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Next-generation sequencing, morphology, and culture-based methods reveal diverse algal assemblages throughout the Florida springs

Alyssa Garvey
University of North Florida, n01177173@unf.edu

NEXT-GENERATION SEQUENCING, MORPHOLOGY, AND CULTURE-BASED METHODS REVEAL DIVERSE ALGAL ASSEMBLAGES THROUGHOUT THE FLORIDA SPRINGS

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Alyssa Desiree Garvey

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The thesis “Next-generation sequencing, morphology, and culture-based methods reveal diverse algal assemblages throughout the Florida springs” submitted by Alyssa Desiree Garvey

Approved by the thesis committee: Date:

_____ _____

Dale Casamatta
Committee Chair Person

_____ _____

Dr. Kelly Smith

_____ _____

Dr. Eric Johnson

Accepted for the Department:

_____ _____

Dr. Cliff Ross
Department Chair

Accepted for the College of Arts and Sciences:

_____ _____

Dr. George Rainbolt
Dean

Accepted for the University:

_____ _____

Dr. John Kantner
Dean of Graduate School

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Abstract

Algae are a group of highly diverse photosynthetic organisms found in variety of habitats. As the primary energy base in ecosystems, knowledge of the diversity and presence of certain algal lineages is paramount to our understanding of the trophic state of aquatic habitats. In recent years, the state of Florida has seen an increase of both marine and freshwater algal blooms. Similarly, filamentous algae have begun outcompeting vascular macrophytes throughout many of Florida's springs as nutrient enrichment from anthropogenic sources increases. Traditionally, the Florida algal spring communities have been assessed using classic morphological methods, which may underrepresent the true biodiversity present. Therefore, the goal of this study was to conduct a more complete diversity assessment implementing next-generation sequencing techniques (NGS) with morphological analyses and culturing methods. While morphological methods identified a wide variety of algal taxa, belonging to 4 phyla (Bacillariophyta, Charophyta, Chlorophyta, and Cyanobacteria), next-generation sequencing techniques provided greater detail of the diatom community. This is particularly important as many diatom taxa are used as indicators of water quality. We noted discrepancies between these two methods, highlighting how NGS techniques may complement the use of morphological analyses when analyzing algal diversity in this system. Culturing methods also revealed the presence of two taxa new to science (*Nodosilinea fontis* and *Brasilonema variegatus*), indicating these springs may represent a potential source of novel cyanobacteria. Taken together, this study showcases Florida springs are rich in algal diversity and a combination of methods is required for more complete biodiversity assessments. Future studies implementing such methods will aid in the preservation and conservation of these ecosystems.

Introduction

Long term monitoring of the composition of biological communities (*i.e.*, biomonitoring) has been essential in providing data for insights into biogeography, conservation, climate change, and water quality assessments. The use of biomonitoring in freshwater systems first began in 1908 by Kolkwitz and Marsson, revolutionizing the concept of using the presence and/or absence of sentinel species (*i.e.*, biological indicators) as indirect indicators for levels of pollution. As the field of biomonitoring progressed, it was noted that biological community composition provides greater insight to environmental conditions over time, rather than measuring individual stressors or ecosystem attributes (Schoolmaster *et al.* 2013). Factors which affect the abundance and/or alpha and beta diversity can be measured by targeting indicator species (Day 2008). Alpha diversity represents the total number of species within a given community and beta diversity can be defined as the extent of species replacement or biotic change along environmental gradients (Whittaker 1972). Beta diversity is a key component to understanding the conservation of biodiversity, ecosystem management, and functioning of ecosystems. In aquatic systems, the key sentinels of ecosystem health and functionality are typically members of the algal community.

Algae are a diverse assemblage of lineages that span four of the six major eukaryotic supergroups (Hampl *et al.* 2009) and a phylum of Bacteria (Cyanobacteria). Together, they constitute approximately 10 phyla across two domains within the Tree of Life. While not a monophyletic group, the term “algae” is used to describe both oxygenic-photosynthetic prokaryotes (cyanobacteria) and eukaryotes (e.g., chlorophytes, eustigmatophytes, rhodophytes) found in freshwater, estuarine, marine, and terrestrial habitats. As algae are found in virtually every aquatic environment, comparisons of species diversity and respective densities have been used in assessments of water quality, urbanization, and climate change (Wehr *et al.* 2015).

Further, taxonomy based on morphological features has been well established, especially with freshwater diatoms, which have been the standard indicators of water quality in Europe for the last 30 years (Lowe 1974). There is also a growing body of reference molecular data for both cyanobacteria and eukaryotic algae. Thus, due to the robustness of taxonomic databases, algae provide good study organisms with which to conduct biological surveys.

In aquatic systems, algae are some of the most species-rich groups (Meyer 2007) and have several advantages over other groups of organisms to be used as bioindicators. First, algae are mostly sessile or have limited movement, and thus cannot migrate to avoid potential pollution. Second, short- and long-term shifts in environmental conditions can be observed due to the short generation times for many taxa (i.e., a few hours for unicellular species to several days for multicellular, filamentous and colonial soft-bodied algae to decades for crustose marine taxa) (Jarlan *et al.* 1996; Whitton 2012). Third, benthic algal communities are typically species-rich and exhibit species-specific environmental tolerances, so small sample sizes provide a wealth of information (Lowe & Pan 1996). Because algae are located at the center of the food web, they are better suited for local-scale or upstream-downstream studies and are better suited for studies examining water chemistry and how land use alters water quality (Johnson *et al.* 2006; Resh 2008). Lastly, when examining intermittent streams in more arid areas, algae may be the only indicator of water quality due to high desiccation tolerances of many species.

Specific algal groups, such as cyanobacteria, green algae, and diatoms, have been shown to be useful general biological indicators of ecosystem stress. For example, the presence of some cyanobacterial taxa, such as *Microcystis*, have often been linked to increased phosphorus loading caused by anthropogenic pollution (Michalak *et al.* 2013). Increased nutrient levels have also been linked to macroalgal blooms of the chlorophyte *Ulva* in Italy (Viaroli *et al.* 2005),

throughout Narragansett Bay in Rhode Island (Guidone & Thornber 2013), and the Yellow Sea in China (Huo *et al.* 2013). As silicate levels increased, blooms of the diatom *Pseudo-nitzschia australis* were observed in Monterey Bay, where they produced considerable amounts of the neurotoxin domoic acid, which was later implicated in health decline and mortality of fish and marine mammal species (Scholin *et al.* 2000). On the other hand, different algal genera from these three major groups have also been found to be indicative of oligotrophic conditions (Lowe 1974).

In order to articulate the algal community structure and taxa present in aquatic environments, morphological methods have traditionally been employed (Almeida *et al.* 2014). These methods were first pioneered with diatoms, a clade whose siliceous cell walls (frustules) provide a clear mechanism for identification. This led to many places, such as Germany and France, exclusively employing diatoms as indicators of water quality. However, recent analyses have shown that diatoms may not be a comprehensive diagnostic for water quality (Tan *et al.* 2017). First, some diatoms exhibit seasonal periodicity (phenology), and thus may not always be present in the system. Second, recent molecular analyses have revealed significant cryptic diversity in the lineage, thus calling into question the taxonomic validity of sentinel species (Lundholm *et al.* 2012). Third, identifying diatoms down to species level requires both significant taxonomic expertise and laborious processing of frustules, thus rendering this technique impractical for many researchers (Barbour *et al.* 1999). As such, recent focus has shifted to more of a community level approach for articulating nutrient status using algae in aquatic systems, highlighting the need for examination of a variety of algal genera (Stevenson 2014).

Relying solely on morphological methods poses an issue as these methods potentially underrepresent true diversity within a given habitat. Many algal genera contain cryptic members, making it difficult to identify species solely based on morphology (Casamatta *et al*. 2003). Another limitation to morphological analyses is the need for highly trained taxonomists, with concurrent time and financial costs. Due to these limitations, we propose a new method for monitoring algal communities within a major Floridian fresh water system, such as karst springs, using a multi-marker metabarcoding approach. Metabarcoding is a fast and cost-effective method used for measuring diversity (Janzen *et al.* 2005; Bourlat *et al.* 2013; Ji *et al.* 2013; de Vargas *et al.* 2015) that combines two technologies: high-throughput sequencing and DNA taxonomy. By using a PCR based method with universal primers, this method allows scientists to mass amplify taxonomically informative genes from a large collection of organisms or environmental DNA. Due to the high levels of cryptic diversity exhibited by many algal taxa, this approach could provide greater insight into the algal communities of the karst springs than morphology alone.

Karst springs are composed of carbonate rocks and occur within a geologically distinct topography where a collection of habitats can be found including springs, sinkholes, and subterranean drainages (Bowles & Arsuffi 1993; Hubbs 1995; Springer & Stevens 2009; Glazier 2009). Many springs exhibit stable temperature and water chemistry and are known to contain a diverse array of organisms, from bacteria to manatees (Ferrington 1995; Lydeard & Mayden 1995; Mattson *et al.* 1995; Andersen 2002; Garcia-Pichel *et al.* 2002; Martiny *et al.* 2011). Due to their stable nature and isolation, springs are hotspots of biological diversity, as well as targets for conservation (Blinn & Prescott 1976; Breitbart *et al.* 2009; Brodie *et al.* 2009; Cantonati & Lange-Bertalot 2010), and have been termed “Imperiled Arks of Biodiversity” (Clements *et al.* 2006). Freshwater springs are increasingly altered by anthropogenic impacts, such as water

removal, agricultural run-off, climate change, and urbanization (Bowles & Arsuffi 1993; Lydeard & Mayden 1995; Erman 2002; Katz 2004; Kløve *et al*. 2014), making them critically threatened freshwater ecosystems.

There are currently over 1,000 karst springs throughout the state of Florida, which provide a significant source of freshwater, create unique habitats for rare taxa, and are economically important (Florida Springs Institute 2019). However, anthropogenic pollution during the past century has severely impacted these fragile ecosystems (Pacheco & Cabrera 1997; White 1988; Wells & Krothe 1989; Molerio & Gutierrez 1999; Katz *et al*. 2001; Veni *et al*. 2001). This has led to both eutrophication and concurrent proliferation of invasive aquatic vegetation (Florida Springs Task Force 2000). Some springs have also been found to contain toxic strains of algae, potentially posing a risk to human health (Foss *et al.* 2012).

In recent decades, blooms of algae have become common features of many aquatic ecosystems throughout the state of Florida. Population growth, coupled with subtropical temperatures, and eutrophication of Florida's lakes, rivers, and estuaries, are linked to the increase in frequency and duration of harmful algal blooms (HABs; e.g., Paerl & Otten 2013). These blooms pose threats to human and animal health, as well as to water quality, ecosystem stability, and surface drinking water supplies. Many of Florida's largest and most important aquatic habitats have been increasingly impacted by HABs, including, but not limited to, Lake Okeechobee (Havens *et al*., 1996), the St. Johns River (SJRWMD 2001), the St. Lucie River (FFWCC 2005), and the Caloosahatchee River (Gilbert *et al.* 2006). It is also now common to the find nuisance bloom forming cyanobacteria "*Lyngbya*" present in many Florida springs and along coastal coral reefs (e.g., Foss *et al.* 2012; Engene *et al.* 2013).

This study proposes to examine algal community diversity throughout some representative Florida springs in order to gain insight into the overall health of these environments. Preliminary results from this study assessing algae from Ichetucknee Springs has shown high diversity, but a potential methodological bias. For example, morphological analyses have shown a variety of cyanobacteria, diatoms, and green algae present; however, culturing has only recovered a small subset of that diversity. Thus, a more robust technique is needed, such as the use of a metabarcode analysis using next-generation sequencing. Previous studies have documented the use of metabarcoding in diversity assessments from a variety of environments (Stein *et al.* 2014; Fahner *et al.* 2016; Lanzen *et al.* 2016; Andrukiewicz *et al.* 2017; Groendahl *et al.* 2017; Stat *et al.* 2017), highlighting how this method can be applied to a myriad of habitats employing a diversity of sentinel taxa. By conducting a diversity assessment of some representative Florida springs, a community "baseline" can be established and used for future monitoring of the springs as they face increasing anthropogenic impact. Understanding the algal communities prior to significant anthropogenic disturbance and ecosystem disruption is critical for protection and preservation.

Chapter 1

Multi-marker metabarcoding complements morphological methods for assessing algal diversity in the Florida springs

ALYSSA D. GARVEY[1], ALEXANDER BESUDEN[1], DALE A. CASAMATTA[1]*

[1] *Department of Biology, University of North Florida, Jacksonville, Florida, 32224, USA*

[*] *Author for correspondence: dcasamat@unf.edu, phone: 904-620-1936, fax: 904-620-3885*

Abstract

Karst springs provide a unique environment from which to study biodiversity given their relatively stable habitats. In Florida, springs are economically and culturally significant, even as these systems are presenting evidence of anthropogenic impacts (e.g., alterations to water flow, nitrate contamination from surrounding land use, etc.). Biodiversity assessments of algal communities (both cyanobacteria and eukaryotic) of Florida springs have relied on traditional morphological analyses alone, thus potentially grossly underestimating the diversity present. In this study, we employed a polyphasic approach to elucidate the algal communities from three "baseline" springs: Ichetucknee, Blue Spring, and Silver Glen. Periphytic algal samples were collected from each spring during fall of 2018. Previous studies have shown that these springs are dominated by cyanobacteria, green algae, and diatoms, thus primers were chosen accordingly. Results from microscopy and metabarcoding via the Illumina MiSeq platform (employing 16S rDNA, *rbc*L, and 18S rDNA primers) revealed that each method individually was not sufficient in capturing the diversity present. For example, morphological analyses underestimated diatom diversity, whereas metabarcoding using the *rbc*L marker showed

substantially more taxa present (13 genera vs. 47, respectively). On the other hand, morphological analyses provided greater taxonomic resolution for the cyanobacteria and green algae present compared to the 16S and 18S rDNA markers. These results suggest metabarcoding may complement the use of morphological analyses. Further, we detected significant differences in algal communities from the sampled springs. For example, Ichetucknee Spring had higher Shannon index values compared to either Blue Spring or Silver Glen. This may have been potentially correlated with lower nutrient concentrations, based on an analysis of various water quality parameters from 2014-2018. Based on these results, we recommend that springs throughout Florida be monitored with combined morphological and molecular techniques to more comprehensively assess biodiversity with on-going and future anthropogenic alterations.

Introduction

North Florida has the largest concentration of first magnitude springs in the world (Scott *et al.* 2008), with more than 1,000 karst springs dependent on groundwater from the Floridan Aquifer System. These systems represent important sites for studying trophic dynamics and ecosystem energetics (e.g., Odum 1956, 1957) and provide significant economic value to the state of Florida (Bonn 2004). However, many Florida springs have seen increased degradation over time due to excessive agricultural, urban, and industrial development, thus leading to increased nitrate contamination and declined flow rates. During the past 40-50 years, nitrate-N concentrations have steadily increased in many spring basins (Upchurch *et al*. 2007). Potential sources of nitrate contamination within spring waters includes fertilizer, atmospheric deposition, livestock, septic systems, and land application of municipal wastewater and biosolids. Along with nitrate contamination, increased human populations throughout the state of Florida have led

to more than 100 million visitors each year, which in turn has severely impacted water quality throughout Florida's springs.

Water quality impairments, such as those caused by nutrient enrichment, can cause an increase in epiphytic algae or benthic algal mats, which in turn may outcompete macrophytes (Scheffer *et al.* 2001). Throughout the world, freshwater and marine habitats are increasingly threatened by proliferations of nuisance algae (Carpenter *et al.* 1998; Lembi 2003). Spring runs in Florida have historically supported dense beds of submerged vascular macrophytes, such as *Sagittaria kurziana* and *Vallisneria americana*; however, filamentous macrolagae have begun to outcompete these macrophytes. A survey of 21 Florida springs indicated that, on average, 50% of all spring bottoms were covered in nuisance macroalgae (Stevenson *et al.* 2007). During this survey, 23 different algal taxa were observed in the springs, with *Lyngbya wollei* and *Vaucheria* spp. being the two most common taxa, occurring in extensive growths. The presence of *L. wollei* is of particular concern, as strains isolated from Blue Hole Spring (Ichetucknee Springs State Park) and Silver Glen Springs have been found to be a potential source of paralytic shellfish toxins (Foss *et al.* 2012).

Studies examining algal communities throughout Florida's springs have relied mainly on morphological based methods (e.g., Phelps *et al.* 2006; Stevenson *et al.* 2007; Quinlan *et al.* 2007), which potentially underestimates the diversity present. Many algal taxa exhibit high levels of cryptic diversity, lack taxonomically informative characters, and contain phenotypic plasticity (Komárek *et al.* 2014). Coupled with the need for highly trained taxonomist and the time-consuming tasks of morphological identifications, a more efficacious approach is needed. To overcome these limitations and challenges, the possibility of using next-generation sequencing

techniques provides a unique opportunity to more accurately assess biological diversity (Marcelino & Verbuggen 2016).

Metabarcoding combines the use of DNA taxonomy with high-throughput sequencing to rapidly assess biodiversity of a given habitat (Janzen *et al*. 2005; Bourlat *et al.* 2013; Ji *et al.* 2013; de Vargas *et al.* 2015). Because this method is PCR-based, universal primers can be used to mass amplify taxonomically informative genes from a large collection of organisms, as well as environmental DNA (eDNA). Along with universal primers, group-specific primers with higher phylogenetic resolution may be used to address organisms containing cryptic species (i.e., diatoms). By using a multi-marker strategy (combining universal and group-specific primers), studies have been able to further elucidate biodiversity in a variety of systems, such as coral reefs and cave biofilms (Marcelino & Verbruggen, 2016; Pfendler *et al.* 2018).

Here we present data collected from a study investigating the potential use of DNA metabarcoding for examining algal community composition from the Florida springs. Due to the high degree of cryptic diversity in various algal genera, we suggest combining traditional morphological analyses with a multi-marker metabarcoding approach. In doing so, we were able to gather valuable information regarding algal species richness, species abundance, and biodiversity measurements. This study is the first to combine morphology-based methods with next-generation sequencing for assessing algal communities from the Florida springs. While we noticed discrepancies between both methodologies, taken together these results highlight how metabarcoding methods may complement morphological analyses. This study only focused on three representative springs, but results showcase how NGS may help provide more comprehensive biodiversity assessments throughout the springs as they face increasing

anthropogenic alterations. More complete biodiversity assessments will facilitate preservation/restoration planning for these fragile ecosystems.

Methods

Sampling site descriptions

Three springs were selected for inclusion in the study: Blue Spring, Ichetucknee Spring, and Silver Glen Spring. Blue Spring is in east-central Florida just north of Orlando and is a tributary to the Middle St. Johns River. This spring is within the Blue Spring State Park and provides various recreational opportunities for visitors, as well as critical habitat for the threatened West Indian manatee (*Trichechus manatus*) and various endemic snail species. Blue Spring is a 1st magnitude spring (flow rate >100 cfs), but has seen significant declines in flow rate since 1980 due to increasing groundwater pumping. Intensive human use, coupled with high manatee populations, has likely contributed to an increase in filamentous algae and a decrease in submerged aquatic vegetation within the spring run.

Ichetucknee Springs encompasses the southern two-thirds of Columbia County in north-central Florida in the Suwannee River Water Management District. This is a 1st magnitude spring (flow rate >100 cfs) comprised of nine named springs that discharge into the spring-fed Ichetucknee River, which flows into the Santa Fe River, eventually ending in the Gulf of Mexico. Visitors of this spring enjoy a variety of recreational activities in the main spring run; however, activities such as swimming, snorkeling, and diving are limited to the Ichetucknee Head Spring. This area of Ichetucknee has seen increasing nitrate levels as groundwater nitrate concentrations have steadily increased in the Ichetucknee Springshed since the 1960s.

Silver Glen Springs is located in Marion County, Florida within the Ocala National Forest. Maintained by the United States Department of Agriculture (USDA) Forest Service, Silver Glen Spring and the spring run flow into Lake George, the second largest lake in Florida. The spring run is comprised of two vents: the eastern spring vent, which is a first magnitude spring, and the southwest vent, which is a 2nd magnitude spring (flow rates 10-100 cfs). The eastern spring vent is frequented by swimmers and used for various recreational purposes. Similar to Blue Spring, Silver Glen also provides a refuge for manatees; however, manatee use has been limited due to damage to aquatic vegetation and increased human activity.

Collection

Periphytic algae were collected from microhabitats in each spring by establishing transects then collecting samples from randomly determined (e.g. rolling dice) locations along the transect. Attached forms were removed from surfaces using syringes, forceps, and toothbrushes (Cantonati, et al., 2007; O'Brien and Wehr, 2010; Wehr, 2013) then placed in 50 mL Falcon tubes containing 20mL of sterile RNase free water. Samples were kept on ice until returning to the laboratory, where they were pooled together based on sampling location and homogenized (centrifuged at 6,500g for 10 min). Each homogenized sample was further subdivided into three samples to be used for DNA extractions, morphological identifications, and voucher specimens. Subsamples for DNA extractions were kept frozen (-20 °C) and voucher specimens were preserved in 5% glutaraldehyde in diH_2O. Samples for morphological identifications were kept at 4 °C.

