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FINALLY, THE MOLECULAR CHARACTERIZATION OF GEITLERIA: RESULTS IN THE FORMATION OF A NEWLY DESCRIBED NOSTOCALES (CYANOBACTERIA) CLADE GEITLERIACEAE FAM. PROV., SP. PROV

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FINALLY, THE MOLECULAR CHARACTERIZATION OF GEITLERIA: RESULTS IN THE FORMATION OF A NEWLY DESCRIBED NOSTOCALES (CYANOBACTERIA) CLADE GEITLERIACEAE *FAM. PROV.*, *SP. PROV.*

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Abstract

Geitleria was described from a limestone cave in Israel, and subsequently reported from caves of France, Romania, Spain, and Florida, Costa Rica, and Cook Islands. It is morphologically unusual in that it has true-branching, but no heterocytes. A morphologically distinct species of *Geitleria* was recently collected from a limestone cave in Great Smoky Mountains National Park, Tennessee, and is herein described as *G. appalachiana sp. prov*. Sequence data for 16S-rRNA and *rpo*C1 loci for the species were obtained from field material using single filament PCR. Phylogenetic evidence indicates that *Geitleria* does not belong to any family in the Nostocales containing true-branching genera, i.e. Hapalosiphonaceae, Chlorogloeopsidaceae, and Symphyonemataceae, and consequently Geitleriaceae *fam. prov.* is established to contain this unique genus.

Introduction

The heterocytous cyanobacteria capable of division in two planes, i.e., of true branching, were until recently all placed in the order Stigonematales (Anagnostidis & Komárek 1990). Phylogenetic analyses have shown that the Nostocales form a monophyletic lineage, but the true-branching genera are scattered in several unrelated families, making Stigonematales polyphyletic (Gugger and Hoffman 2004; Komárek 2013). Consequently, Stigonematales is no longer recognized, and members of the former order are now placed in Nostocales (Komárek 2013). Komárek *et al.* (2014) recognize Symphyonemataceae, Hapalosiphonaceae, Stigonemataceae, Capsosiraceae, and Chlorogloeopsidaceae as the families containing genera with true branching, or division in multiple planes. This newest revision of the heterocytous cyanobacteria is based upon

a phylogenetic analysis utilizing 32 loci, which has good support but lacks representation of many of the heterocytous genera (Komárek *et al.* 2014). More recent phylogenetic analyses utilizing only 16S rRNA data have given the same topology (Singh *et al.* 2013; Mishra *et al.* 2014; Bohunická *et al.* 2015 Mareš *et al.* 2015).

Morphology of the true-branching cyanobacteria still remains important in taxonomic classification of the genera possessing this trait. A combination of morphological and molecular data are now being used in the Nostocales to reveal evolutionary relatedness and understand both the species-level and higher-order taxonomy (Lukešová *et al.* 2009; Hauer *et al.* 2014; Hentschke *et al.* 2016). The use of diverse data sets (morphological, ecological, physiological, molecular) in cyanobacterial taxonomy is called the polyphasic approach (Colwell 1970; Johansen & Casamatta 2005), but has also been referred to as the total evidence approach (Wiley *et al.* 2000; Strunecký *et al.* 2017).

Frequently, morphology and phylogenetic taxonomic placement using molecular data are not congruent (Gugger & Hoffman 2004). Mishra *et al.* (2014) conducted an analysis in which they produced separate phylogenies based on morphological and molecular data, and found as little as 36% agreement in results between the two analyses. However, despite the recent widespread use of molecular data and potential conflict with other character sets, morphological and ecological data should not be neglected (Dvořák *et al.* 2015). Closely related taxa such as genera and species are fairly stable in molecular phylogenies, even when additional sequences are later added. However, the higher-order relationships of cyanobacteria (family and order) are often not clear because of a lack of phylogenetic stability when sequences are added as well as a lack of nodal support along

the backbone of most analyses (Komárek *et al.* 2014). Higher-level taxonomy is consequently more difficult to confirm phylogenetically, requires more extensive gene and taxon sampling, and likely is in more need of revision.

The genera within the heterocytous families Scytonemataceae, Symphyonemataceae, Hapalosiphonaceae, and Stigonemataceae, are placed within their familial group based on morphology. Molecular resolution of these families is still in its infancy. For example, *Loriellopsis* has been placed in the Symphyonemataceae, based on its ability to produce both T-type and V-type branching, although preliminary molecular data indicate it may fall outside of that family (Lamprinou *et al.* 2011). *Iphinoe* and *Brasilonema,* which are true-branching and non-branching, respectively, fall into the Scytonemataceae in most phylogenies (a family defined by having false branching only). Furthermore, in a recent phylogeny with greater taxon sampling, it appears that the genera of the Symphyonemataceae, Scytonemataceae, and Stigonemataceae are interleaved, with *Brasilonema, Iphinoe, Symphyonemopsis, Symphyonema, Scytonema, Stigonema, Loriellopsis,* and *Umezakia* all in a single clade (Dale Casamatta, personal communication). The most recent revision of heterocytous cyanobacteria by Komárek (2013) places *Geitleria* in the Hapalosiphonaceae, a lineage that includes *Hapalosiphon, Fischerella, Mastigocladus, Nostochopsis,* and *Westiellopsis*. *Iphinoe* is commonly found among *Geitleria calcarea* in calcareous caves (Lamprinou *et al.* 2011). Both taxa are capable of forming calcite sheaths and have similar branching, but reside in different families. Both the Symphyonemataceae and Hapalosiphonaceae include genera with T-, V-type branching, as well as genera in which heterocytes were not observed (Komárek 2013). *Geitleria* was thought to have very distinct geographic and habitat limitations, but

strains outside of Europe have been observed (Friedman 1979; Skuja 1937, Johansen *et al.* 2007). *Geitleria clandestina* (Skuja) Bourrelly was recognized by Bourrelly (1970), who transferred *Rosaria clandestina* Skuja into the genus. *Geitleria floridiana* Friedmann, the third species described, was found in a cave system in Marianna, Florida (Friedman 1979). A summary of the morphological and ecological characteristics of the genera in the Symphyonemataceae and *Geitleria* shows that all of these genera have ecological similarities, as well as morphological overlap (Table 1). Formation of heterocytes in *Geitleria* has not been observed (Friedmann 1955, 1979). Little is understood about the evolutionary relationships of non-heterocytous and true-branching cyanobacteria such as *Geitleria* (Gugger & Hoffman 2004). Molecular markers are often lacking in the historical genera of cyanobacteria described before the advent of molecular analyses (Komárek *et al.* 2014). The use of multi-loci analyses has shown increased phylogenetic support in higher-level classification (Wu *et al.* 2011; Sciuto *et al.* 2012; Komárek *et al.* 2014). Due to the presence of multiple ribosomal operons, the addition of multiple molecular markers should be used to better clarify these evolutionary relationships (Sciuto *et al.* 2012).

Herein, I collected *Geitleria* from a location from which it was previously collected in the Great Smoky Mountains National Park (Johansen *et al.* 2007), then completed a thorough morphological analysis of this population to determine if it is morphologically in agreement with the type species, *G. calcarea.* Subsequently, sequence data for multiple molecular markers were targeted, including 16S rRNA with the associated 16S–23S ITS region, *rpo*C1, and *het*C. Phylogenetic analyses of close genera

for which these loci exist were conducted to test higher-level placement of *Geitleria* in the Hapalosiphonaceae, where it currently is placed.

The following hypotheses and goals were central to the questions addressed by this study:

(1) *Geitleria* from the Great Smoky Mountains will be the same species as one of the previously-described *Geitleria* species, e.g. *G. calcarea, G. clandestina,* or *G. floridiana.* Alternatively, it will be a new species.

(2) *Geitleria* is not a member of the Hapalosiphonaceae clade, but rather belongs to the Symphyonemataceae based upon its morphological and ecological similarity to members of that genus, *Loriellopsis* and *Iphinoe*.

(3) *Geitleria* has a *het*C gene, but does not express the gene due to mutations in the gene complex for heterocyte formation. Alternatively, *Geitleria* lacks the gene for heterocyte formation, presumably due to an evolutionary loss.

(4) Multiple loci analysis will be congruent with 16S rRNA phylogeny, but the phylogeny will be better supported.

(5) *Geitleria* is congeneric with either *Loriellopsis* or *Iphino*e, necessitating the transfer of species from one of these more recent genera into the genus with nomenclatural priority, i.e. *Geitleria.*

Materials and Methods

Sample collection. Samples were collected on the 16th of May, 2016 in The Great Smoky Mountain National Park (Study number GRSM-01266, Permit number GRSM-2016-SCI-1266). The site where *Geitleria* occurs is a cave near Cades Cove and White Oak Sink (35°36'40.61"N, 83°46'11.05"W). Samples were collected at the mouth of the cave where light was present. Using a sterilized spatula, I scraped biological material from the limestone walls into 1 ml Eppendorf tubes. Two populations were sampled, one coming from right above the cave entrance, and the other deeper in the cave.

Environmental samples of *Geitleria* were maintained in the laboratory using sterilized and filtered cave water, which was collected on site and subsequently enriched with the addition of 1% nitrogen and phosphorous. I placed natural samples into culture tubes along with sterile marble boiling chips. This culturing effort allowed *Geitleria* to remain viable for almost a year, but I was unable to obtain monocultures.

