

Molecular characterization of *Geitleria appalachiana* sp. nov. (Nostocales, Cyanobacteria) and formation of Geitleriaceae fam. nov.

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Abstract: *Geitleria* was described from a limestone cave in Israel, and subsequently reported from caves of France, Romania, Spain, 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. nov. Sequence data for 16S rRNA and *rpoC1* 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. nov. is established to contain this unique genus.

Key words: Caves, 16S rRNA, Cyanobacteria, *Geitleria*, Nostocales, Great Smoky Mountains National Park, phylogenetics, systematics

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 & 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 31 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, and 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 usually 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, recent phylogenies with greater taxon sampling suggest that the genera of the Symphyonemataceae and Scytonemataceae are interleaved, with *Brasilonema*, *Iphinoe*, *Symphyonemopsis*, *Symphyonema*, and *Scytonema*, all in a single clade (HAUER et al. 2014, BOHUNICKÁ et al. 2015, MAREŠ et al. 2015, HENTSCHEKE et al. 2016). 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- and 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, we 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, *rpoC1*, and *hetC*. 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 family, such as *Loriellopsis* and *Iphinoe*.
- (3) *Geitleria* has a *hetC* 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 *Iphinoe*, 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 16 May 2016, in The Great Smoky Mountains 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, we 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. We 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 we were 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 that were not attached to the slide and noncalcareous 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. We performed cloning, sequencing, analysis of secondary ITS structures, and phylogenetic analysis using the same techniques and methods described in 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, *hetC*, and *rpoC1* 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 followed 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 were 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 *rpoC1* and 16S rRNA sequence data were identified using BLASTX (www.ncbi.nlm.nih.gov/blast). Additionally, we ensured maximum representation of families within the Nostocales,

including members of the following families: Nostocaceae, Gloeotrichiaceae, Rivulariaceae, Symphyonemataceae, Tolypothricaceae, Chlorogloeopsidaceae, Stigonemataceae, and Hapalosiphonaceae. Initial alignments of the 16S rRNA and ITS region were performed using MUSCLE within MEGA6 (Tamura et al. 2013). The nucleotide sequence of the *rpoC1* gene from NCBI was first translated to protein sequences to position the reading frame, so that the correct amino acids could be identified. Then the amino acid sequence was aligned in MEGA6 using MUSCLE, and used to create an alignment of nucleotides for the phylogenetic analysis. We checked both the 16S rRNA gene and *rpoC1* 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 gene sequences containing a maximum of 1,202 characters including nucleotides and indels, which encompassed the closest relatives from NCBI GenBank. The *rpoC1* phylogeny incorporated 1,896 characters including nucleotides and indels. Maximum Likelihood (ML) and Bayesian inference trees were derived using the CIPRES science gateway. The ML analysis with rapid bootstrapping was conducted using RAXML-HP v.8 on XSDE V8.2.9 (Stamatakis 2014). GTR+G+I estimated the proportion of invariable sites with 1000 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. 15 million generations were used for the 16S rRNA gene alignment and 25 million for the *rpoC1* alignment. *Chroococcidiopsis* sp. (AB074809) was the outgroup taxon for the *rpoC1* 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 method recommended by the Census of Freshwater Algae in Australia for sensitive algae (<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. We gave the preserved (uncultured) specimen the code of GSM–WOS–CK01.

Five clones of *Geitleria appalachiana* and one clone of *Loriellopsis* sp. 16S rRNA sequences were deposited into NCBI GenBank and given accession numbers: KY924318–KY924323. The two *rpoC1* 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* from the cave in Great Smoky Mountains National Park was also sequenced. The posterior probabilities and ML bootstrap