Morphological identifications

Provisional identifications and enumeration were made from fresh algae using an Olympus BX51 Brightfield/Phase Compound Microscope (Olympus Corporation, Tokyo, Japan) based on well-documented morphological characters. A minimum of 400 cells were counted in each sample. Enumerations were done using 1) 400 cell count, 2) three sweep scans, and 3) three half slide scans (Beaver *et al.* 2018). Algae were identified to smallest taxonomic unit possible using taxonomical keys (e.g. Schneider & Searles 1991; Cox 1996; Komárek & Anagnostidis 1999; Hindák 2008). Species abundance (# of cells) and relative abundance (%) was determined for each sampling location. Rarefaction curves were calculated to determine enough cells were counted to assess the diversity within a given sample.

DNA extraction and library preparation

A total of six samples were extracted from each collection site using a DNeasy PowerSoil Extraction Kit (Qiagen, Hilden, Germany). Extraction replicates were completed for one spring at a time, with a negative control included for each subset of extractions (autoclaved nanopure H_2O). The negative control was subjected to the same procedures as all samples and later used in the sequence analysis to account for laboratory contamination in order to be removed from the data set.

To prepare samples for Illumina amplicon sequencing, we employed a two-step PCR approach. The initial PCR was completed in order to amplify the three barcode markers in individual reactions using specific primers with the attached Illumina adapter. The three gene regions of interest were the 16S rDNA gene for cyanobacteria (Klindworth *et al.* 2013), 18S rDNA for green algae (Groendahl *et al.* 2017), and *rbc*L for diatoms (Rimet *et al.* 2018). The primary PCR amplification was completed in 25 μl reactions using 12.5 μl of Q5 High-Fidelity

2X Master Mix (New England BioLabs Inc., Ipswich, MA, USA), 1.0 µl of each primer (1µM), 9.5 µl RNase-free H_2O, and 1 µl eDNA. Thermocycler parameters were tailored for the individual primer sets and can be found in Table 1. Following initial PCR amplification, samples were cleaned using Agencourt AmPure XP beads (Beckman Coulter Inc., Indianapolis, IN, USA) and quantified using a Qubit Fluorometer (v.2.0; ThermoFisher Scientific, Waltham, MA, USA). Samples were then normalized and pooled in equimolar ratios based on sampling location.

A second PCR reaction (50 µl) was performed to apply the indexing primers and enriched with Q5 High-Fidelity 2X Master Mix with the following cycling conditions: 95°C for 3 min followed by 10 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, with a final extension of 72°C for 5 min, modified from the 16S protocol (Illumina 2013). A second PCR clean-up was performed and samples were quantified using a Qubit Fluorometer. Prior to sequencing, samples were checked for quality control by the genomics facility at the University of Florida (Gainesville, FL, USA), normalized for DNA concentration, and pooled. Libraries were diluted to 5 pM concentrations and loaded with 25% PhiX clustering control on the Illumina MiSeq platform for 300 b.p. x 2 paired end reads using the V3 kit.

Bioinformatics pipeline

Demultiplexing and adapter removal was performed by the genomics facility at UF, which provided a single fastq file for forward and reverse reads for each sample. DNA reads were parsed by gene and primers were trimmed in python (version 2.7.16) using separate_genes.py (Marcelino & Verbuggen 2016). Reads were quality filtered based on Q30 score and trimmed to remove low quality reads in Qiime2 (Caporaso *et al.* 2010, version 2019.4, https://docs.qiime2.org/2019.4/). Trimmed and filtered reads were denoised and dereplicated

using DADA2 (Callahan *et al.* 2016) to produce Amplicon Sequence Variants (ASVs). ASVs are OTUs which have been assessed based on 100% similarity, as opposed to 97% for traditional OTU clustering. ASVs will be referred to as OTUs throughout the study. OTUs present in the negative control were filtered out of the samples, and singletons and doubletons (OTUs with only or two reads assigned) were also removed from the dataset. The SILVA database (release 132, Quast *et al.* 2013) was employed to assign taxonomy to 16S and 18S OTUs. For *rbc*L OTUs, taxonomy was assigned using the R-Syst::diatom database (Rimet *et al.* 2016, database version v7, https://www.rsyst.infra.fr/en). Reference taxonomy databases were trimmed to contain only the region of the gene amplified by the primers used in this study. A machine learning naïve Bayes classifier was trained and used to assign taxonomy (confidence = 0.7, classify-sklearn: Qiime 2). OTUs identified as chloroplast and/or mitochondria in the 16S dataset after assigning taxonomy were removed, as these are not prokaryotic sequences. Venn diagrams (bioinformatics.psb.ugent.be/webtools/Venn/, 6/12/2019).
were constructed to visualize the number of shared and unique OTUs for each marker in the three springs (Fig. 3).

Statistical analysis

Alpha level diversity was assessed using the Shannon diversity index to account for abundance and evenness of OTUs for each gene marker (Table 3). Shannon index was calculated separately for algal only OTUs in the 16S dataset. A Kruskal-Wallis pairwise test was used after samples were pooled to determine significant differences between Shannon index values (Qiime2, Caporaso *et al.* 2010, version 2019.4). Shannon index was plotted for 16S and *rbc*L markers based on sampling location (Fig. 4). Rarefaction curves were generated in Qiime2

(Caporaso *et al.* 2010, version 2019.4) for each genetic marker to visualize the rate at which additional reads increased number of OTUs and to ensure sequence depth was adequate for each spring (Figs 5-7). Relative abundance and OTU richness of taxa (phylum for 16S and genera for *rbc*L) per sample (combined replicates within a spring) was calculated for each marker (Tables 3 and 5).

In order to determine potential differences in water quality parameters for each spring, data were downloaded from for the Suwannee River Water Management District (http://www.mysuwanneeriver.com/507/Water-Data-Portal) for Ichetucknee Springs and from the St. Johns River Water Management District (https://www.sjrwmd.com/data/water-quality/) for Blue Springs and Silver Glen Springs. Environmental parameters containing data for all three sampling locations during a five-year time period (2014-2018) including dissolved oxygen, ammonium (NH_4), total Kjeldahl nitrogen (TKN), phosphates (PO_4), total phosphorus, water temperature, and pH were analyzed. Analysis of Variance (ANOVA) and Kruskal-Wallis Rank Sum Test (IBM SPSS Statistics for Macintosh, Version 25.0) were calculated to determine environmental parameters that were significantly different among the three springs.

Results

Morphological analysis

For the compilation of taxa lists, a minimum of 400 cells were counted. Counts were then grouped together based on phylum for each sampling location. A total of 23 infra-generic taxa were identified belonging to four phyla: Bacillariophyta (diatoms), Charophyta (green algae), Chlorophyta (green algae), and Cyanobacteria (Table 2). Among the Bacillariophyta three taxa were identified to species level and five cyanobacteria were identified to species level. Only a

few diatoms were identified in all three springs: *Achnanthidium*, *Gomphonema*, *Navicula,* and *Synedra*. Ichetucknee had the greatest overall algal abundance (Fig. 1), and various taxa were unique to this location (i.e., *Synedra delicatissima, Tabellaria, Scenedesmus, Jaaginema, Leptolyngbya* sp., *Lyngbya* cf. *martensina,* various *Oscillatoria* species, and *Tolypothrix* sp.). Silver Glen had the next highest algal abundance, which was largely composed of diatoms and Cyanobacteria, with low green algal abundance (Fig. 1). Silver Glen and Ichetucknee had similar algal assemblages compared to Blue Spring (Fig. 1). *Cymatopleura solea* and *Gyrosigma* were the only algae unique to Silver Glen. Blue Spring was composed of diatoms and green algae only, no cyanobacteria were identified. *Closterium* and *Aulacoseira* were unique to Blue Spring. Rarefaction curves calculated per site showed all samples plateaued at varying counts, indicating enough cells were scored (Fig. 2).

Illumina data analysis

The Illumina MiSeq amplicon sequencing produced a total of 43,171,590 paired end (60,198,928 single) reads and after reads were quality filtered and denoised, a total of 4,816,949 reads remained. Removing chimeric sequences further reduced the dataset to 4,110,472 reads. Of those reads, 701,407 remained in the 16S dataset, 3,408,254 remained the *rbc*L dataset, and 811 in the 18S dataset. Filtered reads were assigned to 2,118 16S OTUs, 25 18S OTUs, and 397 *rbc*L OTUs. Rarefaction curves calculated per site (combined replicates) for 16S and *rbc*L showed OTUs plateauing, indicating enough reads were sequenced for each site (Figs 4 and 6). OTUs did not plateau for Blue Spring in the 18S dataset, indicating not enough reads were sequenced for this marker (Fig. 5).

Analysis of all OTUs

All OTUs for each genetic marker, algal and non-algal, were assigned phyla level taxonomy and the *rbc*L dataset was assigned taxonomy to the genera level. Reads in the 18S dataset could not be identified below kingdom level and were not included for further diversity analysis. Any reads present in the 16S and *rbc*L datasets that could not be identified to the phylum level were also not included for further analysis. In order to compare diversity among and between the three springs, relative abundance was calculated for each marker dataset.

For the 16S dataset, the most abundant bacterial phyla varied based on sampling location (Figure 7, Table 4). For Blue Spring, the Bacteriodetes was the most abundant phyla (47.7%), followed by the Proteobacteria (36.2%), and the Verrucomicrobia (10.6%). Various other phyla were abundant throughout this spring, with Cyanobacteria only comprising 1.3% of the dataset. Ichetucknee Springs was dominated by the Proteobacteria (52.4%), with Cyanobacteria being the next most abundant (20.3%), followed by the Bacteriodetes (15.2%) and various other phyla at lower abundances (<3%). The bacterial community of Silver Glen closely resembled that of Ichetucknee with the Proteobacteria, Bacteriodetes, and Cyanobacteria being the most abundant phyla, but with different abundances. The Cyanobacteria were the most abundant (36.6%), followed by the Proteobacteria (34.8%), and then the Bacteriodetes (21.3%). Analysis of OTUs showed Silver Glen having the greatest number of unique OTUs (908) (Fig. 3, Table 5). Silver Glen and Ichetucknee shared more OTUs than with Blue Spring, and only 13 OTUs were shared among all three springs. The 16S dataset was also examined for Cyanobacteria-specific OTUs, the only prokaryotic algal phylum. A total of 62 individual cyanobacterial OTUs were present in the dataset: 7 OTUs in Blue Spring, 33 OTUs in Ichetucknee, and 46 OTUs in Silver Glen.

A wide range of diatom genera were identified using the *rbc*L marker and a total of 397 individual OTUs were present: 158 OTUs in Blue Spring, 172 OTUs in Ichetucknee, and 152 OTUs in Silver Glen (Table 6). In total, only 40 OTUs were shared between all three springs (Fig. 3). Ichetucknee and Silver Glen shared more OTUs (35) than Ichetucknee or Silver Glen shared with Blue Spring (32 and 28, respectively) (Fig. 3). Specificity of the *rbc*L marker allowed for diatom OTUs to be identified to the genus level with reads belonging to 47 genera. Overall, Blue Spring and Ichetucknee had 32 diatom genera present, whereas Silver Glen had 36 diatom genera present. Even though Blue Spring and Ichetucknee had the same number of diatom genera present, they differed in composition and the overall relative abundance of diatom genera varied greatly depending on location (Fig. 8, Table 5). Blue Spring was mostly dominated by 3 genera in descending order: *Pleurosira*, *Diadesmis*, and *Terpsinoe* (Fig 8A). Ichetucknee had a greater diversity of diatom genera, leading to higher abundances for more taxa. Of those, four genera had abundances greater than 10%: *Navicula*, *Encyonopsis*, *Staurosira*, and *Cymbella* (Fig. 8B). Silver Glen was mostly dominated by only 2 genera: *Nitzschia* and *Pleurosira* (Fig. 8C).

Alpha diversity

In addition to relative abundance, alpha diversity was investigated using the Shannon diversity index, which was analyzed for each dataset based on location (Fig. 9, Table 7). Shannon index for 16S data (algal and non-algal OTUs) showed significantly higher diversity in Ichetucknee compared with Blue Spring, but no significant difference between Ichetucknee and Silver Glen. For algal only OTUs (Cyanobacteria), the Shannon index was highest in Ichetucknee and lowest in Blue Spring, but there was no significant difference based on location.

For the *rbc*L marker, Shannon diversity was significantly ($P < 0.05$) higher for Ichetucknee compared to Blue Spring and Silver Glen, and Blue Spring and Silver Glen were also significantly ($P < 0.05$) different.

Comparison of light microscopy and metabarcoding analyses

The relative abundance of algal phyla or taxa identified varied based on the method used (Figs 10-11). In the 16S dataset, light microscopy identified a greater relative abundance of Cyanobacteria than did metabarcoding for Ichetucknee and Silver Glen. However, the opposite was true for Blue Spring and metabarcoding identified Cyanobacteria OTUs whereas light microscopy did not detect any Cyanobacteria (Fig. 10). In general, metabarcoding identified a greater number of diatom genera than did light microscopy (Table 8, Fig. 11). With the exception of *Cymatopleura, Gyrosigma,* and *Synedra* all diatom genera identified using light microscopy were also found in the *rbc*L dataset. Metabarcoding showed a greater number of diatom taxa common to all three springs: *Cymbella, Diadesmis, Encyonopsis, Geissleria, Gomphonema, Halamphora, Luticola, Mayamaea, Navicula, Nitzschia, Placoneis, Planothidium, Pleurosira, Terpinsoe*, and *Ulnaria.* Whereas light microscopy only showed four genera common to all springs (*Achnanthidium, Gomphonema, Navicula,* and *Synedra*). The overall number of taxa identified for each spring matched both methods used, with Silver Glen having the greatest number of taxa, followed by Blue Spring, and Ichetucknee. It is likely that some diatom genera identified using metabarcoding were missed in light microscopy due to low abundance.

Analysis of water quality parameters

Potential differences in water quality parameters based on monthly concentrations were investigated using a Kruskal-Wallis test for data from 2014-2018 (Figs. 12-14, Table 9). Statistical significance ($P < 0.05$) was detected for all quality parameters analyzed. Ammonium concentrations for Blue Spring had average concentration around 0.04 mg/L, whereas Silver Glen Springs and Ichetucknee had average NH_4 concentrations around 0.002 mg/L. Total nitrogen values were an order of magnitude higher for Blue Springs with an average concentration around 0.13 mg/L compared to the average values for Silver Glen (0.022 mg/L) and Ichetucknee Springs (0.036 mg/L). For phosphates and total phosphorus, Blue Spring has significantly higher concentrations ($P < 0.05$) than either Silver Glen Spring and Ichetucknee. For pH, each spring had significantly different values with Blue Spring having the lowest average pH (7.22), and Silver Glen having the highest average (7.83). Water temperature was not significantly different for Blue Spring and Silver Glen, but Ichetucknee had significantly lower water temperatures, with average temperature of 21.76 ºC compared to Blue Spring and Silver Glen having an average water temperature around 23 ºC. Dissolved oxygen concentrations were also significantly lower in Blue Spring with an average value of 0.89 mg/L compared to either Silver Glen (3.56 mg/L) or Ichetucknee (3.77 mg/L), but not significantly different between Silver Glen and Ichetucknee.

Discussion

This study represents one of the first comparative analyses of algal community structure in the Florida springs, employing both morphological and molecular methods. Discrepancies were noted between the methods used, with each having its own having shortcomings and advantages. For example, metabarcoding revealed greater taxonomic diversity for the

Bacillariophyta compared to morphology. Overall, 13 diatom genera were identified using light microscopy, whereas 47 were identified using molecular methods. This difference is likely due to many diatom taxa being morphologically identical, but having considerable genetic variation (Mann *et al.* 2010, Kermarrec *et al.* 2013; Abarca *et al.* 2014). Most algal groups are also known to contain cryptic diversity, making it difficult to identify genetically distinct genera. Metabarcoding methods also provided a significantly larger dataset of reference sequences compared to the 400 cells counted for the morphological analysis, potentially leading to differences in the number of taxa identified (Stoeck *et al.* 2009).

While metabarcoding provided greater insight into the diatom community, this was not the case for green algae or cyanobacteria. The 18S marker was not successful at amplifying as many sequences as the 16S and *rbc*L marker, and taxonomy could not be assigned below the kingdom level, making this dataset futile for further analyses. However, a few green algal genera were identified using morphology, belonging to the Charophyta (*Scenedesmus, Closterium*, and *Mougeotia*) and Chlorophyta (*Cladophora*). In terms of the 16S dataset, universal primers were used that target a broad range of prokaryotes, including Archaea, Bacteria, and the Cyanobacteria. Due to the primers chosen and the level of sequencing depth, taxonomic resolution could not be made below the phyla level. This likely contributed to lower overall relative abundances when using molecular methods compared to light microscopy. Interestingly, 1.3% of the 16S dataset for Blue Spring was composed of Cyanobacteria, whereas no Cyanobacteria were noted via light microscopy, suggesting more robust sampling is needed. Rarefaction curves were calculated to verify sufficient sampe size, and all curves reached an asymptote with the exception of the 18S dataset in Blue Spring. Even though morphology didn't

recover as much diversity in certain groups as the molecular dataset, this rarefaction curve likely plateaued due to the cryptic nature of many algal taxa.

Metabarcoding has shown to be a powerful tool for assessing biodiversity across a wide range of habitats and taxonomical groups, but a limiting factor for all studies employing this method is the lack of complete curated DNA reference libraries (Kermarrec *et al*. 2013; Zimmermann *et al*. 2015; Leray *et al.* 2015; Cowart *et al.* 2015). There are currently four databases for specialized 16S rRNA sequencing: Greengenes (DeSantis *et al*. 2006), LTP (Yarza *et al.* 2008), RDP (Maidak *et al.* 2001), and SILVA (Pruesse *et al.* 2007), with the SILVA database used in this study. While SILVA is the most robust and widely used database, recent work has shown errors in the taxonomy, with multiple identical sequences having conflicting annotations (Edgar 2018). Therefore, we chose a conservative approach and only assigned taxonomy to the phyla level in our 16S dataset. Perhaps the curation of Cyanobacteria-specific databases using known type strains can help resolve issues presented using the SILVA database to further elucidate cyanobacterial diversity when using a NGS approach. Another limitation to our study was the lack of replicates needed for statistical power. Because all replicates were pooled before DNA extraction, only technical replicates could be analyzed. In addition, we only sampled from an isolated area in each spring, and thus future studies should aim to increase both true and technical replicates, as well as sampling throughout the springshed boundaries.

While the 16S marker lacked taxonomic resolution for the Cyanobacteria, by including a group-specific primer set we were able to assess diatom diversity at the genus level. Previous studies have recommended the *rbc*L gene for barcoding diatoms over 18S and other algal specific markers, such as UPA, because it has greater species distinguishing capabilities (Evans *et al.* 2007; Hamsher *et al*. 2011; Kermarrec *et al*. 2013). Universal primer sets, such as 16S and

18S, have low phylogenetic resolution and potentially underestimate the diversity present, but non-standard markers may have relatively poor datasets for comparison. Because of this, multi-marker approaches are not typically used. However, recent work by Rimet *et al*. (2018) has shown it is possible to enrich the current reference barcoding library (R-Syst::diatom in particular) at a low cost. This can be done using high-throughput sequencing of environmental samples, rather than using isolation, culturing, and single-cell sorting methodologies. The ca. 300 bp sequence generated in standard NGS approaches has been shown to be sufficient for species identifications (Kermarrec *et al.* 2014). As the number of barcoding studies increases, and reference databases continue to improve for both universal and group-specific primers, researchers can unlock the full potential of DNA metabarcoding for ecological assessments (Elbrecht *et al.* 2017).

Using group-specific markers, such as *rbc*L for diatoms, can also aid in gauging ecological conditions by articulation of sentinel or indicator taxa (Porter *et al* 2008; Stevenson 2014; Hausmann *et al.* 2016). Diatoms display substantial taxonomic diversity and are estimated to have over 100,000 extant species (Mann & Vanormelingen 2013). Some diatom taxa have wide distribution ranges and are highly sensitive to their chemical environment, making them excellent ecological indicators (Stevenson 2014). For example, *Cymbella* has been previously used to assess pollution levels related to mining in regions of the Wester Alleghany Plateau (Zalack *et al*. 2010). This genus has been shown to have a negative correlation with impairment, and a similar trend was observed in the Florida springs. Ichetucknee had the highest relative abundance of *Cymeblla* (~11%) and also had significantly lower nutrient concentrations than Blue Spring. However, Silver Glen also had lower nutrient concentrations compared to Blue Spring, but *Cymbella* was less abundant (<1%). This may in part be due to the high abundance of

Nitzschia (~65%), which may have outcompeted *Cymbella*. Interestingly, *Nitzschia* has been observed to show no clear nutrient preference in freshwater systems (Wang *et al.* 2005). Similar to *Cymbella*, *Encyonema* has also been observed in areas with lower nutrients and higher water quality (Hausmann *et al.* 2016), and this genus was found only in Ichetucknee. While *Cymbella* was in high abundance in Ichetucknee, other indicator taxa were present in low abundance or not at all. *Cocconeis*, another genus found to indicate overall water quality (Wang *et al.* 2005) and was not present in Ichetucknee, was found in low abundance in Blue Spring and Silver Glen. While indicator taxa were found in all springs at varying abundances, these results still offer important insights into the overall health of these springs.

