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Molecular and Evolutionary Analysis of Cyanobacterial Taxonomic Methods

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MOLECULAR AND EVOLUTIONARY ANALYSIS OF CYANOBACTERIAL TAXONOMIC METHODS

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Abstract

Cyanobacteria are a group of photo-oxygenic bacteria found in nearly every ecosystem, but much cyanobacterial diversity, in various habitats, has yet to be explored. Cyanobacteria are often conspicuous components of photosynthetic flora, providing significant carbon and nitrogen inputs to surrounding systems. As possible primary colonizers of stone substrates not native to this region, cyanobacteria isolated from headstones may provide biogeographically informative data. An exploratory study of lichen-dominated microbial consortia, growing on headstones, was conducted to isolate and identify novel microaerophytic cyanobacteria, and resulted in the establishment of four novel cyanobacterial taxa. Phylogenetic analyses of photobionts in one tripartite lichen revealed two novel taxa: *Brasilonema lichenoides* and *Chroococcidiopsis lichenoides*. Using a total evidence approach, analyzing ecology, morphology, ITS structure, and molecular data two additional taxa were described: *Brasilonema geniculosus* and *Calothrix dumas*. Analysis of secondary structures of the Internal Transcribed Spacer (ITS) regions of the 16S-23S operon in cyanobacteria are commonly used in cyanobacterial taxonomy studies and were applied to the identification of the new taxa in this study. However, the relationship between ITS structures, hairpin loops (helices) in a region of non-coding DNA, has not been thoroughly evaluated. The 16S-23S operon is one of many in prokaryotes with multiple copies and there is evidence that operons may vary due to differential selective pressures or drift. A study was undertaken analyzing ITS operons from 224 previously published cyanobacterial taxa for domain inclusion and exclusion, intragenomic heterogeneity of ITS operons, and the possible relevance of variable selective pressures affecting individual domains. Analysis revealed highly variable ITS domain inclusion even in complete sequences, as well as high variation between domains containing two or no tRNA sequences. Recommendations were made to standardize

ITS analysis in the future to account for this possible variation. Further study is required to statistically demonstrate to what extent ITS secondary structures correlate with taxonomy.

General Introduction to the Cyanobacteria

Cyanobacteria are a group of photo-oxygenic bacteria found in nearly every ecosystem. Cyanobacteria are often conspicuous components of the photosynthetic microbial flora and provide significant carbon and nitrogen inputs to surrounding systems. Traditionally classified by morphological features, the advent and employment of modern molecular methods (e.g., the 16S rDNA gene sequence) has facilitated a much greater examination of the alpha-level biodiversity in this clade (e.g., Casamatta et al. 2005; Perkerson et al. 2011). While the cyanobacterial components of some habitats (e.g., planktonic) have been well characterized, many others (e.g., terrestrial or subaerial) have been woefully understudied. In addition, the majority of cyanobacteria described have been from temperate regions, with only very recent, scant attention turned to less commonly sampled ecoregions (e.g., tropical, subtropical, polar). However, these recent investigations have produced numerous new genera in marine (e.g., *Roseofilum*, Casamatta et al. 2012), tropical (e.g., *Brasilonema*, Fiore et al. 2007) and terrestrial (e.g., *Calochaete*, Hauer et al. 2014) habitats.

The first taxonomic arrangements for cyanobacteria were suggested in the late nineteenth century (Gomont 1892; Bornet & Flahault 1886-1888). In the twentieth century Frémy (1929) and Geitler (1932) advocated only three orders of cyanobacteria, though Geitler later expanded that to four orders (Geitler 1942). Rippka et al. (1979) expanded this scheme to include five subsections of cyanobacteria, a system which formed the basis of most cyanobacterial taxonomy until recently, and was the basis for the cyanobacterial section of Bergey's Manual of Systematic

Bacteriology. Section I (Chroococcales) includes single celled taxa, Section II (Pleurocapsales) includes simple filamentous taxa with no real sheath formation, Section III (Oscillatoriales) includes filamentous taxa with sheath formation, Section IV (Nostocales) includes filamentous taxa with sheaths and cell differentiation via formation of akinetes or heterocytes, and Section V (Stigonematales) includes filamentous taxa with sheaths, obligatory cell differentiation, and true branching (Castenholtz 2001).

All bacterial genomes contain the 16S-23S rRNA operon, consisting of the 16S gene, Intergenic Spacer region (ITS), 23S gene, and 5S gene (Figure 1) (Schleifer & Kandler 1989; Iteman et al. 2000). Heterotrophic bacteria and cyanobacteria typically have multiple copies of this operon in a genome, though the copy number varies (Tourova 2003). Heterotrophic bacteria may have one to 15 operon copies with copy number varying between and among genera (Klappenbach et al. 2001; Větrovský & Baldrian 2013). Cyanobacteria typically have one to five operon copies, with copy number increasing with morphological complexity (Iteman et al. 2000, 2001; Schirrmeister et al. 2013). The 16S gene for ribosomal RNA functions as scaffolding in the small ribosomal subunit, stabilizing bonding between the small and large ribosomal subunits, as well as stable bonding in the ribosomal A site (Stackebrandt & Goebel 1994; Iteman et al. 2000; Boyer et al. 2001). The structural function of the 16S gene makes it highly conserved, but the ITS region, a regulatory rDNA region located between the 16S and 23S genes, is more variable (Woese 1987; Iteman 2000; Boyer 2001).

With the advent of molecular techniques, Rippka's taxonomic classification scheme for cyanobacteria based on morphology has produced mostly polyphyletic groupings. Thus, as molecular data becomes available, constant taxonomic updating has been required (Komárek 2014; Dvorak et al. 2015). Recent genomic research has demonstrated that many morphological

traits classically used to classify cyanobacteria (e.g., multicellularity, baeocyte formation, presence of akinetes, tapering, polarity, branching patterns) developed in several separate lines, and were subsequently lost in some lineages (Schirrmeister 2011; Komárek 2013, Shih et al. 2013). Phylogenies constructed using taxa included in classic morphology-based taxonomy are often polyphyletic because morphology does not always accurately represent evolutionary relationships. This has led to three very different proposed methodologies for revision of cyanobacterial taxonomy coming from different research camps (Komárek 2014).

One camp of researchers has been suggesting that the number of cyanobacterial taxa must be dramatically reduced to simplify the taxonomic system (Drouet & Daily 1956; Drouet 1973, 1978, 1981; Bourrelly 1970; Otsuka et al. 2001). To the contrary, a second camp of researchers has recommended bifurcation of polyphyletic groups of both genera and species until all taxonomic units are monophyletic (Anagnostidis & Komárek 1985; Casamatta et al. 2005; Johansen & Casamatta 2005; Rehakova et al. 2007; Siegesmund et al. 2008; Perkerson et al. 2011). Lastly it has been suggested that no further taxonomic changes should be implemented at all until a great deal more molecular data is collected by researchers (Hoffman 2005). Komárek (2014) recommended a new taxonomic system that reflects true evolutionary relationships by attaining monophyletic groups with exiguous but evidently monophyletic genera that contain fewer, more closely related species (proposal number two). Over fifty new genera were described between 2000 and 2014, and the new taxonomy presented by Komárek (2014) attempts to incorporate all those with good taxonomic standing.

The monophyletic species concept *sensu* Johansen and Casamatta (2005) derived from a polyphasic approach has been proposed as the standard for cyanobacterial systematics (e.g., Komárek et al. 2014; Komárek, 2016), which requires the description of an apomorphy, along

with ecology and molecular data, to test phylogenetic hypotheses. If a new strain of cyanobacteria has <98% sequence similarity to previously described taxa, some habitat or ecological incongruity, and a documented apomorphy, the case can be made to designate the new strain as a novel species (Johansen & Casamatta 2005). Komárek's (2014, 2016) system reflects true evolutionary relationships by erecting monophyletic groups with exiguous but evidently monophyletic genera that contain fewer, more closely related species.

Describing and elucidating cyanobacterial species, however, can be problematic, even with genetic data. Morphological plasticity and cryptic diversity often mask how akin or divergent various taxa are, stifling attempts to clarify cyanobacterial systematics with genetics (Casamatta et al. 2003; Komárek et al. 2013; Dvořák et al. 2015). Traditionally, non-planctonic cyanobacterial ecology has been woefully understudied, with only recent forays into non-lentic ecologies just beginning, leaving the habitat ranges of many taxa to be revised (Dvořák et al. 2015). Altogether, this means that most cyanobacterial phylogenies remain unresolved (Dvořák et al. 2015; Komárek 2016).

The 16S rDNA gene, however, has recently been shown to lack resolving power at the species level for prokaryotes (Konstantinidis et al. 2006; Goris et al. 2007). The level of 16S rRNA gene sequence similarity that corresponds to the accepted average nucleotide identity (ANI) threshold for prokaryotic species identification has been calculated as 98.65% (Kim et al. 2014). There have been instances where well differentiated populations of phenotypically different cyanobacteria had identical 16S and ITS sequences, though they varied considerably at six nitrogen metabolism loci (Miller et al. 2006). Other potential molecular markers have been suggested for use in prokaryotic taxonomy, such as the *rpoB, nifD, psbA, rbcL* genes (Case et al. 2007; Singh et al. 2014; Dvořák et al. 2014). Concatenation of the 16S marker data with other

molecular markers, collectively known as multilocus sequence analysis (MLSA), has become common in molecular studies to address the taxonomic shortfalls of the 16S rRNA gene (Singh et al. 2014; Wilmotte et al. 2017). However, a robust multilocus phylogenetic analysis of 23 coding genes, across eight cyanobacterial orders, showed that when compared to 16S rRNA phylogenies, only minor differences were noted. This supported the continued use of the 16S marker while confirming that the 16S lacks resolving power beyond the generic level (Mares 2017).

Without the use of morphological characters to act as apomorphies, and with the lack of species level resolution in common molecular markers, differences in ITS secondary structures have developed into a common taxonomic tool to describe new species (Iteman et al. 2001, 2002; Boyer et al. 2001; Johansen et al. 2005, 2011, 2013). Iteman (2000, 2002) pioneered the use of ITS, previously identified as regulatory RNA in other prokaryotes though less highly conserved than the 16S gene, with restriction fragment length polymorphisms of ITS amplicons used to distinguish closely related taxa. Later works by Johansen et al. (e.g., 2005, 2011, 2013) demonstrated how ITS secondary structures, obtained by folding conserved hairpin loop domains within the ITS, could be used to delineate cryptic species, and were thus a powerful tool when applied to phylogenetic studies. ITS secondary structures have become commonly used when describing novel cyanobacterial taxa for the last decade (Johansen 2013; Komárek 2013; Engene et al. 2015; Suradkar et al. 2017). However, protocols for ITS analysis have not been standardized, and thus nomenclature and presentation of folded structures vary from author to author (Johansen 2013; Komárek 2013; Engene et al. 2015; Suradkar et al. 2017).

Morphological plasticity and cryptic diversity have stifled attempts to clarify the cyanobacterial taxonomies constructed using morphology, which has been partially ameliorated

with molecular techniques. Primary producers in most aquatic habitats, cyanobacteria are also ubiquitous in terrestrial habitats, and range from tropical to polar climates around the globe (Holland 1977; Rehakova et al. 2007). Frequently encountered in soils or biocrusts, cyanobacteria are often present due to their capabilities of fixing atmospheric nitrogen, resisting the harmful impacts of UV light, and their ability to enter a quiescent state during times of environmental stress (Adams 2000; Flechtner et al. 2002). Few researchers actively categorize new cyanobacterial taxa in the United States, and fewer still in the southeast. Phylogenetic surveys of cyanobacteria using molecular techniques are still crucial to developing accurate cyanobacterial taxonomy, but studies must begin to focus on ecological considerations as well (Komárek 2014).

The goal of this research was to identify novel cyanobacterial taxa while clarifying enigmatic evolutionary relationships between cyanobacterial taxonomy and ITS secondary structures across all orders of cyanobacteria. This project surveyed lichenized and free-living terrestrial cyanobacteria growing on headstones from the H. Warren Smith cemetery in Jacksonville Beach, Florida. Biocrust samples collected from headstones were used for routine cultural studies, and isolated cyanobacteria were identified via 16S-23S rDNA gene sequencing, and closest related taxa analyzed morphologically and using folded ITS secondary structures.

Previously published (Genbank) sequence data was analyzed by comparing the secondary structures of highly conserved ITS regions by creating a compendium of folded structures across all four major cyanobacterial lineages. Additionally, ITS sequences from 224 samples sourced from Genbank were analyzed by calculating relative sequence partiality as well as the inclusion of individual conserved regions. This research accomplished two important things. First, the survey of novel taxa provided data that aided in the revision of cyanobacterial systematics.

Further, it described some endemic taxa from an under-represented region of the country. Second, compiling a comprehensive data set of folded ITS secondary structures provided a framework of comparable structures for use in future taxonomic studies. From this, recommendations for standardization of these structures and associated nomenclature were proposed.

The results of this work have been submitted as three separate publications, with each publication corresponding to a chapter of this thesis. The first chapter has been published, while the remaining two chapters have been submitted at the time this thesis completion. Chapter one describes two novel cyanobacteria from a single tripartite lichen association. Chapter two describes two novel cyanobacteria from two separate lichen associations. Chapter three is a review of the state of ITS analysis standardization, with recommendations for these analyses in the future.

Chapter 1: *Brasilonema lichenoides* sp. nov. and *Chroococcidiopsis lichenoides* sp. nov. (Cyanobacteria): two novel cyanobacterial constituents isolated from a tripartite lichen of headstones[1]

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Abstract

Cyanolichens are an assemblage of fungi and cyanobacteria from diverse, cosmopolitan habitats. Typically composed of a single species of cyanobacterium, with or without another eukaryotic alga, here we present two novel cyanobionts isolated from an undescribed tripartite lichen. This endolithic lichen was isolated from a granite cemetery tombstone from Jacksonville, Florida, and contains two potentially nitrogen-fixing cyanobionts. Employing a total evidence approach, we characterized the cyanobionts using molecular (the 16S rDNA and ITS gene region), morphological, and ecological data. Phylogenetic analyses revealed two novel taxa: *Brasilonema lichenoides* and *Chroococcidiopsis lichenoides*, both of which fell within well supported clades. To our knowledge, this represents the first instance of a tripartite lichen with two cyanobacterial and no eukaryotic members. These types of lichens may well represent an unexplored reservoir of cyanobacterial diversity. The specific epithets are proposed under the provisions of the International Code of Nomenclature for Algae, Fungi, and Plants.

Key words: 16S rDNA gene, 16S-23S ITS, biodiversity, cyanolichen, taxonomy

Introduction

Cyanobacteria are a group of photooxygenic prokaryotes, and amongst the most important primary producers on Earth. Much of the basic biodiversity of this group is poorly understood, and many habitats have been only cursorily explored for their cyanobacterial members (Dvořák et al. 2015b). Ubiquitous in nearly all known aquatic habitats, they are also common components of numerous terrestrial environments (e.g., Holland 1977; Řeháková et al. 2007). Frequently encountered in soils, cyanobacteria are often present due to their capabilities

of fixing atmospheric nitrogen, resisting the harmful impacts of UV light, and their ability to enter a quiescent state during times of environmental stress (Adams 2000; Flechtner et al. 2002). Cyanobacteria are often also integral components of symbiotic relationships, such as with mosses, angiosperms, and cycads (Rai 1990).

Lichen are composed of an algal or cyanobacterial photobiont incorporated into the body of a fungi, with distribution patterns often reflecting algal climate and substrate preference (Sanders 2001; Peksa and Škaloud 2011). A single fungal symbiont may envelope and direct the growth of multiple photosynthetic endosymbionts, while hosting bacterial symbionts living on fungal surfaces and in intercellular spaces (Grube and Berg 2009; Bjelland et al. 2011; Muggia et al. 2013; Dal Grande et al. 2014). The fungal host, or mycobiont, provides a stable habitat for the photosynthetic member and in exchange, the photobiont provides fixed carbon and, in the case of cyanolichens, fixed atmospheric nitrogen (Sanders 2001). Recently, it has been noted that many common lichens also contain basidomycete yeasts, although their role is currently under study (Spribille et al. 2016). Cyanolichens, or lichens containing a cyanobacterial member, are common components of epilithic and epiphytic habitats throughout the world (Rikkinen 2002). Epilithic lichen populations inhabit stone surfaces, often directly on top of endolithic populations living just within stone, but may host distinct microbial consortia (McNamara and Mitchell 2005; McNamara et al. 2006).

Cyanobacteria photobionts of lichen biocrusts are often studied from a stone biodeterioration perspective, less so as a source of cyanobacterial diversity (Crispim and Gaylarde 2005). The cyanobacterial member of a lichen is typically filamentous and heterocyte forming (e.g., *Nostoc*, *Calothrix*), putatively contributing carbon and nitrogenous compounds (Rai et al. 2002). Other cyanolichens may be coccoid forms that fix nitrogen (e.g.,

Chroococcidiopsis, *Gloeocapsa*), filamentous forms without nitrogen fixing capabilities (e.g., *Oscillatoria*, *Pseudanabaena*, or involved in tripartite interactions with eukaryotic algae (e.g., *Trentepolia*) (Henskens et al. 2012; Pérez-Ortega et al. 2012). In this paper, we describe two novel cyanobacteria isolated from a tripartite lichen containing no eukaryotic algal symbionts, a new species of a filamentous, nitrogen fixing cyanobacterium (*Brasilonema lichenoides*) and a new coccoid species (*Chroococcidiopsis lichenoides*) inhabiting granite headstones (Jacksonville Beach, FL, USA).

Methods

Sampling Site

Isolates were obtained from H. Warren Smith cemetery (Jacksonville Beach, FL, USA). Six individual headstones were sampled, which provided three to five epilithic and five endolithic samples each. Epilithic samples were teased from headstones using a sterile scalpel and transported in 1.5 mL micro centrifuge tubes. Endolithic samples were collected using non-destructive, sterile tape sampling techniques (Cutler et al. 2012). Irradiance was measured at each collection site using a basic quantum meter (Apogee Instrument Inc., Logan UT) and the age and type of stone, the condition of the stone, the presence of effluents or nearby plant growth, as well as the class and color of dominant lichen growth forms, and any color change of growth after wetting was recorded (Supplemental Table 1).

Culturing

Microthallus samples were used to inoculate cultures to isolate photosymbionts. Cyanobacterial isolates were cultured in liquid Z8 medium (Staub 1961) with the addition of 10

µL (1x) fungicide (Amphotericin, Cell Grow Virginia). Cultures were kept on a desktop, at ambient conditions (23 °C, ca. 12:12 light:dark photoperiod). In addition, cultures were grown on nitrogen free Z8 medium to test for nitrogen fixation. Thallus dissection was not possible due to the small size.

Morphological assessment

Morphology of the strains was analyzed via light microscopy (Zeiss AxioImager, objectives EC Plan–Neofluar 40×/1.3 N.A., oil immersion, DIC; Plan–Apochromat 100×/1.4 N.A., oil immersion, DIC). Images were taken with a high-resolution camera (AxioCam HRc 13MPx). Pictures were processed using with Zeiss AxioVision software (version 4.9.1.). During morphological evaluation of natural samples and strains, the following characters were assessed: cell shape, cell dimensions, type of cell reproduction, sheaths, and granulation of cells. Measurements were performed on 100 cells of both natural and culture materials.

Molecular Techniques

DNA from cyanobacterial isolates was extracted with the PowerSoil™ DNA Kit from 0.25g of culture samples (Mo Bio Laboratories Inc., Carlsbad, CA). DNA quality was checked on an ethidium bromide stained 1.5% agarose gel.

PCR amplification of the partial 16S rDNA and the whole 16S–23S ITS was performed using primers forward 27F (5'–AGAGTTTGATCCTGGCTCAG–3'), and reverse B23S (5'–CTTCGCCTCTGTGTGCCTAGG –3') previously described in Lane (1991). The 50 µl PCR reaction contained: 27 µl sterile water, 1 µl of each primer (0.01 mM concentration), 20 µl PCR Master Mix (Promega, Madison, WI) and 1 µl template DNA (50 ng/µl) and PCR amplification

proceeded as detailed in Casamatta et al. (2005). Amplified rDNA was cloned into pGEM® T Vector System I and JM-109 High Efficiency Competent Cells (Promega, Madison, WI) and cultured using carbenicillin infused LB media. Plasmid DNA was purified from eight replicate transformed competent cell colonies per isolate, using QIAprep® Spin Miniprep Kits (QIAGEN, Hilden, Germany). Sequencing of cDNA libraries from two operons of varying size was performed by Eurofins Genomics (MWG Operon Inc., Louisville, KY).

A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to obtain closely related taxa. New 16S sequences were combined with sequences from GenBank having ≥93% sequence similarity via BLAST searches. For both phylogenetic trees, sequences were aligned together using the ClustalX web interface (Thompson et al. 1997) and manually checked and edited using Maclade v.4.06 (Maddison and Maddison 2000). The GTR+I+gamma model was selected using MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (Kumar et al. 2016). An unweighted maximum-parsimony (MP) and maximum-likelihood (ML) analyses were carried out using MEGA 7 (Kumar et al. 2016), and bootstrap support was obtained from 1,000 pseudoreplicate data sets.

The 16S-23S ITS region (ca. 800 bp) was analyzed by determining secondary structure of the following conserved domains: D1-D1' helix, Box-B helix, and the V3 helix. Secondary structures of specific ITS motifs were predicted using comparative analysis combined with confirmation in Mfold (Zuker 2003).

Results

Morphological assessment

Brasilonema was similar to other species, but with several distinctions. First, this strain appeared brownish-orange when initially isolated, but subsequently lost in culture (Fig. 1.1).

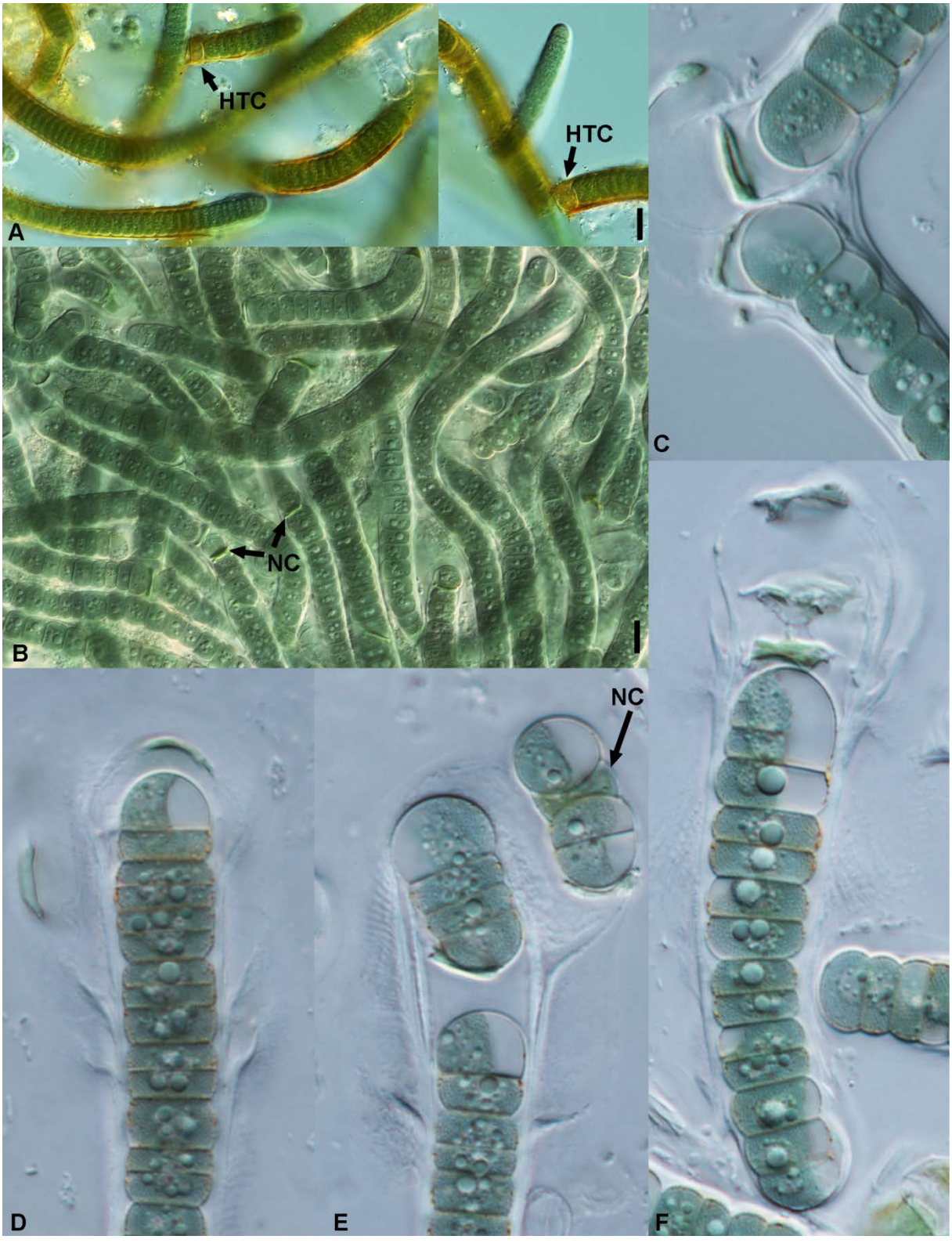

Figure 1.1 Photomicrographic plate of *Brasilonema lichenoides sp. nov.* (A) Natural sample of filaments ensheathed by brownish sheaths, tolypotrichoid false branching at heterocytes (HTC). (B) Freshly isolated strain exhibits a thick, colorless sheath, (NC) necridic cells. This is in contrast to most *Brasilonema* which display a purple color. (C) Formation of false branching after trichome disintegration. (D-F) Hormogonia formation in apical parts of filaments surrounded by widened and layered mucilaginous sheath (>3 weeks old). Scale bars = 10 µm.

