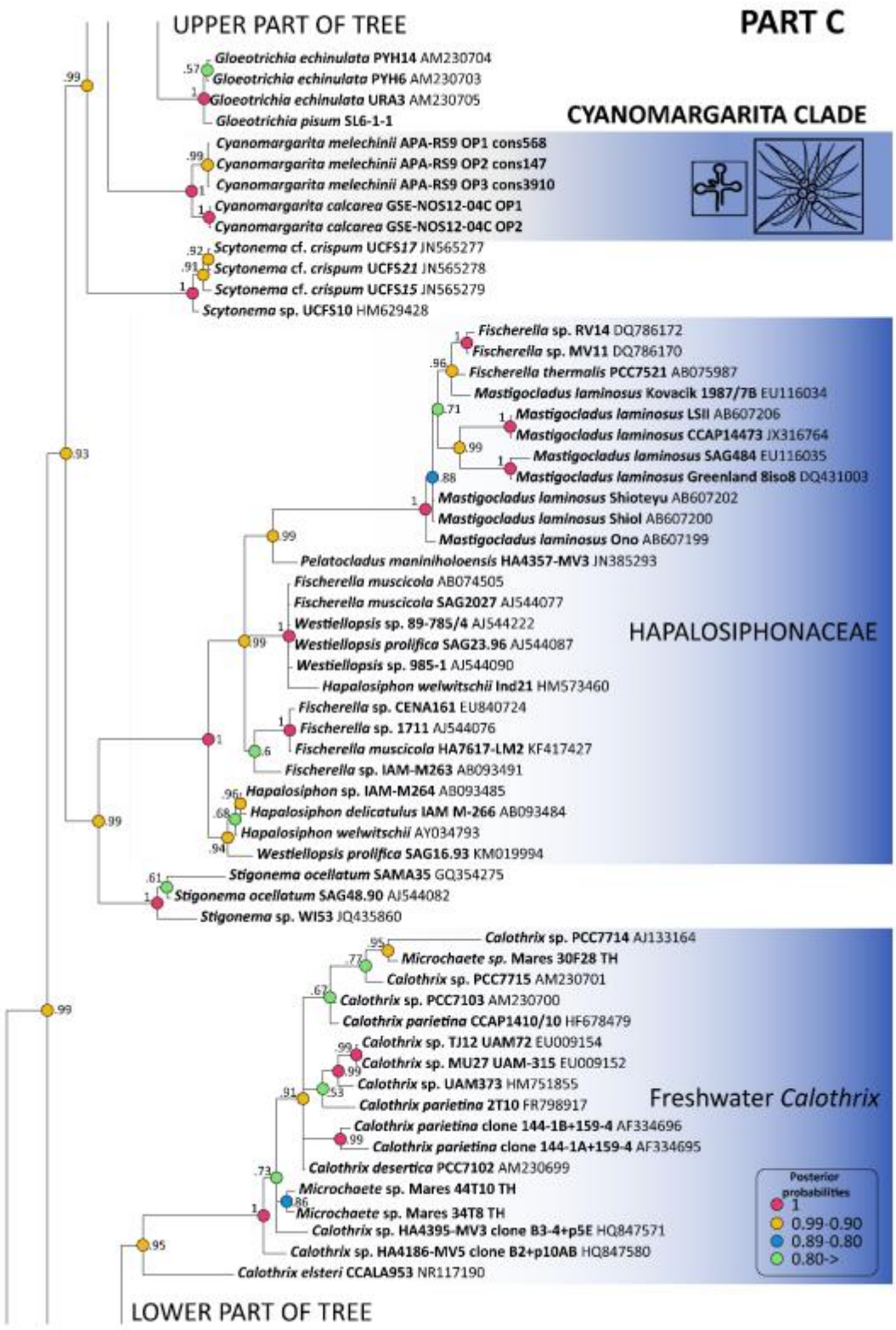
0.80->
LOWER PART OF TREE

Fig. S1B.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Gloeotrichia pisum SL6-1-1
CYANOMARGARITA CLADE
Cyanomargarita melechinii APA-RS9 OP1 cons568
Cyanomargarita melechinii APA-RS9 OP2 cons147
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Cyanomargarita calcarea GSE-NOS12-04C OP1
Cyanomargarita calcarea GSE-NOS12-04C OP2
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Scytonema cf. crispum UCFS21 JN565278
Scytonema cf. crispum UCFS15 JN565279
Scytonema sp. UCFS10 HM629428
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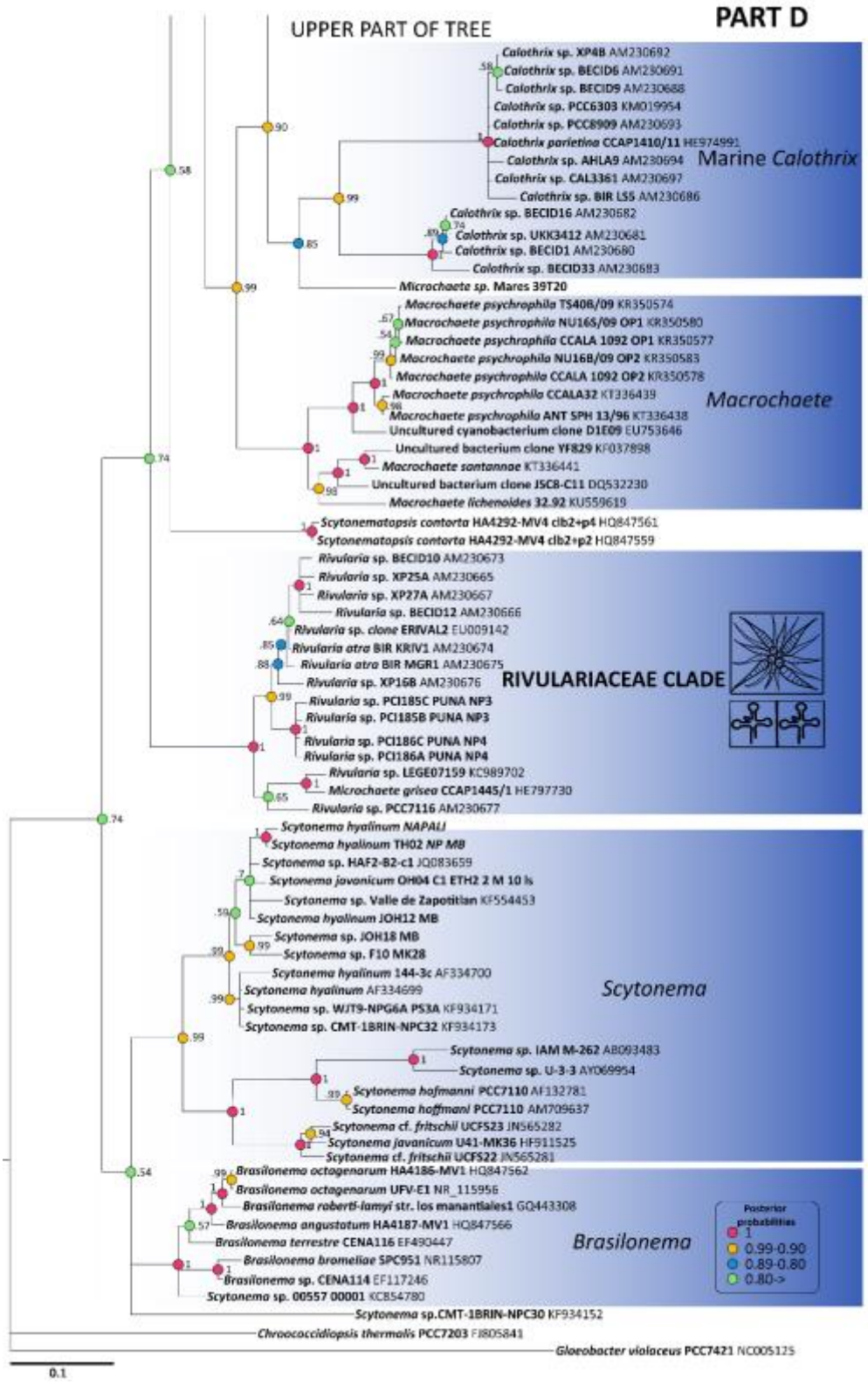
0.89-0.80
0.80->
LOWER PART OF TREE

Fig. S1C.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Marine Calothrix
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Microchaete grisea CCAP1445/1 HE797730
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Scytonema hoffmani PCC7110 AM709637
Scytonema cf. fritschii UCFS23 JN565282
Scytonema javanicum U41-MK36 HF911525
Scytonema cf. fritschii UCFS22 JN565281
Brasilonema octagenarum HA4186-MV1 HQ847562
Brasilonema octagenarum UFV-E1 NR_115956
Brasilonema robertii-lamyi str. los manantiales1 GQ443308
Brasilonema angustatum HA4187-MV1 HQ847566
Brasilonema terrestre CENA116 EF490447
Brasilonema bromeliae SPC951 NR115807
Brasilonema sp. CENA114 EF117246
Scytonema sp. 00557 00001 KC854780
Brasilonema
Scytonema sp.CMT-1BRIN-NPC30 KF934152
Chroococcidiopsis thermalis PCC7203 FJ805841
Gloeobacter violaceus PCC7421 NC005125
Posterior probabilities
1
0.99-0.90
0.89-0.80
0.80->
0.1

Fig. S1D.