Recent studies have highlighted that diatoms alone may not be a comprehensive metric of overall water quality (Tan *et al.* 2017), and that looking at the entire algal community may be a more robust approach (Stevenson 2014). In terms of cyanobacterial abundances, we noted Ichetucknee and Silver Glen had substantially greater abundances than did Blue Spring. Cyanobacterial growth and presence has shown to be limited by pH, with most species having a growth optima between pH 7.5 and 10 (Kallas & Castenholz 1982). Blue Spring had an average pH around 7.2, whereas Ichetucknee had an average pH of 7.5 and Silver Glen 7.8. These differences in pH may have contributed to the varying abundances of cyanobacteria throughout the springs. Among the cyanobacteria identified using light microscopy, *Phormidium* was found in both Ichetucknee and Silver Glen (Table 1). This is a cosmopolitan genus (Sheath & Cole 1992) and shows a wide range of genetic diversity not reflected in its morphology (Casamatta & Vis 2003), making it difficult to distinguish species. This taxon was originally described from oligotrophic streams, and Casamatta & Vis (2004) noted *Phormidium* is potentially specialized at growing in low nutrient conditions. The presence of this cyanobacteria may correspond to the

lower nutrient levels observed in Ichetucknee and Silver Glen (Fig. 12, Table 9). Other genera of cyanobacteria identified belonged to the Synechococcales, which is an order of cyanobacteria that often shows preference to higher pH environments (Kallas & Castenholz 1982). During morphological analyses, we didn't detect any freshwater bloom-forming cyanobacterial genera, which suggest conditions in the springs are currently not conducive to these taxa.

Along with diatoms and cyanobacteria, various green algal taxa were identified. Previous studies have highlighted the use of the18S rDNA gene as a universal barcode for both green algae and diatoms (Marcelino & Verbruggen 2016; Groendahl *et al*. 2017; Wolf 2018). However, the 18S rDNA marker used in our study was unsuccessful. Some algal lineages may be difficult to amplify using standard primer pairs due to potential substitutions at primer binding sites, such as seen in green algae (Marcelino & Verbruggen 2016). A potential alternative to the 18S rDNA gene in this study could be the *tuf*A marker. This marker has been shown to have a higher rate of evolution, yielding many more green algal OTUs in some studies. Recent work has noted the Universal Plastid Amplicon (UPA) primer set, which targets the 23S rDNA gene, to be successful in amplifying a variety of green algal taxa (Marcelino & Verbruggen 2016; Sherwood *et al*. 2017; Wolf 2018). Future studies should implement the use of these markers to specifically target the green algae rather than using a universal primer set for the 18S rDNA gene.

Florida has the largest concentration of first magnitude springs in the world. While they vary in geography, flora and fauna, mineral content, and flow rate, nearly all of the springs are suffering from anthropogenic impacts (Florida Springs Conservation Plan 2018). Many springs have seen declining flow rates and increased water quality impairments, which has caused a decline in submerged aquatic vegetation and an increase in filamentous algae (Scheffer *et al.* 2001). Similar phase shifts have been noted in a variety of habitats, such as tropical coral reefs

(Engene *et al.* 2013) and temperate kelp forests (Filbee-Dexter & Wernberg 2018) as a result of climate change. The algal communities throughout the springs have been studied since the 1950's when Whitford (1956) first began characterizing the algal flora. More recent studies have observed similar algal assemblages (Phelps *et al.* 2006; Stevenson *et al.* 2007; Quinlan *et al.* 2007), but have noted an increase in nuisance taxa, such as "*Lyngbya*" and *Vaucheria*. Algal taxa identified in this study also closely match those found in past studies, but we noted some differences. First, we didn't find any *Vaucheria* and very little cyanobacteria were found in Blue Spring. This is possibly a case of small sample size, and future studies should focus on increased sampling throughout the springs. We also noted substantially more diatom taxa than previous recorded. This is most likely due to previous studies relying on morphological features alone, potentially misidentifying a variety of diatom genera. In addition, diatom systematics has changed greatly over the last fifteen years, resulting in numerous monophyletic lineages created from previous polyphyletic species "swarms" (e.g., *Navicula*) and thus potentially more genera to identify. With the advancements in next-generation sequencing, and the robustness of the R-Syst::diatom database, coupling morphology with barcoding via the *rbc*L marker may provide greater insight into the these diatom communities.

Overall, this study highlights how a multi-marker metabarcoding approach, combined with traditional morphological analyses, can enrich biodiversity surveys of the algal communities in the Florida springs. Results from the 16S dataset show universal primers lack the taxonomic resolution needed for identifying the cyanobacterial community at the genetic level, but by using a group-specific marker we were able to clearly articulate members of the diatom community. We also noted that were our barcode methods failed (i.e., 18S rDNA for green algae and taxonomic resolution of cyanobacterial), microscopy methods resolved gaps in the data. Taken

together, these results highlight how a combination of the two methods provide a more comprehensive diversity assessment than either method in isolation. As these springs continue to face increasing impairments, conducting more complete biodiversity assessments will facilitate preservation/restoration planning for these fragile ecosystems.

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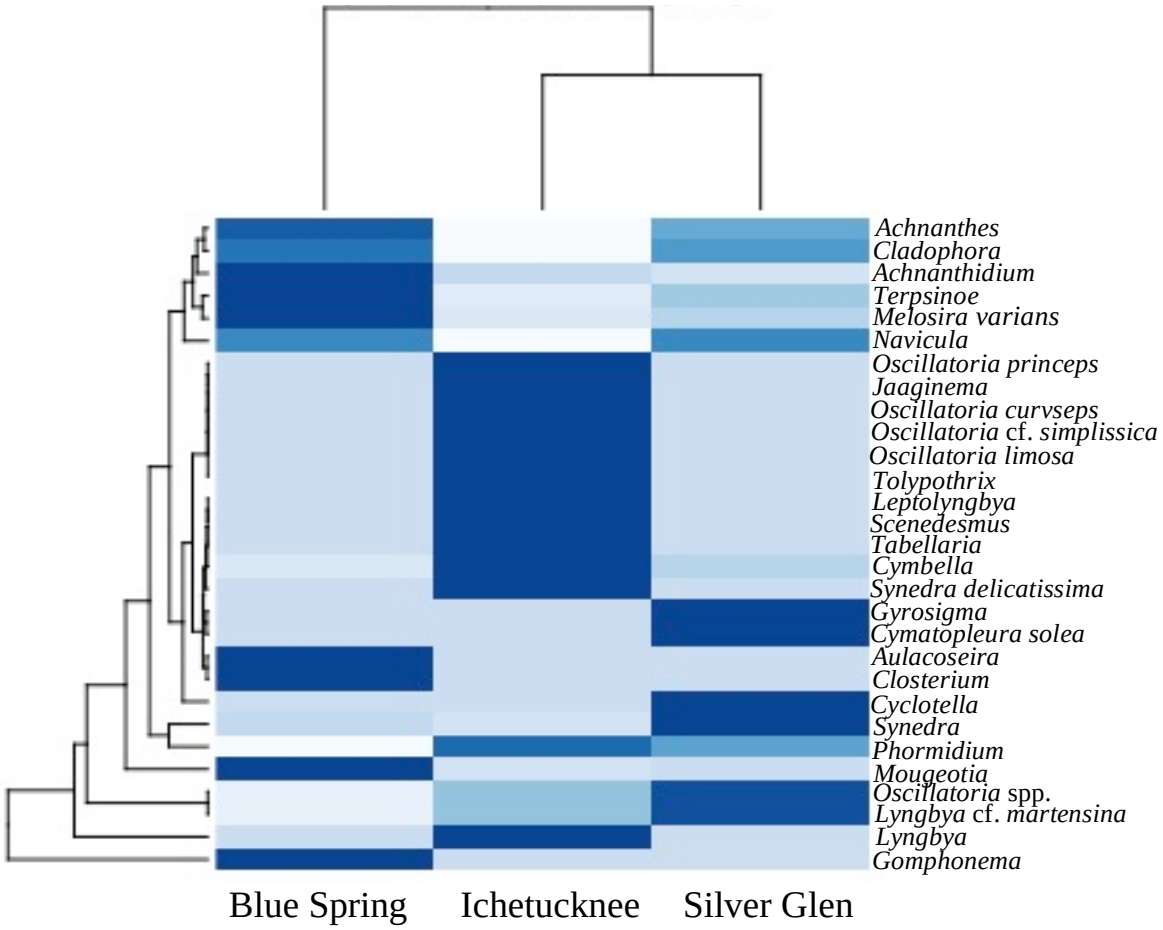

Figure 1. Heatmap showing relative abundance for algal taxa identified during morphological analysis. The dendogram on the top shows the relationship between the abundance of taxa at each spring and the dendogram on the left shows the relationship between the abundance of the different taxa.

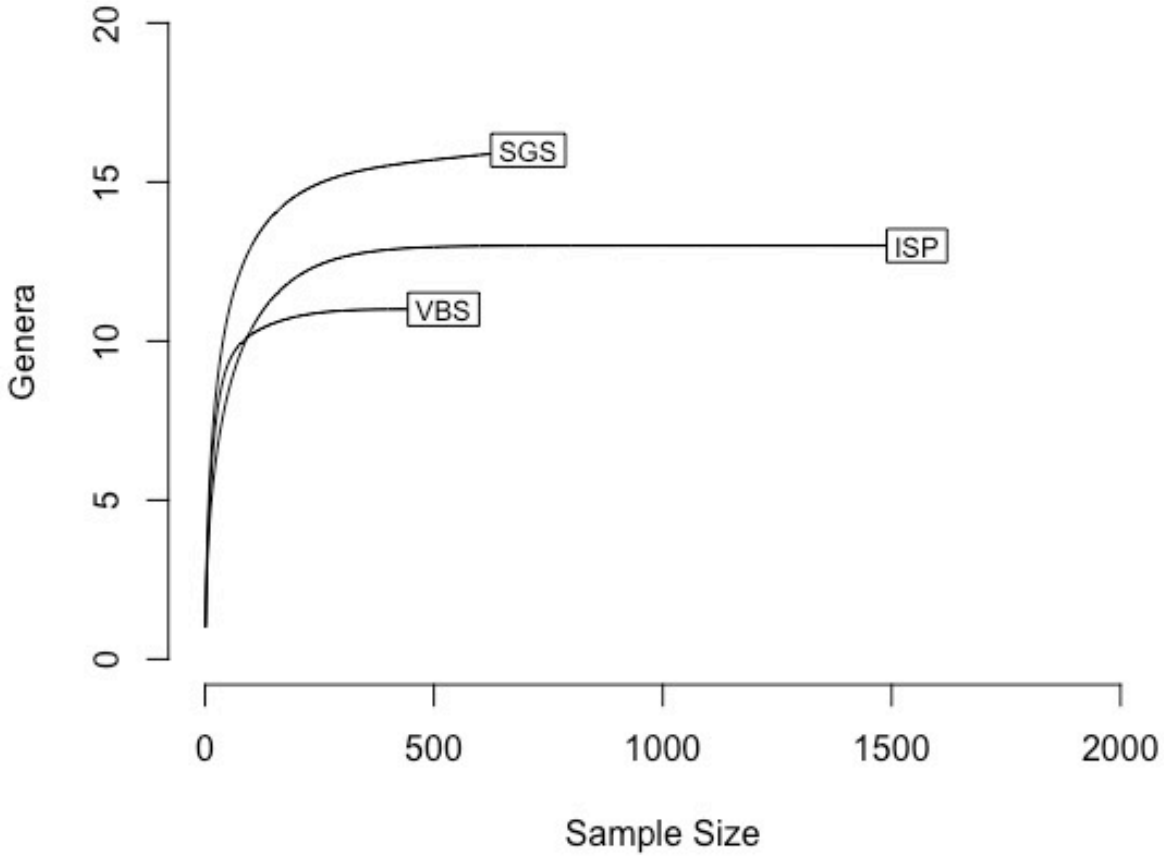

Figure 2. Rarefaction plot for morphological analysis showing the number of genera identified as a function of sample size (number of cells counted) for Blue Spring (VBS), Ichetucknee (ISP), and Silver Glen Spring (SGS).

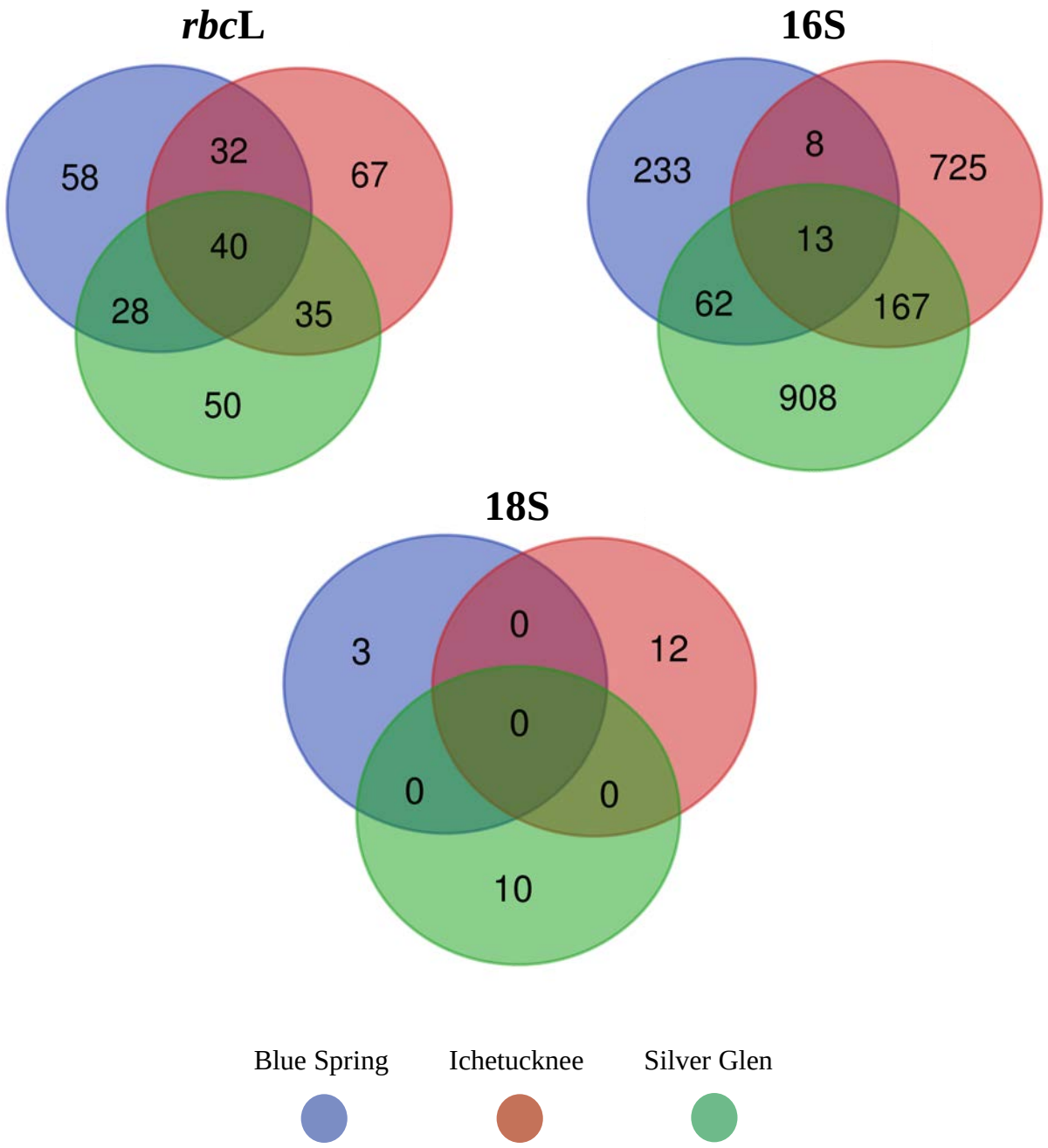

Figure 3. Venn diagrams for each genetic marker showing the number of OTUs unique and shared between and among spring locations.

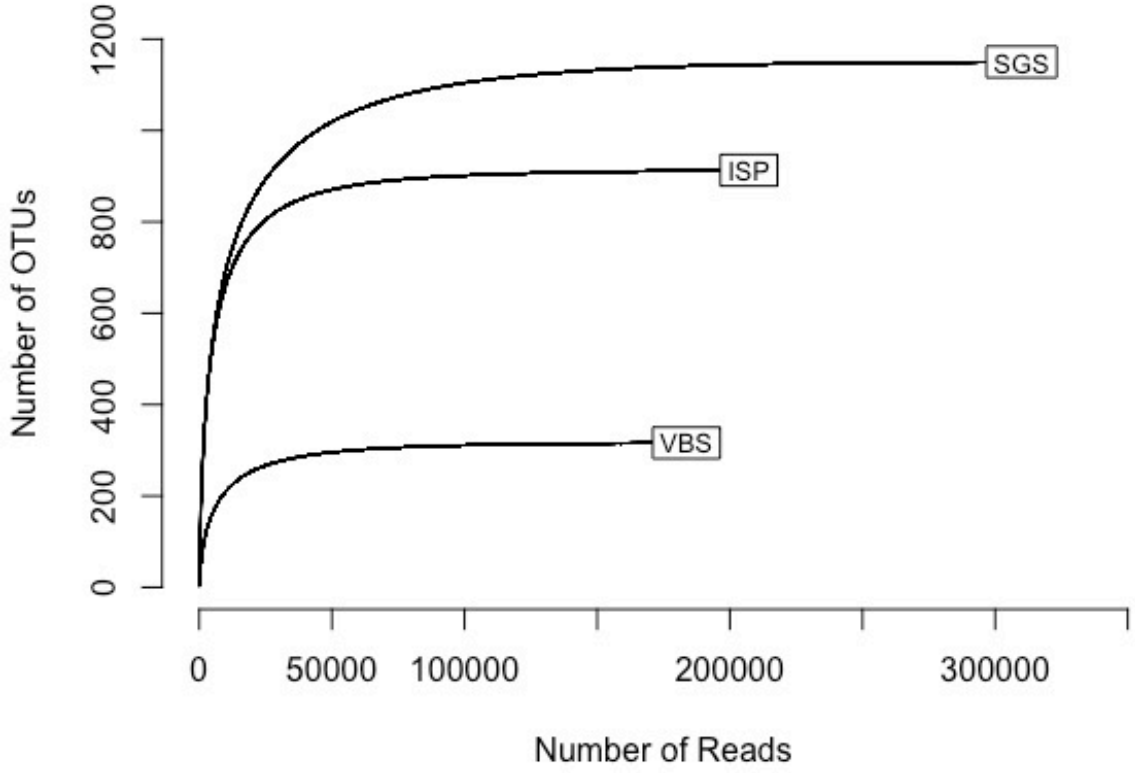

Figure 4. Rarefaction plot for 16S marker showing the number of OTUs as a function of sequence depth (number of reads) for Blue Spring (VBS), Ichetucknee (ISP), and Silver Glen Spring (SGS). Replicates were pooled for each spring.

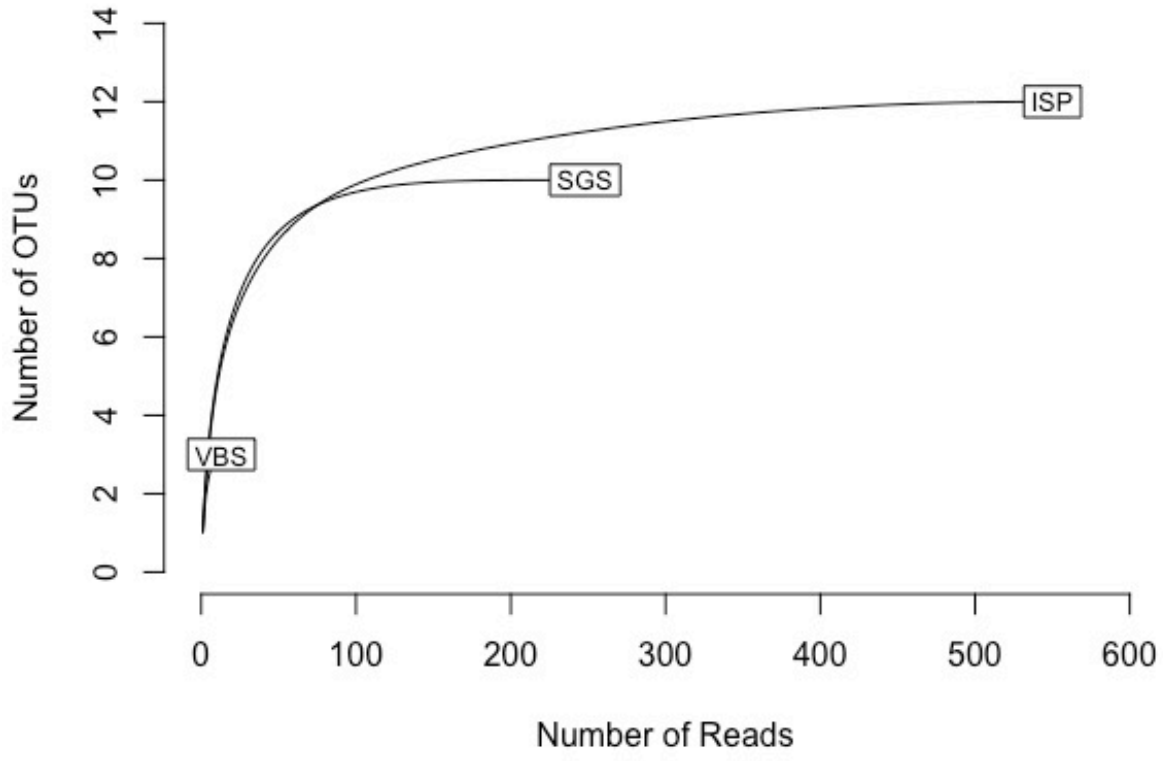

Figure 5. Rarefaction plot for 18S marker showing the number of OTUs as a function of sequence depth (number of reads) for Blue Spring (VBS), Ichetucknee (ISP), and Silver Glen Spring (SGS). Replicates were pooled for each spring.