Second, *B. lichenoides* cells were discoid with very infrequent vacuolization and developed inclusion bodies in culture. Third, heterocytes were rounded and never squared or rectangular (Fig. 1.1). Fourth, *B. lichenoides* exhibited rare heteropolar filaments. Fifth, *B. lichenoides* possessed undulated trichomes (Suppl. Table 2).

The new species of *Chroococcidiopsis* was morphologically similar to other taxa, but with the simultaneous production of both baeocytes and nanocytes (Fig. 1.2). Further, strains produced copious amounts of extracellular vesicles in culture (Fig. 1.2).

Figure 1.2 Photomicrographic plate of *Chroococcidiopsis epilithica sp. nov.* Note the presence of both A) baeocytes and B) nanocytes. Cultures also produced C) many extracellular vesicles of unknown function. Scale bars = 10 µm.

Molecular assessment

For both new taxa, both ML and MP trees were constructed, which yielded similar topologies. A total of 70 taxa (ca. 1400 bp) were used in the construction of each tree, which was subsequently winnowed down to a smaller set of OTUs, including all available species sequences, for assessment in the respective genera.

Brasilonema sp. nov.

Both the winnowed ML and MP trees yielded similar topologies and thus only the ML tree is included employing all available *Brasilonema* strains plus 14 sister taxa (Fig. 3).

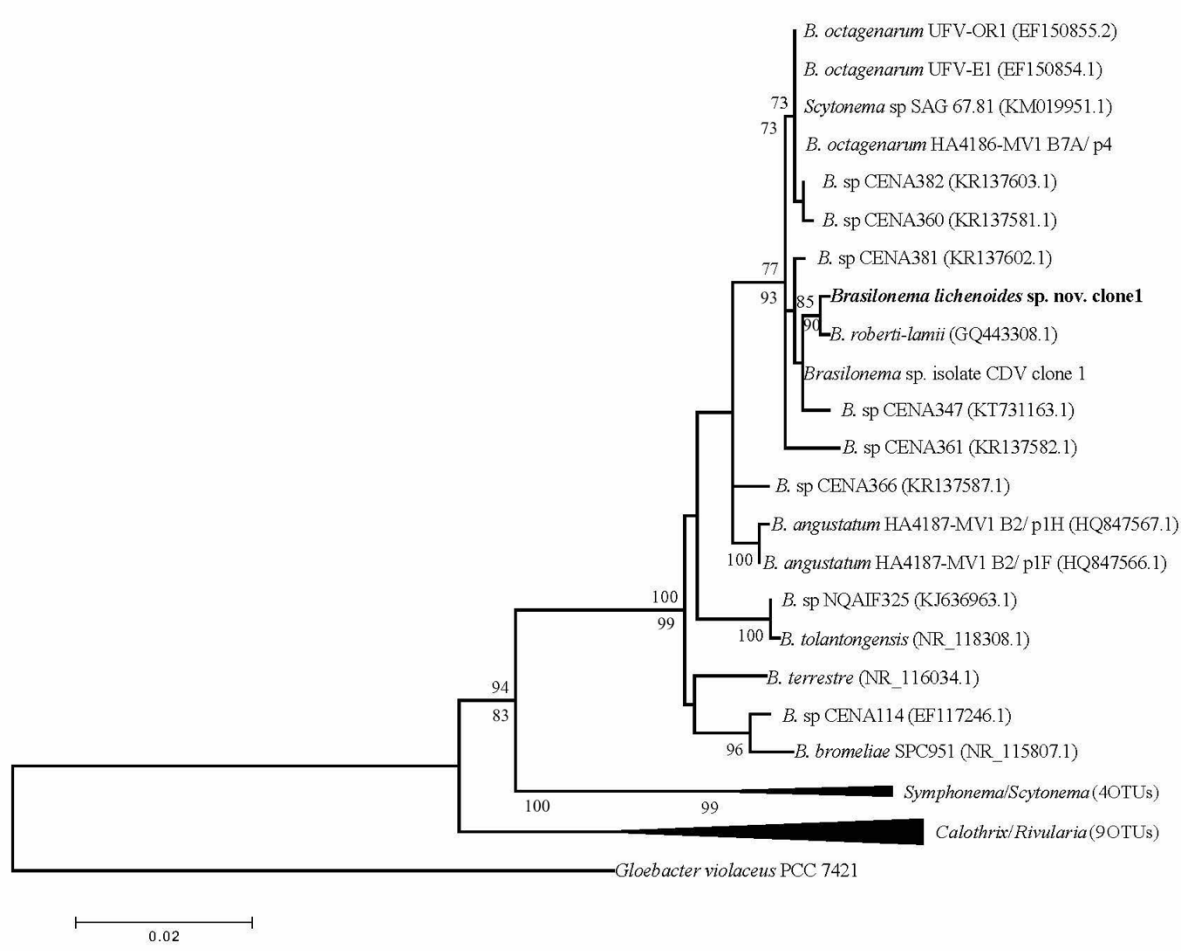

Figure 3. Maximum Likelihood tree of 16S rDNA gene sequence data for *B. lichenoides*. Numbers above the nodes represent Maximum Likelihood bootstrap values, while numbers below are from Maximum Parsimony. The new taxon is in bold.

Brasilonema is a well-supported, monophyletic clade and the closest relative of our strain was *B. roberti-lamii* (Bourrelly) Sant'Anna et al. (85 and 90% support, respectively), which was isolated from central Mexico. The new taxon shared between 98.5-98.7% sequence similarity to the closest relatives, and 95.1% similarity with *B. bromeliae* Fiore et al., the type (Table 1.1).

Table 1.1 Similarity matrix based on the 16S rRNA gene sequence data from *B. lichenoides* sp. nov. and sister taxa.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *B. lichenoides sp. nov*.													
2. *B. octagenarum* EF150855	98.7												
3. *Brasilonema* sp. KR137602	98.7	99.5											
4. *Brasilonema* sp. KT731163	98.7	99.4	99.4										
5. *Brasilonema* sp. KR137603	98.5	99.9	99.4	99.4									
6. *Brasilonema* sp. KR137581	98.4	99.8	99.3	99.3	99.9								
7. *B. roberti-lamii* GQ443308	98.5	99.0	99.1	99.0	99.0	98.9							
8. *Brasilonema* sp. KR137587	98.4	98.7	98.7	98.6	98.6	98.6	98.8						
9. *B. terrestre* NR116034	96.7	97.7	97.6	97.6	97.7	97.6	97.1	98.0					
10. *Brasilonema* sp. KJ636963	97.7	98.4	98.2	98.1	98.2	98.2	98.1	97.9	98.2				
11. *Brasilonema* sp. EF117246	96.6	97.1	97.1	97.1	97.0	96.9	97.0	97.4	97.3	97.9			
12. *B. bromeliae* NR115807	95.1	96.6	96.5	96.4	96.4	96.4	95.9	97.0	97.4	97.6	99.4		
13. *B. tolantongensis* NR118308	97.8	98.5	98.2	98.3	98.4	98.3	98.0	97.7	98.1	99.9	97.7	97.4	
14. *B. octagenarum* EF150854	98.7	100.0	99.5	99.4	99.9	99.8	99.0	98.7	97.7	98.4	97.1	96.5	98.5
15. *Symphyonema* sp. AJ544084	92.8	93.8	93.6	93.4	93.9	93.8	93.7	93.9	93.8	93.1	93.9	93.6	93.6
16. *S. hyalinum* AF334700	94.3	95.1	94.8	94.7	95.0	94.9	94.4	94.1	93.8	93.9	93.7	93.3	93.8
17. *Calothrix* sp. AM230697	87.1	88.2	88.1	87.7	87.9	87.8	88.4	88.1	88.9	88.9	88.5	88.4	89.2
18. *Rivularia* sp. AM230677	89.7	91.0	91.1	90.8	90.9	90.9	90.8	90.9	91.4	91.6	91.0	89.9	91.7

Chroococcidiopsis sp. nov.

The new isolate fell within a highly supported (91%) clade containing other *Chroococcidiopsis* taxa (Fig. 1.4).

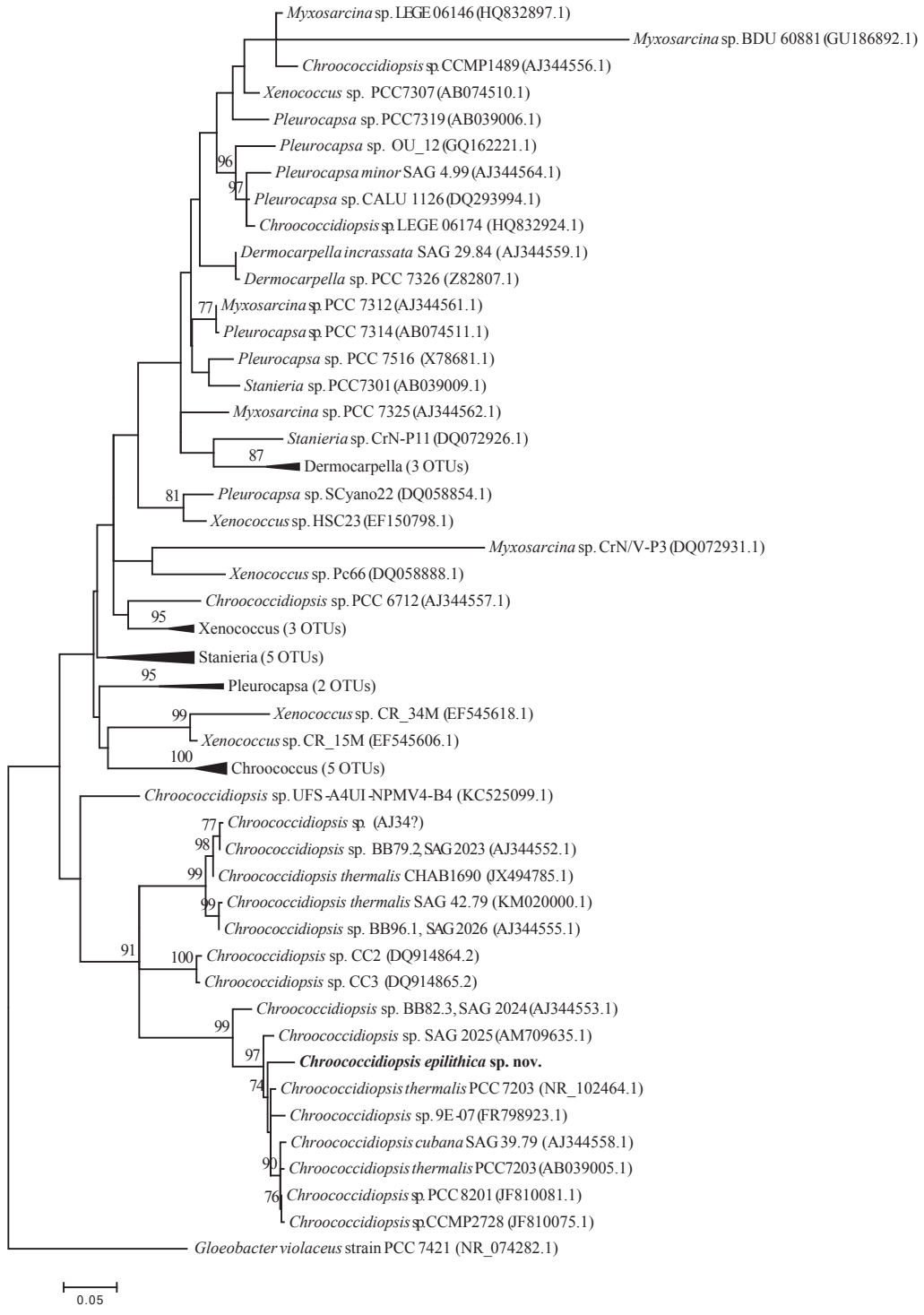

Figure 1.4 Maximum Likelihood tree of 16S rDNA gene sequence data for *C. epilithica*. Numbers above the nodes represent Maximum Likelihood bootstrap values, while numbers below are from Maximum Parsimony. The new taxon is in bold.

Specifically, *C. cubana* Komárek & Hindák SAG39.79, isolated from a soil sample in Cuba, numerous *C. thermalis* Geitler (e.g., CCAP 1423/1 from Roman baths in the United Kingdom, SAG 42.79 from German soils), and assorted *Chroococcidiopsis* spp. (e.g., PCC8201 from mineral springs in Cuba and CCMP2728 from Pennsylvania, USA) fell within this clade. The closest relative to our strain was *C. thermalis* PCC7203, isolated initially from soil near Greifswald, Germany. The new taxon shared between 98.4-99.7 sequence similarity to the closest relatives in the genus *Chroococcidiopsis* (Table 1.2).

Table 1.2 Similarity matrix based on the 16S rRNA gene sequence data from *C. epilithica* and sister taxa.

	1	2	3	4	5	6	7	8	9
1. *C. cubana* SAG 39.79 AJ344558									
2. *C. cubana* SAG 39.79 JF810082	98.4								
3. *C. thermalis* CCAP 1423/1 JX316763	99.4	98.3							
4. *C. thermalis* CHAB 1690 JX494785	99.7	93.4	90.0						
5. *C.* sp. CCMEE29 JF810080	99.6	89.1	99.3	90.1					
6. *Chroococcopsis gigantea* SAG 12.99 KM019987	88.1	89.5	87.4	88.4	88.4				
7. *Myxosarcina* sp. PCC7325 AJ344562	85.2	86.4	84.5	86.5	85.4	90.8			
8. *Xenococcus* sp. AB074510	84.8	86.1	84.2	85.3	84.3	90.4	93.1		
9. *C. thermalis* PCC7203 FJ805841	99.6	91.4	99.7	91.5	100	89.5	85.3	86.4	
10. *C. epilithica sp. nov.*	98.3	97.1	97.9	99.1	98.1	86.8	83.3	84.0	98.2

ITS assessment

Brasilonema

Several sister taxa with available ITS regions were selected to examine the secondary folding structures of the D1-D1' helix. The new taxon, for example, was most similar to *B. octogenarum*, with an A-C side bulge off the initial stem loop and shared the exact same terminal loop, with two nucleotide changes midstem, constraining the side bulge (Fig. 1.5a,d).

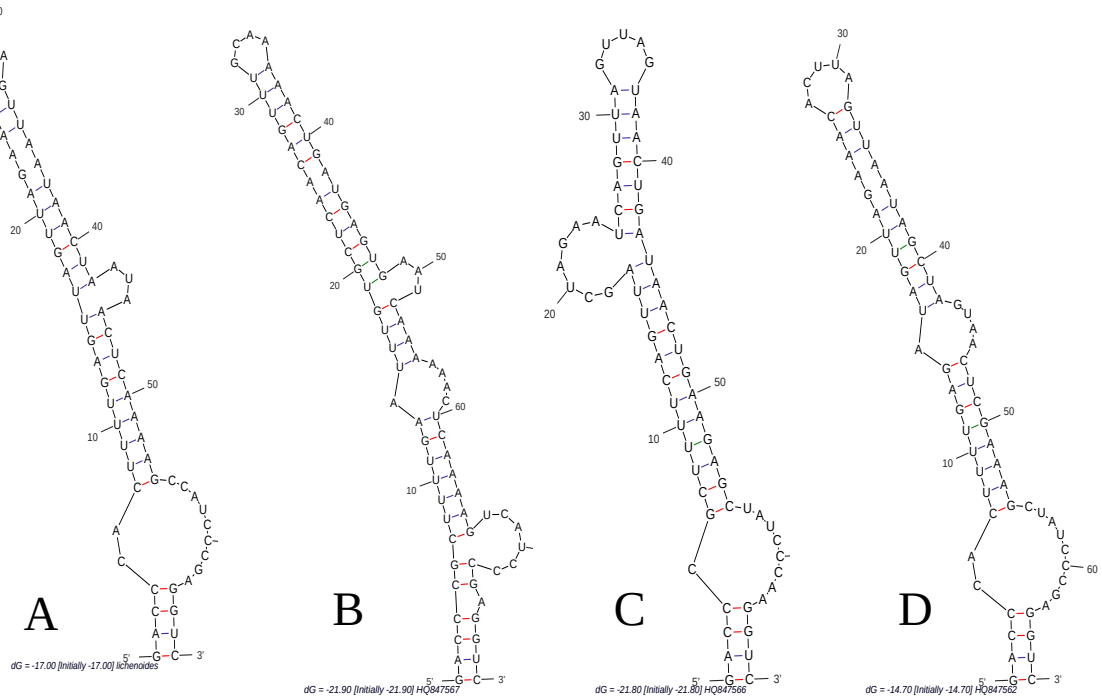

Figure 1.5 D-stem of the 16S-23S ITS region for *Brasilonema lichenoides* and the closest taxa containing available ITS data. A) *B. lichenoides* CDV clone 2, B) *B. angustatum* HA4787-MV1 B2/p1h, C) *B. angustatum* HA4787-MV1 B2/p1f, D) *B. octagenarum* HA4786-MV1 B7A/p4.

The new taxon did not contain any tRNAs, similar to other *Brasilonema* isolates, e.g., *B. angustatum* Vaccarino & Johansen HQ847566 and *B. octagenarum* Aguiar et al. HQ847562 (although other *Brasilonema* isolates do have tRNAs). The D1-D1' length was the same as the other *Brasilonema* that lacked tRNAs (67 bp), as were three other regions (Table 3).

Table 1.3 Comparing nucleotide lengths of conserved ITS domains for *B. lichenoides sp. nov*. and closest relatives that have available ITS data.

Strain	Leader	D1-D1' Helix	spacer+D2+spacer	D3+spacer	tRNA Ile gene	spacer+V2+spacer	tRNA Ala gene	Spacer	Box-B+spacer	Box A	D4+spacer	V3+ITS end (partial)
B. lichenoides CDV strain 1	10	67	38			138			48	11	19	57
B. angustatum HQ847567	10	79	38	11	74	60	73	126	49	11	19	110
B. angustatum HQ847566	10	67	38			129			49	11	19	110
B. octagenarum HQ847562	10	67	38			139			47	11	19	108

While the size of the sequence for the *B. angustatum* with the tRNAs was larger as it contained the sequence, the new taxon was closer to *B. octogenarum* in terms of overall length (139 v. 138) compared to either *B. angustatum* (126 and 129 bp, respectively).

Chroococcidiopsis

The new taxon was structurally identical to the closely related *C. thermalis* PCC7203, with the only difference being the presence of a UUU segment at the terminal tip in our taxon and single nucleotide substitution (G to a C) immediately above the second internal loop, below the A- side bulge (Fig. 1.6a,b).

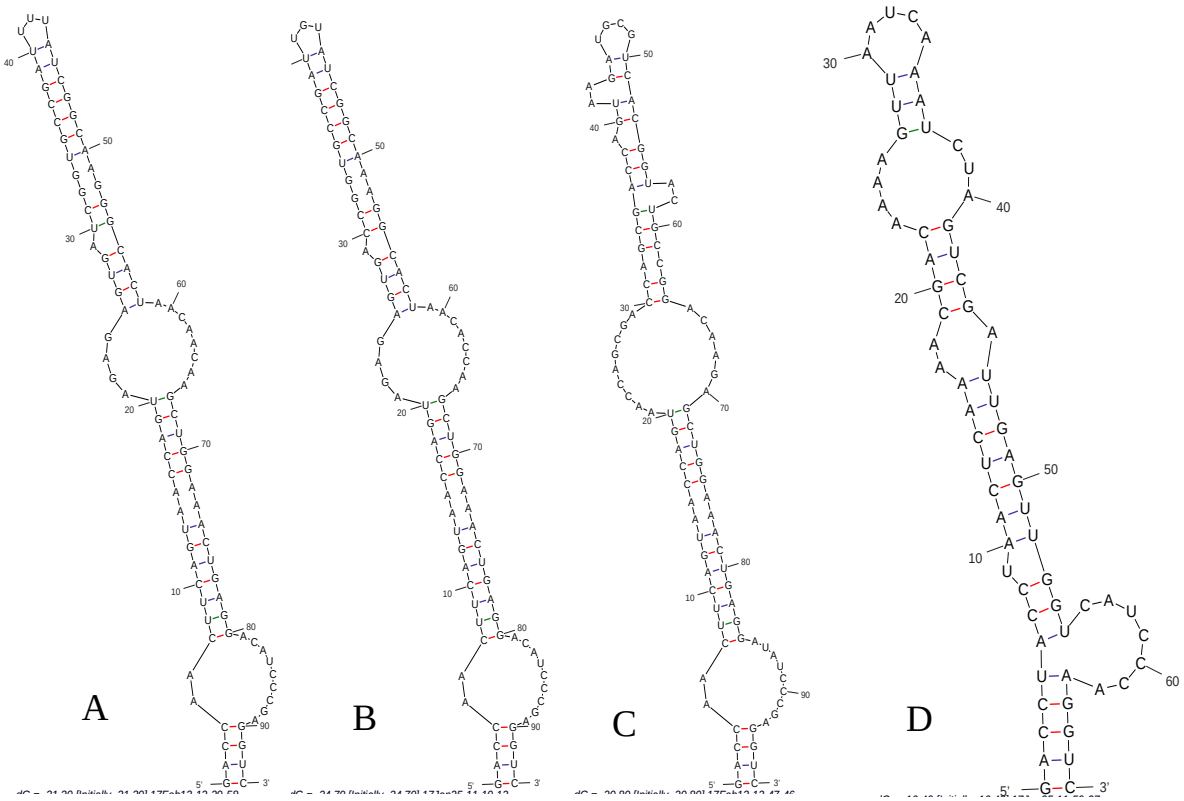

Figure 1.6. D-stem of the 16S-23S ITS region for *C. epilithica* and taxa that are phylogenetically related. A) *C. epilithica*, B) *C. thermalis* PCC7203, C) *C.* sp. SAG2025, D) *C.* sp. UFS-A4UI-NPMV4-B4 clone B4.

A close relative, *C. thermalis* PCC7203 which formed a sister clade to the majority of other *Chroococcidiopsis*, showed a similar folding pattern, but with distinct single nucleotide mutations (Fig. 1.6c). Another *Chroococcidiopsis*, *C.* sp. SAG2025, which fell within this clade, had a very different structure (Fig. 1.6d). Two other outgroup taxa from the sister clade (Fig. 1.6e,f) were markedly different in ITS structure, with corresponding differences in overall conserved segment lengths (Table 1.4).

Table 1.4 Comparing conserved ITS domains for *C. epilithica sp. nov*. and closest relatives that have available ITS data.

Strain	Leader	D1-D1' Helix	spacer+D2+spacer	D3+spacer	tRNA Ile gene	spacer+V2+spacer	tRNA Ala gene	Spacer	Box-B+spacer	Box A	D4+spacer	V3+ITS end (partial)
C. epilithica	8	93	57	23	74		48		106	11	35	
C. thermalis FJ805841	8	93	57	23	74		48		106	11	57	
C. thermalis NR112108	8	93	52	28	74		48		106	11	57	
C. sp. AM709635	8	97	52	28	74		40		108	11	59	
C. sp. KC525099	9	67	37			97			101	11	87	

Description of new taxon

Brasilonema lichenoides Villanueva et al. sp. nov.

Description: Culture (Fig. 1.7): Colonies initially isolated from lichens and consisting of interwoven filaments that occasionally stood erect from the substrate. Trichomes straight, bent, or undulated inside the sheath, constricted at cross walls. Sheaths brown-orange (fresh isolates) to colorless (culture), distinct, firm, becoming thin and colorless or disappearing in culture, up to 2.5 µm wide, seldom layered at the basal part near the false branching. Filaments straight or bent, typically non-tapering (very infrequently heteropolar towards the end), false branched. Meristematic zones if present usually short and located near the apical or basal parts. Cells flattened or barrel-shaped, green to blue-green with peripheral chromatoplasm and central nucleoplasm, frequently granulated in culture, typically non-vacuolated, 4.1 ±0.9 wide (avg. ± STD) × 9.1 ±0.7 µm long, apical cells rounded. Heterocytes common, intercalary, occasionally

flattened but typically spherical or hemispherical, 8.4 ±1.2 wide × 9.7 ±0.9 µm long; akinetes not present. Reproduction by hormogonia and fragmentation of trichomes using help of necridic cells.