Microscopy and PCR amplification. Isolation and manipulation of single filaments was completed using either a SZ-PT Olympus stereo microscope (Tokyo, Japan) or a Leica MZ12.5 stereo microscope (Meyer Instruments, Houston, TX). Observation and characterization was primarily completed with a Zeiss Axioskop with Nomarski DIC optics and a Macrofire digital camera (Optronics, Goleta, CA). SEM micrographs were prepared using standard protocols (Wilde *et al.* 2014). Single filament isolation for PCR was conducted in accordance with Mareš *et al.* (2015). The method of Mareš was modified, in that filaments were selected by spreading the environmental samples on a glass microscope slide that contained VersaTaq direct PCR polymerase buffer (Affymetrix—ThermoFisher, MA, USA). The solution evaporated, leaving behind the calcareous filaments which were not attached to the slide and non-calcareous algae and

cyanobacteria that adhered to the slide. The calcareous filaments were easily picked up with a sterilized dissecting needle, and moved into another area on the slide that contained the buffer for visual confirmation of the taxon. Again, the solution was evaporated, and 3–5 filaments or fragments were selected and placed into a PCR tube containing 1 µL of the VersaTaq direct PCR polymerase buffer.

Protocols for PCR amplification using the Affymetrix VersaTaq™ Direct PCR for environmental samples was followed. I performed cloning, sequencing, analysis of secondary ITS structures, and phylogenetic analysis using the same techniques and methods described by multiple papers from the Johansen lab (Boyer *et al.* 2001; Flechtner *et al.* 2002; Řeháková *et al.* 2007; Lukešová *et al.* 2009; Johansen *et al.* 2014; Mühlsteinová *et al.* 2014; Osorio-Santos *et al.* 2014; Pietrasiak *et al.* 2014; Bohunická *et al.* 2015). Multiple reactions were needed to obtain multiple loci. PCR amplification of the 16S rRNA, 16S–23S rRNA and, *rpo*C1 were conducted using standard primers (Table 2). Each PCR reaction included 2.5 µl VersaTaq™ 10X direct PCR reaction buffer, 0.5 µl 10 mM (dNTPs), 0.5 µl of the primers at 10 µM concentration, 0.25 µl VersaTaq direct PCR polymerase and up to 25 µl of PCR-qualified water. The amplification protocol for 16S amplification was 35 cycles of 94°C for 30 sec; 52°C for 30 sec; 72°C for 1 min; 72°C for 5 min for the final extension. This was performed this by TA-cloning into a pSC-Amp/Kan Plasmid of the Stratagene Cloning kit (La Jolla, CA). Then plasmids were purified using the QIA Miniprep Spin kit (Qiagen, Carlsbad, CA). After purification, the clones were checked using digestion with EcoRI. Six clones was selected for the 16S–23S analysis and two clones for rpoC1 analysis. Sequencing was conducted by Functional Biosciences, Inc. (Madison, WI) using M13 forward and

reverse primers. Ribosomal sequence contigs were assembled using Sequencher software (v4.8, Ann Arbor, MI).

Alignment, Phylogenetic Analysis, and Secondary structure folding. Closest relatives of the *rpo*C1 and 16S rRNA data were identified using BLASTX (www.ncbi.nlm.nih.gov/blast). Initial alignments of the 16S rRNA and ITS region were performed using MUSCLE within MEGA6 (Tamura *et al.* 2013). The nucleotide sequence of the *rpo*C1 gene from NCBI was first translated to protein sequences to position the reading frame, so that the correct amino acids could be identified (i.e. start and stop codon). Then the amino acid sequence was aligned in MEGA6 using MUSCLE, and used to create an alignment of nucleotides for the phylogenetic analysis. I checked both the 16S rRNA gene and *rpo*C1 alignments manually, to ensure secondary structures were maintained (in 16S) and indels were appropriately placed (in both). The ML and Bayesian phylogenies were created from partial 16S rRNA sequences containing 1,202 nucleotides which encompassed the closest relatives from NCBI GenBank. The *rpo*C1 phylogeny incorporated 1,896 nucleotides. Using the CIPRES science gateway, Maximum Likelihood (ML) and Bayesian inference trees were derived. The ML with rapid bootstrapping was conducted using RAxML-HPC v.8 on XSDE V8.2.9 (Stamatakis 2014). GTR+G+I estimated the proportion of invariable sites with 100 bootstrap iterations. Bayesian inference was conducted with MrBayes on XSDE V3.2.3 (Huelsenbeck & Ronquist 2001), applying the GTR+G+I model of nucleotide substitutions with 1000 bootstrap replications. 15 million generations were used for the 16S rRNA gene alignment and 25 million for the *rpo*C1 alignment. *Chroococcidiopsis* sp. (AB074809) was the outgroup taxon for the *rpo*C1 phylogeny and *Chroococcidiopsis*

sp. (FR798923) for 16S rRNA analysis. PAUP was used to calculate uncorrected p-distance for comparative analysis of selected, identified, most closely related strains (Swofford 2003). Secondary structures of the 16S–23S ITS were determined using Mfold v3.2 (Zuker 2003). Editing of both ITS secondary structures and the phylogenetic analyses was completed using Adobe Illustrator CS V5.1.

Preserved Material and GenBank Accession Numbers. Natural material was preserved using a recommended method for sensitive algae by the Census of Freshwater Algae in Australia (http://plantnet.rbgsyd.nsw.gov.au/PlantNet/fwalgae/Introduction/preserve.htm). This method calls for a 6:3:1 solution of water, 90% ethyl alcohol, and 40% formaldehyde, respectively. I gave the preserved (uncultured) specimen the code of GSM-WOS-CK01.

The five clones of *Geitleria appalachiana* and the one clone of *Loriellopsis* sp. 16S rRNA sequences were deposited into NCBI GenBank and given accession numbers: KY924318–KY924323. The two *rpo*C1 clones were given accession numbers: KY924324 and KY924325.

Results

Phylogenetic Analyses

The five analyzed clones of *Geitleria* were sister to the Chlorogloeopsidaceae and the Hapalosiphonaceae clades based on the Bayesian Inference (BI) phylogeny (Fig. 1). A clone belonging to *Loriellopsis* was also sequenced. The posterior probabilities and

ML bootstrap values support recognition of three distinct clades (Chlorogloeopsidaceae, Hapalosiphonaceae, Geitleriaceae) within a single clade (Fig. 1). The two sister families are all freshwater or subaerial, in both thermal and nonthermal habitats. The closest relative to *Geitleria appalachiana* is *Chlorogloeopsis fritschii* (DK431003), with a sequence identity of 93.8% (Table 3). The closest relative within the Hapalosiphonaceae is *Mastigocladus laminosus* (DQ431003) with sequence identity of 93.6% (Table 3). The two close relatives in the Hapalosiphonaceae and Chlorogloeopsidaceae are 92.8% similar. The *rpo*C1 phylogeny suggests *Geitleria* has a closer relationship with Chlorogloeopsidaceae than Hapalosiphonaceae (Fig. 2).

Morphological and ITS Characterization

As in the original description of *Geitleria calcarea,* the most obvious observation is the apparent inability of *Geitleria* to produce heterocytes naturally. I did not observe heterocyte formation during extensive and repeated examinations under LM. Molecular amplification of the *het*C gene was attempted using three different sets of primers, the first two pairs of forward- and reverse-primers were previously published (Khudykov & Wolk 1999; Wang & Xu 2015). The other primer pair was designed using a Clustal Omega Alignment of *Calothrix* sp. CP011382, *Nostoc* sp. U55386, *Cylindrospermum stagnale* CP003642, *Calothrix* sp. CP003943, *Fischerella* sp. AP017305, and *Fischerella* sp. MV11 FJ211388 (Table 2). The amplification of the *het*C gene was not successful in any attempt, suggesting absence or nonfunction of the gene in *G. appalachiana.*

Geitleria appalachiana exhibits true branching, with branches arising laterally (T-branches) and pseudodichotomously (V- branches) without heterocytes. Filaments form

loose tufts with a calcareous incrustation of trichomes. These qualitative characteristics of *G. appalachiana* clearly match the characteristics of *Geitleria calcarea* as described by Friedmann (1955) from caves in Israel. However, its cell sizes are larger. The maximum cell length for *Geitleria appalachiana* is 28.2 µm long, whereas in *Geitleria calcarea* cell length does not exceed 14.7 µm. *Geitleria appalachiana* did not bear a close resemblance to either *G. floridiana* or *G. clandestina. Geitleria appalachiana* specimens were morphologically distinct from most of the other calcareous cyanobacteria in the sample, except for *Loriellopsis,* which possesses true branching and heterocytes.

The two populations of *Geitleria appalachiana* have variation in the 16S–23S ITS region (Figs. 3–6) that could be due to variability in operons, or variation in populations indicative of genetic divergence between populations. The uncorrected p-distance between these ITS sequences of the two populations (four cave sample 22 sequences vs. one cave sample 21 sequence) is 0.025. The V3 helix was variable within populations (Figs. 3–4) with a deletion in the cave sample 21 population, and was very different from that of *Fischerella muscicola* (Fig. 7). The cave sample 21 sequence has a number of indels (missing bases), which elevate the p-distance to 0.115 if the indels are counted as a fifth base, and cause the observed difference in structure in the V3 helix (Fig. 4). The minor variations observed in the D1-D1' helix did not result in a change in secondary structure (Fig. 6), but the basal unilateral bulge that resulted in a large unpaired sequence on the 3' side of the helix was very different from the structure in the ITS region of *Fischerella muscicola* HA7617-LM2 (Fig. 9). The Box B helix was invariant in clones of *G. appalachiana* and similar in size to the Box B helix of *Fischerella* (Figs. 5–8).