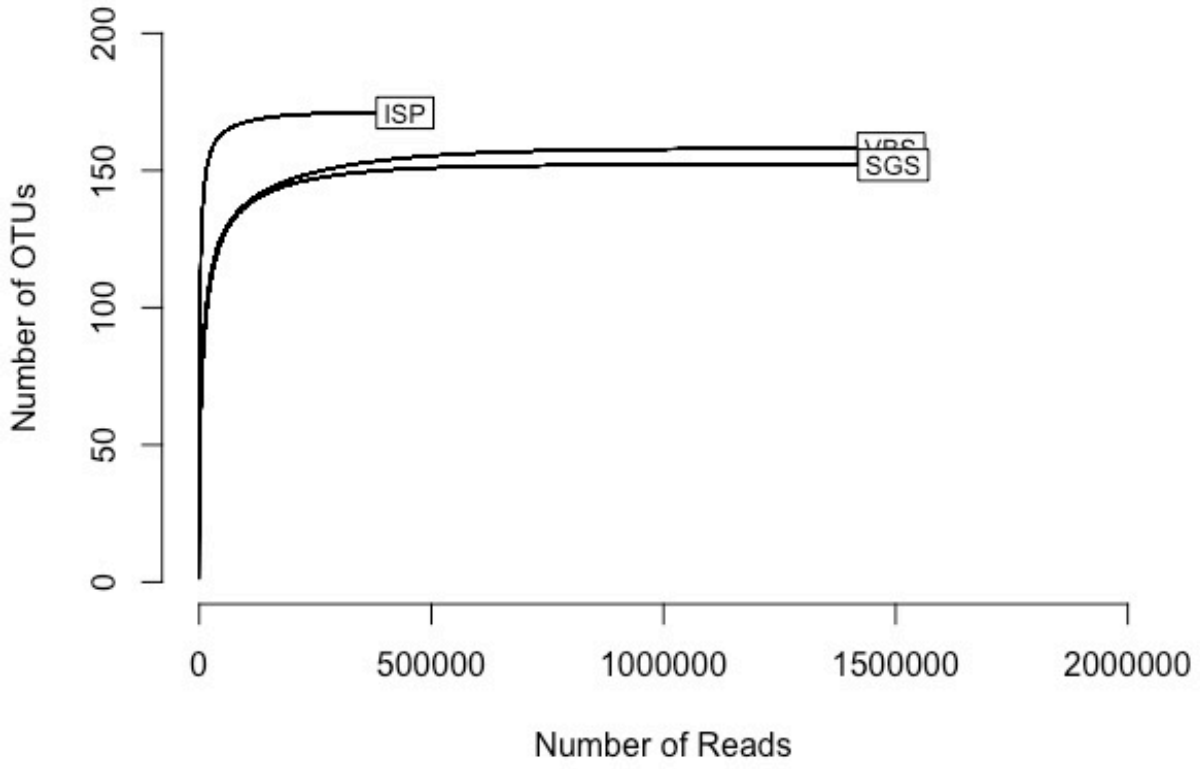

Figure 6. Rarefaction plot for *rbc*L marker showing the number of OTUs as a function of sequence depth (number of reads) for Blue Spring (VBS), Ichetucknee (ISP), and Silver Glen Spring (SGS). Replicates were pooled for each spring.

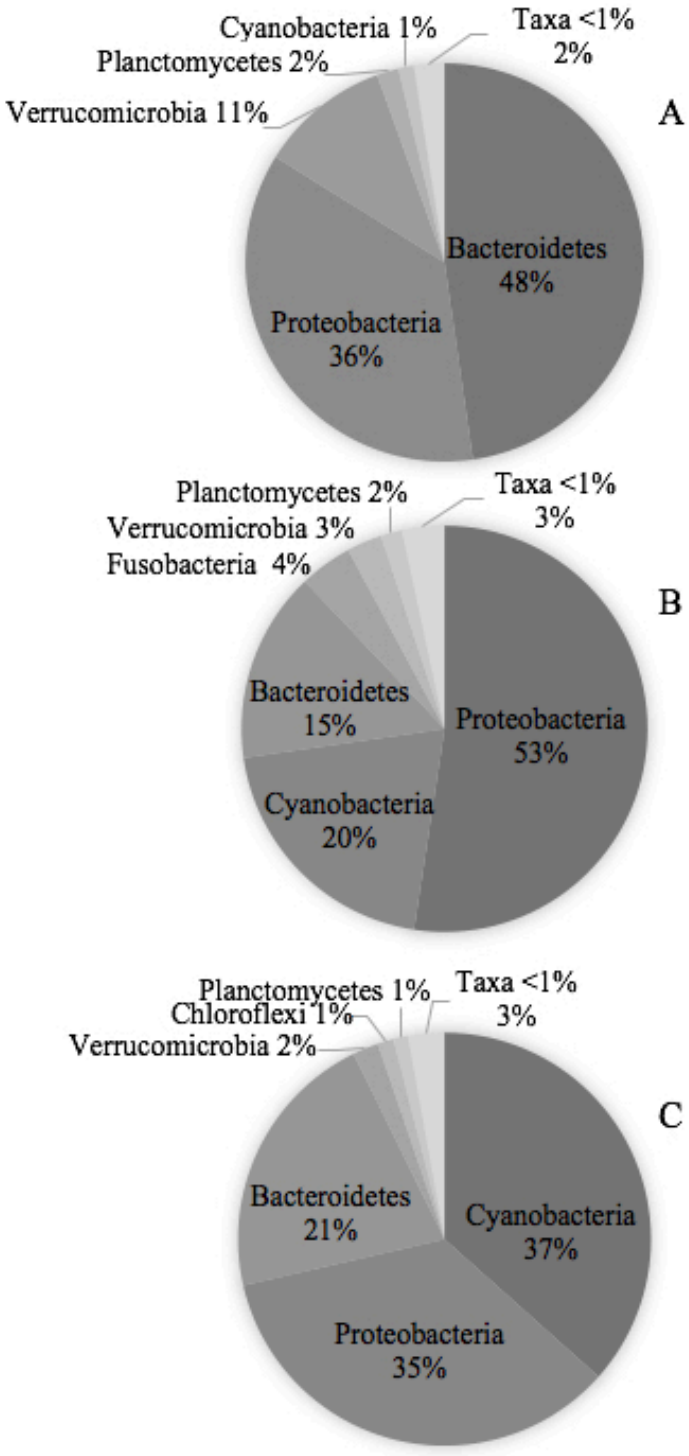

Figure 7. Relative abundance of bacterial phyla for the 16S marker in A) Blue Spring, B) Ichetucknee Spring, and C) Silver Glen Spring.

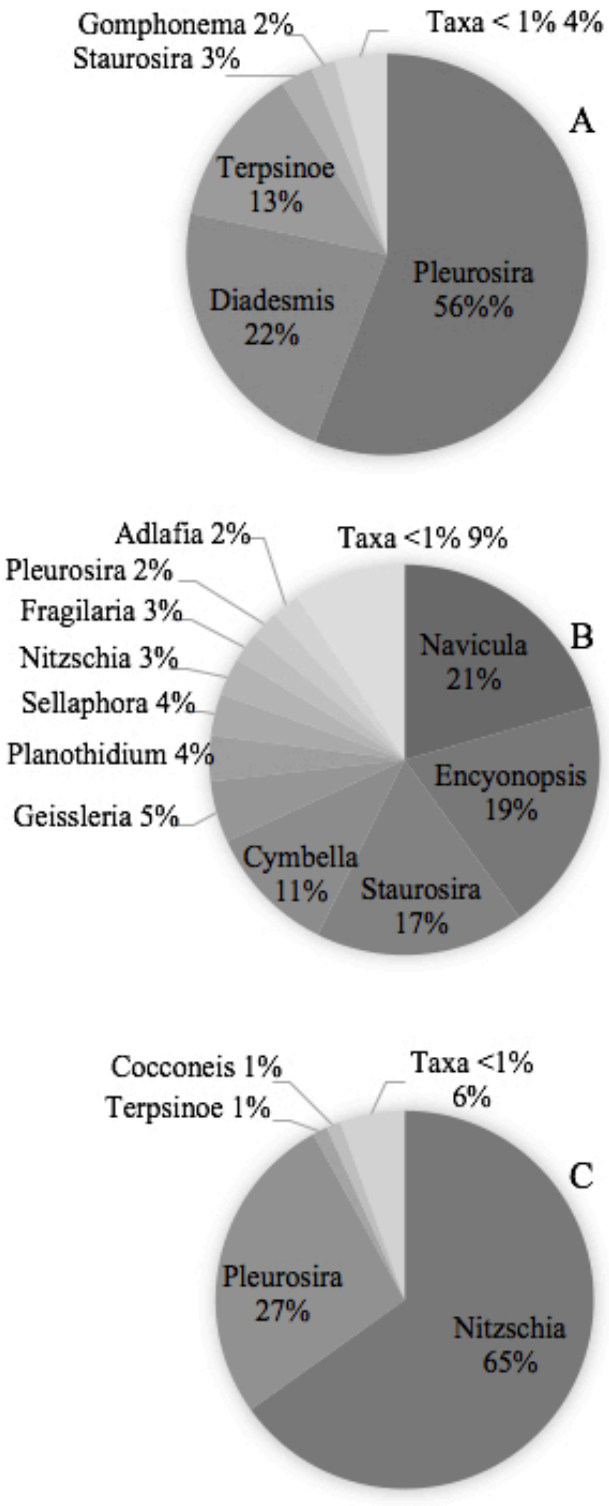

Figure 8. Relative abundance of diatom genera for the *rbc*L marker in A) Blue Spring, B) Ichetucknee Spring, and C) Silver Glen Spring

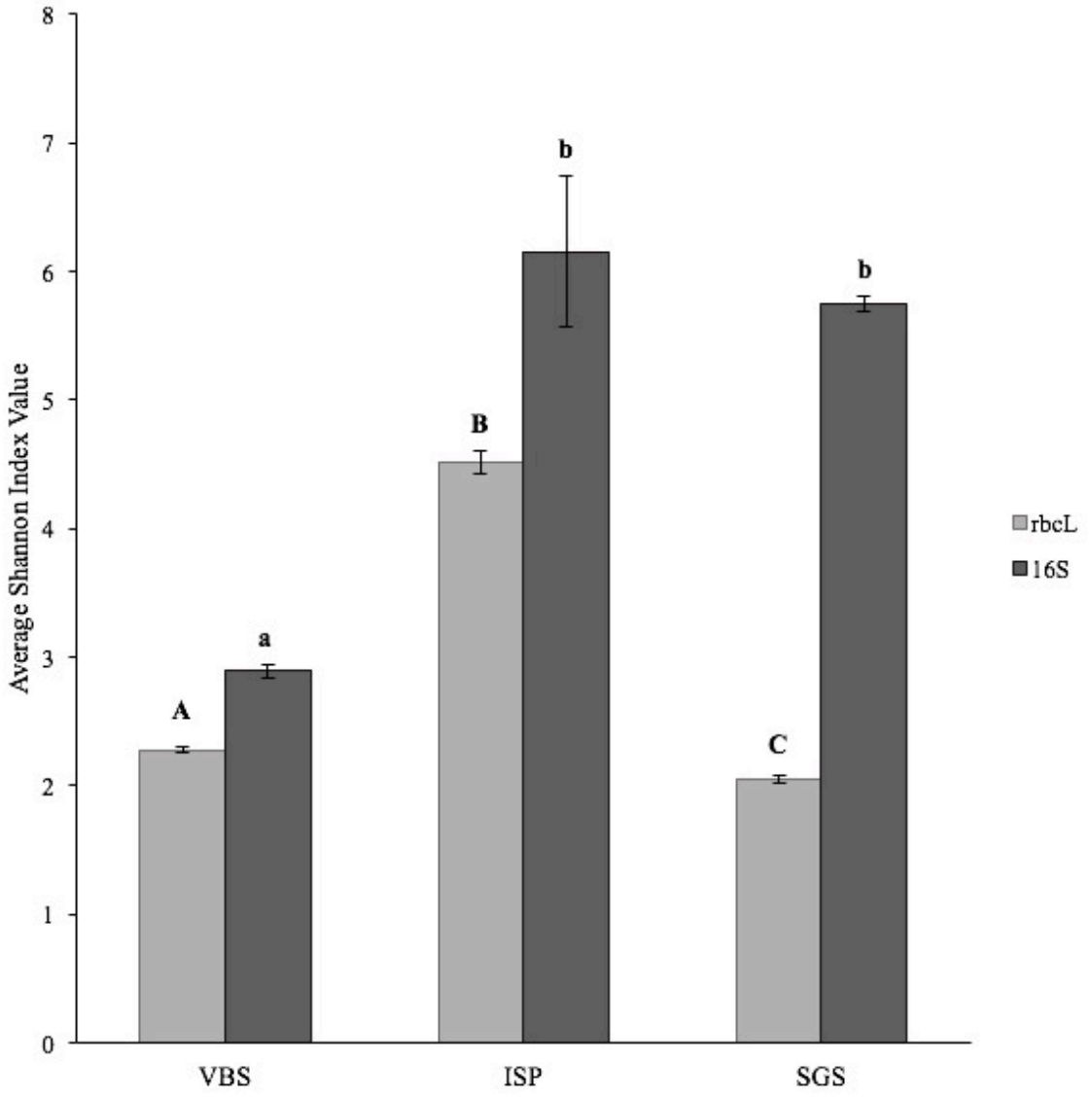

Figure 9. Average Shannon diversity index values for 16S and *rbc*L marker from Blue Spring (VBS), Ichetucknee (ISP), and Silver Glen Spring (SGS). Significant differences among locations were calculated with Kruskal-Wallis H pairwise comparison test ($P < 0.05$) and are denoted by letters on top of the box. Boxes that share the same letter are not significantly different from each other.

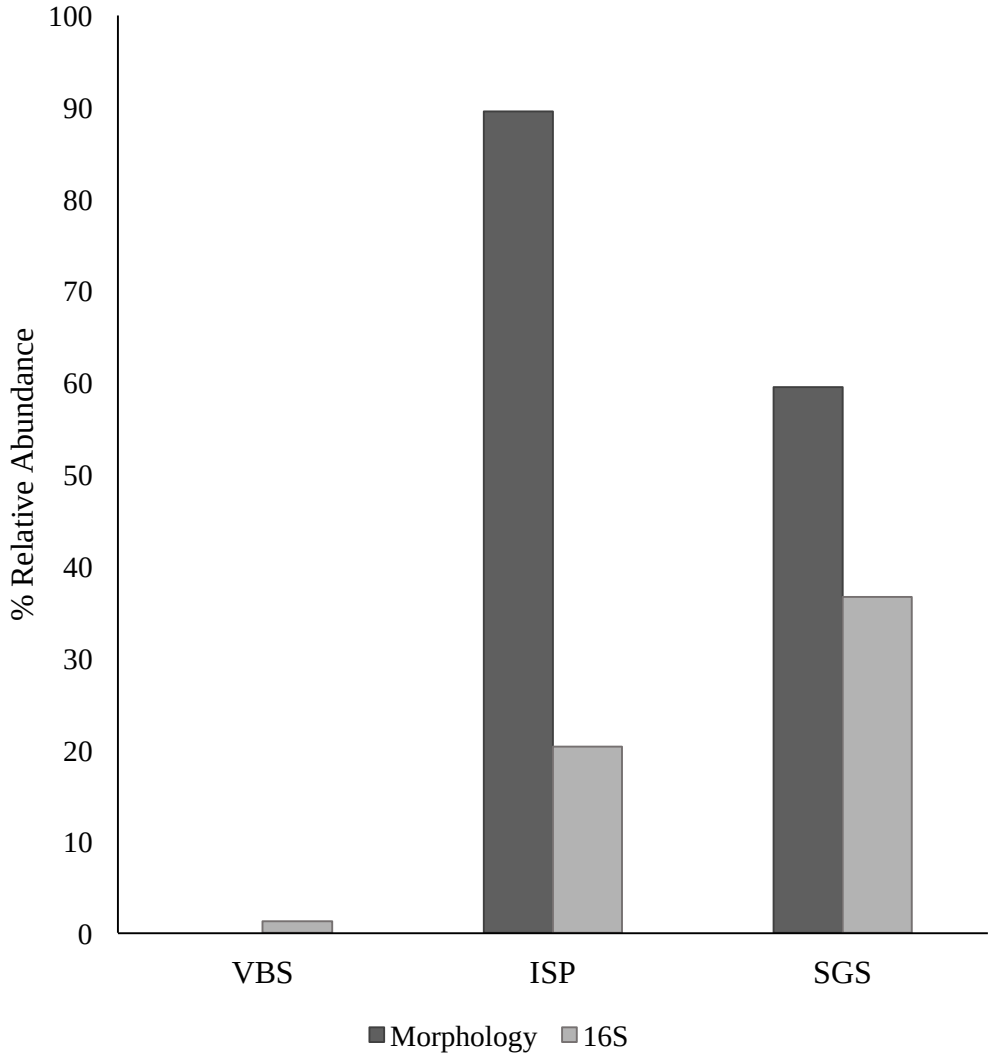

Figure 10. Comparison of cyanobacterial abundance for Blue Spring (VBS), Ichetucknee (ISP) and Silver Glen Spring (SGS) based on morphological analysis and metabarcoding (16S rDNA gene).

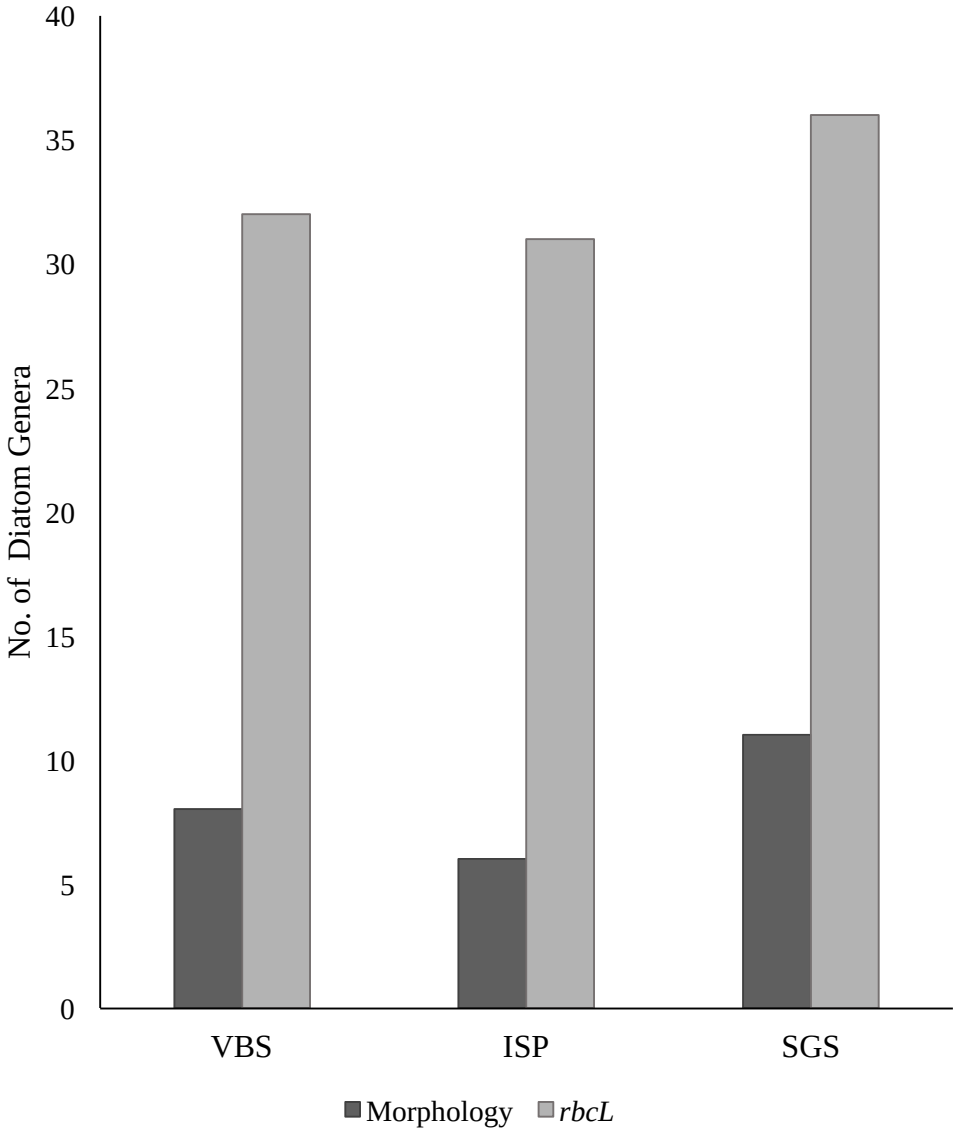

Figure 11. Comparison of the number of diatom genera identified in Blue Spring (VBS), Ichetucknee (ISP), and Silver Glen Spring (SGS) based morphological analysis and metabarcoding (*rbc*L marker).