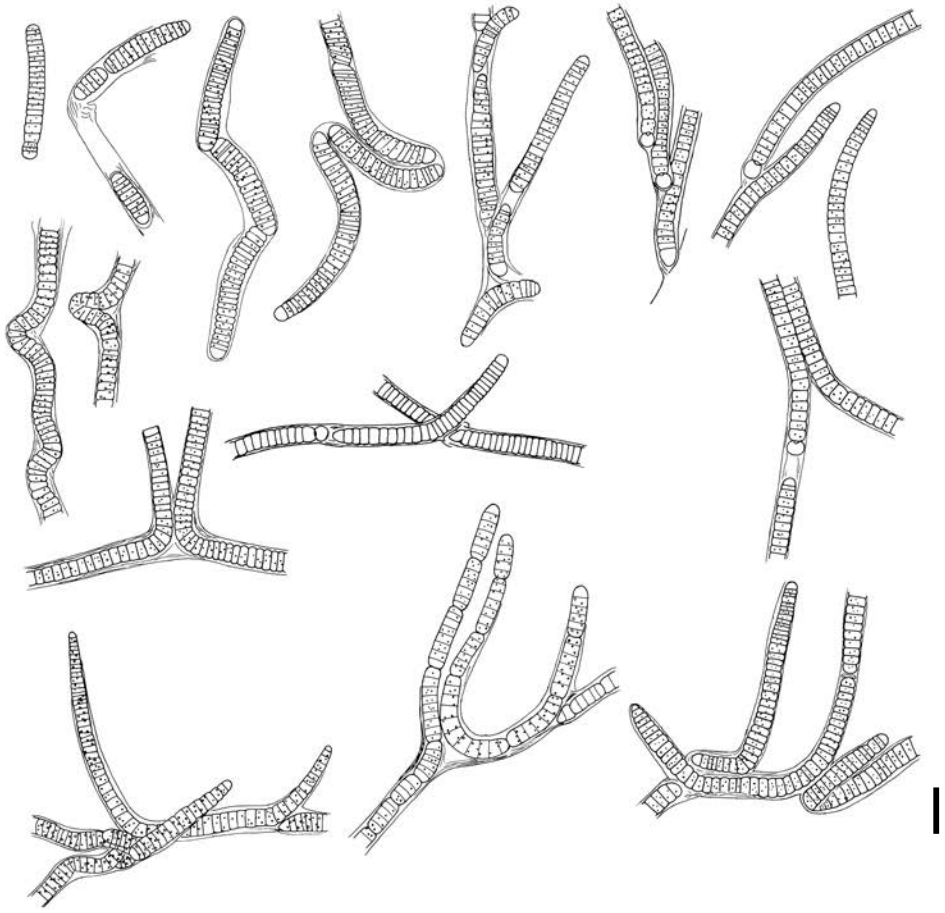

Figure 1.7 Line drawing of *Brasilonema lichenoides sp. nov.* Scale bar = 10 µm.

Holotype: OLM Botany 24: Lichenes and others No. 9226, dried sample is deposited in Regional Museum in Olomouc, Czech Republic.

Type strain: No. 168/2015, deposited at the culture Collection of Department of Botany, Palacký University in Olomouc, Czech Republic.

Type locality: Marble tombstones from the H. Warren Smith Cemetery, Jacksonville Beach, Florida, USA (GPS: 30.2890° N, 81.4071° W).

Genbank accession numbers: MF423720 (16S rRNA) and MF423719 (ITS region)

Etymology: Name is based on habitat of isolation as a cyanobiont of a tripartite lichen.

Habitat: Growing in consortia as a lichen on cemetery tombstones.

Chroococcidiopsis lichenoides Villanueva et al. sp. nov.

Description: Culture (Fig. 8): Thallus microscopic to macroscopic, colonies usually spherical or hemispherical, 31.4 ±4.2 (avg. ± STD) × 26.1 ±5.6 µm, aggregated into thin greenish layer. Mucilaginous envelopes thin, firm, and colorless. Cells variable in shape from almost spherical, oval to irregular, 4.7 ±0.79 wide × 3.3 ±0.5 µm long, bright green or grey-green, densely and irregularly aggregated inside the colony into sarcinoid packages. Reproduction by growth and fragmentation of colonies into subcolonies, by gelatinization and splitting of envelopes and liberation of cells and small oval or irregular baeocytes 3.1 ±0.62 × 2.2 ±0.3 µm.

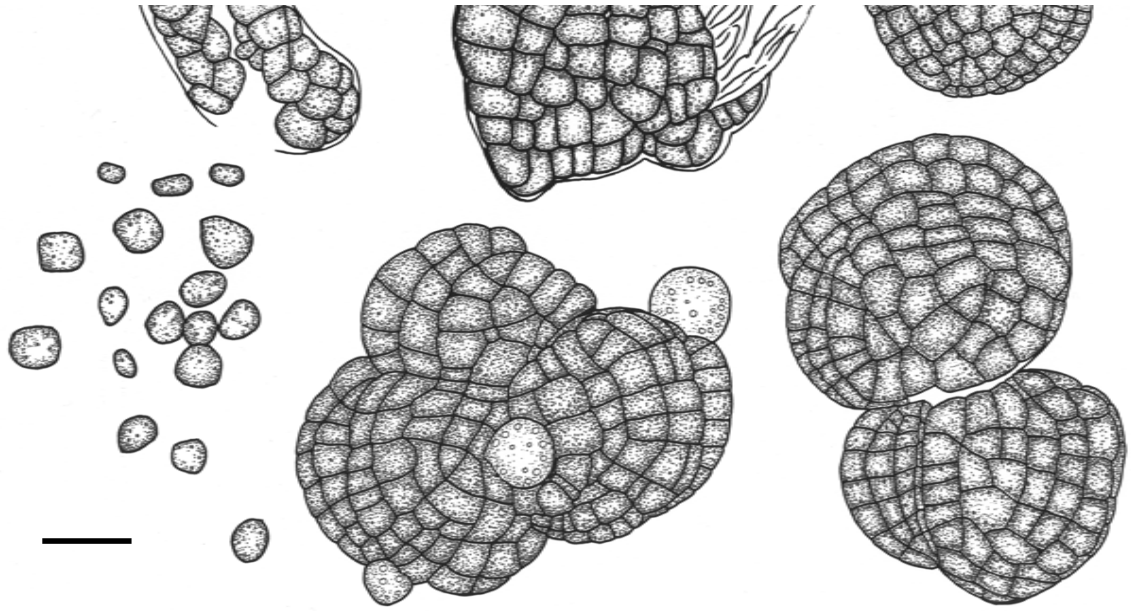

Figure 1.8 Line drawing of *Chroococcidiopsis epilithica sp. nov.* Scale bar = 10 μm.

Holotype: Holotype OLM Botany 24: Lichenes and others No. 9227, dried sample is deposited in Regional Museum in Olomouc, Czech Republic.

Type strain: No. 165/2015, deposited at the culture collection of Department of Botany, Palacký University in Olomouc, Czech Republic.

Type locality: H. Warren Smith Cemetery, Jacksonville Beach, Florida, USA (GPS: 30°17'20.2"N 81°24'24.9"W).

Genbank accession numbers: MF423482 (16S rRNA) and MF423720 (ITS region)

Etymology: Name is based on habitat of isolation as a cyanobiont of a tripartite lichen.

Habitat: Growing in consortia as a lichen on cemetery tombstones.

Discussion

Most cyanobionts are capable of nitrogen fixation (Rai 2002). Cyanobionts may be filamentous or unicellular, with the former employing heterocytes and the later using micro-anaerobic zones to fix atmospheric nitrogen. In the sampled lichen, we note the presence of both forms, which were each capable of growth on nitrogen-free medium. However, it cannot absolutely determine if they both fix nitrogen *in situ*, as has been noted elsewhere (Rai 2002). Likewise, the potential role of associated bacteria cannot be discount. It is important to note that cyanobionts may exhibit phenotypic variability between lichenized and free-living states (e.g., Casamatta et al. 2006), and was also noted in this study.

Brasilonema lichenoides sp. nov. is the first *Brasilonema* species isolated from a lichen thallus. The type species *B. bromeliae* is a member of the Scytonemataceae isolated from subaerophytic habitats in tropical and subtropical Brazil (Fiore et al. 2007). Several aerophytic, epiphytic, and epilithic *Brasilonema* species have been isolated from Hawaii, central Mexico, and Brazil (Aguiar et al. 2008, Vaccarino et al. 2012, Becerra-Absalón et al. 2013, Rodarte et al. 2014). *Brasilonema lichenoides* was most closely related to *B. roberti-lamii*, and both strains were growing epilithically in warm humid climates (Rodarte et al. 2014). One of the defining features of *Brasilonema* is the presence of vacuole-like structures (actually free-spaces within protoplasts surrounded by thylakoids; Fiore et al. 2007), but the new strain did not exhibit such vacuolization. Our strain did exhibit both “C” and “J” shaped trichomes, similar to our closest relative, *B. roberti-lammi* (Rodartel et al. 2014). While *Brasilonema* is described as non-attenuated, our new taxon did exhibit rare heteropolarity. Further, we note that the images of *B. angustatum* show a similar degree of filament heteropolarity and J-shaped trichomes to the new

taxon (Fig. 2, *sensu* Vaccarino and Johansen 2012). The new strain represents a unique taxon based on ecology (photobiont), morphology, and molecular (16S) data.

Chroococcidiopsis is a widely distributed genus of cyanobacteria, often found in xeric habitats with high UV light levels (Dor et al. 1991). They may be found in freshwater, marine, and subaerial habitats (Cumbers and Rothschild 2014). Notoriously difficult to phylogenetically elucidate based solely on morphological characters (e.g., Norris and Castenholz 2006), this genus traditionally belongs to subsection II (Pleurocapsales) of the cyanobacteria (Rippka et al. 2001, Wilmotte and Herdman 2001), which includes cyanobacteria which produce baeocytes. However, recent work by Komárek et al. (2014) have transferred this clade to the *Chroococcidiopsidales* ordo nov. due to baeocyte formation coupled with cell division in three planes. *Chroococcidiopsis* rarely show their typical mode of reproduction while lichenized, even if they would typically do so in a free-living or cultured state (Friedel and Büdel 1996). The new strain clearly showed baeocyte formation and division in three planes in culture (Fig. 3). Further, results of 16S rDNA sequence data and ITS secondary folding patterns showed that this strain fell within the "*Chroococcidiopsis*" *sensu stricto* clade with high bootstrap support. However, the new isolate did not match any morphological description or ecology (e.g., tripartite lichens of headstones in Florida) of any currently circumscribed species. Büdel and Henssen (1983) note similar strains isolated as phycobionts from the lichen family Lichinaceae, but their isolates were of different sizes, different baeocyte arrangements, or different sheath production. *Chroococcidiopsis lichenoides* forms individual spherical colonies or clusters resembling sarcinoid packages. Colonies fragment and continue their growth or produce small spherical, oval, or irregular baeocytes. Shape, colony structure, and symbiotic mode of life are specific for this species.

Species concepts within the cyanobacteria are subject to much debate (Dvořák et al. 2015b). Given the dearth of morphological features from which to choose, coupled with issues related to both phenotypic plasticity and cryptic diversity, describing and elucidating cyanobacterial species may be problematic (Dvořák et al. 2015, Dvořák et al. 2017, Strunecký et al. 2017). The monophyletic species concept *sensu* Johansen and Casamatta (2005) has been proposed as the standard for cyanobacterial systematics (e.g., Komárek 2013), which requires the description of an autapomorphy to test phylogenetic hypotheses. The new strain was only 98% similar to *C. thermalis*, the closest relative based on 16S sequence data. It is proposed that the morphological disjunction, unique ecological setting, 16S sequence dissimilarity, and difference in ITS sequence justifies the erection of a new taxon.

All lichens contain at least a single photobiont, and only ca. 10% of all lichens contain cyanobacteria as their primary photobiont (Friedel and Büdel 1996). The majority of tripartite lichens (those with two photosynthetic members) contain a single cyanobacterial photobiont and a green alga (Tschermak-Woess 1988). In these cases, the cyanobacterial component is sequestered into a separate region of the thallus, the cephalodia. The novel cyanobionts were not separated into cephalodia, but rather loosely organized into the thallus of the endolithic lichen.

Headstones represent an interesting substrate to explore algal diversity and colonization. The sampled headstones were all >50 years old, and provide a stable (e.g., no history of headstone cleaning or preservation) environment for primary colonization. This is also an intriguing habitat to examine patterns of long-distance cyanobacterial dispersal. Many lichens contain photobionts with cosmopolitan distribution (Chua et al. 2012), and some cyanolichen guilds share similar cyanobionts (Rikken 2002). However, it remains unclear if the new taxa have broad or limited distribution as more sampling is needed. Tombstones are also interesting

habitats to answer questions relating to ecological succession and facilitation of microbial communities.

Acknowledgements

The lead author thanks Gordon Rakita for an introduction to the exciting world of cemetery tombstone preservation. The authors gratefully acknowledge financial support from the Department of Biology (UNF), the Coastal Biology Flagship program (UNF), and the Internal Grant Agency of Palacký University in Olomouc No. PrF-2017-001.

Supplemental Table 1: Relevant physical data from cemetery tombstone sample collections.

Sample site	Stone type	Age of stone (yrs)	Surface integrity	Irradiance ($\mu mol \cdot m^{-2} \cdot sec^{-1}$)	Canopy cover	Effluents present	Soil deposition	Aspects showing growth	Aspects sampled	Lichen forms sampled
1	Granite	41	Pitting	44	+	-	-	North	North	Foliose, leprose
2	Granite	41	Pitting	30	+	-	+	All aspects	North, East, & Top	Foliose, crustose
3	Granite	38	Pitting	80	+	-	-	North, East, & West	North, East, & West	Leprose, crustose
4	Granite	38	Pitting	65	+	+	-	North and West	North & West	Foliose, crustose
5	Granite	42	Intact	46	+	+	-	All aspects	North, West & Top	Leprose, crustose
6	Marble	61	Intact	26	+	-	-	All aspects	East, West, & Top	Foliose, crustose

Supplemental Table 2. Comparative morphology of *B. lichenoides* and other members of the genus *Brasilonema*.

	Filament Width (µm)	Sheath Color	False Branching	Trichome shape	Trichomes	Tapering	Cell Color	Cell Shape	Cell Length (µm)	Heterocyte Shape	Heterocyte Position	Habitat
B. lichenoides	8-12	Brown-orange to colorless	Abundant single & geminate	Straight, bent, or undulated	Constricted at cross walls	Rarely hetropolar	Green to blue green	Discoid	2-4	Flattened, round or hemispherical	Intercalary	Endolithic lichen
B. roberti-lamy	12-18	Colorless	Rare Tolypothricoid	Cylindrical	Not or indistinctly constricted at cross walls	Filaments isopolar		Isodiametric		Discoid to cylindrical		Aerophytic
B. angustatum	9.8 - 18	Colorless	Abundant single & geminate		Constricted at cross walls	Filaments isopolar, branches heteropolar	Brownish or purple grey	Isodiametric	3-9	Elongated	Intercalary	Terrestrial, epiphytic
B. bromeliae	10-21	Colorless to yellowish brown	Rare	Cylindrical	Slightly constricted	Not attenuated toward the end	Blue, gray, olive or brownish violet green		1.8-16	Discoid to cylindrical	Intercalary	Small pools, on wooden substartes
B. terrestre	12-17	Colorless to yellowish brown	Coeleodesmoid	Cylindrical	Not constricted at cross walls		Grayish green to blue green	Isodiametric, shorter toward the end		Barrell shaped to cylindrical		Subaerophytic on concrete
B. ornatum	20-23	Colorless	Very rare branching	Cylindrical	Distinctly constricted	Not attenuated toward the end	Dark blue green	Discoid		Discoid		Subaerophytic on bark, possibly lichenized
B. epidendron	12-14	Colorless	Very rare branching	Cylindrical	Not constricted at cross walls	Not attenuated toward the end	Bright blue green	Isodiametric, cylindrical or shortened		Barrell shaped to cylindrical		Subaerophytic on bark
B. octegenarun	9.8 - 18.5	Colorless	Rare Tolypothricoid, Scytonematoid	Cylindrical	Not constricted at cross walls	Isopolar	Olive green or brownish-violet	Cylindrical	1.5- 13.3	Discoid or cylindrical	Basal or intercalary	Epiphytic

Chapter 2: Descriptions of *Brasilonema geniculosus* and *Calothrix dumas* (Nostocales, Cyanobacteria): two new taxa isolated from cemetery tombstones.

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Abstract

Cyanobacteria are common members of epilithic communities, contributing fixed carbon and nitrogen products, providing UV light shielding pigments, helping to retain water, and in general stabilizing particles. Conversely, biofouling by cyanobacteria is of great concern in the U.S. and abroad. The epilithic growth of cyanobacteria on cultural monuments has been long noted, yet the basic systematics and diversity of these organisms is poorly understood. This paper describes two novel cyanobacteria isolated from cemetery tombstones from Jacksonville, Florida (USA). Using a total evidence approach of ecology, morphology, ITS structure, and molecular data we present two taxa new to science: *Brasilonema geniculosus* and *Calothrix dumas*. We note that tombstones represent an intriguing habitat for sampling cyanobacteria due to their ubiquity, stability, and cultural importance.

Keywords: biodiversity, systematics, 16S-23S ITS secondary structure

Introduction

The last decade has seen an explosion in the number of novel cyanobacterial taxa as researchers have begun to unravel the systematic relationships of this lineage. Previous impediments to describing novel cyanobacterial diversity, such as a lack of clear species concepts, elusive character sets, and restricted habitat sampling have all been addressed and ameliorative endeavors undertaken (e.g., Johansen & Casamatta 2005; Dvořák et al. 2015). New assessments of family level characters (Komárek et al. 2016), coupled with new investigations into traditionally understudied habitats (e.g., sub-aerial samples and lichen associated cyanobionts), have facilitated work on alpha-level cyanobacterial diversity.

One potentially rich source of novel cyanobacteria is from stone substrata. These unique taxa may be free-living (e.g., *Geitleria sensu* Kilgore et al. 2018) or in complex consortia with other microbes, such as lichens (e.g., Villanueva et al. 2018). Uher (2008) notes that humidity and microclimate might influence the vertical distribution of cyanobacteria on tombstone substrates in a central European cemetery. Subaerial cyanobacteria are common components of epilithic communities contributing to carbon and nitrogen fixation, water retention, and habitat facilitation (Casamatta et al. 2002, Lopez-Bautista et al. 2007). Conversely, biofouling of monuments by cyanobacteria is a world-wide issue, impacting both efforts at historic preservation and being economically burdensome (Bellazza et al. 2003). del los Ríos et al. (2002) note that cyanobacteria may actually draw ions out of the substrate leading to biomineralization and subsequent deterioration. Cyanobacteria may thrive in subaerial environments due to their ability to form biofilms (Adhikary et al. 2015), which in turn may lead to accelerated weathering of substrata from acid production (Gaylarde & Morton 1999), although

potential ameliorative impacts from the cyanobacterial community on these habitats have been proposed (Carter & Viles 2003).

In this paper we present to novel cyanobacteria isolated from lichen dominated consortia from cemetery tombstones in Jacksonville, FL, USA: *Brasilonema geniculosus* and *Calothrix dumas.* The specific epithets are proposed under the provisions of the International Code of Nomenclature for Algae, Fungi, and Plants.

Methods

Sampling Site

Isolates were obtained from H. Warren Smith cemetery (Jacksonville Beach, FL, USA, collected in May 2013 from ca. 45-76 cm above ground). Six individual headstones were sampled, which provided five epilithic samples each. Epilithic samples were teased from headstones using a sterile scalpel and transported in 1.5 mL micro centrifuge tubes. Irradiance was measured at each collection site using a basic quantum meter (Apogee Instrument Inc., Logan UT) and the age and type of stone, the condition of the stone, the presence of effluents or nearby plant growth, as well as the class and color of dominant lichen growth forms, and any color change of growth after wetting was recorded (Table 1).

Culturing

Scraped samples were used to inoculate cultures to isolate cyanobacteria and grown in liquid Z8 medium (Staub 1961) with the addition of 10 µL (1x) fungicide (Amphotericin, Cell Grow Virginia). Cultures were kept on a desktop, at ambient conditions (23 °C, ca. 12:12 light:dark photoperiod). In addition, cultures were grown in nitrogen free medium to test for the production of heterocytes.

Morphological assessment

Morphology of the strains was analyzed via light microscopy (Zeiss AxioImager, objectives EC Plan–Neofluar 40×/1.3 N.A., oil immersion, DIC; Plan–Apochromat 100×/1.4 N.A., oil immersion, DIC). Images were taken with a high resolution camera (AxioCam D512 12MPx). Pictures were processed using with Zeiss AxioVision software (version 4.9.1.). During morphological evaluation of natural samples and strains, the following characters were assessed: cell shape, cell dimensions, reproduction, sheaths, and granulation of cells. Measurements were performed on 100 cells of both natural and culture materials.

Molecular Techniques

DNA was extracted with the PowerSoil™ DNA Kit from 0.25g of culture samples (Mo Bio Laboratories Inc., Carlsbad, CA). DNA quality and consistence was checked on ethidium bromide stained 1.5% agarose gel.

PCR amplification of the partial 16S rDNA and the whole 16S–23S ITS was performed using primers forward 8F (5´–AGTTGATCCTGGC–3´), and reverse B23S (5´–CTTCGCCTCTGTGTGCCTAGG –3´) previously described in Osorio-Santos et al. (2014). The 50 µl PCR reaction contained: 19 µl sterile water, 2 µl of each primer (0.01 mM concentration), 25 µl PCR Master Mix (Promega, Madison, WI) and 2 µl template DNA (50 ng/µl) and PCR amplification proceeded as detailed in Villanueva et al. (2018). Amplified rDNA was cloned into pGEM® T Vector System I and JM-109 High Efficiency Competent Cells (Promega, Madison, WI) and cultured using carbenicillin infused LB media. Plasmid DNA was purified from eight replicate transformed competent cell colonies per isolate, using QIAprep® Spin Miniprep Kits (QIAGEN, Hilden, Germany). Sequencing of cDNA libraries from two operons of varying size was performed by Eurofins Genomics (MWG Operon Inc., Louisville, KY).

A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to obtain closely related taxa. Our 16S sequences were combined with sequences from GenBank having ≥93% sequence similarity via BLAST searches. For both phylogenetic trees, sequences were aligned and the T3K+I+gamma model was selected using MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (Kumar et al. 2016). An unweighted maximum-parsimony (MP) and maximum-likelihood (ML) analyses were carried out using MEGA 7 (Kumar et al. 2016), and bootstrap support was obtained from 1,000 pseudoreplicate data sets.

The 16S-23S ITS region (ca. 800 bp) was analyzed by determining secondary structure of the following conserved domains: D1-D1' helix, Box-B helix, and the V3 helix. Secondary structures of specific ITS motifs were predicted using comparative analysis combined with confirmation in Mfold (Zuker 2003).

Results

Molecular phylogenies

Our newly isolated strain of *Brasilonema* Fiore et al. (2007: 794) fell out in a highly supported (97%) clade with all other *Brasilonema* strains (Fig. 2.1). The new strain in particular fell within a modestly supported clade (77%) with aerophytic *B. robert-lamii* Sant'Anna & J. Komárek ex Bourrelly (2011: 56) isolates and *B.* sp. CENA 347, isolated from leaves of *Avicennia schaueriana* Stapf & Leechman ex Moldenke (1939: 336) from Brazil (Fig 2.1).

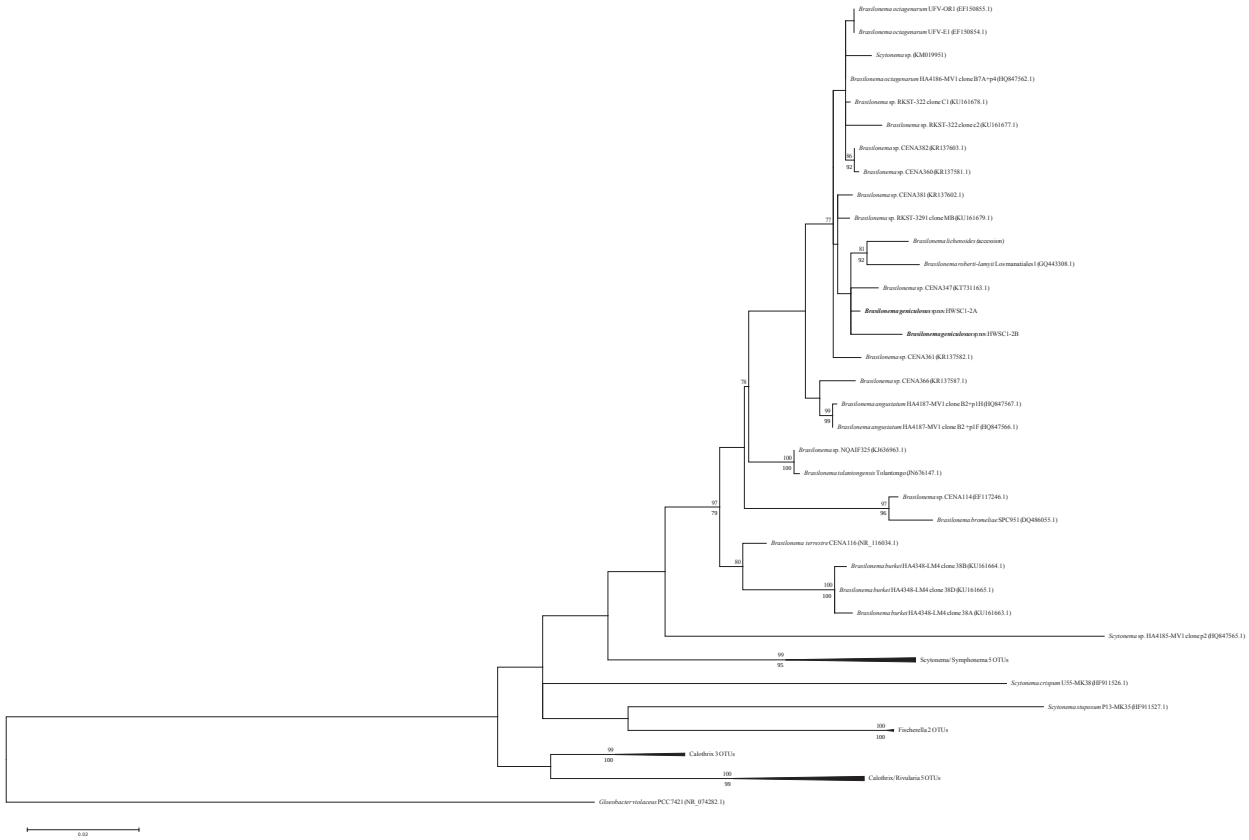

Figure 2.1 Maximum likelihood tree of *B. geniculosus* and the closest relatives based on 16S rRNA gene sequences. Numbers above the line represent ML values, numbers below MP.