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

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

values support recognition of three distinct clades (Chlorogloeopsidaceae, Hapalosiphonaceae, and 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 *rpoC1* 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. We did not observe heterocyte formation during extensive and repeated examinations under LM. Molecular amplification of the *hetC* 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 *hetC* gene was not successful in any attempt, suggesting absence or nonfunction due to mutation 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, 13–14) 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 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. The minor variations observed in the D1–D1' helix did not result in a change in secondary structure (Fig. 3), but the basal unilateral bulge that resulted in a larger unpaired sequence on the 3' side of the helix (8 nucleotides) was different from the same helix in the ITS region of *Chlorogloeopsis fritschii* PCC 6912, *Fischerella muscicola* HA7617–LM2, and *Nostochopsis* sp. HA04292–00001 (Figs. 4–6). *Pelatocladus maniniholoensis* HA4357–MV3 had a very open basal unilateral bulge with four unpaired nucleotides on the 5' side of the helix, a highly unusual structure for the D1–D1' helix in cyanobacteria. Both *Geitleria* and *Pelatocladus* had unusually long D1–D1' helices (Figs 3, 7) in comparison to the other taxa (Figs 4–6). The Box B helix was invariant in clones of *G. appalachiana* and similar in size to the Box B helix of the other taxa (Figs 8–12). The V3 helix was variable within populations (Figs 13–14) with a deletion in the cave sample 21 population, and both structures were very different from those of *C. fritschii* and representatives of the Hapalosiphonaceae (Figs 15–18). Perhaps most significant at the genus/family level was the fact that *Geitleria* did not have the tRNA^{Ala} gene nor the V2 helix in the ITS region. *Chlorogloeopsis fritschii* had both tRNA genes, but only 5 nucleotides between the tRNA genes. Consequently, it lacked the V2 helix.

Table 2. Primers used for the 16S rRNA gene, ITS, and *rpoC1* 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)
<i>rpoC1</i> amplification	Forward/Reverse	GGTGARGTNACNAARCCAGARAC/ CCAGARTAGTCNACCCGTTACC	MAREŠ et al. (2013)
16S–23S ITS	M13F	GTGTA AACGACGCCAG	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)
<i>hetC</i> amplification	Forward/Reverse	ATGAATCCCTCTTCGTCGTAA/ CTATAGTTGCAGTTGAGCT	KHUDYKOV & WOLK (1999)
<i>hetC</i> amplification	<i>hetC11/hetC21</i> (forward/reverse)	AAGAGTTCAGGGAGGGCTG/ GTCGTA- ACCCAGAGGTAAGGCT	WANG & XU (2005)
<i>hetC</i> amplification	<i>hetC1/hetC2</i> (forward/reverse)	GCYCA YTGGAAGGDAWTCA/ CCCARRKAARYMAYYAYCAT	This study