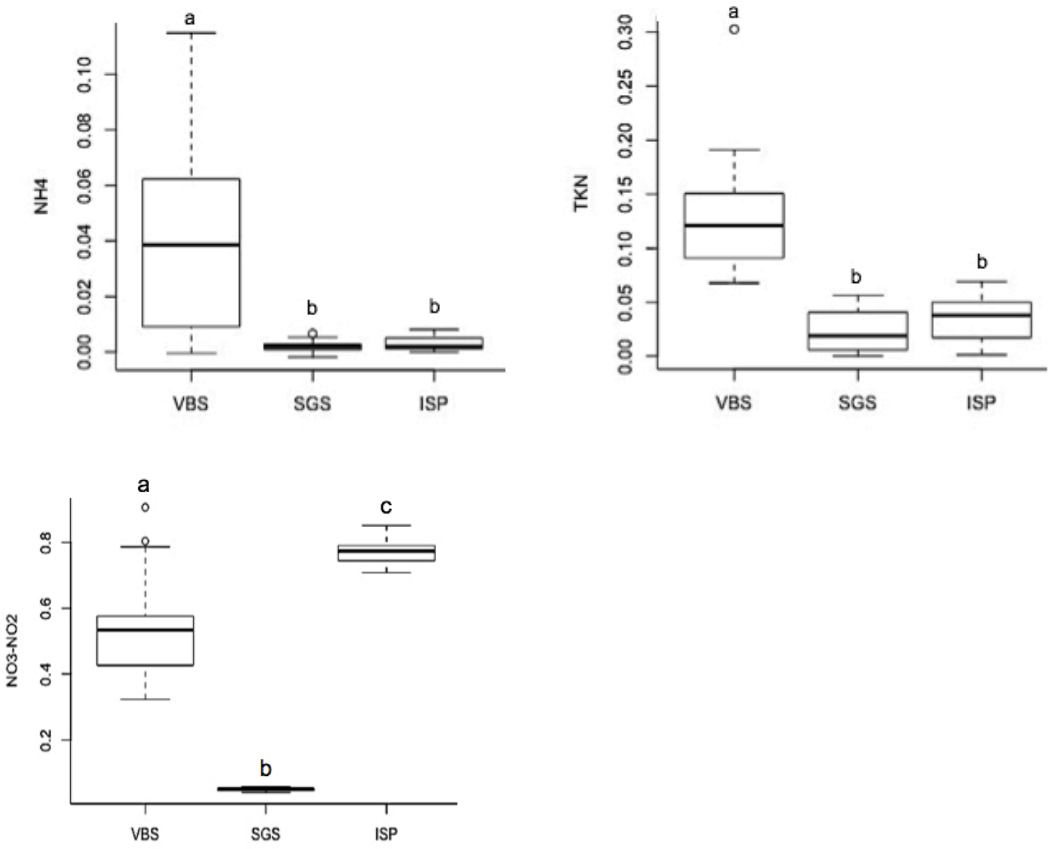

Figure 12. Boxplots for ammonium (NH_4), total Kjeldahl nitrogen (TKN), and nitrate-nitrite (NO_3-NO_2) in mg/L from 2014-2018 for Blue Spring (VBS), Silver Glen Springs (SGS), and Ichetucknee Spring (ISP). Each box represents the lower (25%) and upper (75%) quartiles. Mean values are indicated by the bold line, minimum and maximum values by whiskers, and outliers by circles. Significant differences among locations were calculated with Kruskal-Wallis H pairwise comparison test ($P < 0.05$) and are denoted by letters on top of the box. Boxes that share the same letter are not significantly different from each other.

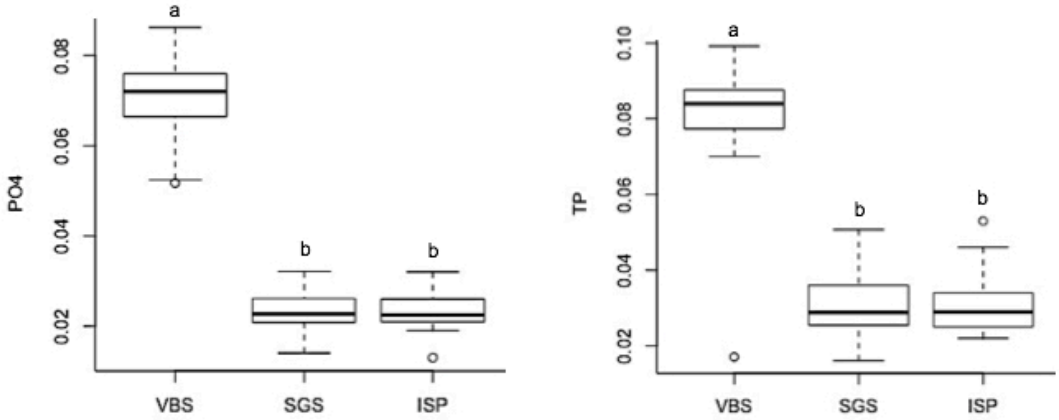

Figure 13. Boxplots for phosphate (PO_4) and total phosphorus (TP) in mg/L from 2014-2018 for Blue Spring (VBS), Silver Glen Springs (SGS), and Ichetucknee Spring (ISP). Each box represents the lower (25%) and upper (75%) quartiles. Mean values are indicated by the bold line, minimum and maximum values by whiskers, and outliers by circles. Significant differences among locations were calculated with Kruskal-Wallis H pairwise comparison test ($P < 0.05$) and are denoted by letters on top of the box. Boxes that share the same letter are not significantly different from each other.

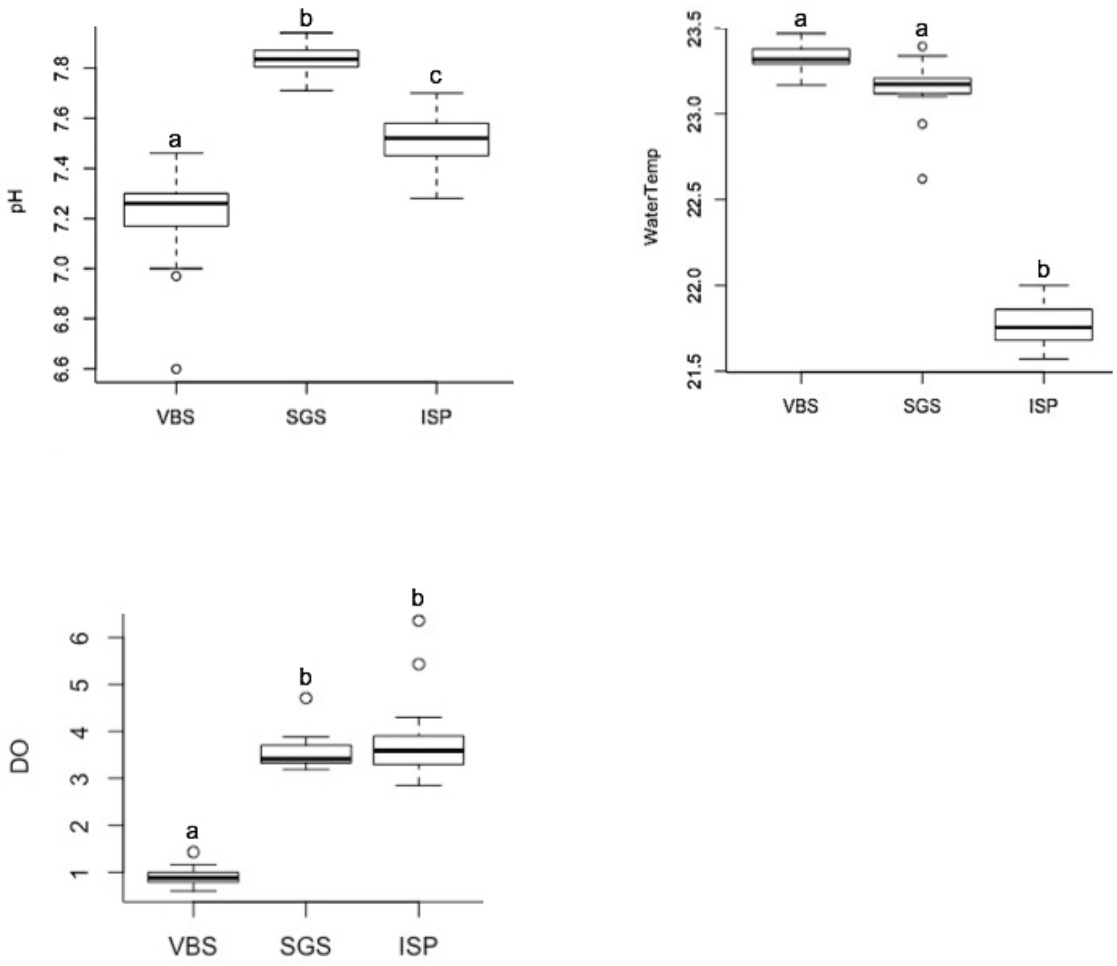

Figure 14. Boxplots for pH, dissolved oxygen (mg/L), and water temperature (°C) data from 2014-2018 for Blue Spring (VBS), Silver Glen Springs (SGS), and Ichetucknee Spring (ISP). Each box represents the lower (25%) and upper (75%) quartiles. Mean values are indicated by the bold line, minimum and maximum values by whiskers, and outliers by circles. Significant differences among locations were calculated with Kruskal-Wallis H pairwise comparison test ($P < 0.05$) and are denoted by letters on top of the box. Boxes that share the same letter are not significantly different from each other.

Table 1. Individual thermocycler settings of amplicon PCR for each marker.

DNA Marker	Amplification Conditions
16S	Initial denaturing = 95°C, 3 min 25 Cycles: Denature = 95°C, 30 sec Annealing = 55°C, 30 sec Extension = 72°C, 30 sec Final Extension = 72°C, 5 min
18S	Initial denaturing = 98°C, 3 min 30 Cycles: Denature = 98°C, 10 sec Annealing = 59°C, 30 sec Extension = 72°C, 30 sec Final Extension = 72°C, 10 min
*rbc*L	Initial denaturing = 95°C, 15 min 40 Cycles: Denature = 95°C, 45 sec Annealing = 55°C, 45 sec Extension = 72°C, 45 sec Final Extension = 72°C, 5 min

Table 2. Algal taxa identified in each sampling location based on morphological analysis. A total of 23 infra-generic taxa were identified, belonging to 4 phyla.

Species	**Phylum**	**Location**
Achnanthes sp.	Bacillariophyta	Silver Glen, Blue Spring
Achnanthidium sp.	Bacillariophyta	Ichetucknee, Silver Glen, Blue Spring
Aulacoseira sp.	Bacillariophyta	Blue Spring
Cyclotella sp.	Bacillariophyta	Ichetucknee, Silver Glen
Cymatopleura solea	Bacillariophyta	Silver Glen
Cymbella sp.	Bacillariophyta	Ichetucknee, Silver Glen
Gomphonema sp.	Bacillariophyta	Ichetucknee, Silver Glen, Blue Spring
Gyrosigma sp.	Bacillariophyta	Silver Glen
Melosira varians	Bacillariophyta	Silver Glen, Blue Spring
Navicula sp.	Bacillariophyta	Ichetucknee, Silver Glen, Blue Spring
Synedra delicatissima	Bacillariophyta	Ichetucknee
Synedra spp.	Bacillariophyta	Ichetucknee, Silver Glen, Blue Spring
Tabellaria sp.	Bacillariophyta	Ichetucknee
Terpsinoe	Bacillariophyta	Silver Glen, Blue Spring
Cladophora sp.	Chlorophyta	Silver Glen, Blue Spring
Closterium sp.	Charophyta	Blue Spring
Mougeotia sp.	Charophyta	Silver Glen, Blue Spring
Scenedesmus sp.	Charophyta	Ichetucknee
Jaaginema sp.	Cyanobacteria	Ichetucknee
Leptolyngbgya sp.	Cyanobacteria	Ichetucknee
Lyngbya cf. *martensina*	Cyanobacteria	Ichetucknee
Lyngbya sp.	Cyanobacteria	Ichetucknee, Silver Glen
Oscillatoria princeps	Cyanobacteria	Ichetucknee
Oscillatoria curvseps	Cyanobacteria	Ichetucknee
Oscillatoria cf. *simplissica*	Cyanobacteria	Ichetucknee
Oscillatoria limosa	Cyanobacteria	Ichetucknee
Oscillatoria spp.	Cyanobacteria	Ichetucknee, Silver Glen
Phormidium sp.	Cyanobacteria	Ichetucknee, Silver Glen
Tolypothrix spp.	Cyanobacteria	Ichetucknee

Table 3. Relative abundance (%) for each phyla in the 16S dataset. Algal phyla are bolded.

Phylum	**Blue Spring**	**Ichetucknee**	**Silver Glen**
Acidobacteria	0.45	0.45	0.27
Actinobacteria	0.16	0.05	0.01
Armatimonadetes	0.39	0.60	0.20
Bacteroidetes	47.72	15.21	21.33
Berkelbacteria	0	0.03	0
Caldiserica	0	0	0.01
Chlamydiae	0	0.15	0.02
Chloroflexi	0.02	0.38	1.24
Cyanobacteria	**1.26**	**20.31**	**36.60**
Deinococcus-Thermus	0	0	0.26
Fibrobacteres	0	0.004	0.05
Firmicutes	0.27	0.08	0.26
Fusobacteria	0	4.20	0.15
Gemmatimonadetes	0	0.74	0.06
Gracilibacteria	0.55	0.05	0.72
Kiritimatiellaeota	0	0	0.01
Melainabacteria	0	0.05	0.04
Microgenomatia	0	0.03	0.02
Nitrospirae	0	0.004	0.003
Parcubacteria	0.64	0.66	0.65
Patescibacteria	0	0.01	0.02
Planctomycetes	1.76	1.70	1.16
Proteobacteria	36.16	52.40	34.8
Saccharibacteria	0	0.10	0.09
Spirochaetes	0	0.02	0
Verrucomicrobia	10.62	2.81	2.02

Table 4. Number of operational taxonomic units (OTUs) assigned to each phylum in the 16S dataset based on replicates.

Phylum	**VBS01**	**VBS02**	**VBS03**	**VBS04**	**ISP01**	**ISP02**	**ISP03**	**ISP04**	**SGS01**	**SGS02**	**SGS03**	**SGS04**
Acidobacteria	1	3	0	2	1	6	5	1	6	6	5	6
Actinobacteria	0	0	0	1	0	3	1	0	0	0	0	1
Armatimonadetes	0	3	1	1	3	4	3	3	4	5	3	4
Bacteroidetes	29	36	48	43	42	81	72	29	111	90	112	103
Berkelbacteria	0	0	0	0	0	1	1	1	0	0	0	0
Caldiserica	0	0	0	0	0	0	0	0	1	0	0	1
Chlamydiae	0	0	0	0	3	4	3	2	1	0	1	2
Chloroflexi	0	0	0	1	3	8	7	1	12	10	13	8
Cyanobacteria	3	3	3	4	19	33	23	7	37	36	46	86
Deinococcus-Thermus	0	0	0	0	0	0	0	0	2	2	2	2
Fibrobacteres	0	0	0	0	0	1	0	0	1	2	1	1
Firmicutes	1	1	1	1	1	2	3	0	3	2	5	4
Fusobacteria	0	0	0	0	1	1	1	2	2	2	2	2
Gemmatimonadetes	0	0	0	0	3	4	4	2	1	1	1	1
Gracilibacteria	2	0	1	1	0	2	2	2	2	2	2	2
Kiritimatiellaeota	0	0	0	0	0	0	0	0	10	0	9	0
Melainabacteria	0	0	0	0	1	5	1	0	3	1	4	2
Microgenomatia	0	0	0	0	1	2	2	0	2	0	2	0
Nitrospirae	0	0	0	0	0	0	0	1	1	0	0	0
Parcubacteria	1	0	0	1	1	3	3	3	3	3	3	5
Patescibacteria	0	0	0	0	7	26	0	0	27	0	29	30
Planctomycetes	4	0	5	4	19	20	16	9	29	15	30	38
Proteobacteria	31	0	34	41	65	116	126	41	106	113	138	154
Saccharibacteria	0	0	0	0	20	83	62	25	40	38	44	38
Spirochaetes	0	0	0	0	1	4	0	0	0	0	0	0
Verrucomicrobia	11	0	6	7	10	30	16	6	15	16	19	16

Table 5. Mean relative abundance (%) of diatom genera based on *rbc*L sequences for each spring. Genera in bold are taxa noted to indicate water quality.

Genus	Blue Spring	Ichetucknee	Silver Glen
Achnanthes	0.24	0	0.04
Achnanthidium	0	1.60	0.002
Adlafia	0	2.27	0
Amphora	0.01	0	0.003
Asterionella	0	0	0.001
Bacillaria	0	0	0.006
Caloneis	0.10	0.38	0.01
Cocconeis	0.14	0	1.22
Conticribra	0	0	0.02
Craticula	0	0.001	0
Cyclotella	0	0.012	0.57
Cymbella	0.06	10.83	0.62
Denticula	0	0.33	0
Diadesmis	22.40	1.12	0.97
Diploneis	0.01	0.004	0
Encyonema	0	1.00	0
Encyonopsis	0.01	19.40	0.04
Entomoneis	0	0	0.03
Eunotia	0	0.02	0
Fallacia	0	0	0.03
Fistulifera	0	0.04	0.004
Fragilaria	0.02	2.46	0.01
Geissleria	0.01	5.08	0.014
Gomphonema	1.94	1.62	0.98
Halamphora	0.03	0.04	0.02
Hippodonta	0.04	0	0.007
Karayevia	0.02	0	0
Lemnicola	0	0.16	0.02
Luticola	0.004	0.02	0.001
Mayamaea	0.07	0.68	0.014
Melosira	0.001	0	0
Navicula	0.81	20.58	0.57
Nitzschia	0.89	3.11	65.13
Nupela	0	0.30	0
Opephora	0.50	0	0.52

Genus	Blue Spring	Ichetucknee	Silver Glen
Pinnularia	0.01	0.34	0
Placoneis	0.50	0.78	0.02
Planothidium	0.11	3.74	0.02
Pleurosira	55.90	2.37	26.84
Schizostauron	0.90	0.20	0.25
Sellaphora	0.58	3.48	0.01
Seminavis	0.02	0	0.22
Staurosira	2.61	17.36	0.39
Tabularia	0.09	0	0.004
Terpsinoe	12.96	0.20	1.26
Tryblionella	0.001	0	0
Ulnaria	0.003	0.47	0.14

Table 6. Number of *rbc*L operational taxonomic units (OTUs) assigned to each diatom genus based on sampling replicates.