The newly isolated *Calothrix* Agardh ex Bornet & Flahault (1886: 345) fell within the highly supported (99%) clade containing a mixture of soil and freshwater strains more distantly related to either of the marine clades (Fig. 2.2). Further, it fell into a highly supported clade (99%) sister to a strain of *Calothrix* sp. isolated as a member of a *Nostoc commune* Vaucher ex Bornet & Flahault (1888: 181) crust from Japanese soils (Fig. 2.2).

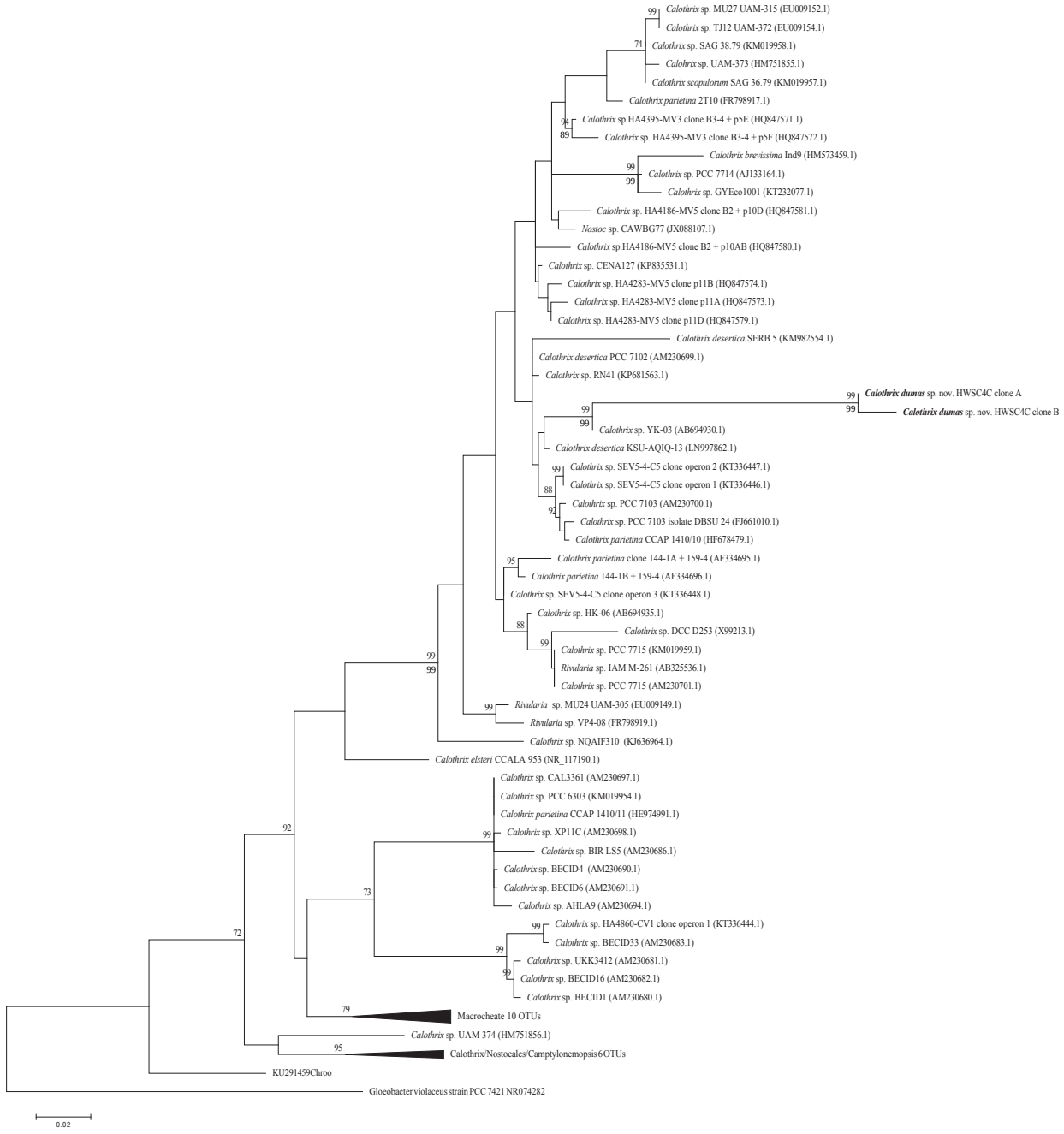

Figure 2.2 Maximum likelihood tree of *C. dumas* and the closest relatives based on 16S rRNA gene sequences. Numbers above the line represent ML values, numbers below MP.

ITS folding patterns

We recovered two operons for *B. geniculosus*. One operon contains two tRNAs while the second had no tRNAs (Table 2.1). *Brasilonema geniculosus* clone IIA showed a similar pattern of ITS domain lengths to other *Brasilonema* strains which also included two tRNAs. The difference came in the length of the spacer region between the tRNAs (Table 2). *Brasilonema geniculosus* clone IIB displayed a similar pattern to other strains lacking tRNAs, with the exception that the V3 and ITS end domains were ca. four times longer than any other recovered known *Brasilonema* sequences at 228 nucleotides (Table 2.1).

Table 2.1 Comparing lengths in nucleotides of conserved ITS domains for *B. geniculosus* sp. nov. and closest relatives with available ITS data.

Strain	Leader	D1-D1' Helix	spacer+D2+spacer	D3+spacer	tRNA Ile gene	spacer+V2+spacer	tRNA Ala gene	Spacer	Box-B+spacer	Box A	D4+spacer	V3+ITS end (partial)
B. geniculosus sp. nov. clone 2A	8	91	38	11	74	78	73	136	48	11	21	60
B. geniculosus sp. nov. clone 2B	8	67	38			138			48	11	21	228
B. lichenoides 5A	8	67	38			138			48	11	21	55
B. sp. RKST-3291 clone MB	8	105	38	11	74	91	73	134	48	11	21	63
B. sp. RKST-322 clone C1	8	67	38			139			47	11	21	61
B. sp. RKST-322 clone c2	8	102	38	11	74	84	73	133	47	11	21	73
B. octagenarum HA4186-MV1 clone B7A+p4	1	67	38			139			47	11	21	61
B. angustatum HA4187-MV1 clone B2+p1F	8	67	38			129			49	11	21	72
B. angustatum HA4187-MV1 clone B2+p1H	8	79	38	11	74	60	73	126	49	11	21	67

The D1-D1' helix for *B. geniculosus* was distinct from other *Brasilonema* strains, but most similar to *B. angustatum* M.A. Vaccarino & J.R. Johansen (Fig. 2.3). The helix of the first operon was 91 nucleotides long, while the helix of the second operon was 67 nucleotides in length. The helices of the two operons were less alike than their closest relative, *B. lichenoides*

Villanueva et Casamatta, which also had no tRNAs (Fig. 2.3). It must be noted that there were significant differences between the two, with two insertions, one deletion, and five nucleotide point mutations. The first operon was not similar to any other taxon of *Brasilonema* for which there was ITS sequence data available.

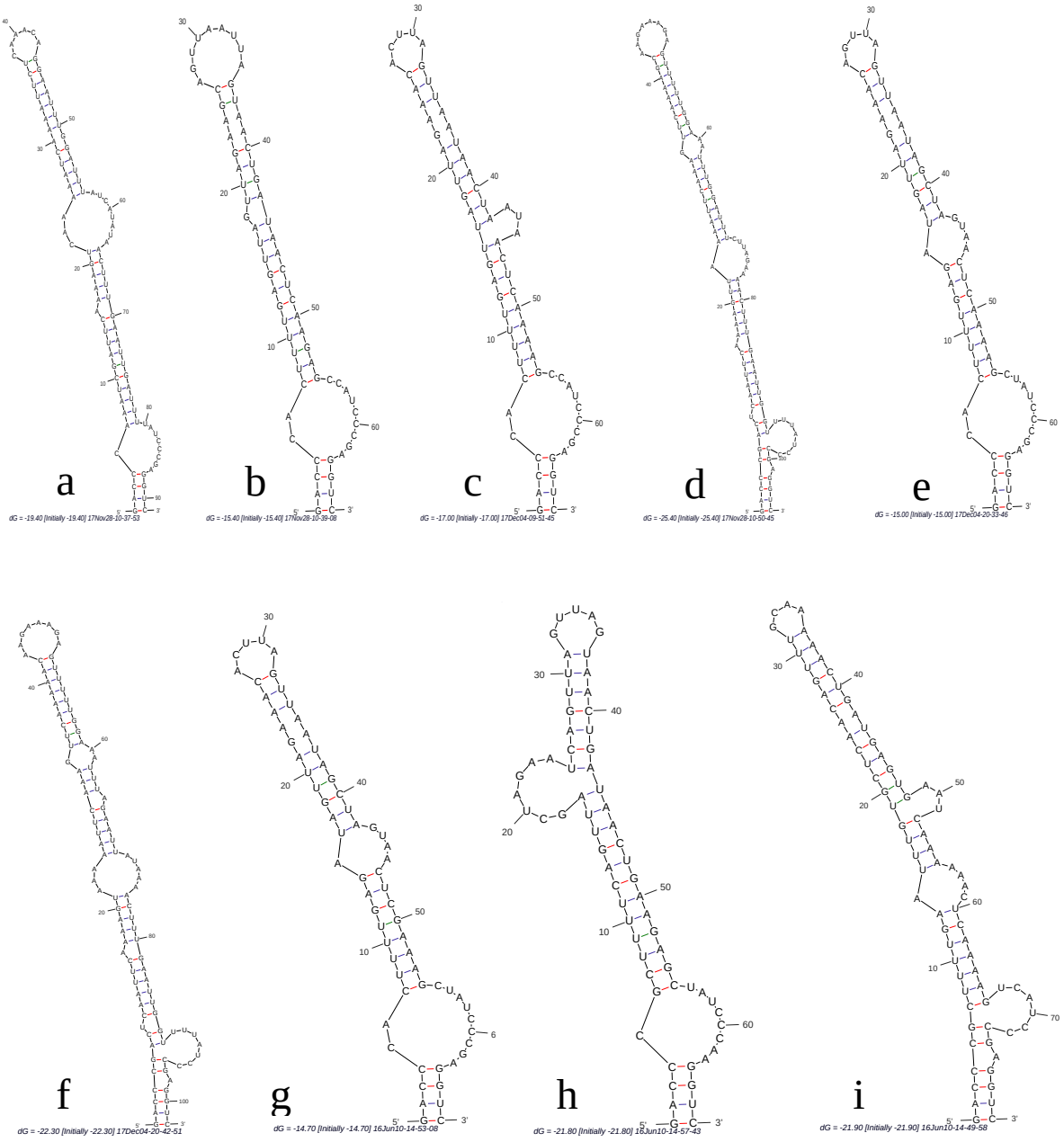

Figure 2.3 D1-D1' helices for *B. geniculosus* and closet relatives for which ITS sequence data is available a) *B. geniculosus* HWSC4C Clone IIA, b) *B. geniculosus* HWSC4C Clone IIB, c) *B. lichenoides* clone 5A, d) B. sp. RKST-3291 clone MB, e) B. sp. RKST-322 clone C1, f) B. sp.

RKST-322 clone c2, g) *B. octogenarum* HA4186-MV1 clone b7a+p4, h) *B. angustatum* HA4187-MV1 clone b2+p1f, i) *B. angustatum* HA4187-MV1 clone b2+p1h.

The structural motif of the Box B helix for *B. geniculosus* clone IIA was identical to other strains, but with nucleotide substations in the terminal bulge (Fig. 2.4). *B. geniculosus* clone IIB, however, was the only Box B helix with a single nucleotide unilateral bulge to the left instead of the right (Fig. 2.4b), also with variability in the nucleotide sequences of the terminal bulge, but with the same number of nucleotides in each.

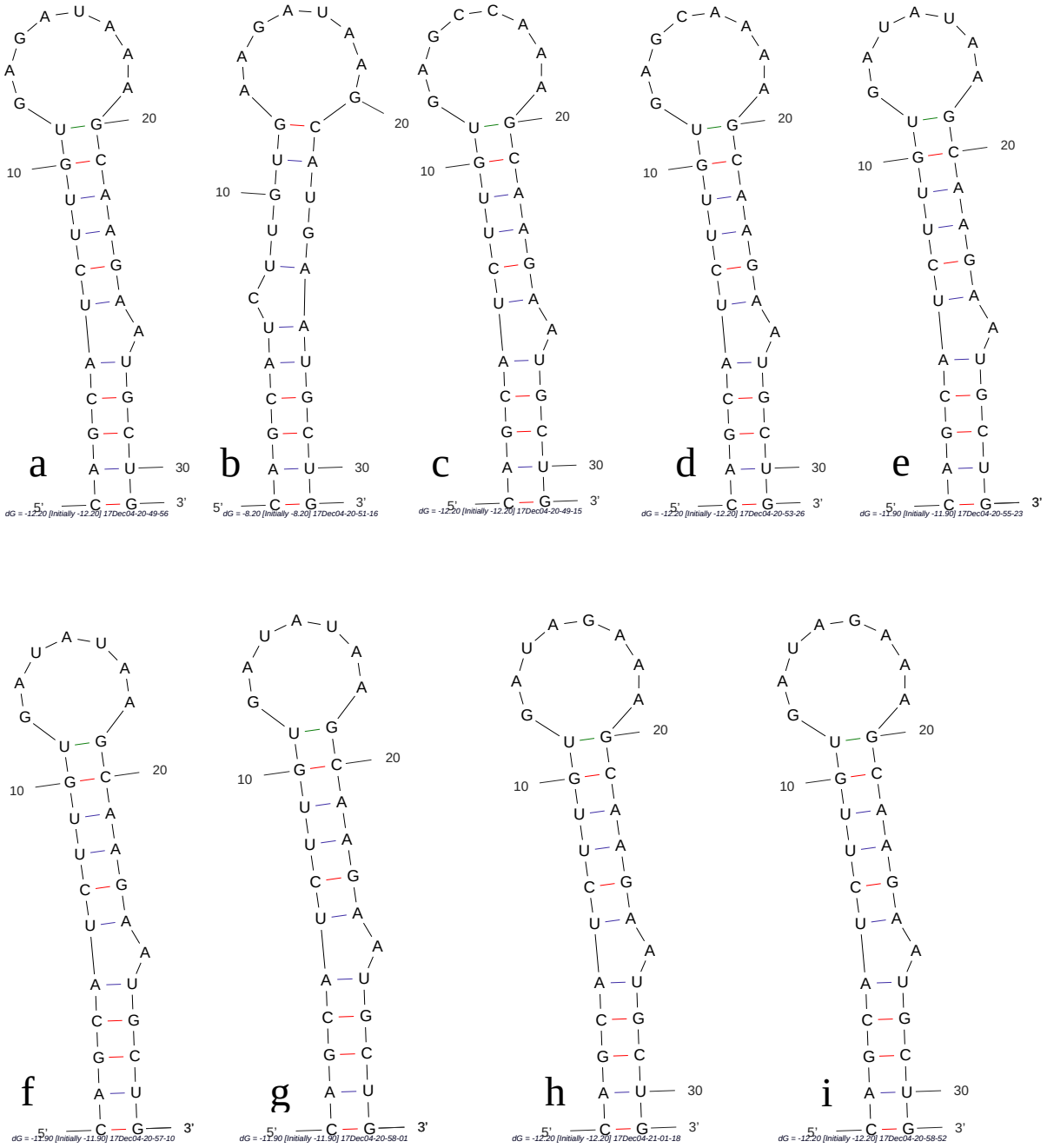

Figure 2.4 Box B helices for *B. geniculosus* and closet relatives for which ITS sequence data is available a) *B. geniculosus* HWSC4C Clone IIA, b) *B. geniculosus* HWSC4C Clone IIB, c) *B. lichenoides* clone 5A, d) B. sp. RKST-3291 clone MB, e) B. sp. RKST-322 clone C1, f) B. sp. RKST-322 clone c2, g) *B. octogenarum* HA4186-MV1 clone b7a+p4, h) *B. angustatum* HA4187-MV1 clone b2+p1f, i) *B. angustatum* HA4187-MV1 clone b2+p1h.

Three V3 helices were identified for *B. geniculosus* clone IIB (Fig. 2.5), which had a large insert in the V3 and spacer region upstream from the 23S gene. The first V3 helix identified in *B. geniculosus* IIB had a nearly identical structure as *B. geniculosus* IIA, but with two additional nucleotides paired in the basal clamp (Fig. 2.5a,b). *Brasilonema geniculosus* was most similar to *B. angustatum* in terms of motif and nucleotides. The second and tertiary motifs (Fig. 2.5c,d) were radically different than all other taxa, and different from each other.

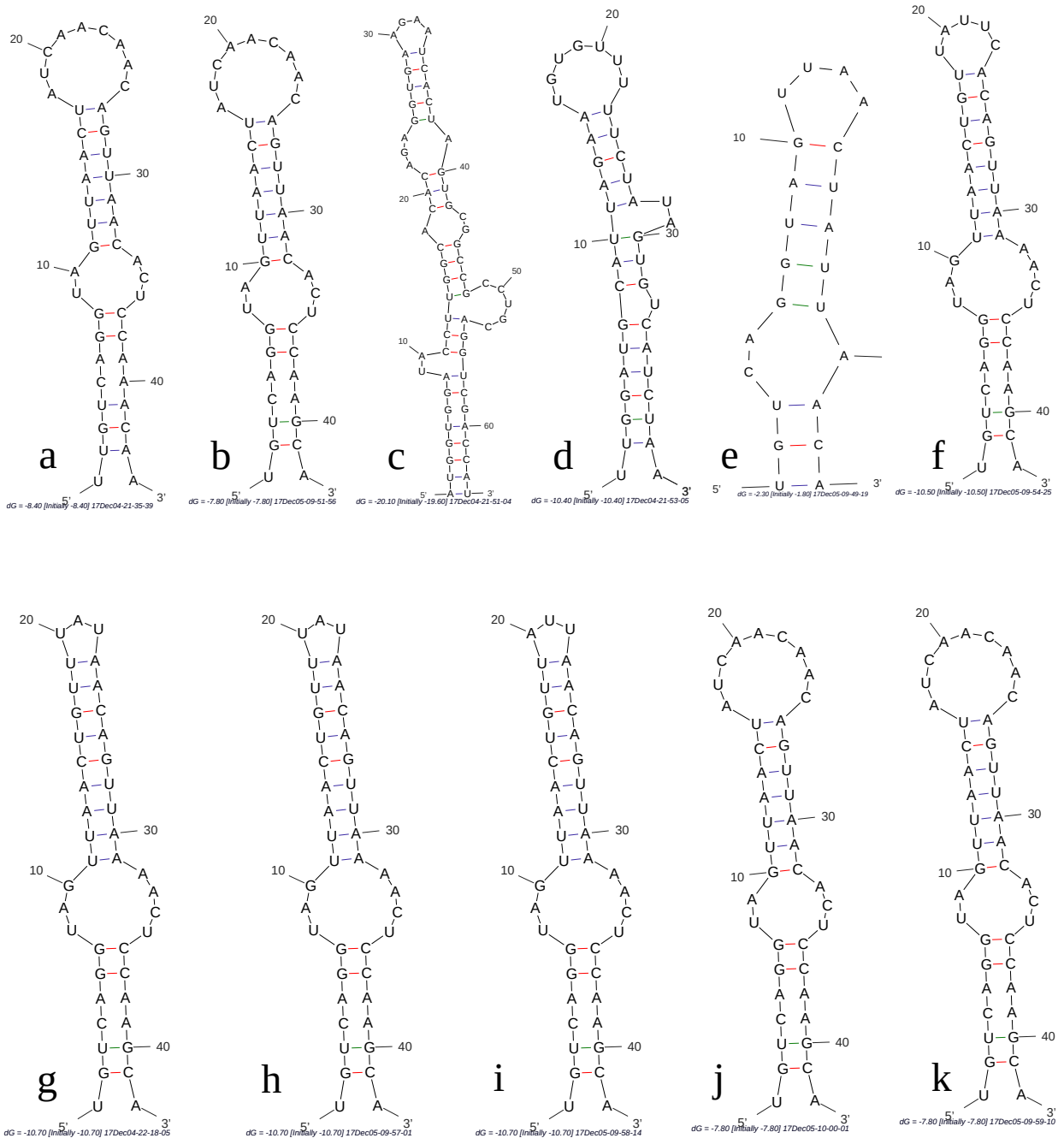

Figure 2.5 V3 helices for *B. geniculosus* and closet relatives for which ITS sequence data is available. a) *B. geniculosus* HWSC4C Clone IIA, b) *B. geniculosus* HWSC4C Clone IIB, c) *B. geniculosus* HWSC4C Clone IIB second helix, d) *B. geniculosus* HWSC4C Clone IIB third helix, e) *B. lichenoides* clone 5A, f) B. sp. RKST-3291 clone MB, g) B. sp. RKST-322 clone C1, h) B. sp. RKST-322 clone c2, i) *B. octogenarum* HA4186-MV1 clone b7a+p4, j) *B. angustatum* HA4187-MV1 clone b2+p1f, k) *B. angustatum* HA4187-MV1 clone b2+p1h.

We recovered two operons for *C. dumas*, both of which contain two tRNAs but with variable inserts between the tRNAs (Table 2.3). *C. dumas* clone A had only 11 nucleotides in the spacer between tRNA genes, while clone B had 77 nucleotides and a variable V2 helix.

Table 2.3 Comparing lengths in nucleotides of conserved ITS domains for *C. dumas* sp. nov. and closest relatives with available ITS data.

Strain	Leader	D1-D1' Helix	spacer+D2+spacer	D3+spacer	tRNA Ile gene	spacer+V2+spacer	tRNA Ala gene	Spacer	Box-B+spacer	Box A	D4+spacer	V3+ITS end (partial)
C. dumas HWSC4 clone A	8	97	37	11	74	11	73	63	54	11	25	86
C. dumas HWSC4 clone B	8	100	39	11	74	77	73	89	44	11	23	85
C. sp. SEV5-4-C5 clone operon 1	8	71			76				52	11	20	97
C. sp. SEV5-4-C5 clone operon 2	8	66			76				52	11	20	97
C. sp. HA4283-MV5 clone p11D	8	98			66				67	11	20	97
C. sp. HA4283-MV5 clone p11A	8	98	38	11	74	79	73	63	53	11	20	80
C. sp. HA4283-MV5 clone p11B	8	98			80				53	11	20	97
C. sp. HA4186-MV5 clone B2+p10AB	8	65			74				51	11	20	88
C. sp. HA4395-MV3 clone B3-4+p5E	8	98	38	11	74	94	73	64	52	11	20	85

The D1-D1’ helix for *C. dumas* was distinct from other *Calothrix* strains (Fig. 2.6). Clone I had a secondary, internal unilateral bulge of six nucleotides, which differed in orientation from other strains (Fig. 2.6a). Clone II had a distinct unilateral bulge directly opposite the basal unilateral bulge, not seen in any other taxa.