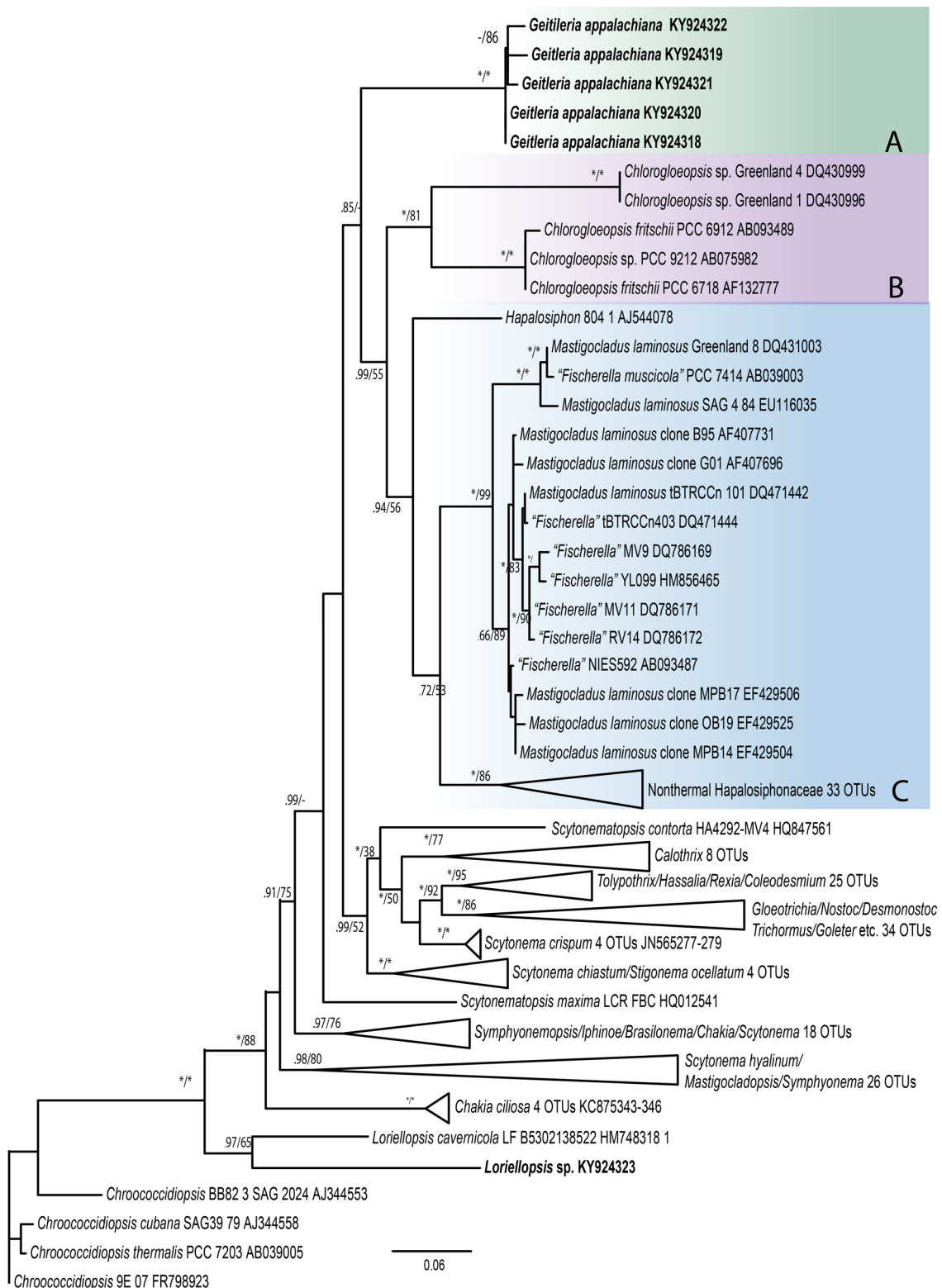


Fig. 1. Bayesian inference analysis using the 16S rRNA gene, with closely related taxa of Geitleriaceae (90 OTUs, 1202 characters). Triangle cartoons represent collapsed branches. Bootstrap values of the nodes (> 50%) are given for Bayesian inference and ML. Asterisks represent bootstrap or probability values of 100 or 1.0, respectively. (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*, *Pelatocladus*, and *Nostochopsis*).

Fig. 2. Bayesian inference analysis of the *rpoC1* gene with closely related taxa of Geitleriaceae (81 OTUs, 1896 characters). Triangle cartoons represent collapsed branches. Bootstrap values of the nodes (>50%) are given for Bayesian inference and ML. Asterisks represent bootstrap or probability values of 100 or 1.00, respectively. (A) Geitleriaceae; (B) Chlorogloeopsidaceae; (C) Hapalosiphonaceae.