Geitleriaceae Kilgore *et* Johansen *fam. prov.*

Description: Thallus in the form of true branched, loosely tufted filaments consisting of single trichomes enveloped in firm sheath encased in calcium carbonate crystals. Trichomes with T, V, and T-type branching. Cells irregularly shaped to cylindrical, longer or shorter than wide, end cells bulbous, irregular, or attenuated. Heterocytes absent. Reproduction by hormogonia. [Type genus, *Geitleria* Friedmann 1955]

***Geitleria appalachiana* Kilgore *et* Johansen *sp. prov.* (Figs. 10–18)**

Description: Thallus loosely tufted, light to dark-grey sometimes faint blue. Filaments fragile, flexuous, 15.1–38.3 µm thick, 38–67.6 µm thick where branching occurs (Figs. 10, 13– 15). Calcite sheaths firm, yellow to golden, sharp, narrowed or roundly truncated near apex, sometimes absent, lattice-like arrangement (Figs. 17–18), with irregular lateral branching, irregular arranged acicular calcite units piercing exteriorly. Sheaths clear, thin around trichomes. Trichomes true branched with T-type (Fig. 10), V-type (Fig. 13), and Y-type (Fig. 14), slightly constricted at crosswalls, more constriction occurs when cells are isodiametric or wider than long (Fig. 12). Cells greyish-green to army-green, irregularly shaped, mostly cylindrical, isodiametric to wider than long or longer than wide, distinct irregular contorted cells (Figs. 10, 14), 4–28.2 µm long x 6–12.5 µm wide, apical cells slightly apically attenuated (Fig. 10), sometimes bent (Fig. 10) or bulbous (Fig. 16). 2–4 granules present, rarely absent. Heterocytes and akinetes absent. Reproduction by hormogonia.

Etymology: *appalachiana,* named for distribution in the Appalachian Mountain Range.

Type locality: Unnamed limestone cave in White Oak Sink, Blount County, Great Smoky Mountains National Park, Tennessee. Growing on the roof in dimly lit portion of cave. Collected 16 May 2016 by J.C. Kilgore.

Holotype here designated: BRY37793, Herbarium of Nonvascular Cryptogams, Monte L. Bean Museum, Provo, Utah, USA.

Discussion

Geitleria appalachiana is differentiated from *G. calcarea* as described in the protologue by cellular size and structure. *G. calcarea* was reported to have cells 4.2–14.7 um long and 3.8–14.7 um wide. In the illustrations of the taxon, most cells are shorter than wide. *G. appalachiana,* on the other hand, has cells 4–28.2 µm long by 6–12.5 µm wide, and most cells are longer than wide. The notable size differences, together with fairly unique biogeography (described from different continents) and habitat (wet, temperate vs. desert climate) are the basis for recognizing this taxon as an independent entity. Because *G. calcarea* has not yet been sequenced, I have no molecular support for identifying the species as different, but it has been routine to erect new species of cyanobacteria based on morphological characters for over 100 years, and I believe morphological and ecological differences still provide sufficient evidence to recognize *Geitleria appalachiana* as a separate species.

Recently, (Osorio-Santos *et al.* 2014; Pietrasiak *et al.* 2014) researchers have come to understand that cryptic species of cyanobacteria can be recognized based on molecular data alone. Morphologically distinct, distantly-distributed populations that are highly similar in their 16S rRNA sequences have not been reported, and I assume that molecular data, were it available, could clearly separate these taxa and confirm the taxonomic conclusions based on morphology, ecology, and biogeography. In a study of 4559 bacterial species for which ribosomal sequences were available, 94.9% genetic identity was the minimum identity between species of the same genus, and identities below that level were considered to belong to species in other genera (Yarza *et al.* 2008). More recently, the cut-off for separation for species has been set to 98.7% genetic identity (Yarza *et al.* 2014); however, named species exist which have 100% identity, so when sequence identity is above 98.7%, it is considered uninformative for taxonomy (Yarza *et al.* 2008). Ideally, a combination of phenotypic, ecological, and molecular evidence will be congruent and clearly support recognition of new species, such as was the case with the recent erection of *Dolichospermum uruguayense (*Kozlíkovám-Zapomělová *et al.* 2016) and *Phormidium etoshi* (Dadheech *et al.* 2013). I must wait for molecular data on *G. calcarea* to confirm that *G. appalachiana* is a new species, but for now it appears that the preponderance of evidence indicates it is a separate lineage.

The 16S rRNA phylogeny supported three hypotheses with regards to family level recognition for *Geitleria*. Monophyletic families could be erected by 1) recognizing a single family, the Hapalosiphonaceae for clades A,B, C (Fig. 1), 2) recognizing two families, the Hapalosiphonaceae (containing clades B, C) and a new family, Geitleriaceae, or 3) recognizing three families, Hapalosiphonaceae,

Chlorogloeopsidaceae and Geitleriaceae (clades A, B, C, Fig. 1). The *rpo*C1 phylogeny supported two hypotheses 1) recognizing two families, the Hapalosiphonaceae (clade C) and Chlorogloeopsidaceae (clades A, B), or 2) recognizing three separate families (clades A, B, C, Fig. 2). The only taxonomy which creates monophyletic families in both gene analyses is the last option, recognizing three families. Even though genetic similarity among members of the families is high (Table 3), I conclude that three families should be recognized to create a taxonomy correctly reflecting evolutionary history based on the evidence currently in hand. While the phylogenetic evidence strongly supports recognition of Geitleriaceae as separate from Chlorogloeopsidaceae, morphological evidence also exists for the separation of these families. *Geitleria* is uniseriate, with obligatory true branching and no heterocytes, whereas *Chlorogloeopsis* is multiseriate or rarely uniseriate (Gugger & Hoffman 2004) but never shows Y-, V-, or T- type branching. True branching was once considered sufficiently important to define a whole subsection (Stigonematales IV), and there is still a focus on branching types in the family descriptions of the Nostocalean lineages. The obligate lack of heterocytes is unique to *Geitleria*, and possibly Geitleriaceae. There are members of the Hapalosiphonaceae for which heterocytes have never been observed (*Colteronema, Albrightia,* and *Mastigocoleopsis*), as well as *Iyengariella* of the Symphyonemataceae. None of these genera have been sequenced to determine if they too could possibly be genera in this family. *Geitleria* shares ecological similarities (e.g. restriction to aerophilic limestone substrates and low light tolerance) with some members of the Symphyonemataceae: *Iphinoe; Loriellopsis; Voukiella; Herpyzonema pulverulentum;* and *Symphyonema cavernicola. Geitleria* additionally shares morphological similarities to the cave-dwelling

Iphinoe (Lamprinou *et al.* 2011). *Loriellopsis* is morphologically similar to *Geitleria* in that it has true branching and a calcareous sheath, but it consistently produces heterocytes and is phylogenetically distant from that taxon (Lamprinou *et al.* 2011). *Iphinoe* forms a sister clade to *Brasilonema* and the two genera bear a strong morphological resemblance (Bohunická *et al.* 2014: Fig. 1). It is phylogenetically distant from both *Geitleria* and *Loriellopsis* (Fig. 1). The rest of the genera in the Symphyonemataceae have yet to be sequenced. Until more members of the Symphyonemataceae are sequenced, it will be unclear whether the family should be collapsed into the Scytonemataceae or continue to be recognized as a separate taxon. Regardless of the fate of genera in this family, *Geitleria* is phylogenetically distinct from these morphologically similar taxa.

The co-occurrence of *Geitleria* and a taxon similar to *Loriellopsis* was interesting. These two populations were highly similar in morphology, and at first it appeared that they might belong to the same taxon. The filaments of *Loriellopsis* sp. were calcified such as *Geitleria*, but heterocytes were clearly visible. *Loriellopsis* sp. was successfully sequenced, and although phylogenetically in the same clade as *Loriellopsis cavernicola*, it was only 92.8% similar to *L. cavernicola* from the type locality in a Spanish cave system. This unusual taxon is likely in a separate, new genus, and it and *Loriellopsis* likely will need to be moved into a new family at the base of the Nostocales (Fig. 1; Lamprinou *et al.* 2011: fig. 4).

With the advent of modern molecular techniques, researchers are now revising polyphyletic taxa into monophyletic taxa that better represent evolutionary history (Bohunická *et al.* 2014; Komárek *et al.* 2014). A thorough investigation based on sequence data for 4559 species in 451 genera and 10 families revealed that 16S rRNA

similarity values below 87.5% between the type specimens of a genus of prokaryotes would indicate that the taxa are in separate families (Yarza *et al.* 2008). If this were a criterion required for recognition of cyanobacterial families, almost all families in the Nostocales would be dissolved into a single family. However, like all molecular cut-off criteria based on 16S rRNA dissimilarity, values below the cut-off indicate separateness, values above the cut-off are uninformative for taxonomy. The family level cut-off of <87.5% is only met between some but not all orders of cyanobacteria. The clades of Geitleriaceae, Chlorogloeopsidaceae, and Hapalosiphonaceae are well defined by phylogeny. I feel that taxon sampling in the *rpo*C1 locus is too low and not available for the same taxa for which 16S rRNA data exist, so phylogenetic analysis of a concatenated alignment of the two loci is not warranted at present.