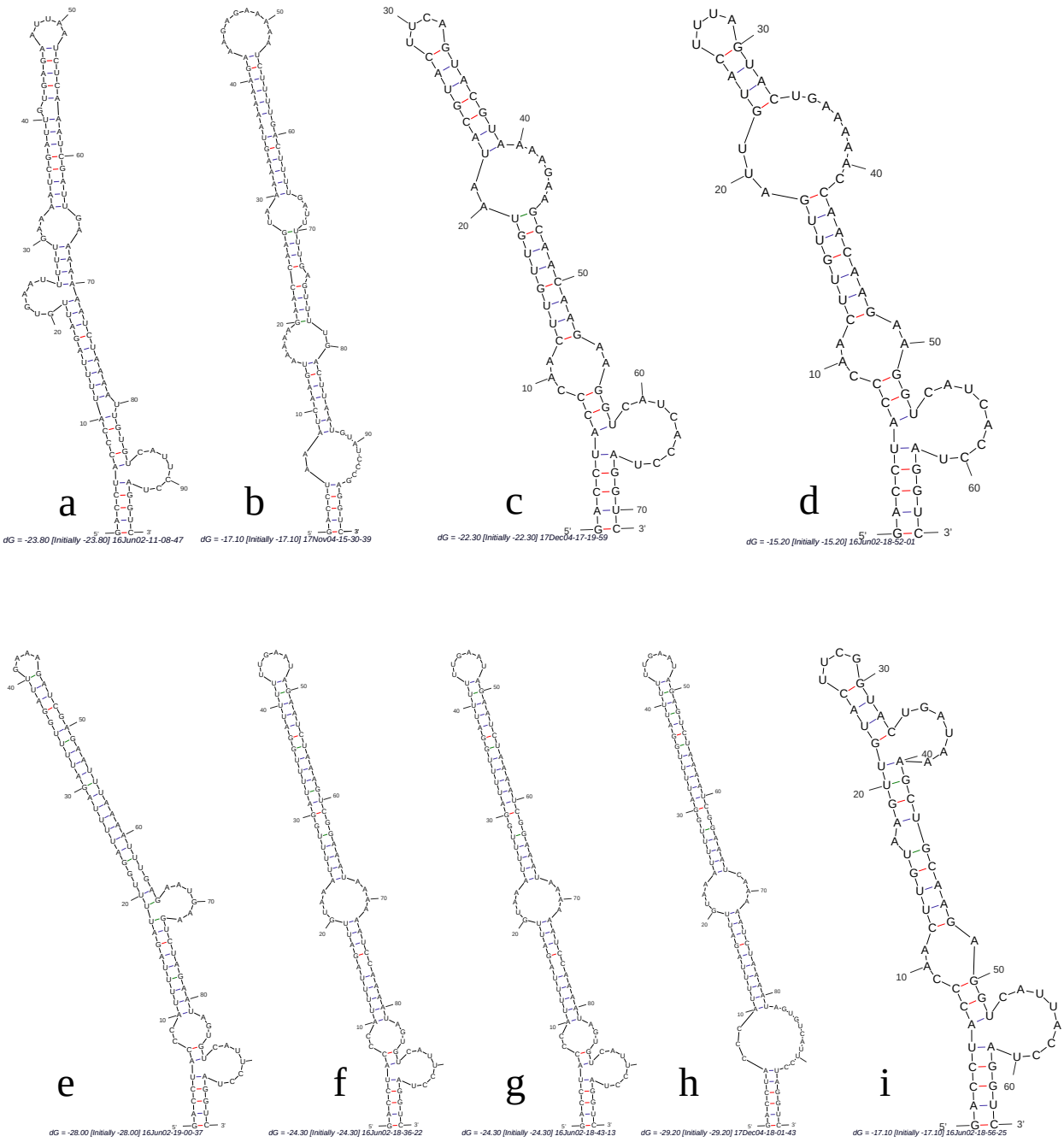

Figure 2.6 D1-D1' helices for *C. dumas* and closet relatives for which ITS sequence data is available. a) *C. dumas* HWSC1B clone A, b) *C. dumas* HWSC1B clone B, c) *C.* sp. SEV5-4-c5 clone operon 1, d) *C.* sp. SEV5-4-c5 clone operon II, e) *C.* sp. HA4395-MV3 clone B3-4+P5e, f) *C.* sp. HA4283-MV5 clone p11B, g) *C.* sp. HA4283-MV5 clone p11D, h) *C.* sp. HA4283-MV5 clone p11A, i) *C.* sp. HA4186-MV5 clone B2+P10ab.

The structural motif of the Box B helix for *C. dumas* clone I had two internal, bilateral bulges, while there are only single bilateral bulges in clone II (Fig. 2.7a,b).

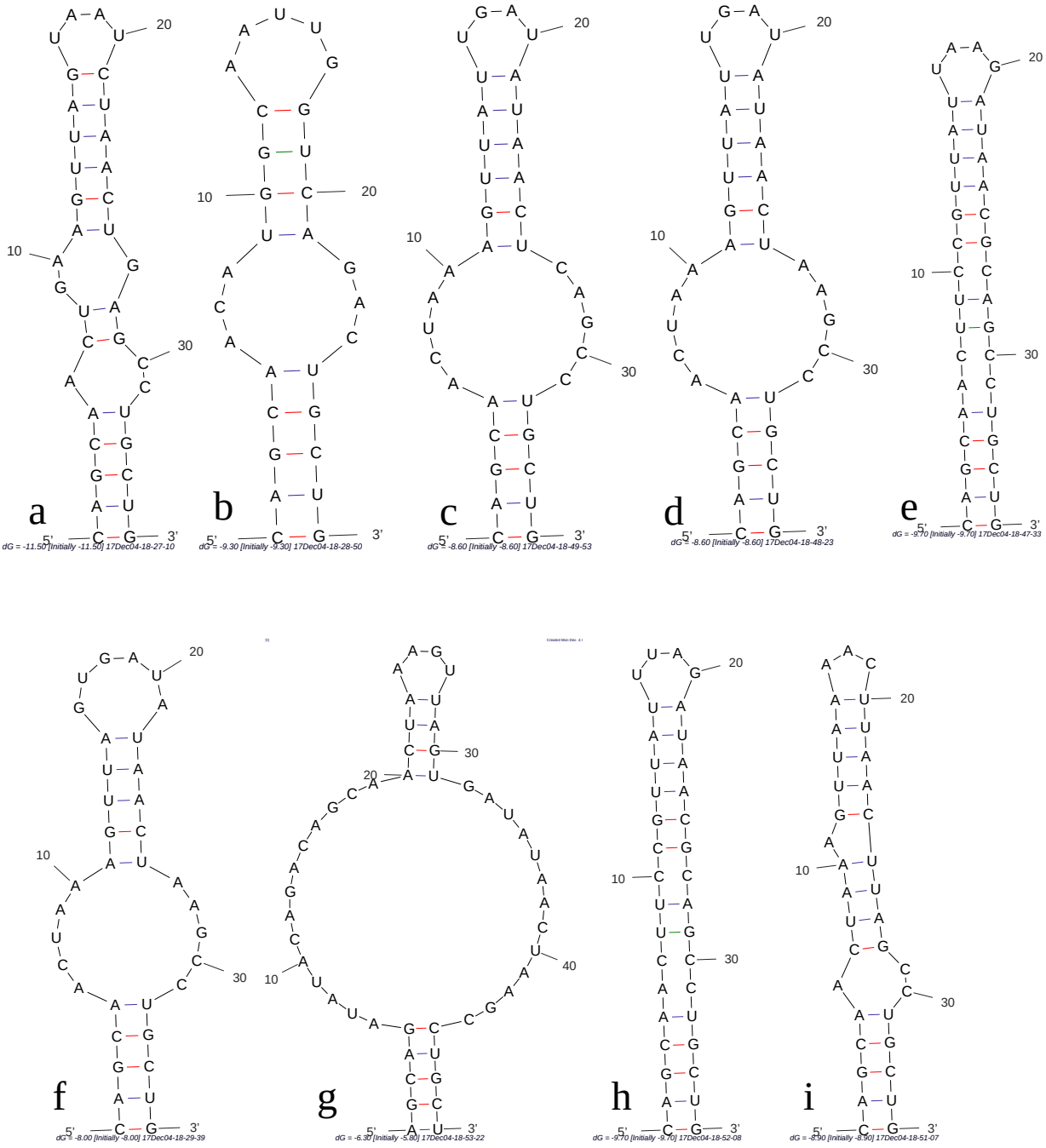

Figure 2.7 Box B helices for *C. dumas* and closet relatives for which ITS sequence data is available. a) *C. dumas* HWSC1B clone A, b) *C. dumas* HWSC1B clone B, c) *C.* sp. SEV5-4-c5 clone operon 1, d) *C.* sp. SEV5-4-c5 clone operon II, e) *C.* sp. HA4395-MV3 clone B3-4+P5e, f)

C. sp. HA4283-MV5 clone p11B, g) *C.* sp. HA4283-MV5 clone p11D, h) *C.* sp. HA4283-MV5 clone p11A, i) *C.* sp. HA4186-MV5 clone B2+P10ab.

The V3 helices were different between the two clones (Fig. 2.8a,b). Clone I mostly closely resembled *Calothrix* sp. SEV4-5-c5 while clone II shared no common motif with any other folded motifs. Both of the basal clamps, though, were extended (e.g., seven and eight nucleotides vs. four to five for all others, Fig. 2.8).

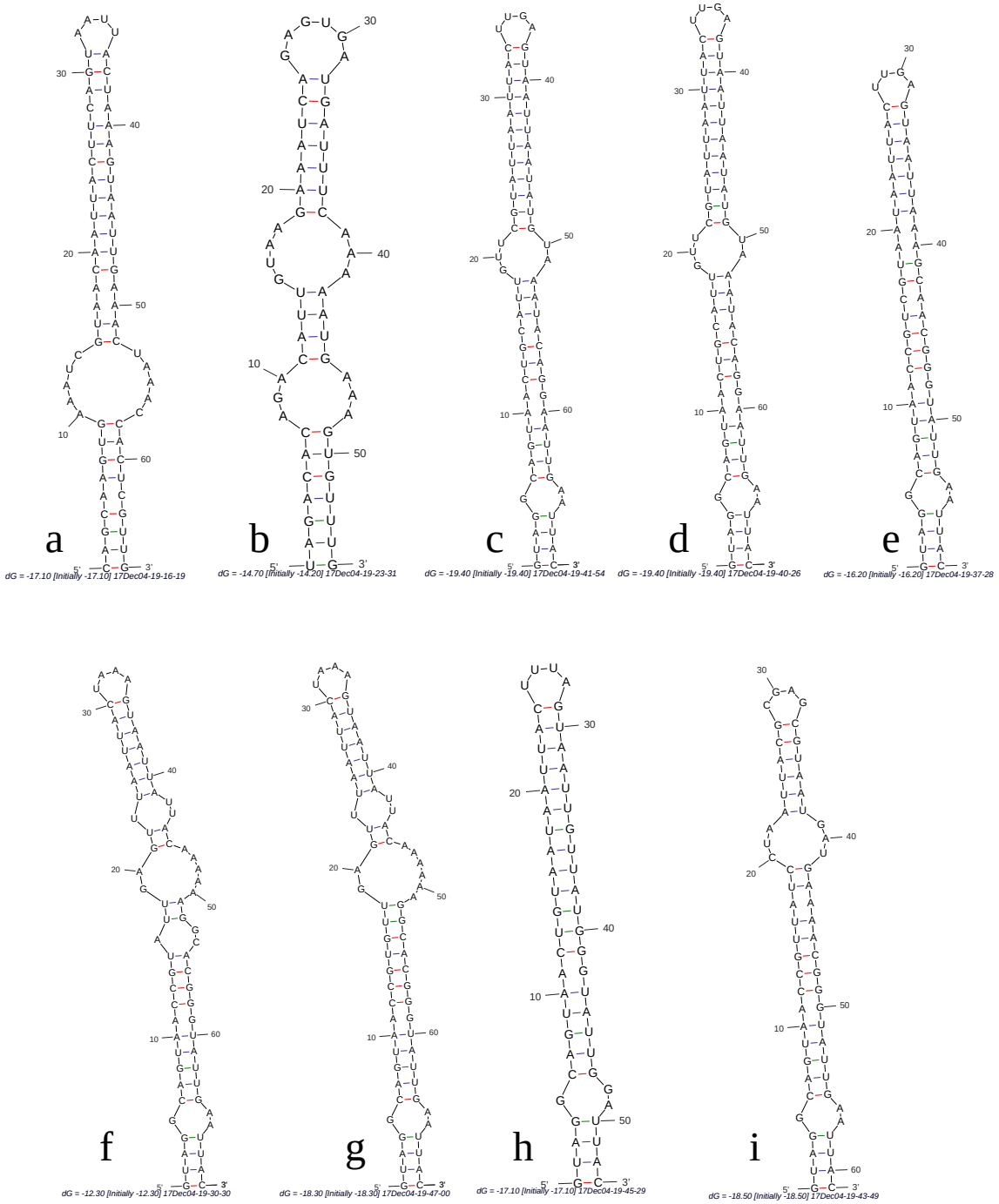

Figure 2.8 V3 helices for *C. dumas* and closet relatives for which ITS sequence data is available. a) *C. dumas* HWSC1B clone A, b) *C. dumas* HWSC1B clone B, c) *C.* sp. SEV5-4-c5 clone operon 1, d) *C.* sp. SEV5-4-c5 clone operon II, e) *C.* sp. HA4395-MV3 clone B3-4+P5e, f) *C.* sp. HA4283-MV5 clone p11B, g) *C.* sp. HA4283-MV5 clone p11D, h) *C.* sp. HA4283-MV5 clone p11A, i) *C.* sp. HA4186-MV5 clone B2+P10ab.

Morphological assessments

The newly described *B. geniculosus* is morphologically similar to other species in the genus (Fig. 2.9). *Brasilonema geniculosus* differs from the type species, *B. bromeliae,* in trichome width, color of sheath, and only rarely slightly attenuated trichomes. *Brasilonema tolantongensis* Becerra-Absalón & Montejano (shares similar cell dimensions and purple-brownish color), and both species are capable of producing external pigments which may be released under stress. However, they differ in trichome attenuation. The closest relative in terms of 16S sequence identity, *B. roberti-lamii*, differed in fascicular arrangement (e.g., erect fascicles), sheath characteristics (*B. robert-lamii* possessed infirm, occasionally lamellated sheaths), and heterocytes (*B. robert-lamii* possessed single, intercalary heterocytes, while *B. geniculosus* occasionally exhibited paired heterocytes).

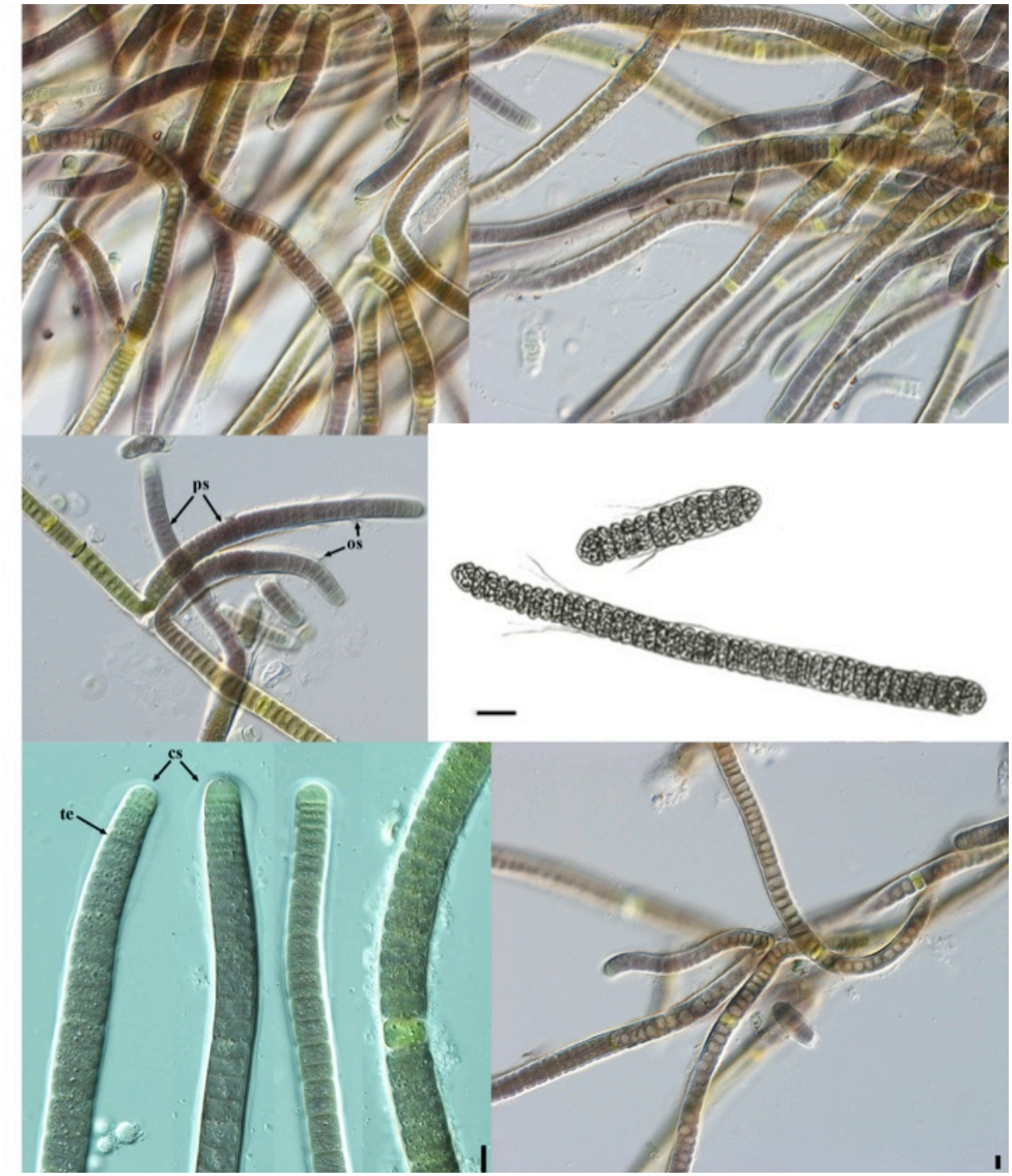

Figure 2.9 *Brasilonema geniculosis* sp. nov., morphological variability and developmental stages. (cs) closed sheath, (os) opened sheath, (ps) purple sheath, (te) tapering end. Scale bars 10 μm.

The newly described taxon, *C. dumas*, possessed a highly knotted growth form with intertwined trichomes and undulating filament growth within the trichome (Fig. 2.10). *Calothrix dumas* forms numerous types of branches, including scytonematoid, tolypotrichoid (*Microchaete*-like), and rivulariacean (Fig. 2.10). *Calothrix dumas* is morphologically similar to other taxa in *Calothrix* but can be uniquely identified based on a combination of characters (see discussion).

Figure 2.10 *Calothrix dumas* sp. nov., morphological variability and developmental stages. (h) hormogonium, (hd) heteropolar development of hormogonium, (htc-b) basal heterocyte, (htc-i) intercalary heterocytes, (mg) microchaetoid growth, (nc) necridic cell, (s) sheath, (sb) scytonematoid branching. Scale bar 10 μm.

Description of new taxon

Brasilonema geniculosus Villanueva et Casamatta

Description: Thallus macroscopic, purple-brown in nature (and culture), growing in mats on granite substrate. Filaments often in fascicles, straight or bent, slightly tapering, false branched. Sheaths purple-brown (fresh isolates and in culture, later colorless), distinct, firm, up to 3.5 µm wide, seldom layered at the basal part near the false branching. Trichomes straight, bent, constricted at cross walls. Cells usually flattened to almost isodiametric, green to blue-green or brownish with peripheral chromatoplasm and central nucleoplasm, frequently granulated, highly vacuolated, 3.2-8.6 µm long × 12.1-20.2 µm wide, apical cells rounded without calyptra. Heterocytes common, intercalary single or in pairs, occasionally flattened but typically square to hemispherical, 5.7-10.1 µm long × 16.7-18.2 µm wide, akinetes not present. Reproduction by hormogonia and fragmentation of trichomes via necridic cells. Meristematic zones if present usually short and located near the apices.

Holotype: OL-100180 (Herbarium at the Department of Botany, Faculty of Science, Palacký University), micrograph from culture Fig. 2.9.

Type strain: No. UPOC 151/2014, deposited at the culture Collection of Department of Botany, Palacký University in Olomouc, Czech Republic.

Type locality: Granite tombstones from the H. Warren Smith Cemetery, Jacksonville Beach, Florida, USA (GPS: 30.2890° N, 81.4071° W).

Genbank accession numbers: MG674086 (16S rRNA and ITS for clone IIA) and MG674085 (16S rRNA and ITS for clone IIB)

Etymology: Name is based on knotted growth form (geniculatus (L.) = knotty, jointed).

Habitat: Growing in a lichen-dominated consortium on cemetery tombstones.

Calothrix dumas Villanueva et Casamatta

Description: Thallus macroscopic, blue-green, in mats on granite substrates. Filaments straight or bent, solitary or in dense clusters, variable false branched (scytonematoid, tolypotrichoid as well as rivulariacean solitary branching). Sheaths colorless (in culture and as fresh samples), distinct, firm, up to 3.5 μm wide, seldom layered at the basal part near the false branching, opened and layered at the apical part. Trichomes straight, bent, or undulated inside the sheath, constricted at cross walls, slightly tapering at the ends. Cells green to blue-green, flattened or barrel-shaped, frequently granulated, non-vacuolated, 3-4.5 μm long × 9-10.5 μm wide at basal part, 2-3.5 μm long × 6.5-7.1 μm wide at the apical part, apical cells rounded or conical. Heterocytes common, typically spherical or hemispherical, 5.1-5.4 μm long × 7.8-8.9 μm wide, often basal, sometimes developing intercalary in pairs before trichome breakage and formation of heteropolar arrangement of trichomes, akinetes not present. Reproduction by hormogonia and fragmentation of trichomes using help of necridic cells. Meristematic zones if present usually short and located near the apical or basal parts.

Holotype: OL-100181 (Herbarium at the Department of Botany, Faculty of Science, Palacký University), micrograph from culture Fig 2.10.

Type strain: No. UPOC 168/2015, deposited at the culture Collection of Department of Botany, Palacký University in Olomouc, Czech Republic.

Type locality: Granite tombstones from the H. Warren Smith Cemetery, Jacksonville Beach, Florida, USA (GPS: 30.2890° N, 81.4071° W).

Genbank accession numbers: MG674088 (16S rRNA and ITS for cloneA) and MG674087 (16S rRNA and ITS for cloneB)

Etymology: Name is based on the tufted appearance of the colonies (dumas (L.) = thorn-bush, bramble).

Habitat: Growing in lichen dominated consortia on cemetery tombstones.

Discussion

Recently exploration of aerophytic cyanobacteria has yielded a bounty of new taxa (e.g., Vilanueva et al. 2018, Alverenga et al. 2016, Hentschke et al. 2016, Sherwood et al. 2015). Aerophytic and aquatic cyanobacterial populations often share similar morphologies and the cyanobacteria as a lineage may be rife with convergent morphological features (Dvorák et al. 2014). However, ecological and molecular data is useful for revealing cryptic taxa. Further, recent employment of ITS data (both sequence and folding patterns) has allowed researchers to untangle the phylogenetic relationships amongst the cyanobacteria, even in cryptic lineages (e.g., Buch et al. 2017, Genuário et al. 2015). It is this approach that we employed in the erection of our two new taxa from cemetery tombstones.

Brasilonema was erected to describe a collection of strains isolated primarily from tropical habitats, and includes taxa traditionally included in *Scytonema* (Fiore et al. 2007). Perhaps common in tropical and subtropical habitats, molecular analyses have confirmed the well-supported molecular position of this genus (Sant'Anna et al. 2011). Characterized by macroscopic, fasiculated colonies consisting of (mostly) reddish filaments with false branching, vacuolized cells (typically), and (typically) aerophytic or sub-aerophytic habitats, this genus has been found in tropical habitats (e.g., Fiore et al. 2007), Hawaii (Vaccarino & Johansen 2012), southern Europe, and as a member of a tripartite lichen from Florida, USA (Villaneuva et al. 2018). Given the dearth of sampling from tropical and subtropical habitats, it is expected that

numerous new taxa are likely (Dvorak et al. 2015). Our new strain fell within a highly supported cluster of other *Brasilonema*, further confirming the monophyletic placement of this lineage. Our strain was closest to *Brasilonema* isolates from mangrove trees (*Avicennia schaueriana*) from Brazil (Alvarenga et al. 2016). It is noteworthy, though, that another recently described taxon isolated from a nearby tombstone, *B. lichenoides* Villanueva & Casamatta (2018: xxx), was most similar to *B. roberti-lamii*, isolated from central Mexico.

The secondary folding patterns of the ITS region present an interesting dilemma for phylogenetic assessment. Typically, patterns in ITS ultrastructure provide characters that may be used as evidence to erect new taxa (e.g., Osorio-Santos et al. 2014). In the case of *B. geniculosus*, though, the two recovered D1-D1' helices of the clones were very different from each other (Fig. 2.3a,b). In addition, the one helix was 24 nucleotides longer than the other, thus impacting the folded structure. Similarly, three helices were identified in the V3 region for *B. geniculosus* clone IIB which yielded different structures (Fig. 2.5b,c,d). The first helix was identical to that found in clone IIA, and it is evident that an insert in this region occurred in a second operon. How these differences may impact the phylogeny of this genus as a whole, though, is currently unclear. Very few strains of *Brasilonema* have sequenced ITS regions available in Genbank, so it is difficult to make conclusions about patterns without more extensive sequences from sister taxa available. Additionally, very few clonal replicates are published, thus potentially missing some of the heterogeneity that may be present in this, and possible other, cyanobacterial lineages.

The genus *Calothrix* contains a high number of species predominantly inhabiting aquatic biotopes all over the world. However, *Calothrix* has recently underdone significant revision, with several distinct lineages being identified based on ecology (Berrendo et al. 2016). Hair-like

formation in the apical parts of trichomes represents one of the most important diacritic features. Our strain fell within a highly supported clade containing fresh-water and subaerial taxa. *Calothrix dumas* belongs to the group of species without terminal hairs and an aerophytic ecology. Our strain did not produce terminal hairs in culture, but it must be noted that we can not say for certain that it is incapable of hair production given an appropriate medium and growth (e.g., Chu medium). *Calothrix conica* N.L. Gardner (1927: 66), a morphologically similar taxon, was described as subaerophytic in Puerto Rico (Gardner 1927). However, *C. conica* is rarely false branched, while *C. dumas* forms numerous branches, including scytonematoid, tolypotrichoid (*Microchaete*-like), and rivulariacean types. *Calothrix flamulorum* C.L. Sant'Anna, S.M.F. Silva & L.H.Z. Branco (1992: 85), another subaerophytic species isolated from wet rocks in Brazilian caves, also differs in similar features to *C. conica* (cell width, formation of sheath, mode of branching). The thallus and filament habitus of *C. elenkinii* Kossinskaja (1924: 11) is similar to *C. dumas* (dense clusters, opened sheath, and intercalary heterocytes) as well. However, *C. elenkinii* forms thinner and isodiametric to slightly flattened cells and differs from *C. dumas* in cell width, formation of sheath (closed/opened), and mode of branching. It is also worth noting that our new taxon displayed a long branch in the maximum likelihood analysis, with a strain from Japan as our closest relative. Though the distance was great, the bootstrap support was very high, and it may be that with additional sister taxa sequenced our new taxon may fit more neatly into a clearly delineated soil clade of *Calothrix*. *Calothrix* as currently accepted is polyphyletic, but with increased sampling and sequence availability the phylogenetic relationships amongst this lineage may be resolved.