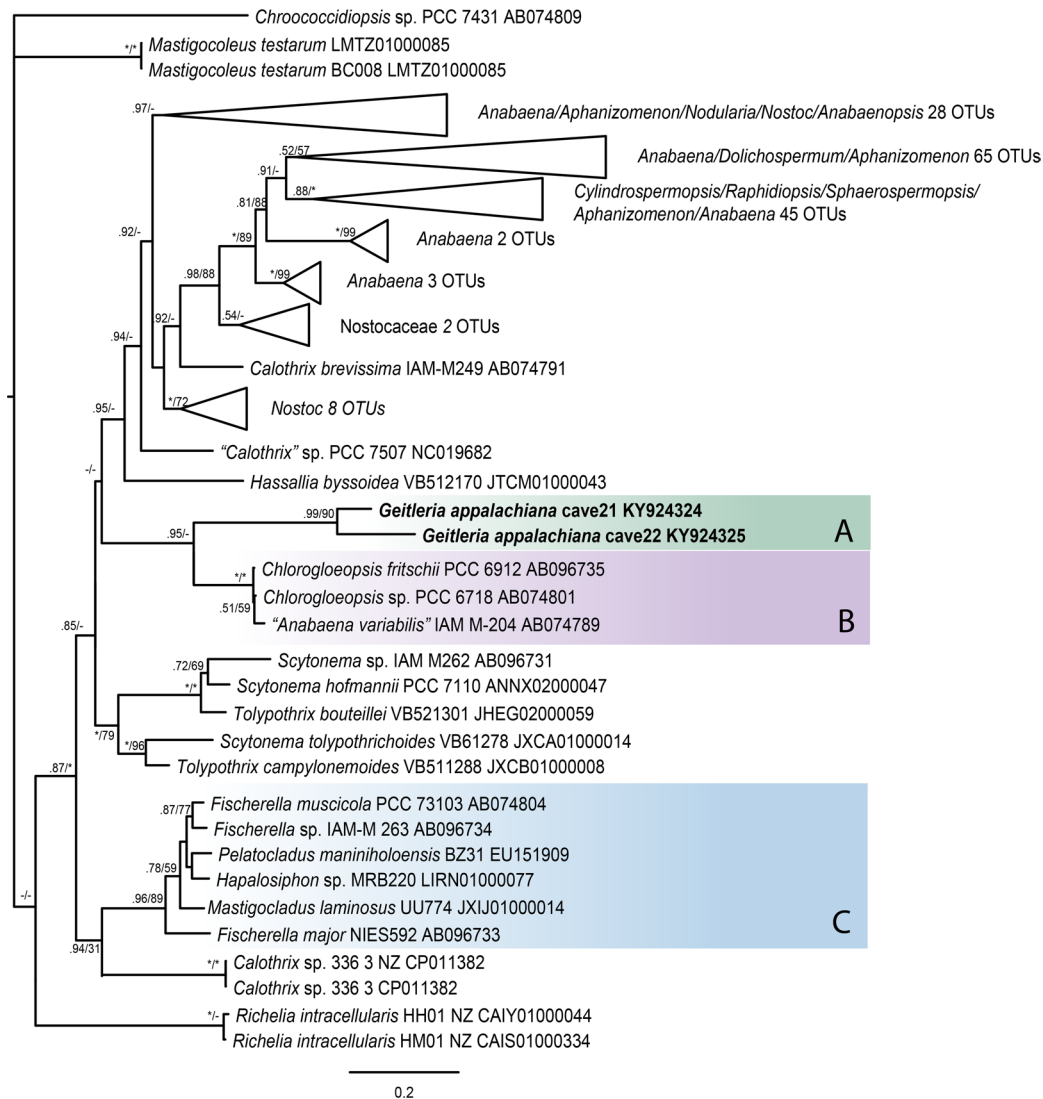
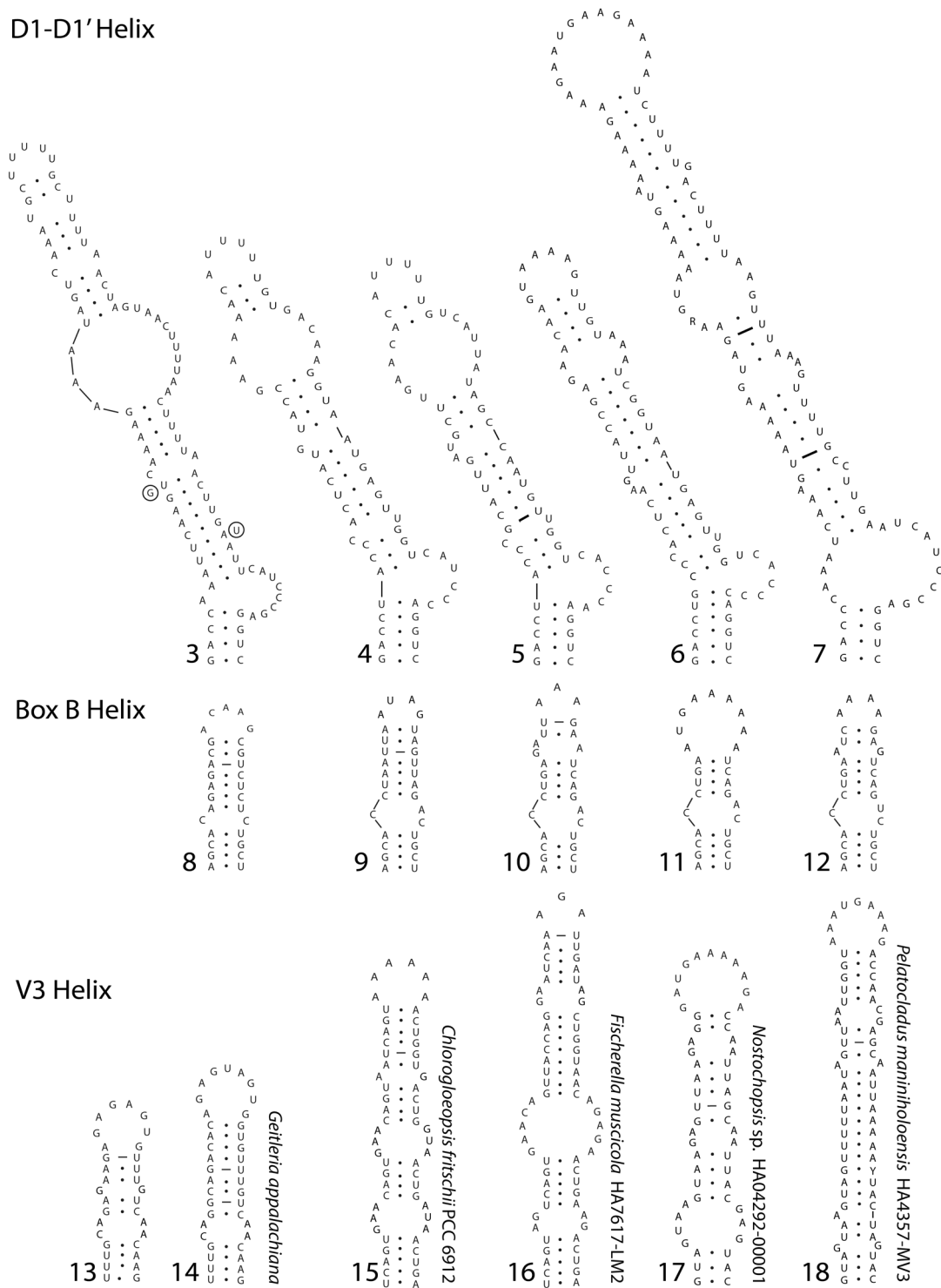