Most of the studies of evolutionary history of select groups of cyanobacteria is based upon single locus ribosomal phylogenies. I assume the *rpo*C1 gene has the same evolutionary history as the 16S rRNA gene history, but with the quickly growing knowledge in molecular phylogenomics this assumption may be challenged in the future. Concatenated sequence alignments have produced trees with a well-supported phylogenetic signal, especially when a high number of loci are used (Komárek *et al.* 2014; Sciuto *et al.* 2012). However, even if sophisticated techniques such as supertrees, concatenated sequences, and consensus trees were acquired for this study, possible conflicting topologies from genes believed to be orthologs still arise (Shi & Falkowski 2007). The 16S rRNA and photosynthetic gene sequences (e.g., *rbcL*X) are believed to be conserved for cyanobacteria, but they do not always give congruent results. Chance

events, such as lateral gene transfer are known to occur among the photosynthetic genes (Mulkidjanian *et al.* 2006), leading to incongruence with the ribosomal genes.

I was unable to induce heterocyte formation or confirm molecular evidence of the capability to produce heterocytes. Some cyanobacteria can fix nitrogen without the formation of heterocytes (Bergman *et al.* 1997). Most Nostocales have the ability to fix nitrogen, and the absence of that ability has not been definitively proven. Cave environments are limited in nitrogen and Nostocalean lineages in caves, such as *Scytonema* and *Gloeocapsa*, are known to fix atmospheric nitrogen (Asencio & Aboal 2011). Nitrogen fixation is normally accomplished by spatial separation of the enzyme nitrogenase from oxygen by the formation of a thick-walled heterocyte. Nitrogen fixation in the absence of heterocytes (e.g., outside of the Nostocales) is rare, and must occur during periods of darkness. Some Nostocalean lineages are known to have lost the ability to produce heterocytes, e.g. *Raphidiopsis mediterranea* (McGregor *et al.* 2011). Consequently, caution must be exercised before using loss of heterocyte as the sole criterion for diagnosis of a higher level taxon. I hypothesize that the thick calcareous sheath may provide a means for creating anaerobic conditions in *Geitleria* during darkness. This would appear to limit atmospheric nitrogen as well, but the permeability of N_2 is known to be slightly greater than O_2 in some membranes and this permeability can vary depending on microstructure (Matsukata *et al.* 1994).

References

ANAGNOSTIDIS, K. & KOMÁREK, J. (1990): Modern approach to the classification system of cyanophytes, 5 – Stigonematales. – Algol. Stud. 59: 1–73.

ASENCIO, A.D; & ABOAL, M. (2011): In situe nitrogen fixation by cyanobacteria at the Andraulla Cave, Spain. – J. Cave Karst Stud. 73: 50–54.

BERGMAN, B.; GALLON, J.R.; RAI, A.N. & STAL J.L. (1997): N_2 fixation by non-heterocystous cyanobacteria. – FEMS Microbiol. Rev. 19: 139–185.

BOYER, S.L.; FLECHTNER, V.R. & JOHANSEN, J.R. (2001): Is the 16S–23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in cyanobacteria. – Mol. Bioi. Evol. 18:1057–1069.

BOHUNICKÁ, M.; JOHANSEN, J.R.; MAREŠ, J. & BERRENDER-GOMEZ, E. (2014): Reassessment of the cyanobacterial family Microchaetaceae and establishment of new families Tolypthrichaceae and Godleyaceae. – J. Phycol. 50: 1089-1100.

BOHUNICKÁ, M.; PIETRASIAK, N.; JOHANSEN J.R.; GÓMEZ E.B., HAUER, T.; GAYSINA L. & LUKEŠOVÁ, A. (2015): *Rohetiella,* gen. nov. (Nostocales, Cyanobacteria)─a tapering and branching cyanobacteria of the family Nostcaceae. – Phytotaxa 197: 84–103.

BOURRELLY, P. (1970): Les algues d'eau douce. III. – Éditions N. Boubée & Cie., Paris. 512 pp.

COLWELL, R.R. (1970): Polyphasic taxonomy of the genus *Vibrio*: Numerical taxonomy of *Vibrio cholerae, Vibrio parahaemolyticus,* and related *Vibrio* species. – J. Bacteriol. 104: 410–433.

DADHEECH, P.K.; CASAMATTA D.A.; CASPER, P. & KRIENITZ, L. (2013): *Phormidium etoshii* sp. nov. (Oscillatoriales, Cyanobacteria) described from the Eosha Pan, Namibia, based on morphological, molecular and ecological features. – Fottea 13: 235–244.

DVORAK, P.; POULICKOVA, A.; HASLER, P.; BELLI, M.; CASAMATTA, D.A. & PAPINI A. (2015): Species concepts and speciation factors in cyanobacteria with connection to the problems of diversity and classification. Biodivers. Conser. 24: 739–757.

FLECHTNER, V.R.; BOYER, S.L.; JOHANSEN J.R. & DENOBLE, M.L. (2002): S*pirirestis rafaelensis* gen. et sp. nov. (Cyanophyceae), a new cyanobacterial genus from arid soils. – Nova Hedwigia 74: 1–24.

FRIEDMANN, I. (1955): *Geitlerea calcarea* n. gen. et n. sp. A new atmophytic lime-incrusting blue-green alga. – Bot. Notiser 108: 439–445.

FRIEDMANN E. (1979): The genus *Geitleria (Cyanophyceae or Cyanobacteria):* Distribution of *G. Calcarea* and *G. floridiana* n. sp. – Plant Syst. Evol. 131: 169–178.

GUGGER, M.F. & HOFFMAN, L. (2004): Polyphyly of true branching cyanobacteria (Stionematales). – Int. J. Syst. Evol. Micr. 54: 349–357.

HAUER, T.; BOHUNICKA, M.; JOHANSEN, J.R.; MAREŠ, J. & BERRENDERO-GOMEZ, E. (2014): Reassessmentof the cyanobacterial family Microchaeataceae and establishment of new families Tolypothrichaceae and Godleyaceae. – J Phycol. 50: 1089–1100.

HENTSCHKE, G.S.; JOHANSEN, J.R.; PIETRASIAK, N.; FIORE, M.; RIGONATO, J.; SANT'ANAA, C.L. & KOMÁREK, J. (2016): Phylogenetic placement of *Dapisostemon* gen. nov. and *Streptostemon*. – Phytotaxa 245: 129–143.

HUELSENBECK, J.P. & RONQUIST, F. (2001): MrBayes: Bayesian inference of phylogeny. – Bioinformatics 17: 754–755.

JOHANSEN, J.R. & CASAMATTA, D. (2005). Recognizing cyanobacterial diversity through adoption of a new species paradigm. – Algol. Stud. 17: 17–93.

JOHANSEN, J.R.; BOHUNICKÁ, M.; LUKEŠOVÁ, A.; HRČKOVÁ, K.; VACCARAINO, M.A. & CHESARINO, N.M. (2014): Morphological and molecular characterization within 26 strains of the genus *Cylindrospermum* (Nostacaceae, Cyanobacteria), with deserciptions of three new species. – J. Phycol. 50: 187–202.

JOHANSEN, J.R.; LOWE, R.; CARTY, S.; FUCIKOVA, K.; OLSEN, C.; FITZPATRICK, M.; RESS, J. & FUREY, P. (2007): New algal species records for great smoky mountains national park, with an annotated checklist of all reported algal taxa for the park. The Great Smoky Mountains national Park all taxa biodiversity inventory: A search for species in our own backyard. – Southeast Nat. 1: 99–134.

KHUDYKOV, I. & WOLK, C.P. (1999): *het*C, a gene coding for protein similar to bacterial ABC protein exporters, is involved in early regulation of heterocyst differentiation in *Anabaena sp.* Strain PCC7120. – J Bacteriol. 179: 6971–8.

KOMÁREK, J. (2013): *Cyanoprokaryota 3. Teil/ Part 3: Heterocytous Genera.* Springer-Verlag GmbH, Berlin, Heidelberg. Springer Spektrum.

KOMÁREK, J.; KOSTOVSKY, J.; MAREŠ, J. & JOHANSEN, J. (2014): Taxonomic classification of cyanoprokaryotes (cryanobacterial genera) 2014, using a polyphasic approach. – Preselia 86: 295–335.

KOZLÍKOVÁM-ZAPOMĚLOVÁ, E.; FERRARI G. & PÉREZ, M. (2016): *Dolichospermum uruguayense* sp. nov., a planktic nostocacean cyanobacterium from the Lower Uruguay River, South Africa. – Fottea 16: 189–200.

LAMPRINOU, V.; HERNANDEZ-MARINE, M.; CANALS, T.; KORMAS, K.; ECONOMOU-AMILLI, A. & PANTAZIDOU, A. (2011): Morphology and molecular evaluation of *lphinoe spelaeobios* gen. nov., sp. nov. and *Loriellopsis cavernicola* gen. nov., sp nov., two stigonematalean cyanobacteria from Greek and Spanish caves. – Int. J. Syst. Evol. Micr. 61: 2907–2915.