The patterns of ITS folding for *Calothrix* present some potentially interesting notes for phylogenetics. Many of the clonal replicates for all secondary structures showed extensive

heterogeneity. For example, the Box B helix for some clones are identical (both in structure and nucleotide sequences, e.g., Fig. 2.7c,d) while other clones exhibit different (structure and nucleotide sequences, e.g., Fig. 2.7f,g,h) helices. The clones of *C. dumas* were similar in ultrastructure but different in nucleotides (e.g., 36 vs. 29). Thus, we advocate caution when comparing structures from multiple operons as these structures themselves may be under different evolutionary pressures (e.g., variable expression at different temperatures). In future studies, it may be useful to address this data gap by more sequencing of multiple operons and their associated ITS regions.

We note that cemetery tombstones may represent an untapped reservoir of sampling environments for describing novel cyanobacterial diversity. As noted in Lopez-Bautista et al. (2006), the total biodiversity of subaerial, cyanobacterial diversity is woefully understudied and should be a priority for future research. We echo these sentiments and note that we have recovered four novel (including two from Villanueva et al. 2018) taxa from a single cemetery, thus spurring future taxonomic inquiries.

Acknowledgement

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Chapter 3: Standardizing analysis of Internal Transcribed Spacer regions in multiple 16S-23S operons: a new evolutionary review of ITS paralog patterns in Cyanobacteria

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Keywords: Secondary structures, systematics

Abstract

Cyanobacterial taxonomy first developed over a century ago and was based almost entirely on morphology, which may be taxonomically uninformative. Modern cyanobacterial species concepts, however, requires an apomorphy, either ecological, molecular, or morphological, to define new species. The use of the 16S-23S Internal Transcribed Spacer (ITS) region secondary structures have been proposed as suitable apomorphies for phylogenetic reconstructions. However, the relationship between ITS structures, often visualized as hairpin loops (referred to as helices) in regulatory DNA, has not been thoroughly evaluated or quantified. Further, the use of these secondary structures is not necessarily standardized amongst cyanobacterial systematists. To address this, we employed previously published sequence data from Genbank to evaluate secondary structures of common ITS motifs both within and between cyanobacterial genera. This endeavor is confounded by the presence of multiple operons, which may be under differential selective pressures and thus yield different structures. This is coupled with inconsistent labeling of partial or full sequences into Genbank, a lack of standards for annotation of ITS domains, and lack of clear naming terminologies for predicted structures. We note that ITS structures are reliable markers for use within genera but are not necessarily appropriate for comparisons between genera. We provide recommendations to standardize ITS secondary structure analysis going forward.

Introduction

Early taxonomic proposals for cyanobacteria, suggested in the late nineteenth century, employed morphology and ecology to catalog cyanobacterial orders (Bornet & Flahault 1886-1888; Gomont 1890, 1892). In the twentieth century, Frémy (1929) and Geitler (1932) advocated only three orders of cyanobacteria grouped by morphological characters considered taxonomically informative, later amended to four orders (Geitler 1942). Differences in cyanobacterial fatty acid composition prompted the development of chemotaxonomy in the 1970s having either polyunsaturated fatty acids, mono unsaturated fatty acids, alpha-linolenic acid, or gamma-linolenic acid (Kenyon 1972; Kenyon et al. 1972; Murata et a. 1992). Subsequent research demonstrated that this classification system could neither account for all fatty acid variants, nor for culturally induced changes in composition (Lawry & Simon 1982; Cohen et al. 1995).

Rippka et al. (1979) expanded the morphological classification scheme to include five subdivisions based on multicellularity, branching patterns, and the presence of specialized cells. Rippka's system replaced chemotaxonomy and formed the basis for cyanobacterial taxonomy into the twenty first century and was included in Bergey's Manual of Systematic Bacteriology (Castenholtz 2001). However, this scheme relied on characters (e.g., specialized cells) that either were subject to multiple evolutionary innovations or were not phylogenetically informative.

All bacterial genomes contain the 16S-23S rRNA operon, consisting of the 16S gene, Internal Transcribed Spacer (ITS) region, 23S gene, and 5S gene (Figure 1) (Schleifer & Kandler 1989; Iteman et al. 2000). Heterotrophic bacteria and cyanobacteria typically have multiple copies of this operon, though the copy number varies (Tourova 2003). Heterotrophic bacteria may have one to 15 operons, varying between and among genera (Klappenbach et al. 2001;

Větrovský & Baldrian 2013). Cyanobacteria typically have one to five operon copies, with copy number typically increasing with morphological complexity (Iteman et al. 2000, 2001; Schirrmeister et al. 2012). The 16S gene serves as a constituent component of the bacterial small subunit of the ribosome, stabilizing bonding between the small and large ribosomal subunits, as well as providing stable bonding for the A site of bacterial ribosomes (Stackebrandt & Goebel 1994; Iteman et al. 2000, Boyer et al. 2001). The structural function of the 16S gene makes large segments of it highly conserved, with episodic variable regions (Johansen et al. 2015). However, the ITS region, that regulatory rDNA segment located between the 16S and 23S genes, is more variable (Woese 1987; Iteman 2000; Boyer 2001).

The ITS regulatory domains are involved in transcriptional control of the flanking 16S and 23S genes and contain anti-termination and processing sites (Iteman 2000). Additionally, there may be Ile and Ala tRNA genes located within the ITS (Iteman 2000). Inclusion of tRNA genes in the ITS region is also variable even within a single genome. One operon copy may have both tRNA genes, while a second operon copy within the genome may have neither (Boyer et al. 2001). Some taxa may have multiple operons with both tRNA genes, or one tRNA gene within the ITS, though very few have neither in any copies (Johansen et al. 2011). The mosaic nature of the conserved and variable regions of the 16S gene and ITS region, along with its highly-conserved functionality, and presence in all bacteria made it the gold standard for bacterial systematics, as defined by the bacteriologic code (Vandamme et al. 1996; Stackebrandt et al. 2002).

The accumulation of molecular data has catalyzed continual revision of classic cyanobacterial taxonomy (e.g., Komárek et al. 2014; Dvorak et al. 2015). Phylogenies constructed using taxa included in classic morphology-based taxonomies are often polyphyletic

(Komárek 2014). Genomic research has demonstrated that many morphological and metabolic traits classically used for cyanobacterial classification (e.g., multicellularity, baeocyte formation, presence of akinetes, tapering, polarity, branching patterns, etc.) have complex evolutionary histories, with serial acquisition and loss, and thus may not be taxonomically informative (Schirrmeister 2011; Shih et al 2013). For example, analysis of whole genome data has suggested that multicellularity developed independently in up to four cyanobacterial lineages and was subsequently lost in at least one (Schirrmeister et al. 2011, 2013). Further, some metabolic characters such as nitrogen fixation are likely ancestral traits that have been subsequently lost in many lineages (Larsson et al. 2011). This has led to three very different proposed methodologies for revision of cyanobacterial taxonomy coming from different research camps (Komárek 2014).

For over fifty years one camp of researchers have been suggesting that the number of cyanobacterial taxa must be dramatically reduced to simplify the taxonomic system (Drouet & Daily 1956; Drouet 1973, 1978, 1981; Bourrelly 1970; Otsuka et al. 2001). To the contrary, a second camp of researchers has recommended bifurcation of polyphyletic groups of both genera and species until all taxonomic units are monophyletic (Anagnostidis & Komárek 1985; Casamatta et al. 2005; Johansen & Casamatta 2005; Rehakova et al. 2007; Siegesmund et al. 2008; Perkerson et al. 2011). Lastly, it has been suggested that no further taxonomic changes should be implemented at all until a great deal more molecular data is collected by researchers (Hoffman 2005).

The monophyletic species concept *sensu* Johansen and Casamatta (2005) derived from a polyphasic approach has been proposed as the standard for cyanobacterial systematics (e.g., Komárek et al. 2014; Komárek 2016), which requires the description of an apomorphy, along with ecology and molecular data, to test phylogenetic hypotheses. If a new strain of

cyanobacteria has <98% sequence similarity to previously described taxa, some habitat or ecological incongruity, and a documented apomorphy, the case can be made to designate the new strain as a novel species (Johansen & Casamatta 2005). Over fifty new genera were described between 2000 and 2014, and the new taxonomy presented by Komárek (2014, 2016) attempts to incorporate all those with taxonomic standing. The Komárekian (2014, 2016) system reflects true evolutionary relationships by erecting monophyletic groups with exiguous but evidently monophyletic genera that contain fewer, more closely related species as determined by 16S sequence data.

Describing and elucidating cyanobacterial species, however, can be problematic, even with genetic data. Morphological plasticity and cryptic diversity often mask how akin or divergent various taxa are, stifling attempts to clarify cyanobacterial taxonomy with genetics (Casamatta et al. 2003; Komárek et al. 2013; Dvořák et al. 2015). Traditionally, non-planctonic cyanobacterial ecology has been woefully understudied, with only recent forays into non-lentic ecologies just beginning, leaving the habitat ranges of many taxa to be revised (Dvořák et al. 2015). Thus, most cyanobacterial phylogenies remain unresolved (Dvořák et al. 2015; Komárek 2016).

The 16S rDNA gene, however, has been shown to lack resolving power at the species level for prokaryotes (Konstantinidis et al. 2006; Goris et al. 2007). The level of 16S rRNA gene sequence similarity that corresponds to accepted average nucleotide identity (ANI) threshold for prokaryotic species identification has been calculated as 98.65% (Kim et al. 2014). There have been instances where well differentiated populations of phenotypically different cyanobacteria had identical 16S and ITS sequences, though they varied considerably at six nitrogen metabolism loci (Miller et al. 2006). Other potential molecular markers have been suggested for use in

prokaryotic taxonomy, such as the *rpoB, nifD, psbA, rbcL* genes (Case et al. 2007; Singh et al. 2014; Dvořák et al. 2014). Concatenation of the 16S data with other molecular markers, collectively known as multilocus sequence analysis (MLSA), has become common in molecular studies to address the taxonomic shortfall of the 16S rRNA gene (Singh et al. 2014; Wilmotte et al. 2017). However, a robust multilocus phylogenetic analysis of 23 coding genes, across eight cyanobacterial orders, showed that when compared to 16S rRNA phylogenies, only minor differences exist, confirming the utility of the 16S at the generic level, but not so at the species level (Mares 2017). Thus, the MLSA is of no greater utility than the 16S alone.

Without the use of morphological characters to act as apomorphies, and with the lack of species level resolution in common molecular markers, differences in ITS secondary structures have developed into a common taxonomic tool to describe new species (Iteman et al. 2001, 2002; Boyer et al. 2001; Johansen et al. 2005, 2011, 2013). Iteman (2000, 2002) pioneered the use of ITS, with restriction fragment length polymorphisms of ITS amplicons to distinguish closely related taxa. Later works by Johansen et al. (e.g., 2005, 2011, 2013) demonstrated how ITS secondary structures, obtained by folding conserved hairpin loop domains within the ITS, could be used to delineate cryptic species, and were thus a powerful tool when applied to phylogenetic studies. ITS secondary structures have become the new gold standard for describing novel cyanobacterial taxa for the last decade (Johansen 2013; Komárek 2013; Engene et al. 2015; Suradkar et al. 2017; Villanueva et al. 2018). However, protocols for ITS analysis have not been standardized, nomenclature and presentation of folded structures vary from author to author, and taxonomic differences of conserved ITS domains across cyanobacterial lineages have not been quantified (Johansen 2013; Komárek 2013; Engene et al. 2015; Suradkar et al. 2017).

The identification and annotation of whole ITS sequences and conserved domains in Genbank are inconsistent. Some ITS sequence submissions to Genbank are added separately from the 16S gene, making their evaluation difficult without knowing the submitter's cut points for the two separate entries, while others are submitted as one continuous sequence. Additionally, many 16S and ITS submissions have no annotation as to possible clonal replicates sequenced from the same isolate. Because the ITS region is more variable, and repeats are common especially between conserved domains (Fig 3.1), it is necessary to have flanking regions to be sure that a sequence is in fact the conserved domain. For example, the D1' sequence (usually AGGTC), part of the basal clamp for the D1-D1' helix, can often have a repeat just upstream of the correct D1' location. To identify which of the AGGTC repeats represents the actual D1' sequence can require evaluation of its location in relation to the flanking D2 sequence (GGT).

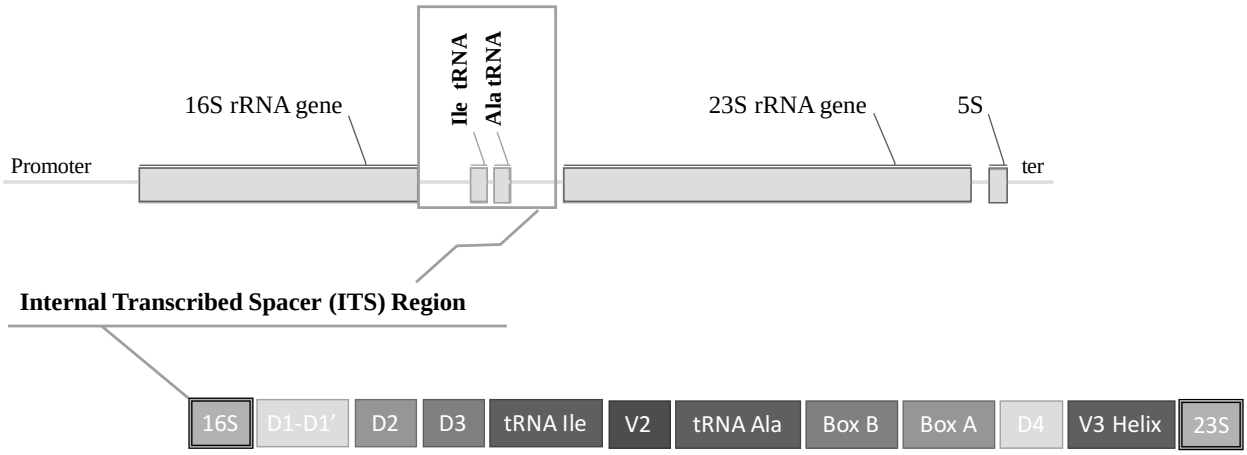

Figure 3.1 The 16S-23S rRNA operon, consisting of the promoter region, 16S gene, Intergenic Transcribed Spacer (ITS) region, 23S gene, 5S gene, and terminator sequence. Denoting the location of the ITS within the operon and the conserved domains of the ITS region flanked by the 16S and 23S genes.

The 16S-23S operon is one of many in prokaryotes with multiple copies (Pei et al., 2010; Sun et al., 2013). Intragenomic heterogeneity of the 16S rRNA gene has been reported in prokaryotes, including cyanobacteria (Pei et al. 2010; Engene et al. 2011; Sun et al., 2013; Johansen et al. 2017). Analysis of 37 cyanobacterial genomes suggested that of the 62.7% of genomes containing multiple 16S-23S operons, only 35.1% displayed intragenomic 16S rRNA heterogeneity. However, the analysis employed genome assembly programs that typically formed consensus sequences of those with <6% difference (far above the 1.9% difference in 16S rRNA that separates species) and the authors noted that the formation of consensus sequences may have overlooked variation between paralogous 16S copies (Engene & Gerwick, 2011). Major patterns of intragenomic ITS heterogeneity have been identified in prokaryotes, including length, tRNA inclusion, and nucleotide divergence (Iteman et al. 2000, 2001; Stewart et al. 2006).

There is evidence, however, that operons may either vary due to differential selective pressures or drift or may undergo concerted evolution due to gene homogenization (Stewart et al., 2006) Though the ITS region is regulatory, the conservation of domains across all orders of cyanobacteria indicates that there is active selection. Further, patterns of tRNA inclusion or exclusion in ITS regions indicates the possibility that there are differential selective pressures for individual operons. Many of the helices from ITS operons of differing patterns of tRNA inclusion, from within the same genome, are significantly different from one another. Additionally, other patterns of domain deletions and duplications within the ITS have been identified in various cyanobacterial taxa. This suggests varying evolutionary history for individual ITS operons. Evolutionary differentiation may have preceded speciation events in some genera if the duplication event was ancient enough. Thus, comparing ITS domains to

paralogous ITS operons from closely related species may not be informative for differentiating species, analogous to comparisons of apples to oranges, evolutionarily.

Inconsistencies in analyses of ITS secondary structures usually relates to inconsistent handling of multiple 16S-23S operons. Some researchers clone replicate ITS operons and publish consensus structures for each paralog to objectively eliminate the possibility of error as the cause of sequence differences (Johansen et al. 2017; Shalygin et al. 2017). Some groups publish only one operon as a cloned consensus structure, while others publish only single operon data without cloning isolates to check for multiple operons. Primers may selectively amplify only certain 16S and ITS operons, leading to the sequencing of only a single operon when multiples are present if cloning is not employed (Osorio-Santos et al. 2015). Very few researchers are actively selecting ITS operons with similar evolutionary patterns to compare to one another or noting patterns of domain inclusion in publications of new taxa. To address these issues, a pilot study was undertaken analyzing ITS operons from 224 previously published cyanobacterial taxa from Genbank for domain inclusion and exclusion, intragenomic heterogeneity of ITS operons, and the possible relevance of variable selective pressures affecting individual domains.

Methods

A total of 224 ITS sequences from all cyanobacterial orders were sourced from Genbank (http://blast.ncbi.nlm.nih.gov) and conserved ITS domains annotated as of January 2018. Evaluation of ITS data began by calculating the percentage of unusable, partial, and full ITS domains. Usable domains were those defined as having the full conserved domain sequence as well as the flanking domains, those domains directly upstream and downstream. Full ITS region sequences were defined as possessing both the flanking 16S gene ending sequence and 23S gene

starting sequence. Partial ITS sequences were defined as having at least one usable domain, including both flanking domains. Unusable ITS sequences did not include any usable domains.

A total of 216 sequences possessed a usable D1-D1' helix, D2, & D3 domains. Of those, only 207 included the 23S starting sequence. Sampled sequences with no usable domains were excluded. Secondary structures were determined for all usable ITS sequences for the D1-D1' helix, Box-B helix, and the V3 helix using Mfold (Zuker 2003), including non-canonical base pairs *sensu* Das et al. (2006). Secondary structures for all families within the order Nostocales were assembled and organized by family then genus to produce a visual data set of secondary structures motifs for comparison. To qualitatively assess intragenomic heterogeneity, unweighted maximum-likelihood (ML) phylogenetic analysis, comparing only the ITS sequences for taxa with multiple operon sequences available within two Nostocalean clades, *Nostoc* and *Brasilonema*, with *Leptolyngbya boryana* as an outgroup, was computed using MEGA 7 (Kumar et al. 2016), and bootstrap support was obtained from 1,000 pseudoreplicate data sets. Evolutionary analysis of ITS domains with different patterns of tRNA inclusion proceeded by calculating Tajima's D, with the assumption of non-coding DNA. Individual ITS domains were compiled and aligned, then Tajima's D calculated using MEGA7 (Kumar et al. 2016). Sample sets used to calculate the test statistic contained either two or no tRNAs, as too few samples contained a single tRNA to calculate the test statistic with a high enough power. Significance level for the Tajima's D calculations was set at 1.0.

Results and Discussion

Of 224 Genbank sequences denoted as having either a full or partial ITS, submitted with 16S gene sequences, 5.6% had no usable ITS domains, where no flanking domains could be

identified (usable domains being those with flanking regions, allowing for indisputable identification of the sequence as that domain) (Figure 3.2). Further, 59.2% contained only partial ITS sequences with usable domains. Only 35.2% of the samples contained the full ITS sequence flanked by the upstream 23S gene (Figure 3.2).

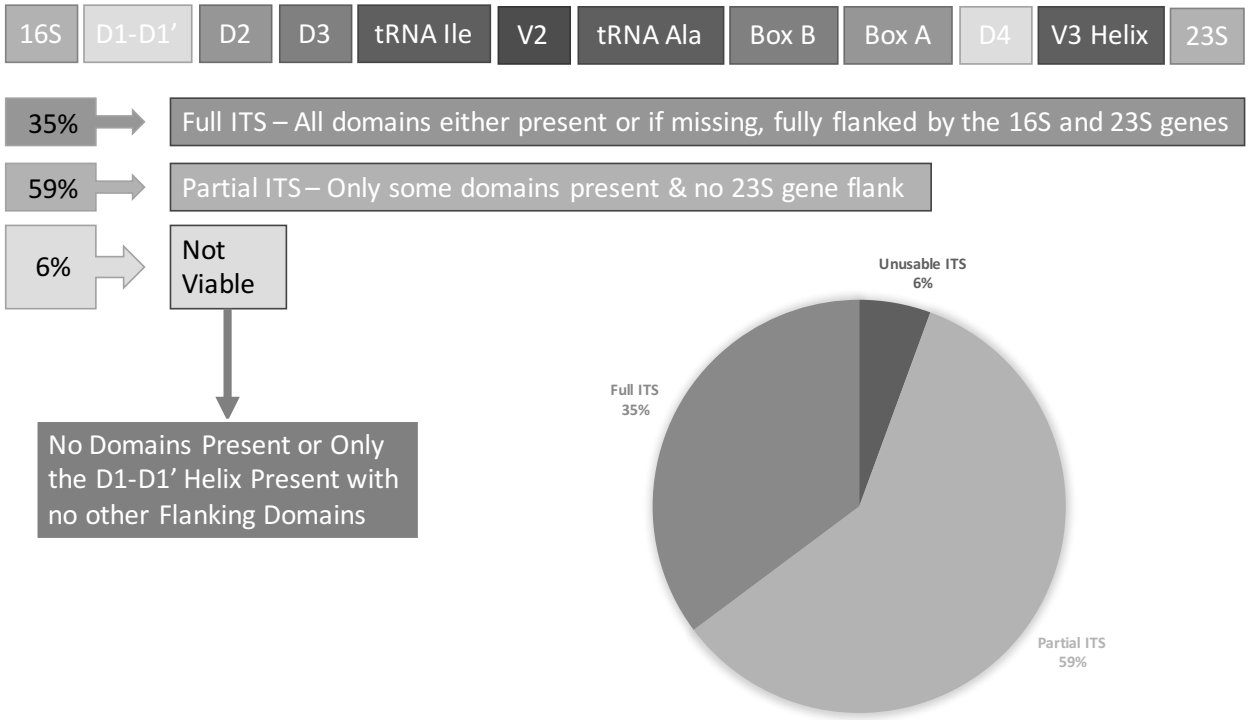

Figure 3.2 Of 224 the 16S-23S ITS sequences (denoted as having a full or partial ITS) acquired from Genbank, the percentages of Full, Partial, and Unusable ITS regions.

The percentage of samples including both the Ile and Ala tRNA genes was calculated (n =207), as well as those with neither, and those with only one tRNA gene (Fig 3.3). Operons with both tRNAs made up 55% of the sample set, while those with neither made up 41%, and those containing only the Ile tRNA made up only 4% of the sample. Those operons containing only one tRNA gene always included the Ile tRNA gene.

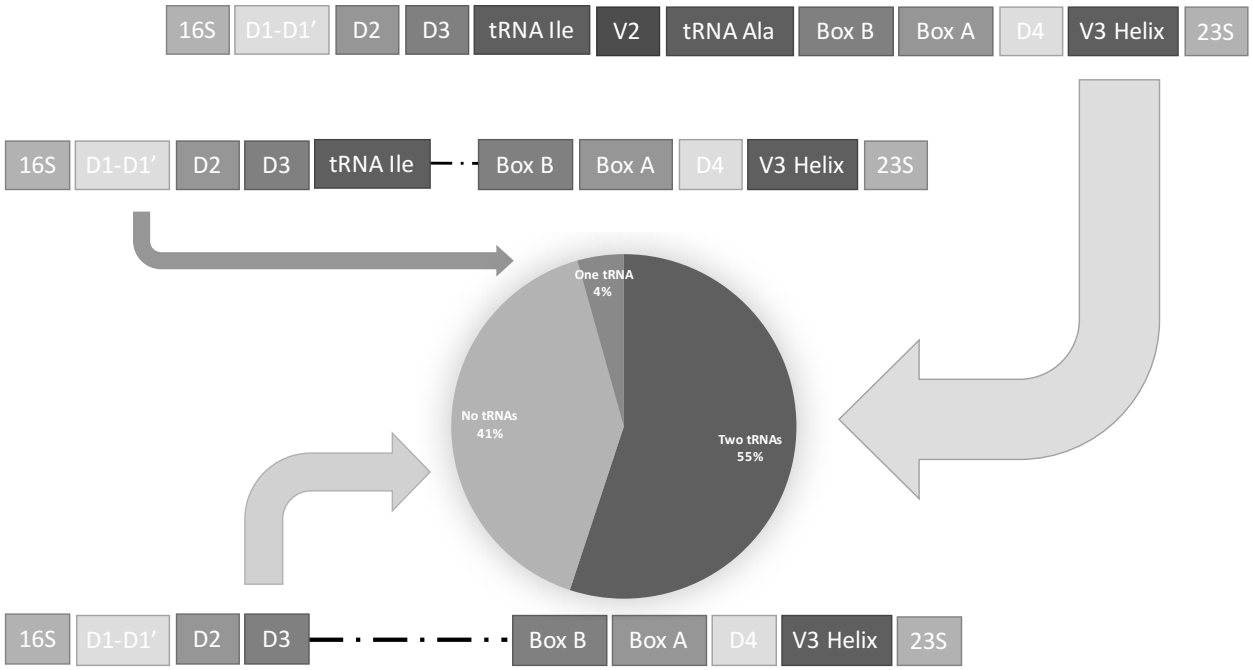

Figure 3.3 Percentage of 207 ITS sequences with both Ile and Ala tRNA genes, no tRNA genes, or one tRNA within the ITS.