	Location											
Genus	**VBS01**	**VBS02**	**VBS03**	**VBS04**	**ISP01**	**ISP02**	**ISP03**	**ISP04**	**SGS01**	**SGS02**	**SGS03**	**SGS04**
Achnanthes	1	1	1	1	0	0	0	0	1	1	1	1
Achnanthidium	0	0	0	0	4	4	3	3	1	1	1	0
Adlafia	0	0	0	0	1	1	1	1	0	0	0	0
Amphora	2	2	2	2	0	0	0	0	1	1	1	1
Asterionella	0	0	0	0	0	0	0	0	1	1	0	0
Bacillaria	0	0	0	0	0	0	0	0	1	1	1	1
Caloneis	2	2	2	2	2	4	3	2	1	1	1	1
Cocconeis	1	1	1	2	0	0	0	0	2	2	2	2
Conticribra	0	0	0	0	0	0	0	0	1	1	1	1
Craticula	0	0	0	0	2	1	1	0	0	0	0	0
Cyclotella	0	0	0	0	1	1	1	0	2	2	2	2
Cymbella	1	1	1	0	4	4	4	4	2	2	3	2
Denticula	0	0	0	0	1	1	1	1	0	0	0	0
Diadesmis	2	2	2	2	3	3	3	1	2	2	2	2
Diploneis	1	1	1	1	1	1	0	0	0	0	0	0
Encyonema	0	0	0	0	1	1	1	1	0	0	0	0
Encyonopsis	1	0	0	0	1	1	1	1	0	0	1	1
Entomoneis	0	0	0	0	0	0	0	0	1	1	1	1
Eunotia	0	0	0	0	1	1	1	1	0	0	0	0
Fallacia	0	0	0	0	0	0	0	0	1	1	1	1
Fistulifera	0	0	0	0	1	1	1	0	0	1	2	1
Fragilaria	1	1	1	1	2	2	2	2	1	1	1	1
Geissleria	1	1	1	1	1	1	1	1	1	1	1	1
Gomphonema	5	5	5	4	7	7	6	5	5	5	5	5
Halamphora	1	1	1	1	2	2	2	2	2	2	2	2

Genus	VBS01	VBS02	VBS03	VBS04	ISP01	ISP02	ISP03	ISP04	SGS01	SGS02	SGS03	SGS04
Hippodonta	1	1	1	1	0	0	0	0	1	1	1	1
Karayevia	1	1	1	1	0	0	0	0	0	0	0	0
Lemnicola	0	0	0	0	2	2	2	2	1	1	1	1
Luticola	1	1	1	1	1	1	1	0	1	1	1	0
Mayamaea	1	1	1	1	2	2	2	2	1	1	1	1
Melosira	0	0	1	1	0	0	0	0	0	0	0	0
Navicula	7	6	6	6	12	12	13	8	3	3	4	6
Nitzschia	9	11	11	10	8	7	7	6	9	14	14	12
Nupela	0	0	0	0	1	1	1	1	0	0	0	0
Opephora	1	2	2	1	0	0	0	0	1	1	1	1
Pinnularia	1	1	1	1	3	3	3	3	0	0	0	0
Placoneis	2	2	2	2	2	3	2	2	1	1	1	1
Planothidium	2	2	2	2	3	3	3	3	2	3	2	1
Pleurosira	1	1	1	1	1	1	1	1	1	1	1	1
Schizostauron	1	1	1	1	1	1	1	1	1	1	1	1
Sellaphora	7	7	8	6	9	9	7	6	2	2	2	2
Seminavis	1	1	1	1	0	0	0	0	1	1	1	1
Staurosira	2	2	2	2	7	7	5	4	4	4	5	5
Tabularia	1	1	1	1	0	0	0	0	1	1	1	1
Terpsinoe	1	1	1	1	1	1	1	1	1	1	1	1
Tryblionella	1	1	2	1	0	0	0	0	0	0	0	0
Ulnaria	0	1	1	1	2	2	2	2	2	2	2	2

Table 7. Shannon index values for 16S, 16S Algae Only, and *rbc*L for each replicate within a spring. Index values are provided for each DNA marker as well as algal only OTUs in the 16S dataset.

	Shannon Index		
Location	**16S**	**16S Algae Only**	***rbc*L**
VBS01	2.84	0.94	2.31
VBS02	2.90	0.57	2.27
VBS03	2.87	0.96	2.28
VBS04	2.95	1.03	2.25
ISP01	6.04	1.12	4.57
ISP02	6.69	1.39	4.58
ISP03	6.51	1.25	4.53
ISP04	5.37	0.93	4.38
SGS01	5.74	0.99	2.06
SGS02	5.71	1.02	2.01
SGS03	5.83	1.04	2.08
SGS04	5.70	1.02	2.06

Table 8. Comparison of diatom genera identified using light microscopy (LM) and next-generation sequencing (NGS) for each spring. Replicates within a site were pooled for NGS.

Blue Spring		**Ichetucknee**		**Silver Glen**	
LM	**NGS**	**LM**	**NGS**	**LM**	**NGS**
Achnanthes	*Achnanthes*	*Achnanthidium*	*Achnanthidium*	*Achnanthes*	*Achnanthes*
Achnanthidium	*Amphora*	*Cymbella*	*Adlafia*	*Achnanthidium*	*Achnanthidium*
Aulacoseira	*Caloneis*	*Gomphonema*	*Caloneis*	*Cyclotella*	*Amphora*
Gomphonema	*Cocconeis*	*Navicula*	*Craticula*	*Cymatopleura*	*Asterionella*
Melosira	*Cymbella*	*Synedra*	*Cyclotella*	*Cymbella*	*Bacillaria*
Navicula	*Diadesmis*	*Tabellaria*	*Cymbella*	*Gomphonema*	*Caloneis*
Synedra	*Encyonopsis*		*Denticula*	*Gyrosigma*	*Cocconeis*
Terpsinoe	*Fragilaria*		*Diadesmis*	*Melosira*	*Conticribra*
	Geissleria		*Dilponeis*	*Navicula*	*Cyclotella*
	Gomphonema		*Encyonema*	*Synedra*	*Cymbella*
	Halamphora		*Encyonopsis*	*Terpsinoe*	*Diadesmis*
	Hippodonta		*Eunotia*		*Encyonopsis*
	Karayevia		*Fistulifera*		*Entomoneis*
	Luticola		*Geissleria*		*Fallacia*
	Mayamaea		*Gomphonema*		*Fistulifera*
	Melosira		*Halamphora*		*Fragilaria*
	Navicula		*Lemnicola*		*Geissleria*
	Nitzschia		*Luticola*		*Gomphonema*
	Opephora		*Mayamaea*		*Halamphora*
	Pinnularia		*Navicula*		*Hippodonta*
	Placoneis		*Nitzschia*		*Lemnicola*
	Planothidium		*Nupela*		*Luticola*
	Pleurosira		*Pinnularia*		*Mayamaea*
	Schizostauron		*Placoneis*		*Navicula*
	Sellaphora		*Planothidium*		*Nitzschia*
	Seminavis		*Pleurosira*		*Opephora*
	Staurosira		*Staurosira*		*Placoneis*
	Tabularia		*Terpsinoe*		*Planothidium*
	Terpsinoe		*Ulnaria*		*Pleuosira*
	Tryblionella				*Schizostauron*
	Ulnaria				*Sellaphora*
					Seminavis
					Staurosira
					Tabularia
					Terpinsoe
					Ulnaria

Table 9. Mean values ± one standard deviation for dissolved oxygen (DO), ammonium (NH_4), total Kjeldahl nitrogen (TKN), nitrate-nitrite (NO_3-NO_2) phosphate (PO_4), total phosphorus (TP), water temperature (ºC), and pH from 2014-2018 for each spring sampled.

	Location		
Parameter	**Blue Spring**	**Ichetucknee**	**Silver Glen**
DO (mg/L)	0.893±0.17	3.77±0.79	3.56±0.34
NH_4 (mg/L)	0.043±0.04	0.003±0.002	0.002±0.002
TKN (mg/L)	0.128±0.05	0.036±0.02	0.022±0.02
NO_3-NO_2 (mg/L)	0.520±0.16	0.770±0.03	0.049±0.004
PO_4 (mg/L)	0.071±0.008	0.023±0.005	0.0234±0.004
TP (mg/L)	0.082±0.01	0.031±0.008	0.031±0.009
Water Temp(ºC)	23.32±0.07	21.77±0.12	23.16±0.16
pH	7.22±0.16	7.51±0.11	7.84±0.05

Chapter 2

Nodosilinea fontis sp. nov. (Leptolyngbyaceae, Synechococcales) and *Brasilonema variegatus sp. nov.* (Scytonemataceae, Nostocales): two novel cyanobacteria isolated from a karst spring in Northeast Florida, USA

ALYSSA D. GARVEY[1], EMILY A. GANSERT[1], ANDREW CHAPMAN[2], STEPHANIE M. WILLETTE,[1] PETR HAŠLER,[3] PETR DVOŘÁK,[3] DALE A. CASAMATTA[1]*

[1] *Department of Biology, University of North Florida, Jacksonville, FL, 32224, USA*

[2] *GreenWater Laboratories, 205 Zeagler Dr, Palatka, FL 32177, USA*

[3]*Department of Botany, Faculty of Sciences, Palacký University Olomouc, Šlechtitelů 27, CZ-771 46 Olomouc, Czech Republic*

[*] *Author for correspondence: dcasamat@unf.edu, phone: 904-620-1936, fax: 904-620-3885*

Abstract

Cyanobacteria are common components of aquatic habitats and are ubiquitous in nearly all ecosystems. However, alpha-level diversity among this lineage remains poorly understood due to cryptic diversity and phenotypic plasticity. This study examined cyanobacterial species diversity from a karst spring system (Ichetucknee Springs, Branford, FL, USA) and found two new species. Using a total evidence approach combining ecology, morphology, ITS structure, and molecular data (16S rDNA gene), we identified two new species: *Nodosilinea fonits* (Leptolyngbyaceae, Synechococcales) and *Brasilonema variegatus* (Scytonemataceae, Nostocales). Karst springs support vast amounts of biological diversity and may represent a potentially rich source of novel cyanobacteria.

Key words: Florida springs, 16S rRNA, biodiversity

Introduction

Cyanobacteria are a group of photo-oxygenic bacteria, and globally important contributors to the oxygen, carbon and nitrogen cycles (Rippka *et al*. 1979; Rippka 1988; Castenholz 1992; Whitton & Potts 2000). Cyanobacteria are often noted for their ubiquity and their significant role in freshwater harmful algal blooms (Hallegreaff 1993; Huisman *et al*. 2005; Paerl & Otten 2013). Recently, the increase of cyanobacterial abundances and distribution ranges have been attributed to changes in abiotic factors, such as temperature and climate change (Paul 2008; Paerl & Huisman 2009; Lopes *et al.* 2012). Anthropogenic impacts such as nutrient additions and habitat alteration are also postulated as contributing factors (MacGregor & Fabbro 2000; Paerl & Paul 2012).

While cyanobacteria are amongst the oldest lineages of Bacteria, their total biodiversity is poorly understood (MacGregor & Rasmussen 2008; Dvorák *et al*. 2015a). There is currently debate pertaining to the total number of cyanobacterial taxa, and many authors have pointed out that the total diversity has been only cursorily addressed (e.g., Ripkka 1988; Sherwood *et al.* 2014). This is especially true in seldom sampled habitats (e.g., benthic, epilithic, or subaerial habitats) or regions, such as the tropics or subtrops (Brito *et al*. 2012; Dvorák *et al.* 2015b). This is further exacerbated by morphological limitations in characters, with some forms (e.g., filaments) potentially masking significant cryptic diversity (Nelissen *et al.* 1996; Turner 1997; Casamatta *et al.* 2003). Molecular tools have greatly expanded our ability to identify and erect novel taxa, and led to a proliferation in the elucidation of basic alpha-level biodiversity (Rudi *et al.* 1997; Turner *et al.* 1999; Wilmotte *et al*. 2001; Teneva *et al*. 2005; Komárek 2010; Shih *et al.* 2013; Komárek *et al.* 2014; Stoyanov *et al.* 2014). It is now common for studies to combine

molecular tools with morphological methods when assessing cyanobacterial diversity to tease apart phylogenetic relationships (Johansen & Casamatta 2005; González-Resendiz *et al.* 2019).

Florida is home to over 1,000 karst springs, which may provide a source of novel cyanobacterial diversity. Karst springs are a unique set ecosystems in that they exhibit relatively stable conditions (i.e., water chemistry and temperature) year-round, supporting vast amounts of biological diversity (Ferrington 1995; Lydeard & Mayden 1995; Mattson *et al.* 1995; Andersen 2002; Garcia-Pichel *et al.* 2002; Martiny *et al.* 2011). Florida springs are highly valued for both their aesthetic and recreational value (Bonn & Bell 2003), with millions of visitors each year. These springs also represent a significant source of drinking water. Algal communities are routinely monitored throughout the Florida springs; however, algal diversity is assessed using morphological methods. Relying on morphological methods can be problematic when identifying cyanobacterial due to the lack of distinguishing characteristics for many genera, as well as phenotypic plasticity (Komárek *et al.* 2014).

During a survey of freshwater algae from Ichetucknee Springs, we found two novel taxa belonging to different genera. One species, described as *Nodosilinea fontis*, was found growing in an epipelic algal consortia on mud substrates, whereas *Brasilonema variegatus* was found growing periphytically on calcium carbonate rocks. These newly erected taxa are unique in ecology, 16S rDNA gene sequence, and ITS secondary folding patterns. Results from this study highlight the importance of combining morphological and molecular methods to more accurately identify cyanobacterial diversity.

Methods

Sampling location

The Ichetucknee Head Spring is located in the Ichetucknee Springs State Park in north central Florida in the Suwannee River Water Management District. Ichetucknee Springs is a 1st magnitude spring comprised of nine named springs that feed into the Ichetucknee River system, which empties into the Santa Fe River. The Ichetucknee Springs and River support a diverse assemblage of insects, amphibians, fish, reptiles, birds, and mammals. There are also a variety of recreational activities for visitors (e.g., swimming, snorkeling, diving, canoeing, and tubing). The Florida Springs Institute monitors the physical and chemical parameters of the springs, noting an increase in groundwater nitrate concentrations, which has led to elevated nitrate concentrations throughout the Ichetucknee Springshed. Along with increased nitrate levels, filamentous algae now cover much of the submerged aquatic vegetation and can be found throughout the springs.

Isolation and morphology

Algal samples were collected from various substrates within the Ichetucknee Head Spring during fall 2018. Attached forms of algae were removed from surfaces using syringes, forceps, and toothbrushes (Cantonati *et al.* 2007; O'Brien & Wehr 2010; Wehr 2013) then placed in 50 mL Falcon tubes containing 20 mL of sterile RNase free water. Samples were kept on ice until returning to the laboratory, where they were further subdivided for use in microscopy, preservation (5% glutaraldehyde), and culturing. Cultures were grown on Z8 medium (Staub 1961) under fluorescent and natural light (23°C, ca. 12:12 light: dark photoperiod). In addition, samples were grown on nitrogen-free Z8 medium to select for nitrogen fixing genera. Morphology of samples were analyzed using a light microscope Zeiss AxioImages (objectives EC Plan-Neofluar 40x/1.3 N.A., oil immersion, DIC, Plan-Apochromat 100x/1.4 N.A., oil immersion, DIC). Images were taken with a high-resolution camera (AxioCam HRc 13MPx).

Line drawings were also made to optimize the representation of characters such as nodules and thylakoids.

DNA extraction and sequencing

Whole genomic DNA was extracted using the DNeasy PowerSoil Extraction Kit (Qiagen, Hilden, Germany) in accordance to the manufacturer's instructions. DNA bands were visualized 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 8F (5' –AGTTGATCCTGGC– 3'), and reverse B23R (5' –CTTCGCCTCTGTGTGCCTAGG– 3') previously described in Osorio-Santos *et al*. (2014). The PCR amplification was completed in 50 µl reactions containing 19 µl sterile water, 2 µl of each primer (10 µM concentrations), 25 µl PCR Master Mix (Promega, Madison, WI), and 2 µl template DNA (50 ng/µl). The following thermocycler parameters were used for amplification: 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 57°C for 45°C s, 72°C for 4 min, and a final extension at 72°C for 10 min. Amplified 16S rDNA was cloned into pGEM® T Vector System I and JM-109 High Efficiency Competent Cells (Promega) and cultured using carbenicillin-infused LB medium. Plasmid DNA was purified from eight replicate transformed competent colonies per isolate using QIAprep® Spin Mini-prep kits (Qiagen). PCR purification and sequencing of cDNA from operons of varying sizes were performed by Eurofins Genomics (MWG Operon, Inc., Louisville, KY, USA).

A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to obtain closely related taxa. 16S sequences were combined with sequences from GenBank having ≥93% sequence similarity via BLAST searches. Additional outgroups from genera within the same

family were added for increased support. For all phylogenetic trees, sequences were aligned using MUSCLE (Edgar 2004) and manually checked and edited using MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger data sets (Kumar *et al.* 2015). The K2+G+I model was selected for *Nodosilinea* sequences and the K2+G model was selected for *Brasilonema* sequences and an unweighted maximum-parsimony (MP) and maximum-likelihood (ML) analysis was carried out for each data set with bootstrap support obtained from 1,000 pseudoreplicate data sets.

The 16S-23S ITS region was analyzed by determining secondary structures for the following conserved domains: D1-D1' helix and Box-B helix. Secondary structures of the specific ITS motifs were predicted using comparative analysis combined with confirmation in Mfold (Zuker 2003). Structures were compared to sequences from closely related taxa available in GenBank.

Results

16S phylogeny

Results of both ML and MP trees for *Nodosilinea* were largely congruous, but overall poorly supported (Fig. 2). A total of 66 taxa (ca. 1270 bp) were used to construct the trees, including all known *Nodosilinea* strains. All four of our strains fell within a single clade, with "*Leptolyngbya*" LEGE06308, a taxon isolated from a temperate estuary in Portugal. It seems likely that this strain is also a *Nodosilinea*, but the presence of nodules under low light conditions has not been confirmed. The new strains are also sister to the recently erected taxon *Nodosilinea ramsarensis* (Heidari *et al.* 2018) and as well as the type, *N. nodulosa*. While the lineage as a

whole is well supported, the poor internal bootstrap support indicates our lack of knowledge concerning the phylogenetic relationships of species at this time.

ML and MP trees for *Brasilonema* Fiore *et al.* (2007: 794) were well supported (Fig. 3). A total of 49 taxa (ca. 1410 bp) were used in tree construction, including all available *Brasilonema*. All three of our strains fell withtin a highly supported clade (100% support, Fig. 3) aligned with *B. tolantongensis* Becerra-Absalón & Montejano (2013: 27) (74/89% bootstrap support, Fig. 3) isolated from a subtropical, subaerophytic limestone environment (Becerra-Absalon *et al.* 2013). Our strains were also aligned with the type, *B. bromeliae* Fiore, Sant'Anna, de Paiva Azevedo, Komárek, Kástovsky, Sulek & Lorenzi (2007: 796), initially isolated from the bromeliad leaves (Fiore *et al.* 2007), but with little support.

Partial similarity matrix (p-distance) values for our newly sequenced *Nodosilinea* strains generated using 16S rDNA gene sequences ranged from 99.5-100 identical (Table 1). Further, our strains ranged from 99.1-99.5% similar to both the most closely aligned sister taxon (*N. ramsarensis*) as well as the type (Table 1).

P-distance values for our *B. variegatus* strains generated using 16S rDNA gene sequences ranged from 96.9-100 identical (Table 2). Further, our strains ranged from 96.9-97.1% similar to the most closely aligned sister taxon, *B. tolantongensis* (Table 2). The type, *B. bromeliae*, was 96.2-96.3 % similar to our strains.

ITS secondary structures

Two of our strains contained ITS regions with both tRNAs and were compared to six closely related *Nodosilinea* D1-D1´ structures. Our new strains were identical to each other, and shared an identical structure with other *Nodosilinea* strains, but differed in nucleotide sequence

(Fig. 4a,c). *N. fontis* strains (Figs. 4a,b) and *N. eplithica* (Fig. 4f) shared a common feature not noted in other strains: the substitution of a cytosine for a uracil side bulge in position 48. Likewise, while our strains were structurally identical to the type and other *Nodosilinea*, there were differences in substitutions in the large, terminal bulge (e.g., Fig. 4a vs. Fig. 4c).
As with the D1-D1´ structures, the Box B structure of our strains shared identical sequences with virtually the same ultrastructure as *N. epilithica* (with the difference being a single nucleotide insert in the terminal bulge of *N. epilithica*, but otherwise identical structures Fig. 5a,b and 5f). This terminal bulge was comprised of uracil and adenine for *N. fontis*, whereas *N. epilithica* contained guanine and adenine. There appears much more heterogeneity of both structure and sequences in the Box B between the species of *Nodosilinea*, with the type sharing an identical structure to *N. epilihtica* Ru-6-11, yet with different nucleotides (Fig. 5c,d).

We recovered two operons for *B. variegatus* and ITS sequences contained both tRNAs When comparing D1-D1´ helices to other know strains, we note significant differences both in structure and nucleotide sequence within and amongst strains (Fig. 6). One of our strains, IS-ADG3 clone 2C, was 89 nucleotides in length versus 63 for ADG-2, illustrating the heterogeneity of this helix. The structural motifs for the two operons for *B. variegatus* did not resemble other D1-D1´ structures, nor did the other strains show similar folding patterns (Fig. 6). Less variability was noted for the Box B structures for *Brasilonema* (Fig. 7). The greatest difference between any of the motifs was recovered from IS-ADG2, which was both substantially larger (45 nucleotides) and of a different topology than any other Box B in the genus (Fig. 7a). The second structure, recorded from IS-ADG3, was similar to other Box B helices, but with two differences. It contained fewer nucleotides and thus presented a smaller

terminal bulge (Fig. 7b), but had similar overall structure. Also, IS-ADG3 possessed a cytosine side bulge at position 25 in contrast to the typical adenosine side bulge at position 25.

Morphological assessments

Nodosilinea fontis strains formed nodules when grown in low light conditions (Fig. 8c), a feature lost when grown under ambient light levels. Filaments either produced kinks or physically wound around themselves. Filaments were cylindrical with distinct cell constrictions (Fig. 8a,b). Sheaths, when formed, were elaborate and inflated, with yellowish coloration as they aged. Trichomes were immotile and cells mainly slightly longer (1.4-3.8 ± 0.59 µm) than wide (1.0-2.0 ± 0.25 µm, Table 3). When grown under ambient light levels, older cultures tended to form fascicles (Fig. 8b).