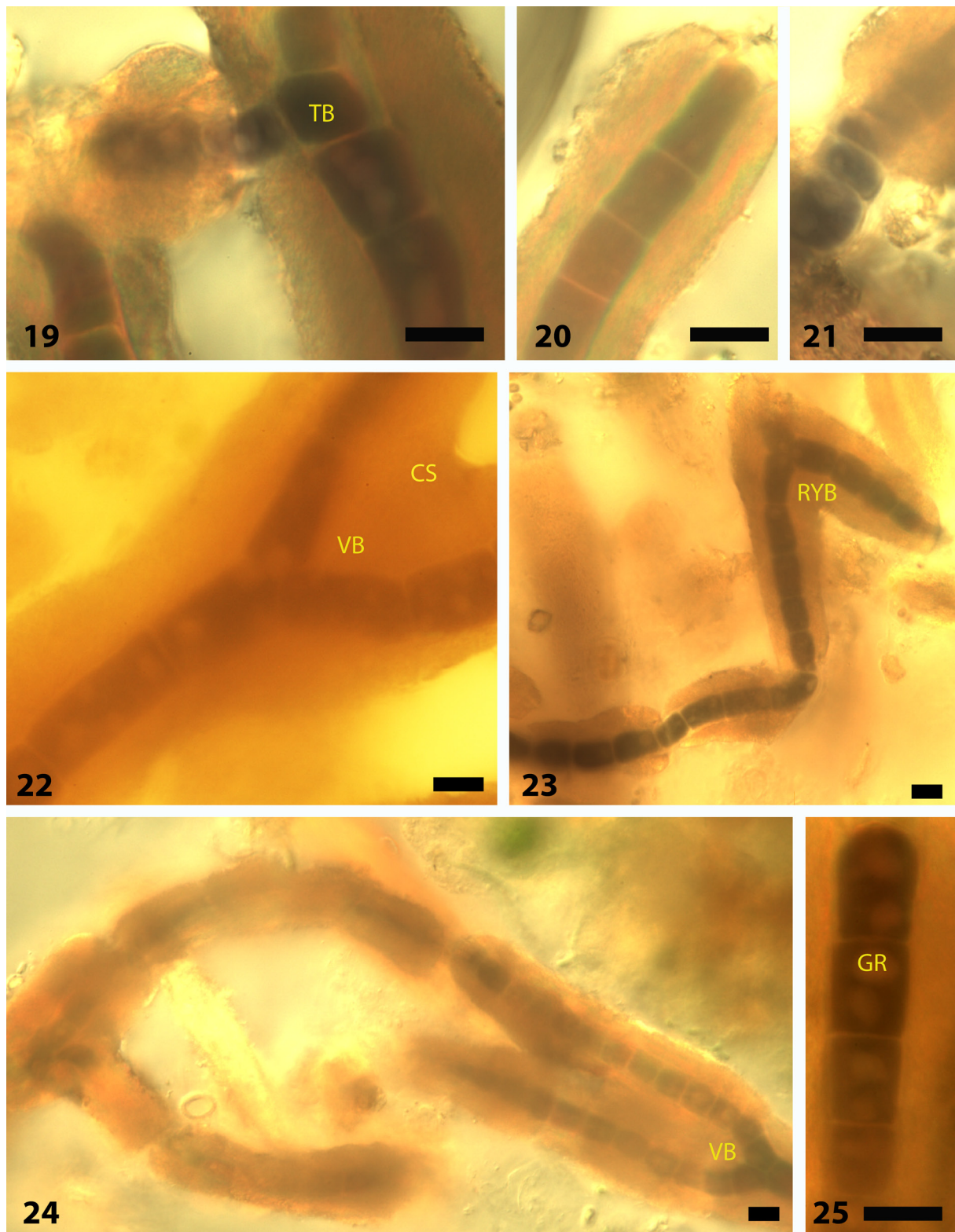