Inclusion of all conserved tRNA domains was calculated and visualized (Figure 3.4). The end sequence of the 16S gene, and the D1-D1' helix and D2 sequence conserved domains in the ITS were all included in 99.07% of the sample set (n = 216) (Fig 3.4). The D3 sequence, which is only 3 to 4 nucleotides in length, could only be positively identified in 89.8% of the sampled sequences (n = 216) (Fig 3.4). Because all sequences containing one tRNA contained the Ile tRNA, inclusion of the Ile tRNA occurred in 60.39% of the sample, while the Ala tRNA was only included in 56.04% of sampled sequences (n = 207) (Fig 3.4). The V2 helix located between the tRNA domains was only included in 47.34% of the sampled sequences (Fig 3.4). The Box B helix, Box A sequence, and D4 sequence were all included in 90.34% of the sampled sequences (n = 207). While 77.78% of the sampled sequences of the usable expanded sample set (n = 207) contained the leader sequence for the 23S gene, only 73.91% included a V3 helix (Fig 3.4).

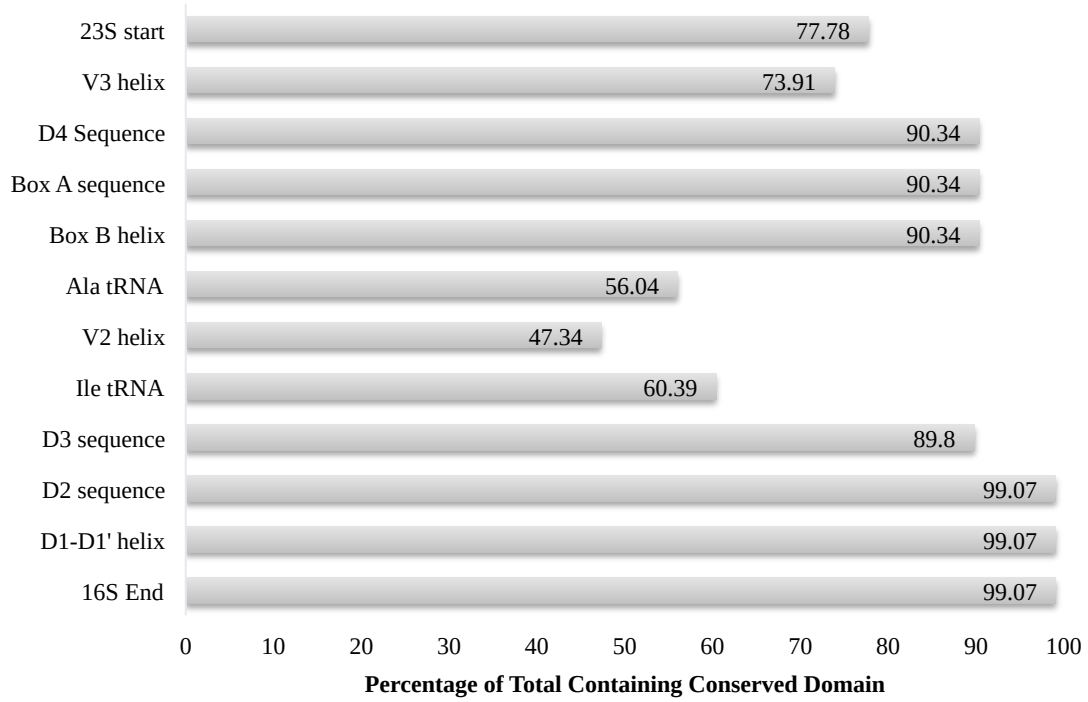

Figure 3.4 Individual conserved domains within the ITS for 216 usable ITS sequences for domains D1-D1’ helix, D2, & D3, and 207 usable sequences for the remaining domains.

Assembled D1’D1’ helix secondary structures within the order Nostocales were visualized taxonomically, for two families, Nostocaceae and Tolypothricaceae (Figure 3.5) (Figure 3.6). Box B helices were similarly displayed for the same families (Figure 3.7) (Figure 3.8). There were found to be distinct patterns of secondary structures included in many of the families within the Nostocales data subset, which makes comparison of structures outside of individual genera uninformative (Fig 3.5) (Fig 3.6) (Fig 3.7) (Fig 3.8). Of the usable Nostocales data, nearly a quarter of the sample set included no V3 helix, although many samples included the beginning sequence of the flanking 23S gene. Secondary structures for V3 helices were not visualized as few samples within a single genus were available for comparison, owing to the uninformative nature of comparisons between genera.

Nostocaceae

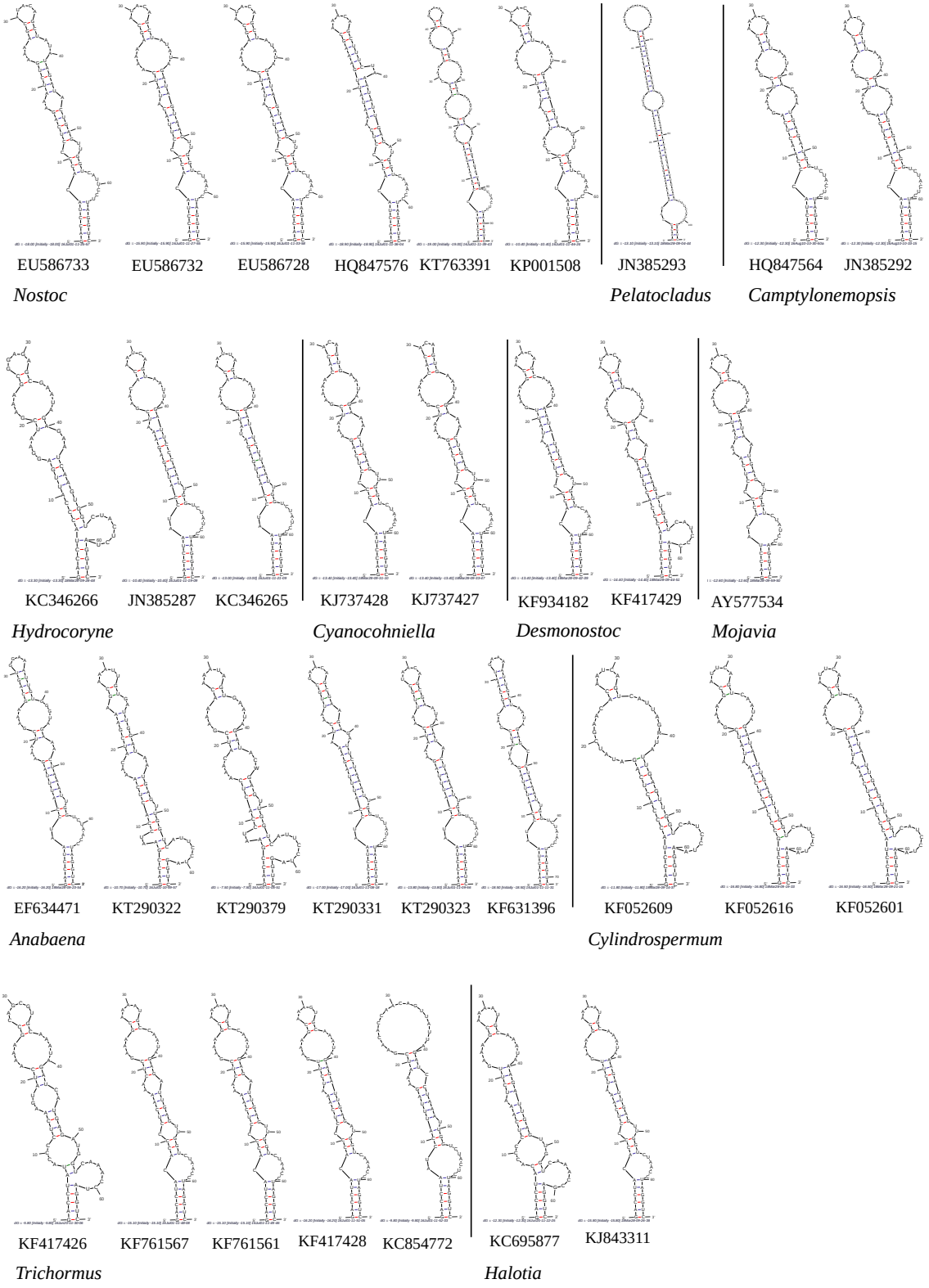

Figure 3.5 All D1-D1′ helices, folded secondary structures, predicted using Mfold, for the Nostocaceae.

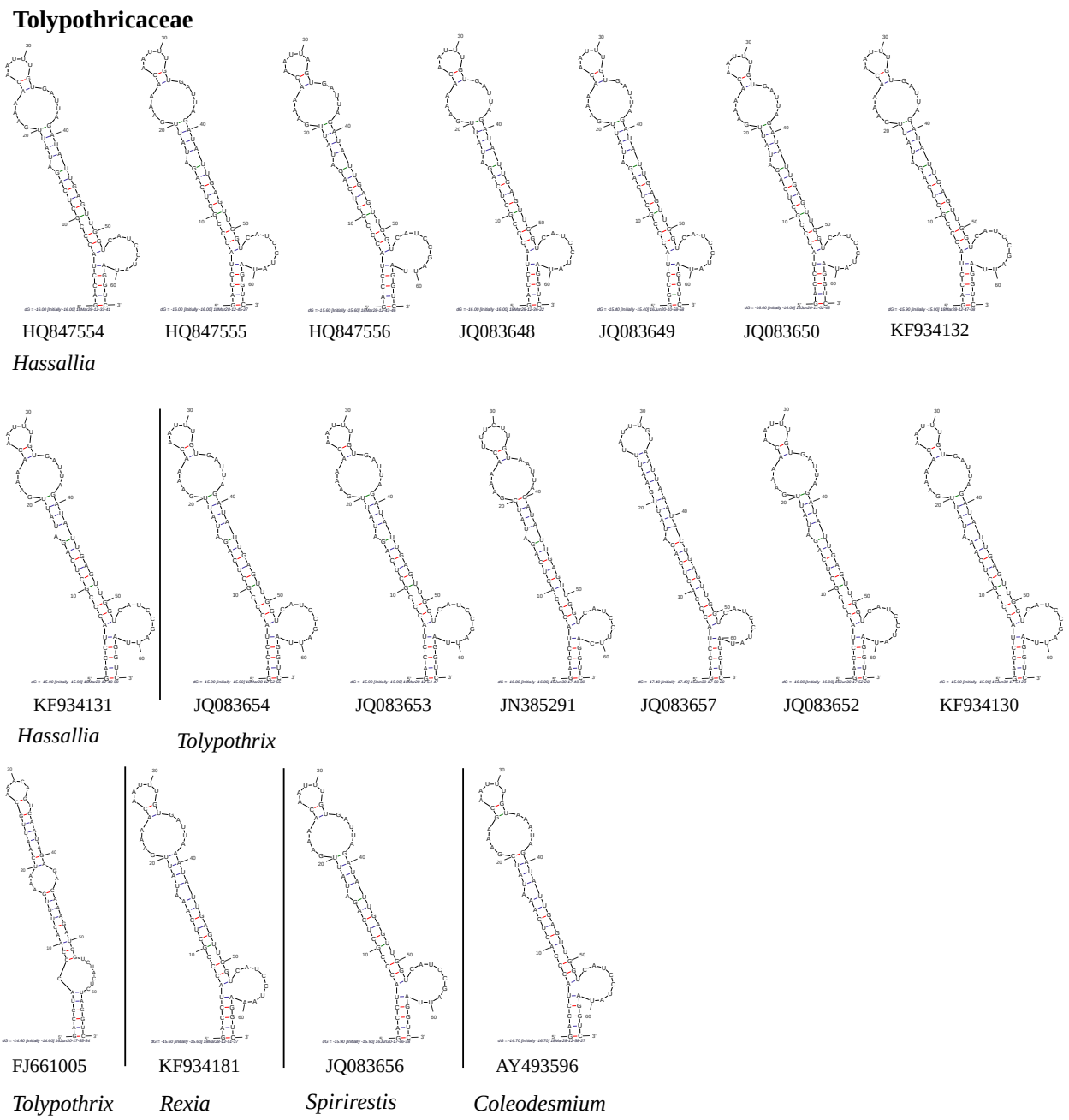

Figure 3.6 All D1-D1′ helices, folded secondary structures, predicted using Mfold, for the Tolypothricaceae.

Nostocaceae

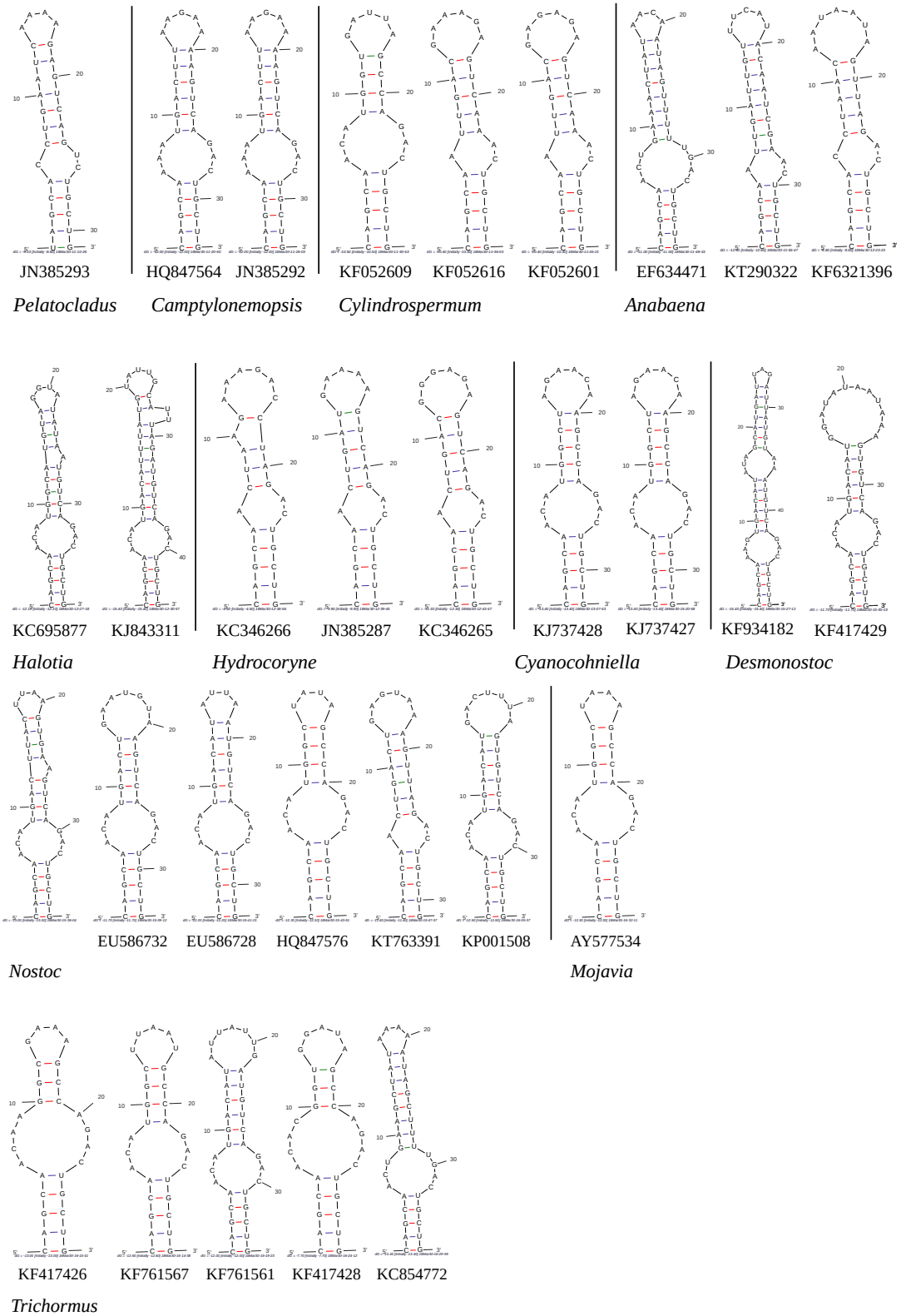

Figure 3.7 All Box B helices, folded secondary structures, predicted using Mfold, for the Nostocaceae.

Tolypothricaceae

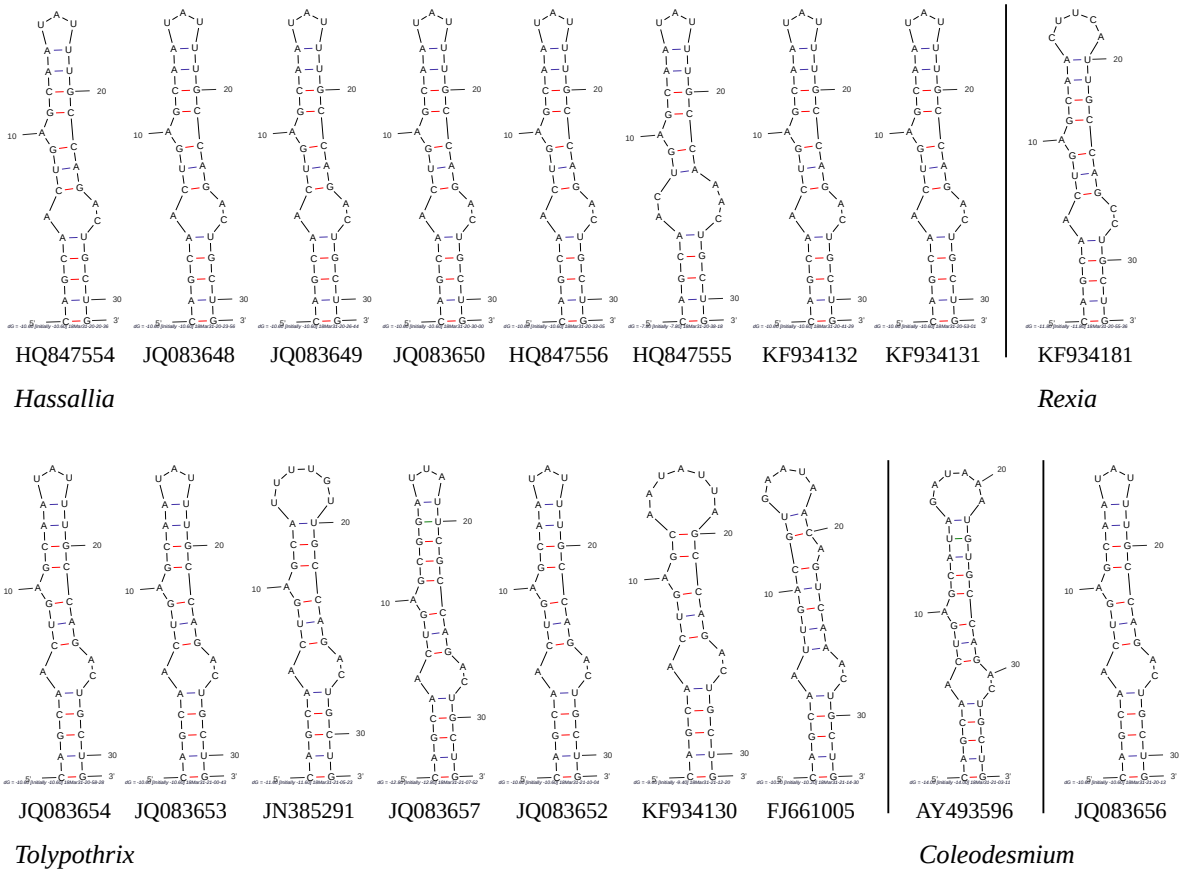

Figure 3.8 All Box B helices, folded secondary structures, predicted using Mfold, for the Tolypothricaceae.

Maximum likelihood (ML) phylogenetic analysis of was calculated for sequences with clonal replicates from the same strains of varying copy number and intragenomic heterogeneity (Figure 3.9). The tree was computed using MEGA 7, with bootstrap support obtained from 1,000 pseudoreplicate data sets. Clades of operons with either both (B), neither (N), or one (1) tRNA marked on the phylogeny.

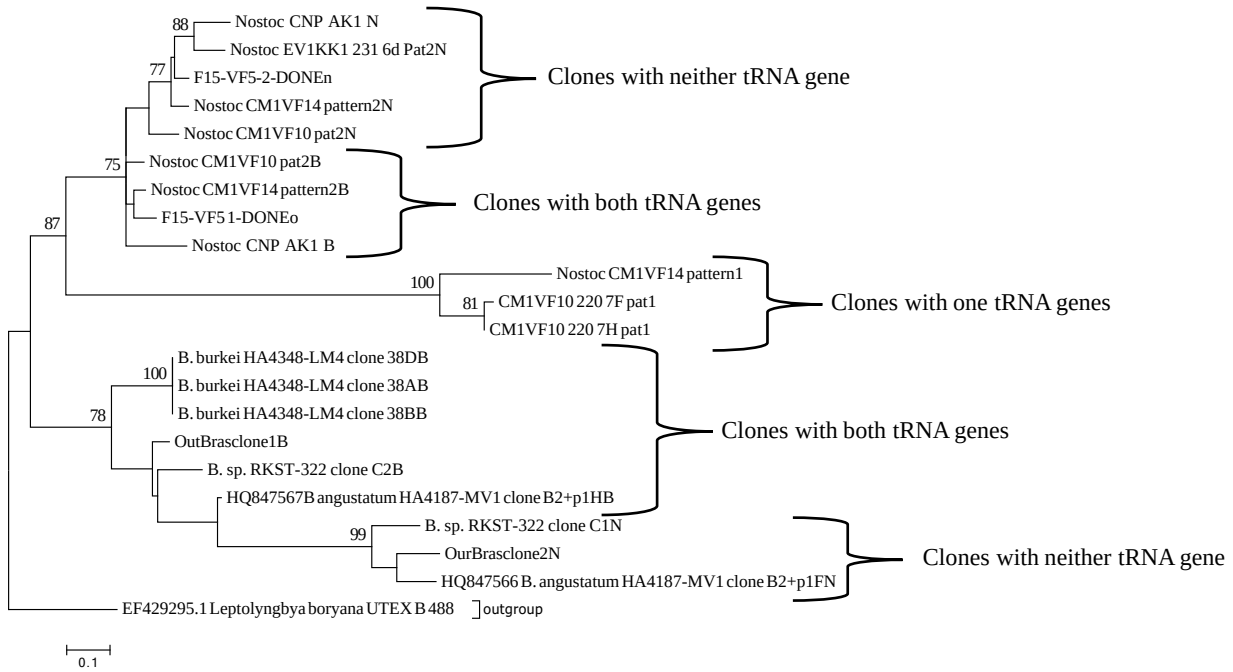

Figure 3.9 Unweighted maximum-likelihood (ML) phylogenetic analysis, comparing ITS sequences for taxa with multiple operon sequences available within two Nostocales clades, *Nostoc* and *Brasilonema*, with an Oscillatoriaceaen outgroup, computed using MEGA 7, with bootstrap support obtained from 1,000 pseudoreplicate data sets. Clades of operons were marked as having either both (B), neither (N), or one (1) tRNA.

Operon sequences in both the *Nostoc* and *Brasilonema* fell out into clades of operons with similar patterns of tRNA inclusion versus clades including multiple operons from individual strains, except for those strains with multiple operons with the same pattern of tRNA inclusion (Fig 3.9). Tajima's D was calculated for both the D1-D1' and Box B helices, as well as the full ITS region for intragenomic multiple operons (Table 3.1).

Table 3.1 Results from Tajima's Neutrality Test

Sample Set	*m*	*S*	*Ps*	θ	π	*D*
D1-D1' Helix Both tRNAs	24	77	0.647059	0.173275	0.154518	-0.426579
D1-D1' Helix Neither tRNA	37	89	0.679389	0.162745	0.197029	0.777132
Box B Helix Both tRNAs	25	24	0.648649	0.171784	0.205135	0.713618
Box B Helix Neither tRNA	37	35	0.507246	0.121509	0.138747	0.500298
Full ITS Both tRNAs	25	481	0.558653	0.14795	0.118533	-0.798165
Full ITS Neither tRNAs	34	477	0.584559	0.142966	0.135046	-0.212097

Sample size ranged from n = 24 to n = 37 (Tab. 3.1). For the D1-D1' helix operons containing both tRNAs demonstrated a higher occurrence of rarer alleles (D = -0.426579), while operons with neither tRNA (D = 0.777132) demonstrated greater than average heterozygosity. Both Box B helices (two tRNAs D = 0.713618; neither tRNA D = 0.500298) displayed balancing selective pressures. Both full ITS sequences displayed positive selection though by different degrees (two tRNAs, D = -0.798165; neither tRNA, D = -0.212097). The results however were not statistically significantly at the significance level of 1. Thus, all populations were found to be evolving neutrally. While prokaryotes cannot have a heterozygous advantage, as multiple operons are not obligatory across all orders, there may be an evolutionary advantage to intragenomic heterogeneity through differential expression of multiple operons.