Brasilonema variegatus strains had macroscopic thalli and were grass green to gray-green with erect growth on nitrogen-free medium. Drastic color changes were noted along a single filament, becoming purple with age in nature (and culture) (Fig. 8), growing in mats on granite substrate. Filaments are mostly straight but occasionally kinked, abruptly tapering towards the end, and were frequently false branched. Sheaths were firm, thin, colorless, and attached to the filament. Cells are usually wider than long with extensive chromatoplasm and central nucleoplasm, frequently granulated, highly vacuolated, 3.2-4.0 µm long (2.5-5) × 12.5-14 µm wide (11.5-15), with apical cells rounded and occasional calyptra. Heterocytes are common, yellowish green, multiple per filament (not adjacent), often flattened but typically square to hemispherical, 10.0-13.3 µm long × 15.0-18.5 µm wide, akinetes not present. Reproduction occurred by hormogonia and fragmentation of trichomes via necridic cells. Meristematic zones if present are usually short and located near the apices.

Discussion

Florida karst springs may represent a potential source of novel cyanobacterial diversity. Previous studies of these systems have relied on traditional morphological analyses alone (e.g., Phelps *et al.* 2006; Stevenson *et al*. 2007; Quinlan *et al.* 2007), potentially underrepresenting algal diversity. Freshwater springs are unique in that they maintain relatively stable conditions year-round (e.g., temperature and water chemistry), thus supporting vast amounts of biological diversity (Blinn & Prescott 1976; Breitbart *et al*. 2009; Brodie *et al.* 2009; Cantonati & Lange-Bertalot 2010), and have been termed "Imperiled Arks of Biodiversity" (Clements *et al.* 2006). However, in recent decades the springs have seen an increase in both nitrate contamination and filamentous algal proliferation (Upchurch *et al.* 2007; Stevenson *et al.* 2007).

By using a polyphasic approach during a survey of algal communities from Ichetucknee Springs, we identified two species new to science, one of which belongs to the genus *Nodosilinea. Nodosilinea* is a recently described genus characterized by the presence of nodules when cultures are grown in low light conditions (Perkerson *et al*. 2011). The purpose of these nodules is currently unknown, but has been hypothesized to play a role in nitrogen fixation or anti-herbivory (Li & Brand 2007). The ecology of this genus seems quite broad, with taxa from marine, freshwater, polar, sub-tropical, aerophytic habitats, saline/alkaline lakes, and Brazilian mangroves (e.g., Andreote *et al.* 2014; Stoyanov *et al.* 2014). Our new strains represent the first occasion of *Nodosilinea* being isolated from sub-tropical, freshwater springs.

A second taxon was identified belonging to the genus *Brasilonema.* This genus was first described by Fiore *et al.* (2007) from subaerophytic habitats in subtropical and tropical Brazil. Later studies have described more species isolated from Hawaii (Vaccarino & Johansen 2012),

southern Europe, central Mexico (Becerra-Absalón *et al*. 2013), and from a tripartite lichen and cemetery tombstones in northern Florida (Villaneuva *et al.* 2018; Villaneuva *et al.* 2019), thus suggesting a cosmopolitan distribution. This genus is characterized by macroscopic, fasciculated filaments, with coloration ranging from violet to blackish-violet with false-branching, generally colorless rigid sheaths, and vacuolized cells (Fiore *et al.* 2007). Our isolated strains were most closely related to *B. tolantongensis*, isolated from central Mexico (Becerra-Absalón *et al.* 2013), where it was found in a subaerophytic habitat. While our ITS secondary structures varied from other species of *Brasilonema*, the two D1- D1´ helices for our strains also differed from each other. This was also the case for clonal replicates for *B. geniculatum* (Villaneuva *et al.* 2019), suggesting potential heterogeneity in this lineage. Very few species of *Brasilonema* have sequenced ITS regions and clonal replicates available in GenBank, making it difficult to draw definitive conclusions about folding patterns in this lineage.

One of the chief difficulties in understanding the basic biodiversity of cyanobacteria stems from issues surrounding the species concepts. Cyanobacteria have long been known to exhibit cryptic biodiversity, which may be exacerbated by ambiguous morphological characters (Casamatta *et al.* 2003; Larsson *et al.* 2011, Dvořák *et al*. 2015b). Further, questions pertaining to the species concepts in cyanobacteria may inform whether or not there exists a justification to erect a new taxon (e.g., Johansen & Casamatta 2005; Komárek 2006; Komárek 2010). A recent study by González-Resendiz *et al.* (2019) urges researchers to address several questions before placing a novel strain to an existing or new species, which we address below.

We propose the erection of two new taxa based on the phylogenetic species concepts, which serves as a model for cyanobacterial systematic endeavors (Komárek *et al*. 2014). In regard to the questions posed in González-Resendiz *et al.* (2019), our *Nodosilinea* and

Brasilonema strains resemble morphological characteristics of other described species; however, they differ in ecology. *N. fontis* was found growing epipssamically, whereas other species were found in marine, terrestrial, or thermal spring locations. It is worth noting, our *Nodosilinea* strains were closely related to *N. ramsarensis*, a strain isolated from thermal springs; however, *N. fontis* differed in sheath morphology, D1- D1´ nucleotide sequence, and Box B ultra-structure. Our isolated strains of *Brasilonema* were found growing periphytically on Ca_2CO_3 rocks in a freshwater environment, whereas other strains were isolated from aerophytic, subaerophytic, and epiphytic environments. Along with difference in ecology, strains of *B. variegatus* shared <98.7% 16S rRNA genetic identity with other named *Brasilonema* strains (Table 3), and nucleotide sequence and ultra-structure of both the D1- D1´ and Box B helices deviated from other species. Taken together, we feel these results that provide sufficient evidence for the erection of novel species within *Nodosilinea* and *Brasilonema.*

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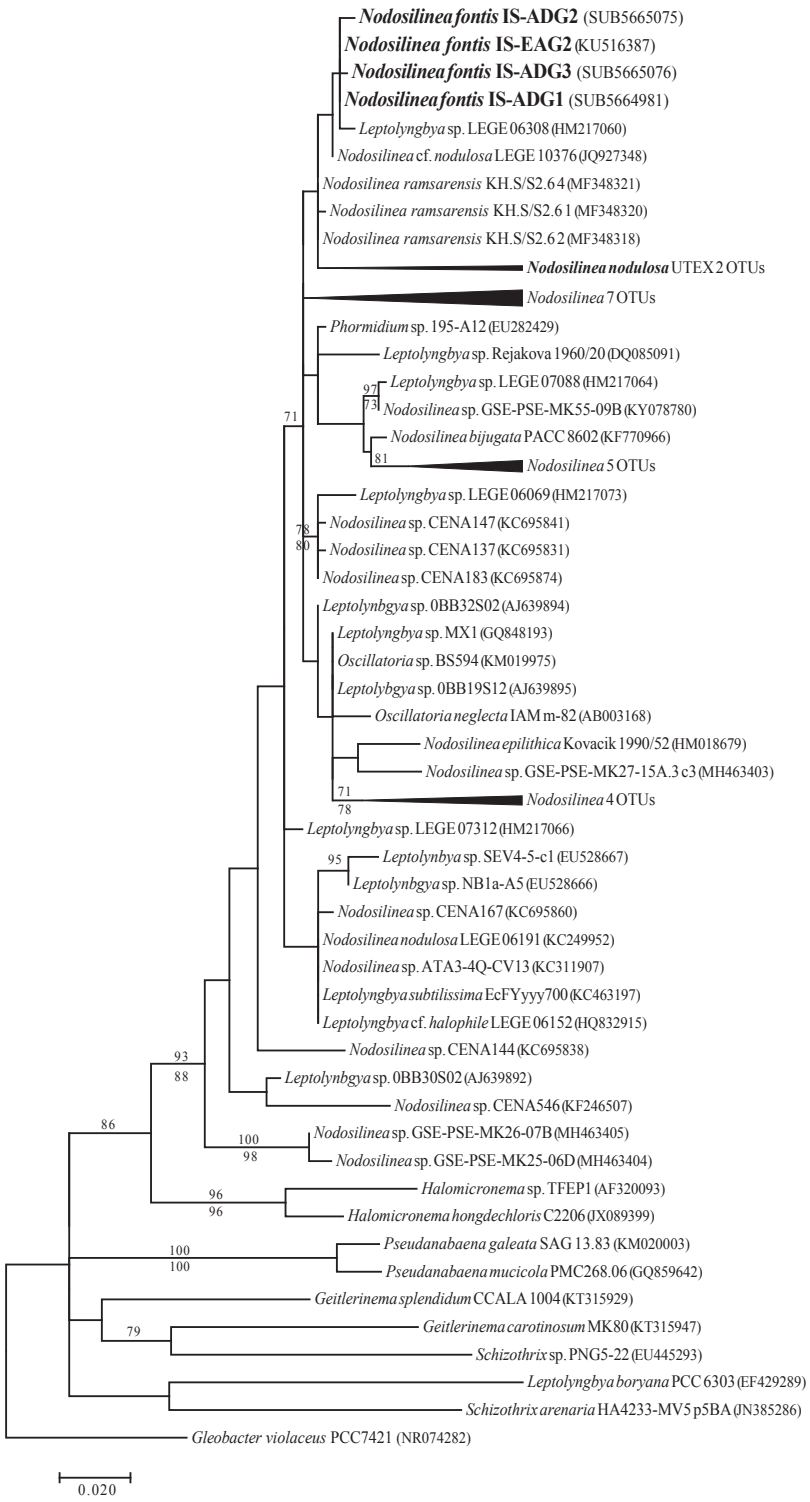

Figure 1. Maximum likelihood tree of *N. fontis* and the closest relatives based on 16S rRNA gene sequences. Numbers above the line represent ML values, numbers below MP.

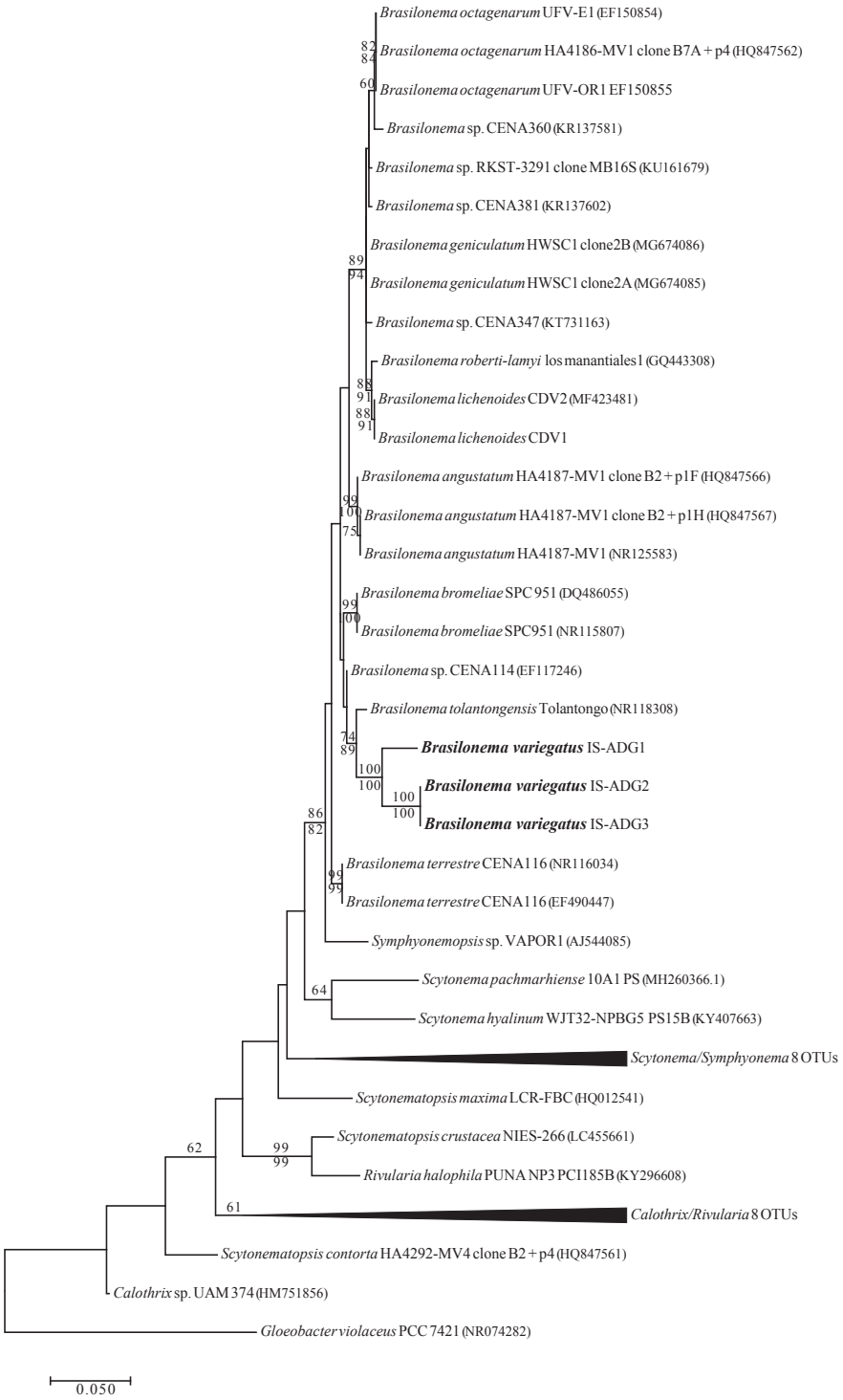

Figure 2. Maximum likelihood tree of *B. variegatus* and the closest relatives based on 16S rRNA gene sequences. Numbers above the line represent ML values, numbers below MP.

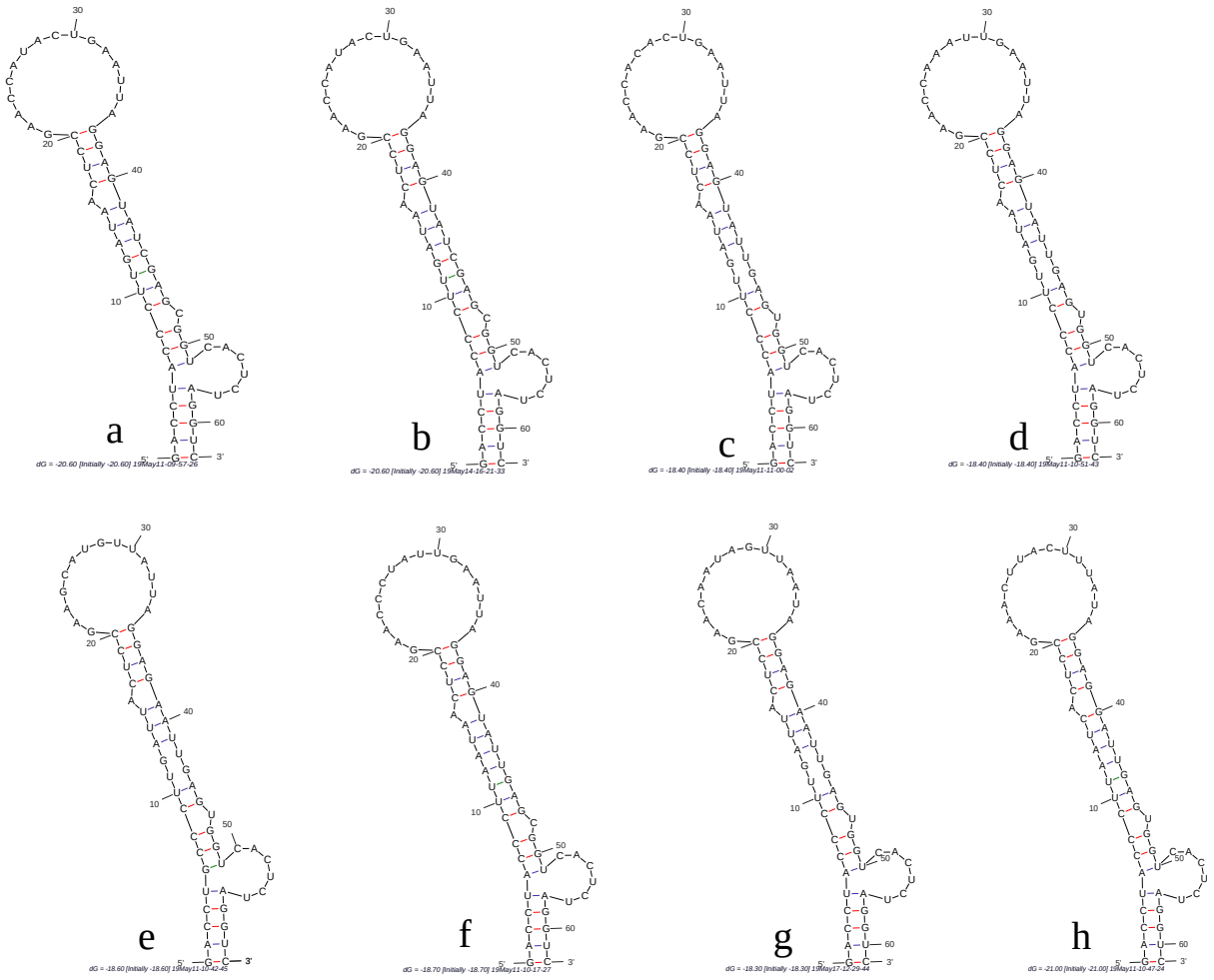

Figure 3. D1-D1´ helices for *N. fontis sp. nov.* and closet relatives for which ITS sequence data is available a) *N. fontis* IS-ADG2, b) *N. fontis* IS-ADG3, c) *N. nodulosa* UTEX 2910, d), *N.* cf. *epilithica* Ru-6-11, e) *Nodosilinea* sp. GSE-PSE-MK25-06D, f) *N. epilithica* Kovacik, g) *Nodosilinea* sp. GSE-PSE-MK55-09B, h) *N. ramsarensis* KH.S/S2.6 2.

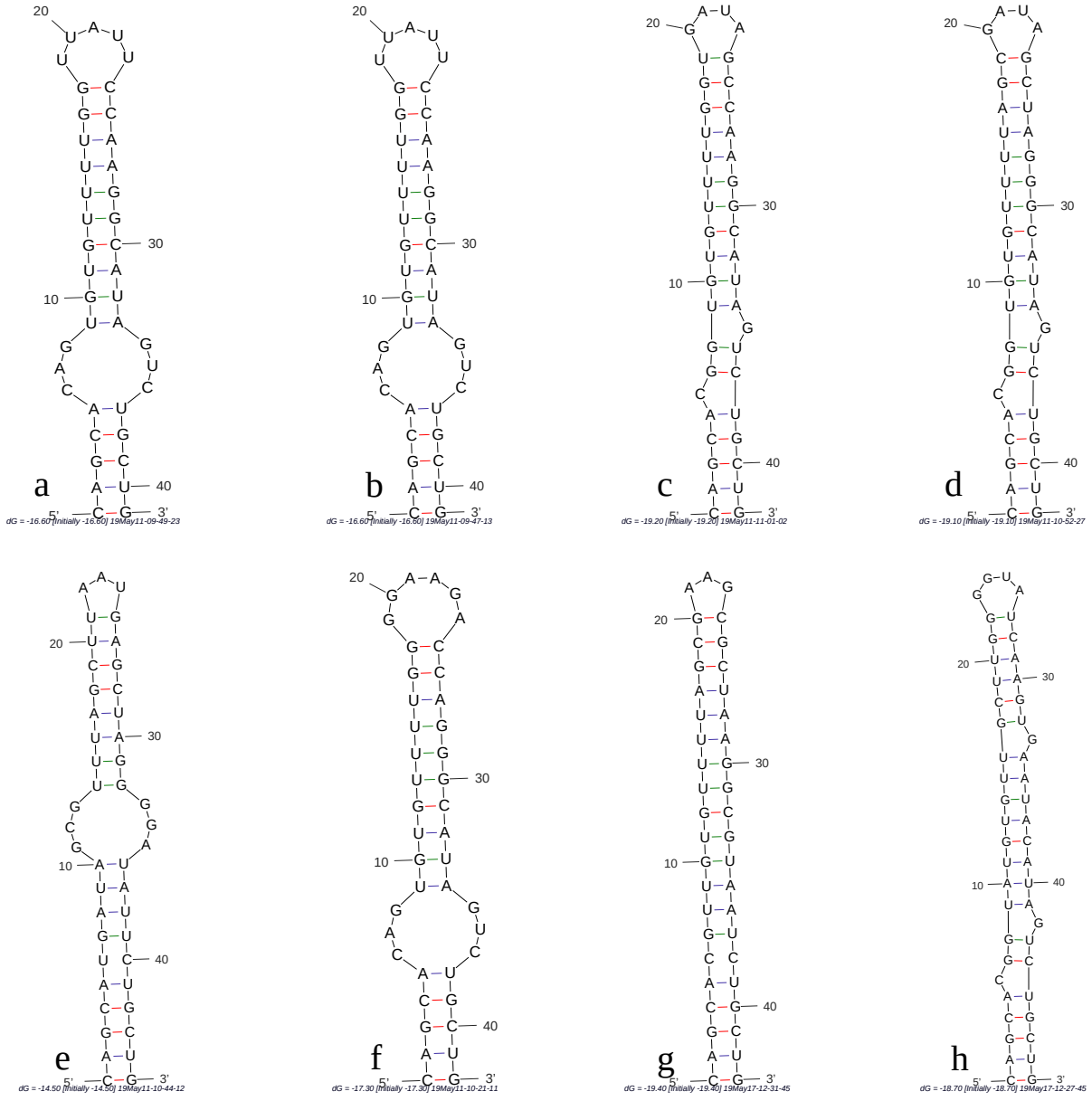

Figure 4. Box B helices for *N. fontis sp. nov.* and closet relatives for which ITS sequence data is available a) *N. fontis* IS-ADG2, b) *N. fontis* IS-ADG3, c) *N. nodulosa* UTEX 2910, d) *N.* cf. *epilithica* Ru-6-11, e) *Nodosilinea* sp. GSE-PSE-MK25-06D, f) *N. epilithica* Kovacik, g) *Nodosilinea* sp. GSE-PSE-MK55-09B, h) *N. ramsarensis* KH.S/S2.6 2.