LUKEŠOVA, A.; JOHANSEN, J.R.; MARTIN, M. & CASAMATTA, D.A. (2009): *Aulosira bohemnsis* sp. nov.: further phylogenetic uncertainty at the base of the Nostocales (Cyanobacteria). – Phycologia 48: 118–129.

MAREŠ, J.; HROUZEK, P.; KAŇA, R.; VENTURA, S.; STRUNECKÝ, O.; KOMÁREK, J. (2013): The primitive thylakoid-less cyanobacterium *Gloeobacter* ss a common rock-dwelling organism. – Plos one 8: e66323. doi:10.1371/journal.pone.0066323

MAREŠ, J.; LARA, Y.; DADÁKOVÁ, I.; HAUER, T.; UHER, B.; WILMOTTE, A. & KAŠTOVSKÝ. (2015): Phylogenetic analysis of cultivation resistant terestrial cyanobacteria with massive sheaths (*Stigonmena* spp. And *Petalonema alatum,* Nostocales, Cyanobacteria) Using single cell and filament sequencing of environmental samples. – J. Phycol. 51: 288–297.

MATSUKATA, M.; NISHIYAMA, N. & UEYAMA K. (1994): Zeolitic membrane synthesized on a porous alumina. – J. Chem. Soc. Chem. Commun. 3: 339–340.

MCGREGOR B.G.; SENDALL B.C.; HUNT L.T. & EAGLESHAM, G.K. (2011): Report of the cyanotoxins cylindrospermopsis and deoxycylindropspermopsin from *Raphidiopsis mediterranea* Skuja (Cyanobacteria/Nostocales). – Harmful Algae 10: 402–410.

MESSING, J. (1983): New M13 vectors for cloning. – Methods Enzymol. 101: 20–78.

MISHRA, S.; BHARGAVA, P.; ADHIKARY, S.P.; PRADEEP, A. & RAI, L.C. (2014): Weighted morphology: A new approach towards phylogenetic assessment of Nostocales (Cyanobactria). – Protoplasma 252: 145–163.

MÜHLSTEINOVÁ, R.; JOHANSEN, J.R.; PIETRASIAK, N.; & MARTIN, M.P. (2014): Polyphasic characterization of *Kastovskya adunca* gen. nov. et comb. nov. (Cyanobacteria: Oscillatoriales), from desert soils of the Atacama Desert, Chile. – Phytotaxa 163: 216–228.

MULKIDJANIAN, A.Y.; VOONIN, E.V.; MAKAROVA, K.S.; MEKHDOV, S.L.; SOROKIN, A.; WOLF, Y.I.; DUFRSENE, A.; PARTENSKY, FRÉDÉRIC, P.; BURD, H.; KANADZEY, D.; HASELKORN, R. & GALPERIN, M.Y. (2006): The cyanobacterial genome core and the origin of photosynthesis. – P. Natl. Acad. Sci-Biol. 103: 13126–13131.

OSORIO-SANTOS, V.; BRANDÃO, M.M. & BITTENCOURT-OLIVEIRA, M.C. (2014): Phylogenetic study of *Geitlerinema* and *Microcystis* (Cyanobacteria) using PC-

IGS and 16S–23S ITS as markers: investigation of horizontal gene transfer. – J. Phycol. 50: 736–743.

PIETRASIAK, N.; MUHLSTEINOVÁ, R.; SIEGESMUND, M.A. & JOHANSEN, J.R. (2014): Phylogenetic placement of *Symplocastrum* (Phormidiaceae, Cyanophyceae) with a new combination *S. californicum* and two new species: *S. flechtnerae* and *S. torsivum*. – Phycologia 53: 529–541.

ŘEHÁKOVÁ K.; JOHANSEN, J.R.; CASAMATTA, D.A.; XUESONG, L. & VINCENT, J. (2007): Morphological and molecular characterization of selected desert soil cyanobacteria: three species new to science including *Mojavia pulchra* gen. et. sp. nov. – Phycologia 46: 481–502.

SCIUTO, K.; ANDREOLI, C.; RASCIO, N.; ROCCA N.L. & MORO, I. (2012): Polyphasic approach and typification of selected *Phormidium* strains (Cyanobacteria). – Cladistics 28: 357–374.

SHI, T. & FALKWOSKI, P.G. (2007): Genome evolution in cyanobacteria: The stable core and the variable shell. – P. Natl. Acad. Sci-Biol. 105: 2510–2515.

SINGH, P.; SHILA, S.; ELSTER, J. & MISHRA, A.K. (2013): Molecular phylogeny, populationgenetics and, evolution of heterocystous cyanobacteria using *nif*H gene sequences. – Protoplasma 250: 751–764.

SKUJA, H. (1937): Symbolae Sinicae. In: Handel-Mazzetti, H. (ed.): Botanische Ergebnisse der Expedition der Akademie der Wissenschaften in Wien nach Südwest-China 1914/1918. Vol. 1, pp. 1–106, Verlag J. Springer, Wien.

STAMATAKIS, A. (2014): RAxML Version 8: A tool for phylogenetic analysis and post-analysisof large phylogenies. Bioinformatics 10.1093/bioinformatics/btu033 http://bioinformatics.oxfordjournals.org/content/early/2014/01/21/bioinformatics.btu033.

STRUNECKÝ, O.; MARKÉTA B.; JOHANSEN, J.R.; ČAPKOVÁ, K.; RAABOVÁ, L.; DVOŘÁK, P. &KOMÁREK, J. (2017): A revision of the genus *Geitlerinema* and a description of the genus *Anagnostidinema* gen. nov. (Oscillatoriaphycidae, Cyanobacteria). – Fottea 17: 114–126.

SWOFFORD, D.L. 2003. PAUP*. *Phylogenetic Analysis Using Parsimony (*and Other Methods) Version 4.* Sinauer Associates, Sunderland, MA.

TAMURA, K.; STECHER, G.; PETERSON, D.; FILIPSKI, A. & KUMAR, S. (2013): MEGA6: Molecular evolutionary genetics analysis version 6.0 – Mol. Biol. Evol. 30:2725–2729.

WILEY, E.O.; JOHNSON, D.G. & DIMMICK W.W. (2000): The interrelationships of Acanthomorph fishes: A total evidence approach using molecular and morphological data. – Biochem. Syst. Ecol. 28: 319–350.

WILDE, S.B.; JOHANSEN, J.R.; WILDE, H.D.; JIANG, P.; BARELME, B.A. & HAYNIE, R.S. (2014): *Aetokthonos hydrillicola* gen. et sp. nov.: Epiphytic cyanobacteria on invasive aquatic plants implicated in Avian Vacuolar Mylinopathy. – Phytotaxa 181: 243–260.

WANG, Y. & XU, X. (2015): Regulation by *hetC* of genes required for heterocyst differentiation and cell division in *Anabaena* sp. strain PCC7120. – J. of Bacteriol. 187: 8489–8493.

WILMOTTE, A.; VAN, D.A. & WACHTER, R.D. (1993): Structure of the 16 S ribosomal RNA of the Thermophilic cyanobacterium *Chlorogloeopsis* HTF ('*Mastigocladus laminosus* HTF') strain PCC7518, and phylogenetic analysis. – FEBS J. 317: 96–100.

WU, Z.; SHI, J.; XIAO, P.; LIU, Y. & LI, R. (2011): Phylogenetic analysis of cyanobacterial genera *Cylindrospermopsis* and *Raphidiopsis* based on multi-gene sequences. – Harmful Algae 10: 419–425.

YARZA, P.; RICHTER, M.; PEPLIES, J.; EUZEBY, J.; AMANN, R.; SCHLEIFER, K.; LUDWIG, W.; GLÖCKNER, F.O. & ROSELLÓ-MÓRA, R. (2008): The all-species living tree project: A 16S rRNA-based phylogenetic tree of all sequenced type strains. – Syst. Apply. Microbiol. 31: 241–250.

YARZA, P.; YILMAZ, P.; PRUESSE, E.; GLÖCKNER, F.O.; LUDWIG, W.; SHLEIFER, H.; WHITMAN, W.B.; EUZÉBY, J.; AMANN, R. & ROSELLÓ-MÓRA, R. (2014): Uniting the classification of cultured and uncultured bacteria and archaea using 16S 267 rRNA gene sequences. Nat. Rev. Microbiol. 12: 635–645.

ZUKER, M. (2003): Mfold server for nucleic acid folding and hybridization prediction. – Nucleic Acid Res. 31: 3406–34015.

Table 1. Characteristics of *Geitleria* (Hapalosiphonaceae?) and the genera in the family Symphyonemataceae as recognized in Komárek (2013).