Recommendations

First Recommendation: Sequence data added into Genbank should be standardized in several respects. First ITS sequences should be included with 16S gene sequence data, not added in separate files. Secondly, ITS domains should be annotated in uploaded sequences, and the partiality of ITS domains should be analyzed prior to sequence addition, and correctly noted given that ITS domains with no flanking 16S and 23S gene regions cannot be considered full, while those with flanking genes should be considered full even if some motifs are missing. The inclusion of only a single ITS domain should not be considered even as partial because when flanking domains are absent there can be no assurance that the included domain has been correctly identified. Lastly, multiple operons from a single newly sequenced or described taxa should also be noted and added in Genbank. Published results where cloning has been described

in the methods, but no clonal replicate data can be found in Genbank or the published manuscript will not allow for thorough evaluation of ITS operon evolution going forward.

Second recommendation: The proposed nomenclature should be standardized for all publishing of ITS. Standardization of nomenclature associated with the ITS is recommend by the authors reflecting the nomenclature presented by Boyer et al. (2001) especially regarding the secondary structures of the helices. Conserved ITS domains should be noted per Boyer et al. (2001) (Figure 3.10).

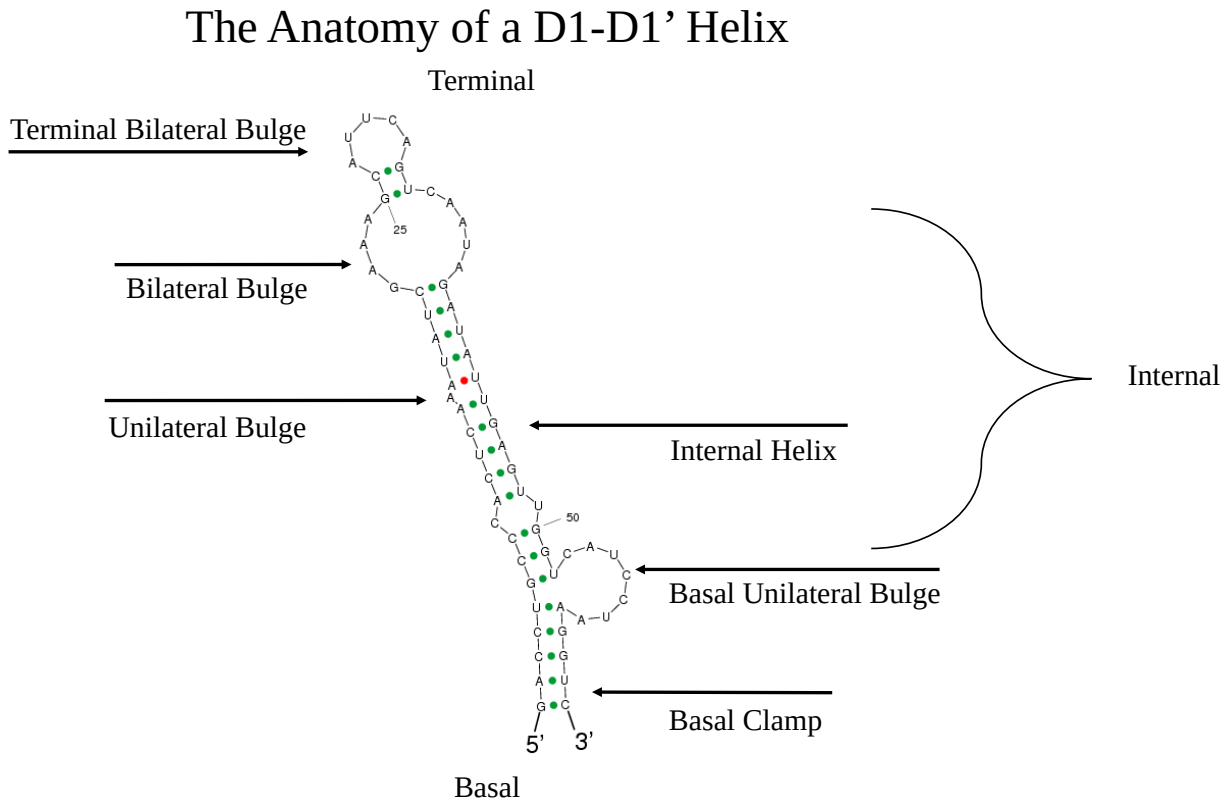

Figure 3.10 Standardized nomenclature for helical secondary structures of ITS hairpin loops as presented in Boyer et al. (2001) and visualized using a common motif of D1-D1’ helices marked with nucleotide numbers.

Third recommendation: Cloning should be the gold standard for assessing ITS, to identify homologous and paralogous consensus sequences (Figure 3.11).

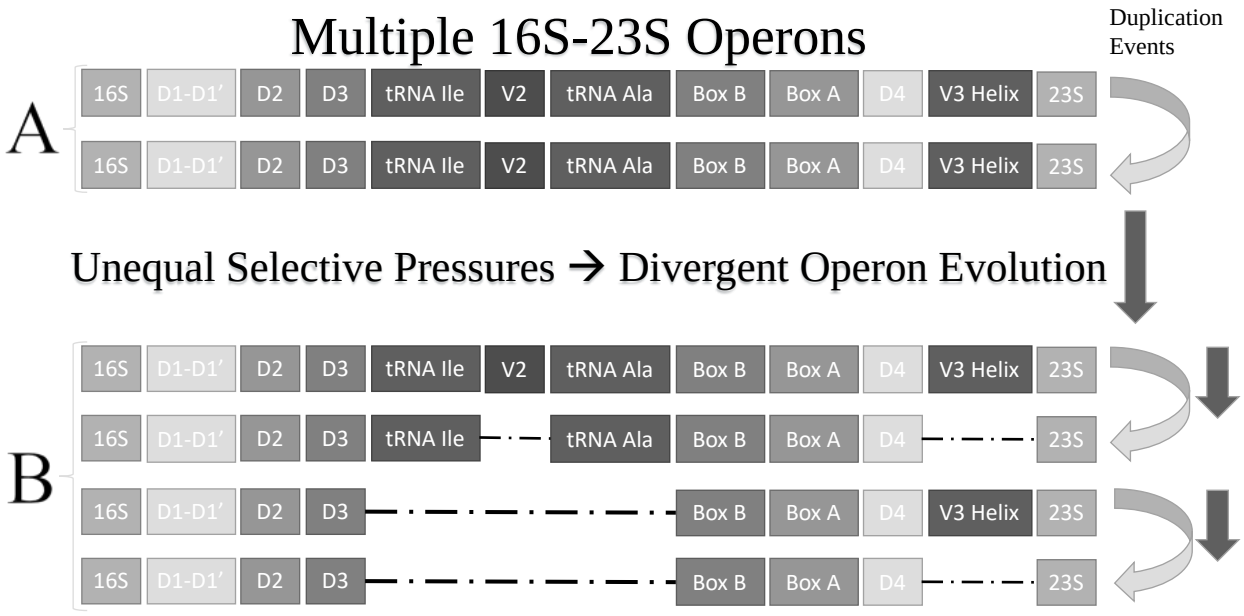

Figure 3.11 Possible ITS operon variants within a single genome following ancient duplication events and variable selective pressures.

A minimum of eight clonal replicates per isolate should be sequenced if cloning is performed. Sequencing clonal replicates in greater numbers allows for the identification of consensus operon sequences. Consensus sequence data should be obtained through the sequencing of multiple clonal replicates to verify nucleotide identity for individual operons within a genome. In addition, consensus secondary structures of helices should be verified as in the given example (Figure 3.12).

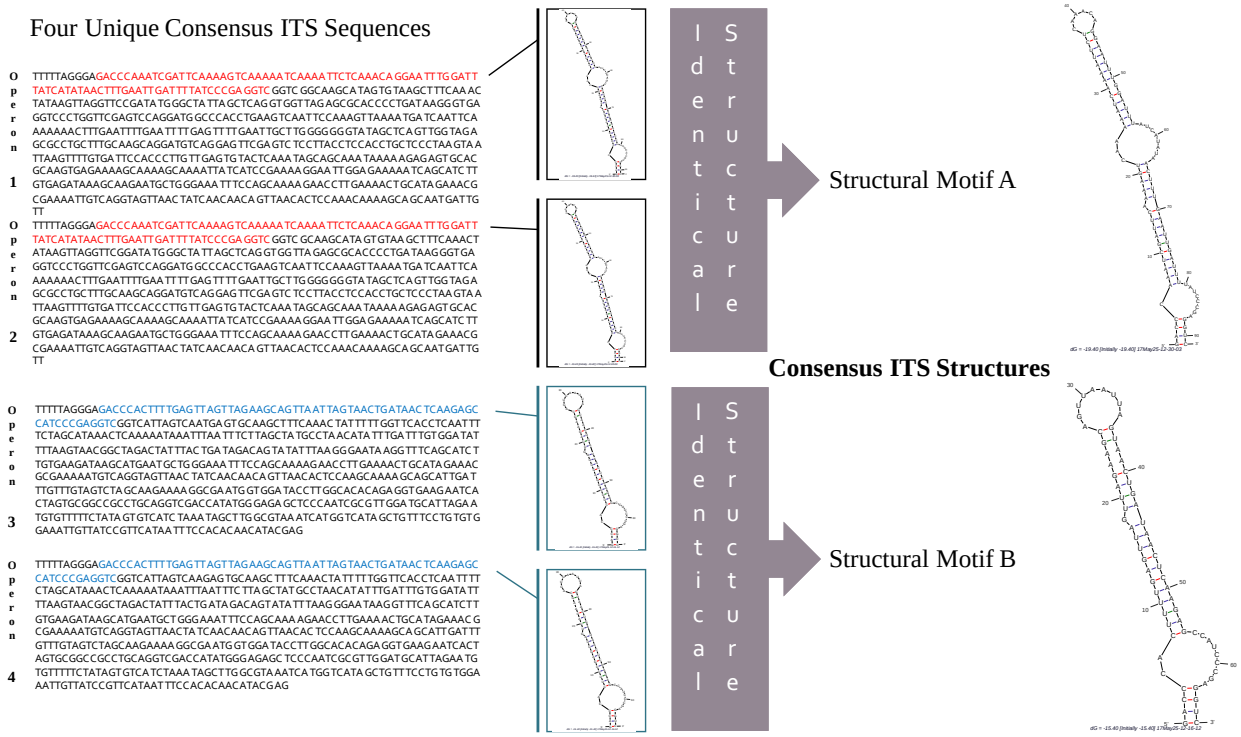

Figure 3.12 Consensus helical structural motifs identified from consensus multiple operons sequences.

All cloning data should be reported to Genbank and included in manuscripts to substantially increase the amount of clonal replicate data for cyanobacterial taxonomy. This data will eventually allow taxonomists to effectively discern evolutionary patterns of operon descent, and their extent of variation at the extragenomic and intragenomic level.

Fourth recommendation: Standardization of folding protocols is recommended per Boyer et al. (2001) and Johansen et al. (2005, 2011). First, sequence data should be coded for conserved ITS domains by hand in a word program. Identification the end of the 16S gene [5'-CCTCCTT-3'] and the beginning of the 23S gene [5'-GGTCAA-3'], with care taken to confirm the beginning of the 23S gene is 45 nucleotides downstream of the sequence [5'-CACACAGAG-3'], should precede annotation of ITS domains. All commonly conserved sequence domains both in the ITS or in the 16S-23S operon at large are variable to some degree and must be identified even if close

to the suggested sequence. Folding of the conserved helices in Mfold can proceed by copying and pasting helical sequences into the RNA folding form. On the RNA folding form find the STRUCTURE DRAW MODE and select ‘Untangle with loop fix’. Often several structures will be presented by Mfold for the same sequence, the structure with the most negative delta free energy has the highest likelihood of forming. Folded structures may not always reflect the actual helix that forms, and some sequences require the forcing of base pairs or preventing of bases from pairing to produce a correct structure. This requires a variable amount of trial and error, working with the structure in Mfold, and often result from knowing the typical helical motifs for the given helix being folded. For example, the D1-D1’ helix always has a basal unilateral clamp, almost always opening to the right of the helix, just above the basal clamp.

Occasionally, there is a unilateral bulge just opposite the right basal unilateral bulge, with one to three unpaired nucleotides, which may interfere with the structure of the right basal unilateral bulge, opening the helix up so that there is a bilateral bulge just above the basal clamp or forming and secondary helix extending from within the right basal unilateral bulge. Knowing that this structure is not typically correct and forcing a single base pair to form, corrects the entire helix structure (Figure 3.13). Folded structures should be presented in manuscripts in a standardized form. While some authors have access to expensive programs such as adobe illustrator, to present structures, many do not. Thus, downloading secondary structures in a picture format directly from Mfold website should also be considered a reasonable representation for publishing.

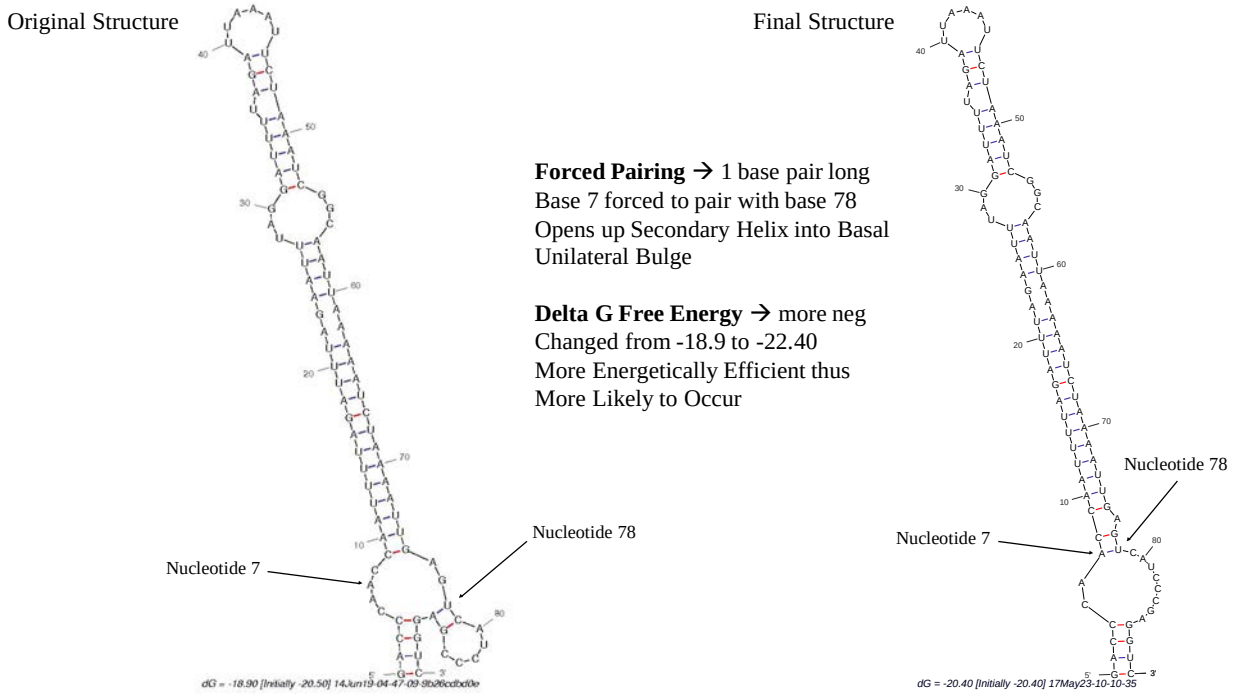

Figure 3.13 Forced pairing of a single base pair (nucleotides 7 and 78) to produce a D1-D1' helix more likely to occur (with more negative delta G free energy) and a more typical helix motif.

Conclusion

Cyanobacterial taxonomists are working to restructure over a century of systematics, using molecular data to compensate for morphological distinctions which are not taxonomically informative. The use of ITS secondary structures as apomorphies to distinguish between cryptic species has a great potential to solidify the systematic restructuring of the cyanobacteria. But, for this tool to have diverse applications, it needs to be standardized. Additionally, the possibility that multiple operons within individual cyanobacterial genomes are undergoing variable selective pressures, and thus evolving differentially, must be addressed in the collection and publishing of future ITS secondary structure data. The results of this study suggest that only secondary structure comparisons between species within individual genera may be informative. However, not accounting for the variable evolutionary patterns of multiple operons when making comparisons may also produce specious conclusions. Recommendations have been made to

standardize the process of producing secondary structures in the future so that both of these issues may be addressed. Further study is required to demonstrate to what extent ITS secondary structures statistically correlate with taxonomy.

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Conclusions

The goal of this research was to identify novel cyanobacterial taxa while clarifying enigmatic evolutionary relationships between cyanobacterial lineages. Four novel taxa were identified using a total evidence approach. Ecological, molecular, and morphological differences were used in concert to identify *Calothrix dumas*, *Brasilonema lichenoides*, *Brasilonema geniculosus*, and *Chroococcidiopsis lichenoides* sp. nov. Cyanobacterial systematics is undergoing a revolution owing to the advent of molecular data. Because much of traditional cyanobacterial morphology may not be informative, often the burden for identifying novel taxa rests on the molecular data. ITS secondary structures were also used to identify novel taxa because the 16S gene sequence, which is the standard for bacterial phylogenetic studies, lacks resolution at the species level in cyanobacteria. These secondary structures, however, are not analyzed in a standard fashion. Many genera within a family may produce similar secondary structures for the same domain, and it is important that ITS structures are only compared within a genus. Additionally, ITS domains that include two tRNA genes may have variable evolutionary histories from those without tRNA genes present. Future phylogenetic studies should compare structures of homologous domains. Further, it is imperative to clone samples in order to recover all possible operonic variants and compare only homologous (vs. analogous) structures. Recommendations have been made in this work to standardize folding procedures, data labelling, and the structural vocabulary used in analysis for ITS comparisons in the future. Further study is required to calculate quantifiably the relationship between ITS secondary structures and taxonomy.

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EDUCATION

2018 **M.S. Biology Candidate** (expected)
University of North Florida, Jacksonville, FL
Thesis: *Novel microaerophytic cyanobacteria isolated from a symbiotic, epilithic consortia identified with polyphasic approach, using molecular and morphological markers.*
Advisor: Dale Casamatta, Ph.D.

2015 **B.S. Biology**
University of North Florida, Jacksonville, FL
Molecular and Cellular Biology and Biotechnology
Minor: Anthropology

2011 **A.A. General Education**
Florida State College at Jacksonville, Jacksonville, FL

PUBLICATIONS

Peer-reviewed

Villanueva C.D., Hásler P., Dvořák P., Poulíčková A., & Casamatta D.A. 2018. *Brasilonema geniculosus* sp. nov. and *Calothrix dumas* sp. nov. (Cyanobacteria) two new taxa isolated from cemetery tombstones. *Phytotaxa* (In Review)

Villanueva, C.D., Hásler, P., Dvořák, P., Poulíčková, A., & Casamatta, D.A. 2018. *Brasilonema lichenoides* sp. nov. and *Chroococcidiopsis epilithica* sp. nov. (Cyanobacteria): Two novel cyanobacterial constituents isolated from a tripartite lichen of marble headstones. *J. Phycol.* 54:224-233.

Suradkar, A., **Villanueva, C**., Gaysina, L.A., Casamatta, D.A., Saraf, A., Dighe, G., Mergu, R., & Singh, P. 2017. *Nostoc thermotolerans* sp. nov., a soil dwelling species of *Nostoc* (Cyanobacteria) isolated from Madhya Pradesh, India. *Int. J. Syst. Evol. Microbiol*. 67:1296-1305.

In prep

Casamatta D.A., **Villanueva C.D**., Johansen J.R. 2018. *Reptodigitus chapmanii* (Stigeonematales, Cyanobacteria) gen. nov.: a unique Stigeonematalean (Cyanobacteria) genus based on a polyphasic approach. *J. Phycol.*

Villanueva, C.D., Johansen, J.R., Casamatta, D.A. 2018. Standardizing analysis of Internal Transcribed Spacer (ITS) regions in multiple 16S-23S operons: a new evolutionary review of ITS paralog patterns in Cyanobacteria. *Int. J. Syst. Evol. Microbiol.*

Brown, A.O., Foss, A., Garvey, A.D., Gibson, Q.A., **Villanueva, C.D**., & Casamatta, D.A. 2018. *Komárekiella delphikthonos* sp. nov. (Cyanobacteria): an epidermal cyanobacterium implicated in an estuarine bottlenose dolphin (*Tursiops trancatus*) fatality. *Harmful Algae*

Non-Peer-reviewed
Villanueva, C.D. 2015. 5 Reasons why no one is talking about the revolution going on in evolutionary science. *Gainsayer online magazine.* <http://www.gainsayer.me/5-reasons-why-no-one-is-talking-about-the-revolution-going-on-in-evolutionary-science/>

PROFESSIONAL EXPERIENCE

Teaching Experience

Graduate Teaching Assistant – Department of Biology, UNF **2016 – 2017**
- General Biology I – Laboratory
- General Biology II – Laboratory

Supplemental Instruction Leader – Academic Center for Excellence, UNF **2015 – 2016**
- General Biology I

Distance Learning Coach – Department of Biology, UNF **2014 – 2016**
- Biology in the Movies

Guest Lecturer – Department of Anthropology, UNF **2014**
- Anthropology of Death

Research Experience
Aeronautic sampling of cyanobacterial bioaerosols: Elucidating local cyanobacterial biogeography, and demonstrating microbial dispersal along the Atlantic coast of northeast FL and the St. Johns River basin (Jacksonville, USA)

Qualitatively and quantitatively establishing that highly conserved ITS 16S-23S rRNA domain secondary structures are taxonomically informative.

Exploratory study of lichen dominated microbial consortia growing on headstones for isolation and identification of novel microaerophytic cyanobacteria

PRESENTATIONS

Abstracts (as contributed posters/ oral presentations)
8. Villanueva, CD; Jeffrey R. Johansen; Dale A. Casamatta. 2017. *Quantitatively establishing that highly conserved ITS 16S-23S rRNA domain secondary structures are taxonomically informative at the species level*. In: PSA 2017 Annual Meeting – Monterey, CA

7. Villanueva, CD; Casamatta Dale A. 2017. *Brasilonema lichenoides* sp. nov. and *Chroococcidiopsis epilithica* sp. nov. *(Cyanobacteria): Two novel species, one tripartite lichen.* In: SOARS – University of North Florida, Jacksonville, FL

6. Villanueva, CD; Casamatta Dale A. 2016. *Is everything everywhere? An investigation into the Global Distribution Theory with subaerial cyanobacteria*. In: Southeastern Phycological Society Colloquy – Valdosta State University, Valdosta, GA

5. Villanueva, CD; Casamatta Dale A. 2016. *Novel endolithic and epilithic microaerophytic cyanobacteria isolated from cemetery headstones in northeastern Florida, USA*. In: 20th IAC Cyanophyte/Cyanobacteria Research Symposium – University of Innsbruck, Innsbruck, Austria

4. Villanueva, CD; Casamatta Dale A. 2016. *Assessing the application of the Internal Transcribed Spacer (ITS) region secondary structures to cyanobacterial systematics*. In: PSA Annual Meeting - John Carroll University, OH

3. Villanueva, CD; Casamatta Dale A. 2015. *Tolypothrix* sp. nov. And *Chroococcidiopsis epilithica* sp. nov. *(Cyanobacteria): Two novel cyanobacterial constituents isolated from a tripartite lichen of marble headstones.* In: PSA Annual Meeting – Drexel University, PA

2. Villanueva, CD; 2014. *A novel Chroococcidiopsis species (cyanobacteria) isolated from an endolithic lichen inhabiting headstones in Jacksonville Beach, FL, USA*. In: Southeastern Phycological Society Colloquy – UNC Wilmington, NC

1. Villanueva, CD. 2013. *Cemetery Preservation*. In: Department of Anthropology & Sociology Undergraduate Symposium – UNF, FL

GRANTS

Hoshaw Travel Grant $1000 - Phycological Society of America **2017**

Summer Graduate Research Grant $1000 - Department of Biology, University of North Florida **2017**
"Aeronautic sampling of cyanobacterial bioaerosols demonstrate microbial dispersal along the Atlantic coast of northeast FL and St. Johns River basin (Jacksonville, USA)"

Coastal Biology Research grant $500 - COAS, University of North Florida **2017**
"Exploratory study identifying novel symbiotic cyanobacteria from coastal habitats"

Graduate Research Grant $500 - Department of Biology, University of North Florida **2016**

Hoshaw Travel Grant $750 - Phycological Society of America **2016**

Hoshaw Travel Grant $450 - Phycological Society of America **2015**

Undergraduate Research Grant of $1000 - Department of Undergraduate Student Research, UNF **2014**
"Typifying epilithic lichen communities"

AWARDS

Department of Anthropology & Sociology Undergraduate Student Symposium, UNF **2013**

PROFESSIONAL AFFILIATIONS

Phycological Society of America (Student)

LANGUAGES

Native: English Studied: Latin