Table 3. Percent similarity matrix which includes the two sample locations within the cave of *Geitleria appalachiana* and closely related taxa: (3) SAG 23.96, AJ544087; (4) AY034793; (5) UTEX 1903, KJ768871; (6) HA4207–MV1 clone 2, JN385294; (7) 92.1, AJ544080 (8); Greenland 8, DQ431003; (9) AF132777;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

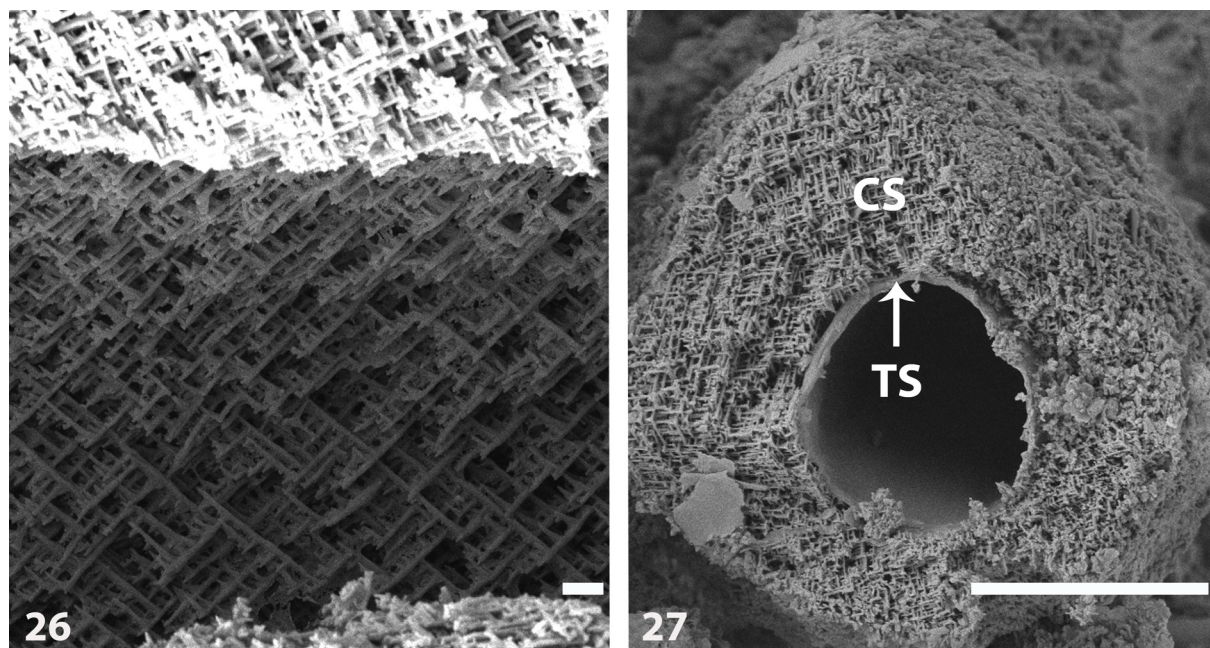
	1	2	3	4	5	6	7	8	9	10
1. <i>Geitleria appalachiana</i> Cave 22	–									
2. <i>Geitleria appalachiana</i> Cave 21	99.3	–								
3. <i>Westiellopsis prolifica</i>	92.8	92.3	–							
4. <i>Hapalosiphon welwitschii</i>	91.9	91.4	98.1	–						
5. <i>Fischerella ambigua</i>	92.6	92.0	98.6	98.1	–					
6. 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