Genus	Branching and Heterocyte Characteristics	Habitat	16S rRNA data sequenced
Geitleria	Branched laterally or pseudodichotomously, without prostrate basal system and without differentiation into main and lateral branches, Sheaths lime-encrusted, firm, containing single filament; cells irregular; heterocytes not defined, akinetes not present, reproduction by hormogonia	Calcareous substrates in limestone caves	This study
Iphinoe	Sheaths finely to heavily calcified, branching T-type or V-type; heterocyst intercalary, rarely terminal;	Epilithic on calcareous substrate in limestone caves	Yes
Symphyonema	Branching T-type or V-type, heterocyte rare and intercalary	Epilithic, chasmoendolithic in limestone caves	Yes
Adrianema	Branching reverse Y-shape or V-shape or T-shaped, Heterocytes and akinetes unknown		No
Mastigocladopsis	Branching reverse Y- and V-shape; sheaths thin not laminated; barrel shaped cells; heterocytes intercalary and bipored;	Stones from running streams, and soils	Yes
Herpyzonema	Branching reverse Y-shaped, sheaths thick heterocytes intercalary, elongated; cells divide at cross walls for reproduction; hormogonia not observed	Calcareous substrate	No
Voukiella	V- or T-type branching, s; heterocytes common intercalary or terminal,	Aerophytic on calcareous rock	No
Symphyonemopsis	Branching T-type, V-type or reverse Y-type, numerous true branching false branching rare; terminal heterocytes. Akinetes not present	Found in multiple habitats	Yes
Loriellopsis	T- and V-type branching, rare false branching; heterocytes intercalary; akinetes isolated or in chains;	Calcareous substrates in limestone caves	Yes
Parenchymorpha	Branching lateral to pseudodichotomous, T-, V-, and reverse Y-type; heterocytes not observed; hormogonia and akinetes present	Shells of marine large mollusks	No
Iyengariella	Branching in upper parts free after simple, lateral or pseudodichotomous reversely Y- or T-shaped; Sheath not present and present; heterocyte absent, intercalary akinetes	Epilithic and endolithic on freshwater carbonate substrates	No

Table 2. Primers used for the 16S rRNA gene, ITS, and *rpo*C1 gene amplification and sequencing.

Genes	Primer designation	Primer sequence (5'–3')	Reference
16S-23S ITS amplification	CY8F	AGTTGATCCTGGC	Lukešová *et al. (*2009)
16S-23S ITS amplification	VRF1	CTCTGTGTGCCTAGGTATCC	Wilmotte *et al. (*1993)
*rpo*C1 amplification	Forward	GGTGARGTNACNAARCCAGARAC/ CCAGARTAGTCNACCCGTTTACC	Mareš *et al. (*2013)
16S–23S ITS	M13F	GTGTAAAACGACGCCAG	Messing (1983)
16S–23S ITS	M13R	GGAAACAGCTATGACCATG	Messing (1983)
16S–23S ITS	Primer 5	TGTACACACGGCCCGTC	Boyer *et al. (*2001)
16S–23S ITS	Primer 7	AATGGGATTAGATACCCAGTAGTC	Wilmotte *et al. (*1993)
16S–23S ITS	Primer 8	AAGGAGGTGATCCAGCCACA	Wilmotte *et al.* (1993)
*het*C amplification	Forward/Reverse	ATGAATCCCTCTTCGTCGTTAA/ CTATAGTTGCAGTTGAGCT	Khudyakov and Wolk (1999)
*het*C amplification	*het*C11/*het*C21	AAGAGTTCAGGGAGGGCTG/ GTCGTAACCCAGAGGTAAGGCT	Wang & Xu (2005)
*het*C amplification	*het*C1/*hetC*2	GCYCAYTGGCAAGGDAWTCA/ CCCARRKAARYMAYYAYCAT	This study

Table 3. Percent similarity matrix which includes the two sample locations within the cave of *Geitleria appalachiana* and closely related taxa: (3) SAG 23.96, AJ544087; (4) AY034793; (5) UTEX 1903, KJ768871; (6) HA4207-MV1 clone 2tcon, JN385294; (7) 92.1, AJ544080 (8); Greenland 8, DQ431003; (9) AF132777; (10) Greenland, DQ430999; and (11) HQ012541

	1	2	3	4	5	6	7	8	9	10
1. *Geitleria appalachiana* Cave 22	-									
2. Geitleria appalachiana Cave 21	99.3	-								
3. *Westiellopsis prolifica*	92.8	92.3	-							
4. *Hapalosiphon welwitzschii*	91.9	91.4	98.1	-						
5. *Fischerella ambigua*	92.6	92.0	98.6	98.1	-					
6. *Nostochopsis* sp.	93.4	92.9	98.5	96.9	98.0	-				
7. *Nostochopsis lobatus*	92.7	92.1	96.9	97.2	97.7	97.9	-			
8. *Mastigocladus laminosus*	93.6	93.1	94.4	94.4	94.0	94.6	94.4	-		
9. *Chlorogloeopsis fritschii*	93.8	93.2	93.0	92.8	92.8	93.6	93.3	93.7	-	
10. *Chlorogloeopsis* sp.	91.3	90.8	93.0	93.3	93.5	92.1	91.8	91.3	93.1	-
11. *Scytonematopsis maxima*	92.5	91.9	91.9	92.8	92.4	92.3	92.8	91.7	92.8	91.4

Fig. 1. Bayesian inference analysis using the 16S rRNA gene, with closely related taxa of Geitleriaceae. Triangle cartoons represent collapsed branches. Bootstrap values of the nodes (> 50%) are given for Bayesian inference and ML. An asterisk represents a bootstrap or probability value of 100. (A) Geitleriaceae; (B) Chlorogloeopsidaceae; (C) Hapalosiphonaceae. The Hapalosiphonaceae contains two clades, thermal strains (all should be placed in *Mastigocladus*) and nonthermal strains (*Hapalosiphon, Fischerella, Westiellopsis,* and *Nostochopsis*).

Fig. 2. Bayesian inference analysis of the *rpo*C1 gene with closely related taxa of Geitleriaceae. Triangle cartoons represent collapsed branches. Bootstrap values of the nodes (> 50%) are given for Bayesian inference and ML. An Asterisks represents a bootstrap or probability value of 1.00. (A) Geitleriaceae; (B) Chlorogloeopsidaceae; (C) Hapalosiphonaceae.

Figs 3–9. Secondary ITS structures of *Geitleria appalachiana* using multiple environmental clones from two different populations in the cave and *Fischerella musicale* HA7617-LM2: (3,4,7) V3 helix; (5,8) box B; (6,9) D1-D1'; (4) represents a V3 structure of a clone from a different location in the cave (sample 21) that has a deletion resulting in a shorter V3; (6) circled letters represent nucleotide substitutions in two clones differentiated by a black and green circle.

Figs. 10–16. Nomarski interference contrast micrographs of *Geitleria appalachiana.* All scale bars represent 10 µm: (10) showing T type branching with bent apical cell; (11) apical cell attenuated; (12) cells rarely wider than long; (13) V-type branching; (14) reverse Y-type branching; (15) Y-type and T-type branching on the same filament; (16) apical cell sometimes are bulbous at the end.

Figs. 17–18. *Geitleria appalachiana* SEM photos distinctly showing the lattice shaped calcareous deposits; (18) with the mucilaginous sheath still attached. Scale bars 1 µm (17); 10 µm (18).

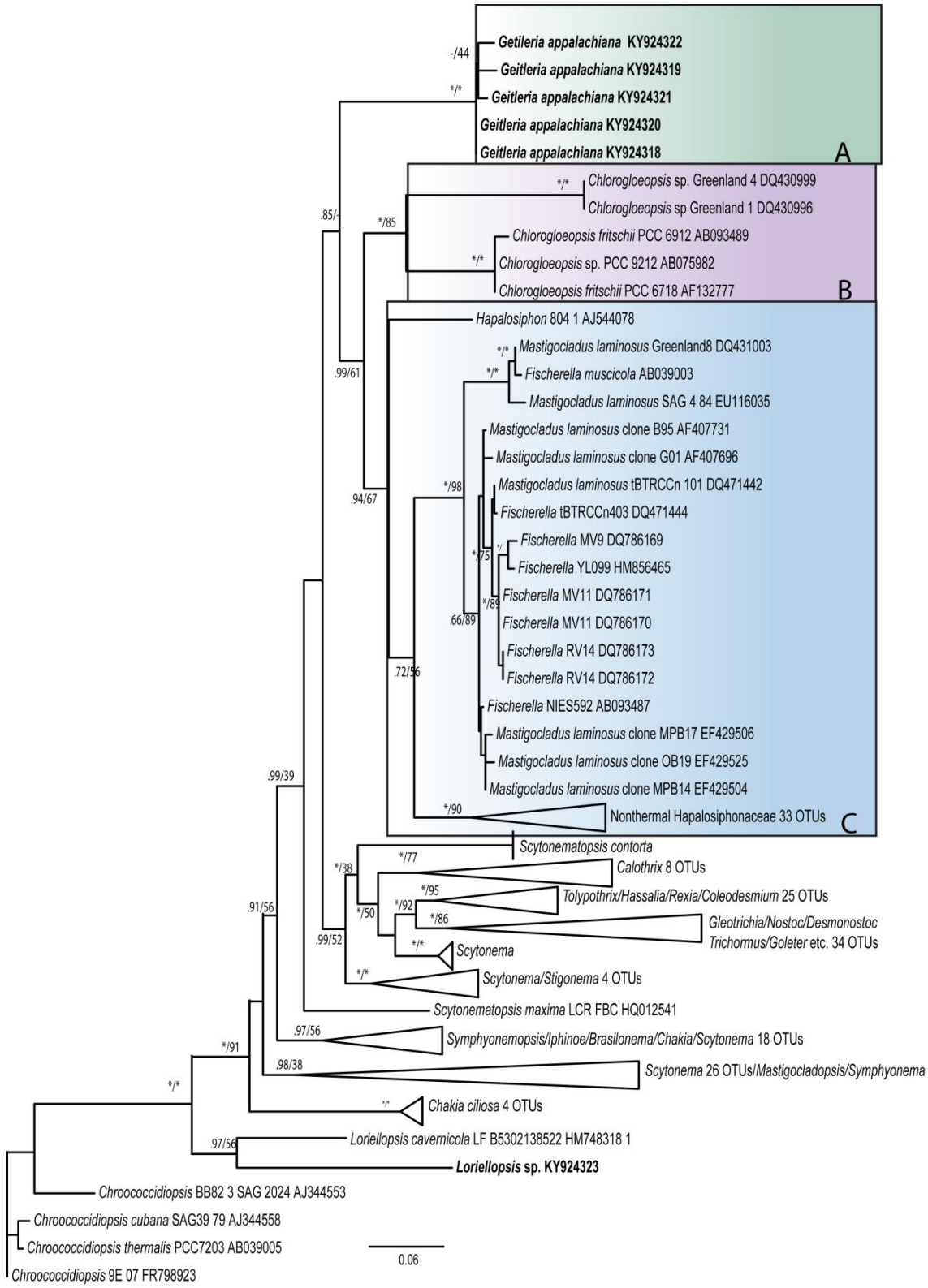
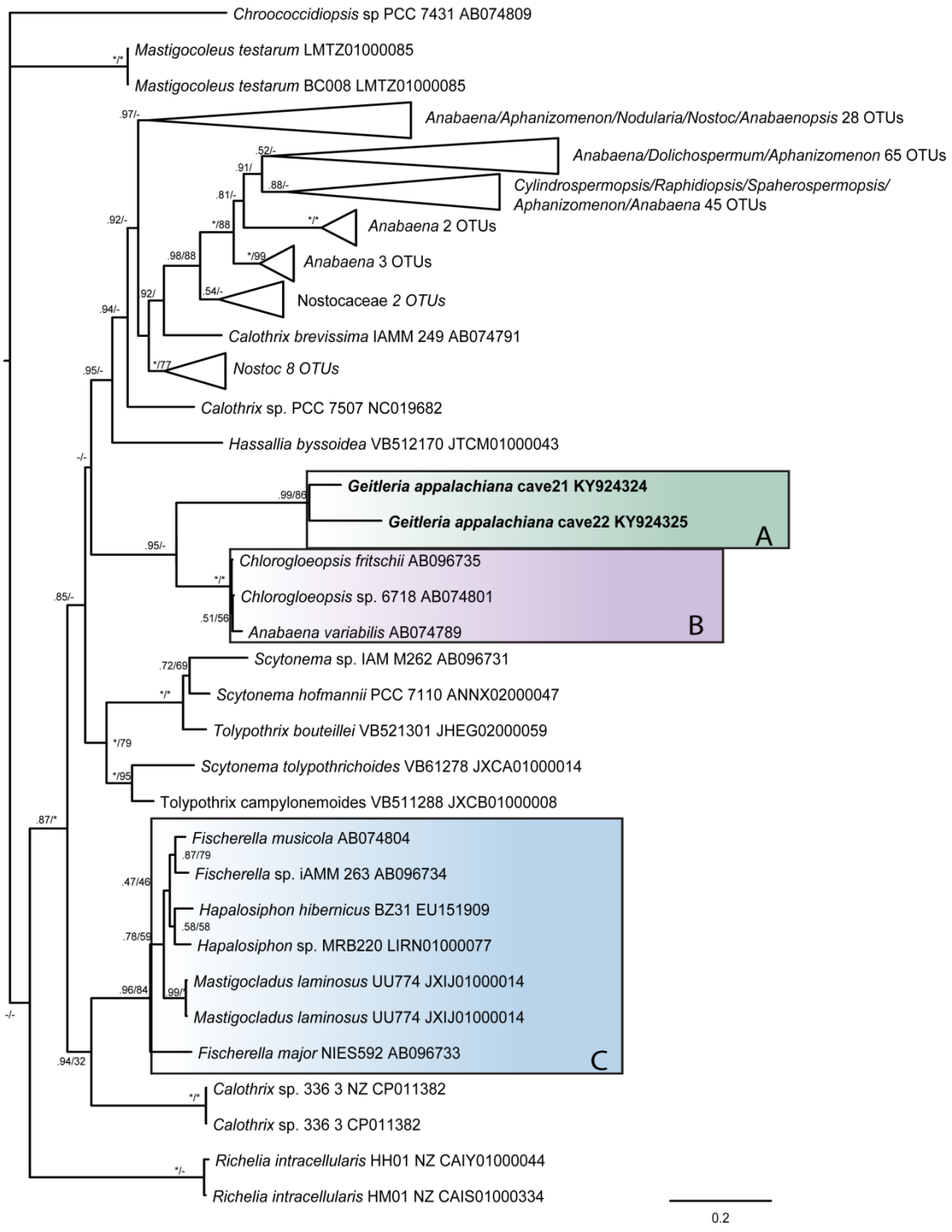

Getileria appalachiana KY924322
Geitleria appalachiana KY924319
Geitleria appalachiana KY924321
Geitleria appalachiana KY924320
Geitleria appalachiana KY924318
A
Chlorogloeopsis sp. Greenland 4 DQ430999
Chlorogloeopsis sp Greenland 1 DQ430996
Chlorogloeopsis fritschii PCC 6912 AB093489
Chlorogloeopsis sp. PCC 9212 AB075982
Chlorogloeopsis fritschii PCC 6718 AF132777
B
Hapalosiphon 804 1 AJ544078
Mastigocladus laminosus Greenland8 DQ431003
Fischerella muscicola AB039003
Mastigocladus laminosus SAG 4 84 EU116035
Mastigocladus laminosus clone B95 AF407731
Mastigocladus laminosus clone G01 AF407696
Mastigocladus laminosus tBTRCCn 101 DQ471442
Fischerella tBTRCCn403 DQ471444
Fischerella MV9 DQ786169
Fischerella YL099 HM856465
Fischerella MV11 DQ786171
Fischerella MV11 DQ786170
Fischerella RV14 DQ786173
Fischerella RV14 DQ786172
Fischerella NIES592 AB093487
Mastigocladus laminosus clone MPB17 EF429506
Mastigocladus laminosus clone OB19 EF429525
Mastigocladus laminosus clone MPB14 EF429504
Nonthermal Hapalosiphonaceae 33 OTUs
C
Scytonematopsis contorta
Calothrix 8 OTUs
Tolypothrix/Hassalia/Rexia/Coleodesmium 25 OTUs
Gleotrichia/Nostoc/Desmonostoc
Trichormus/Goleter etc. 34 OTUs
Scytonema
Scytonema/Stigonema 4 OTUs
Scytonematopsis maxima LCR FBC HQ012541
Symphyonemopsis/Iphinoe/Brasilonema/Chakia/Scytonema 18 OTUs
Scytonema 26 OTUs/Mastigocladopsis/Symphyonema
Chakia ciliosa 4 OTUs
Loriellopsis cavernicola LF B5302138522 HM748318 1
Loriellopsis sp. KY924323
Chroococcidiopsis BB82 3 SAG 2024 AJ344553
Chroococcidiopsis cubana SAG39 79 AJ344558
Chroococcidiopsis thermalis PCC7203 AB039005
Chroococcidiopsis 9E 07 FR798923
0.06