Figs. 3–18. Secondary ITS structures of *Geitleria appalachiana* using multiple environmental clones from two different populations in the cave and representatives of Chlorogloeopsidaceae (*Chlorogloeopsis fritschii*) and nonthermal Hapalosiphonaceae (*Fischerella muscicola*, *Nostochopsis* sp., and *Pelatocladus maniholoensis*): (3–7) D1–D1' helix; (5–12) Box B helix; (13–18) V3 helix; Fig. 13 represents the V3 structure of a *Geitleria* clone from a different location in the cave (sample 21); circled letters in Fig. 3 represent nucleotide substitutions in the D1–D1' helix of the *Geitleria* clone from sample 21.



Figs. 19–25. Nomarski interference contrast micrographs of *Geitleria appalachiana*. All scale bars represent 10 μm : (19) showing T type branching with bent apical cell; (20) apical cell attenuated; (21) cells rarely wider than long; (22) V-type branching; (23) reverse Y-type branching; (24) Y-type and T-type branching on the same filament; (25) apical cells sometimes are bulbous at the end. (TB) T-type branching, (VB) V-type branching, (RYB) reverse Y-branching, (GR) granule, (CS) calcite sheath.



Figs. 26–27. *Geitleria appalachiana* SEM photos distinctly showing the lattice shaped calcareous deposits; (18) with the mucilaginous sheath still attached. Scale bars 1 μm (26); 10 μm (27). (CS) calcite sheath, (TS) trichome mucilaginous sheath.

All members of the Hapalosiphonaceae had both tRNA genes and a long V2 helix.

Geitleriaceae Kilgore et Johansen fam. nov.

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 reverse Y-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. nov. (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). Mucilaginous sheaths clear, thin, tightly-adherent around trichomes. Calcite sheaths external to mucilaginous sheaths, firm, yellow to golden, narrowed or roundly truncated near apex of trichomes, sometimes absent, with lattice-like arrangement (Figs 17–18), calcite crystals with irregular lateral branching (Figs 17, 18). 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 \times 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 μm long and 3.8–14.7 μm 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 distinctive 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, we 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 we believe morphological and ecological differences

still provide sufficient evidence to recognize *Geitleria appalachiana* as a separate species. The secondary structure of conserved domains in the 16S–23S ITS region is best used to distinguish species within genera, but since we only have ITS sequence information for one species in *Geitleria*, we have provided these structures for *Chlorogloeopsis fritschii* PCC 6912 in Chlorogloeopsidaceae and *Fischerella muscicola* HA7617–LM2, *Nostochopsis* sp. HA04292–00001, and *Pelatocladus maniniholoensis* HA4357–MV3 in the nonthermal Hapalosiphonaceae. At present, no ITS sequences exist for *Mastigocladus*, *Hapalosiphon*, or *Westiellopsis*, other members of the Hapalosiphonaceae.

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 we 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* (KOZLÍKOVÁ–ZAPOMĚLOVÁ et al. 2016) and *Phormidium etoshi* (DADHEECH et al. 2013). We 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 relevant 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 *rpoC1* 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), we 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 (LAMPRINOU et al. 2011) and is phylogenetically distant from *Geitleria*. *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 similar to *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. We feel that taxon sampling in the *rpoC1* 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. We assume the *rpoC1* 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 can arise (SHI & FALKOWSKI 2007). The 16S rRNA gene and photosynthetic gene sequences (e.g., *rbcLX*) 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.

We were 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 cyanobacterial 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 (WASMUND et al. 2001). 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. We hypothesize that the thick calcareous sheath and its inherent arrangement may provide a means for creating anaerobic conditions in *Geitleria* during darkness (Figs. 17, 18). This would appear to limit atmospheric nitrogen as well, but the permeability of N₂ is known to be slightly greater than O₂ in some membranes and this permeability can vary depending on microstructure (MATSUKATA et al. 1994).

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