Fig. 1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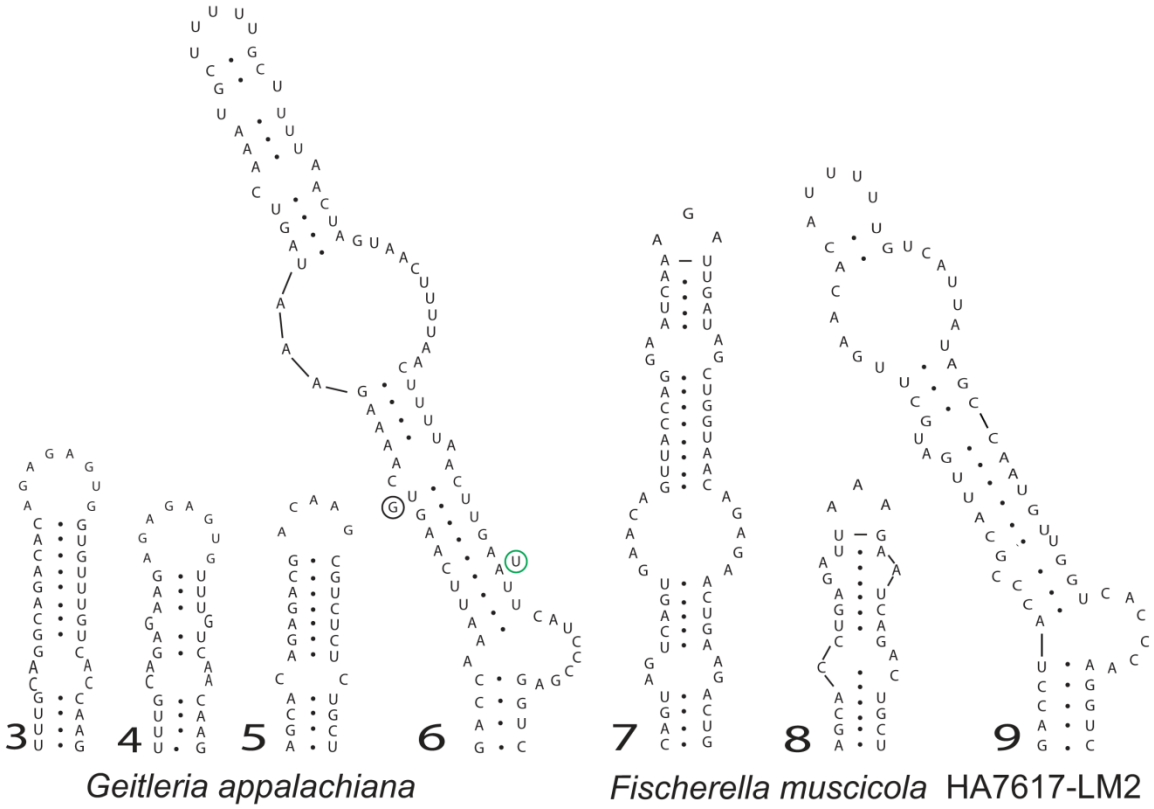
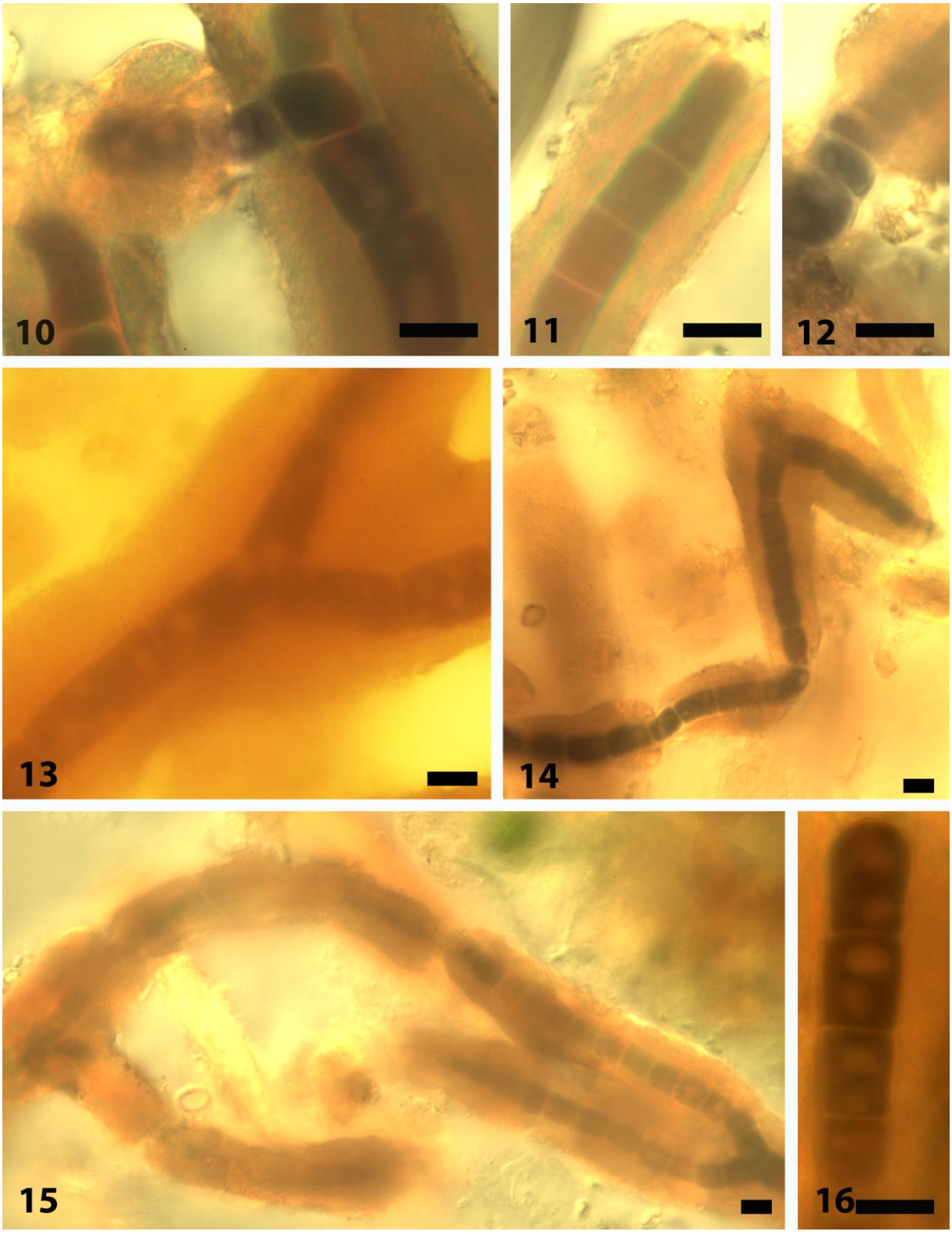
Mastigocoleus testarum LMTZ01000085
Mastigocoleus testarum BC008 LMTZ01000085
Anabaena/Aphanizomenon/Nodularia/Nostoc/Anabaenopsis 28 OTUs
Anabaena/Dolichospermum/Aphanizomenon 65 OTUs
Cylindrospermopsis/Raphidiopsis/Spaherospermopsis/ Aphanizomenon/Anabaena 45 OTUs
Anabaena 2 OTUs
Anabaena 3 OTUs
Nostocaceae 2 OTUs
Calothrix brevissima IAMM 249 AB074791
Nostoc 8 OTUs
Calothrix sp. PCC 7507 NC019682
Hassallia byssoidea VB512170 JTCM01000043
Geitleria appalachiana cave21 KY924324
Geitleria appalachiana cave22 KY924325
A
Chlorogloeopsis fritschii AB096735
Chlorogloeopsis sp. 6718 AB074801
Anabaena variabilis AB074789
B
Scytonema sp. IAM M262 AB096731
Scytonema hofmannii PCC 7110 ANNX02000047
Tolypothrix bouteillei VB521301 JHEG02000059
Scytonema tolypothrichoides VB61278 JXCA01000014
Tolypothrix campylonemoides VB511288 JXCB01000008
Fischerella musicola AB074804
Fischerella sp. iAMM 263 AB096734
Hapalosiphon hibernicus BZ31 EU151909
Hapalosiphon sp. MRB220 LIRN01000077
Mastigocladus laminosus UU774 JXIJ01000014
Mastigocladus laminosus UU774 JXIJ01000014
Fischerella major NIES592 AB096733
C
Calothrix sp. 336 3 NZ CP011382
Calothrix sp. 336 3 CP011382
Richelia intracellularis HH01 NZ CAIY01000044
Richelia intracellularis HM01 NZ CAIS01000334
0.2

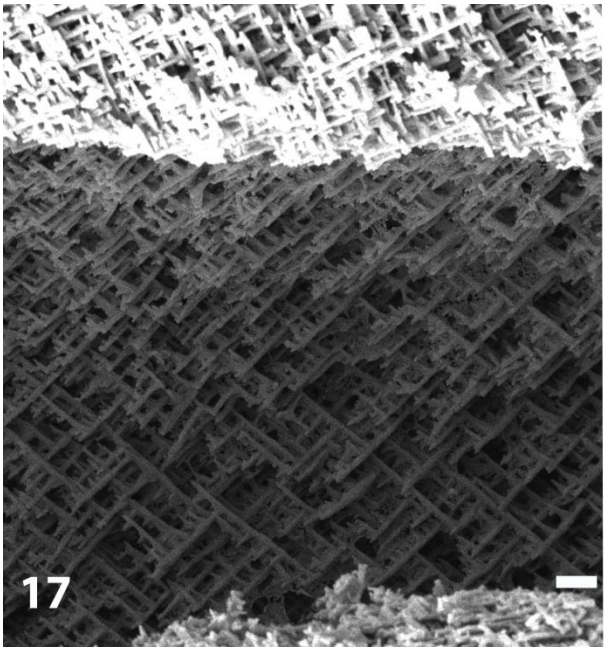
Fig. 2

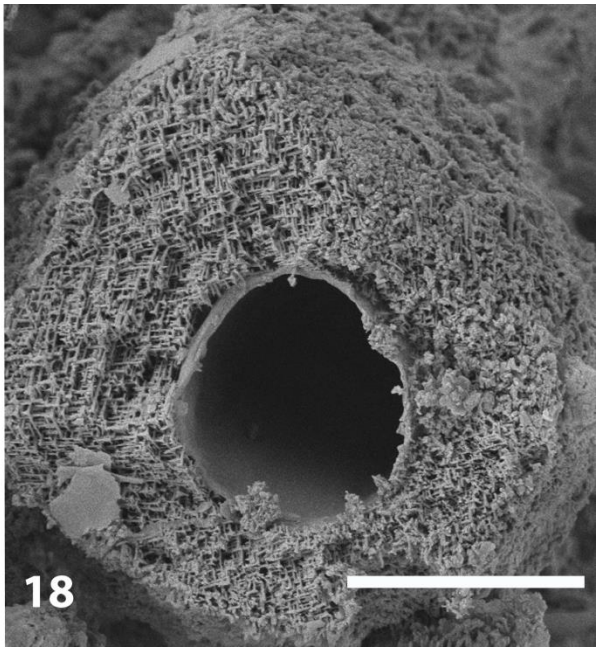

3
4
5
6
7
8
9
Geitleria appalachiana
Fischerella muscicola HA7617-LM2

Figs. 3–9



Figs. 10–16





Figs. 17–18