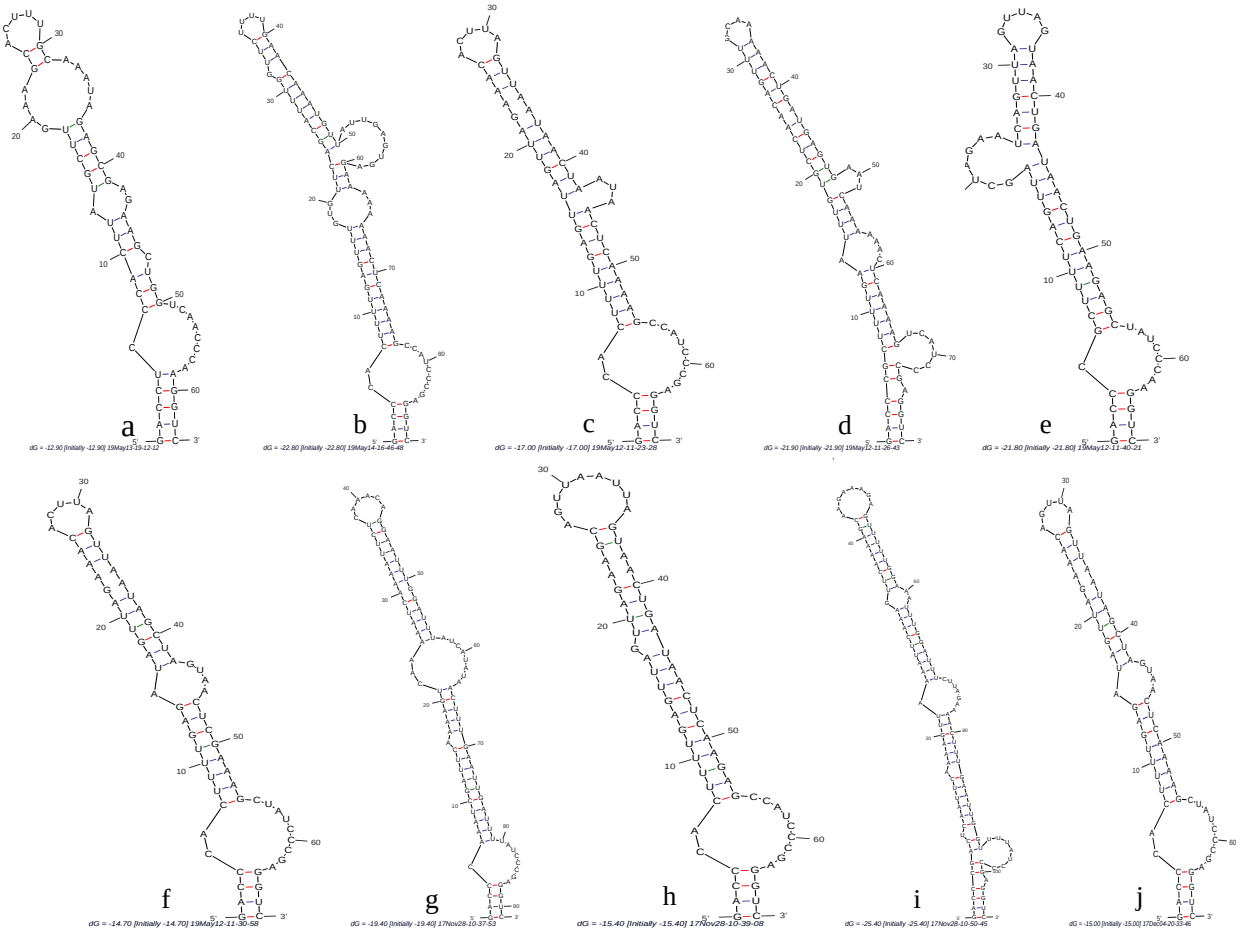

Figure 5. D1-D1´ helices for *B. variegatus* and closet relatives for which ITS sequence data is available a) *B. variegatus* IS-ADG2, b) *B. variegatus* IS-ADG3 clone 2C, c) *B. lichenoides* clone 5A, d) *B. angustatum* HA4187-MV1 clone b2+p1h, e) *B. angustatum* HA4187-MV1 clone b2+p1f, f) *B. octogenarum* HA4186-MV1 clone b7a+p4, g) *B. geniculosus* HWSC4C Clone IIA, h) *B. geniculosus* HWSC4C Clone IIB, i) *B.* sp. RKST-3291 clone MB, j) *Brasilonema* sp. RKST-322 clone C1.

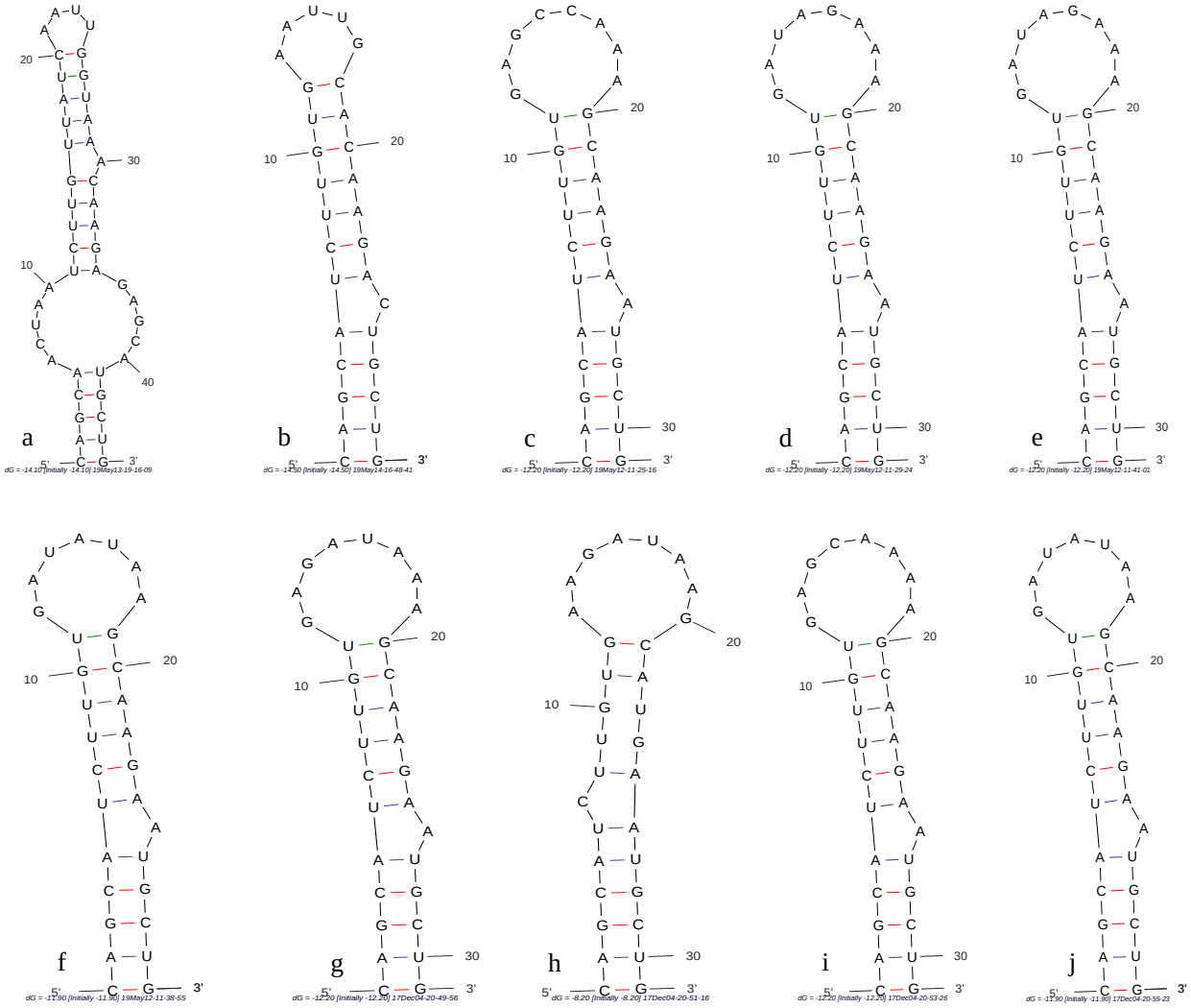

Figure 6. Box B helices for *Brasilonema variegatus* and closet relatives for which ITS sequence data is available a) *B. variegatus* IS-ADG2, b) *B. variegatus* IS-ADG3 clone 2C, c) *B. lichenoides* clone 5A, d) *B. angustatum* HA4187-MV1 clone b2+p1h, e) *B. angustatum* HA4187-MV1 clone b2+p1f, f) *B. octogenarum* HA4186-MV1 clone b7a+p4, g) *B. geniculosus* HWSC4C Clone IIA, h) *B. geniculosus* HWSC4C Clone IIB, i) *B.* sp. RKST-3291 clone MB, j) *B.* sp. RKST-322 clone C1.

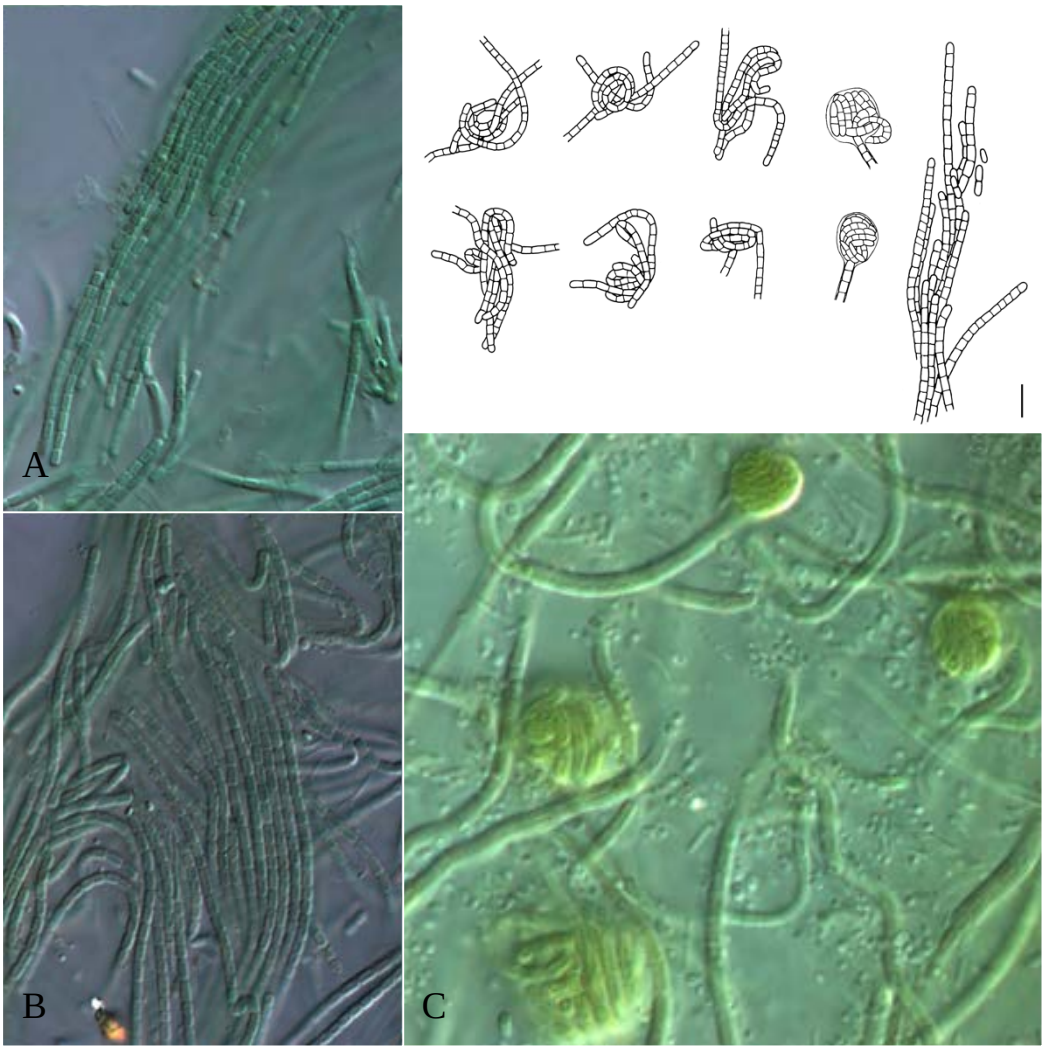

Figure 7. Photomicrographs of *N. fontis*. The unique character of this genus, the presence of copious nodules, is only seen when cultures are grown in low light levels for 5-7 days a) *N. fontis* IS-EAG2 (week old culture under low light), b) *N. fontis* IS-EAG2 (week old culture under low light), c) *N. fontis* IS-ADG1 (ambient light). Scale bar 10 μm.

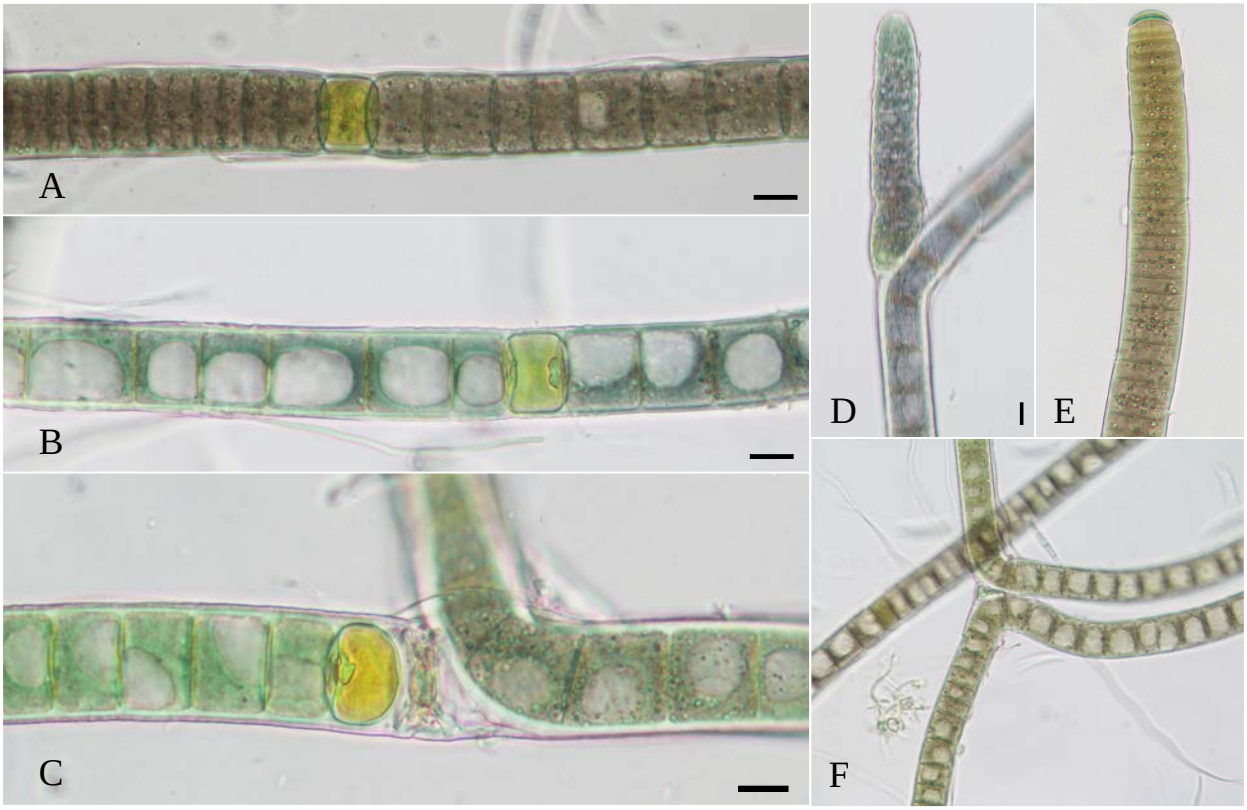

Figure 8. Photomicrograph of new taxon *B. variegatus*. Morphological variability in heterocytes, false branching, and filament coloration. Scale bar 10 μm.

Table 1. Partial similarity matrix (p-distance) for *Nodosilinea* generated using 16S rDNA gene sequences.

Taxa	1	2	3	4	5	6	7	8	9	10	11
1. *N. fontis* IS-ADG01											
2. *N. fontis* IS-ADG02	99.6										
3. *N. fontis* IS-ADG03	99.8	99.5									
4. *N. fontis* IS-EAG2	100	99.6	99.8								
5. *N. nodulosa* UTEX 2910- Type	99.3	98.9	99.1	99.3							
6. *N. bijugata* PACC 8602	97.2	96.8	97.0	97.2	97.9						
7. *N. epilithica* str Kovacik 1990/52	97.9	97.5	97.7	97.9	97.5	96.5					
8. *N.* cf. *epilithica* RU-6-11	96.3	95.9	96.1	96.3	97.0	99.1	96.3				
9. *Nodosilinea* sp. GSE-PSE-MK55-09B	98.0	97.7	97.9	98.0	98.4	99.1	96.6	98.2			
10. *N. ramsarensis* KH.S/S2.6 2	99.5	99.1	99.3	99.5	99.8	97.7	97.7	96.8	98.6		
11. *Nodosilinea* sp. GSE-PSE-MK25-06D	95.4	95.0	95.2	95.4	95.4	96.1	93.8	95.2	97.0	95.6	
12. *Leptolyngbya boryana* PCC 6303	89.9	89.5	89.7	89.9	89.3	89.5	90.0	89.0	90.0	89.3	91.6

Table 2. Partial similarity matrix (p-distance) for *Brasilonema* generated using 16S rDNA gene sequences.

Taxa	1	2	3	4	5	6	7	8	9	10	11
1. *B. variegatus* IS-ADG1											
2. *B. variegatus* IS-ADG2	96.9										
3. *B. variegatus* IS-ADG3	100.0	96.9									
4. *B. tolantongensis*	96.9	97.1	96.9								
5. *B. bromeliae* SPC951	96.2	96.3	96.2	98.4							
6. *B. geniculatum* HWSC1 clone 2A	96.4	96.6	96.4	98.2	98.2						
7. *B. lichenoides* CDV2	96.2	96.3	96.2	98.5	97.9	99.6					
8. *B. robert-lamyi* los manatiales1	96.1	96.2	96.1	98.4	97.8	99.5	99.6				
9. *B. angustatum* HA4187-M1 clone B2	95.9	96.1	95.9	98.2	98.5	98.9	98.6	98.5			
10. *B. octagenarum* UFV-OR1	96.3	96.4	96.3	98.6	98.0	99.6	99.4	99.3	98.8		
11. *B. terrestre* CENA116	96.1	96.2	96.1	98.0	98.6	98.0	97.8	97.7	98.4	98.0	
12. *Brasilonema* sp. CENA360	96.2	96.3	96.1	98.3	97.8	99.3	99.0	99.1	98.5	99.6	97.8

Table 3. Morphological and ecological comparisons for novel taxa, *N. fontis*, and sister taxa. Includes type species, *N. nodulosa*.

Character	*Nodosilinea fontis* (all strains)	*Nodosilinia nodulosa* UTEX 2910	*Nodosilinea nodulosa* PCC7104	*Nodosilinea epilithica*	*Nodosilinea bijugata*	*Nodosilinea conica* SEV4-5c1	*Nodosilinea ramsarensis*
Cell width (μm)	1.0-2.0	1.1-1.5	1.0-1.5	1.5-2.5	1.5-1.7	2.5-2.7	1.0-1.5
Cell length (μm)	1.4-3.8	1.2-2.4		1.2-8.0	1.5-6.2	0.9-2.4	1.0-2.0
Constricted	Distinct	Distinct	Distinct	Distinct	Distinct	Distinct	Distinct
Apical cell	Rounded	Rounded	Bluntly rounded	Rounded	Rounded	Bluntly conical	Rounded
Collection location	Benthic mat in freshwater spring Ichetucknee Springs, Florida, USA	South China Sea (marine), plankton tow, 10 m depth	Rocky shoreline (marine), Montauk Point, New York, USA	Vieste, Pennisula Gargano, Itlay, green biofilm on wall of a house	Eutrophic Lake Piaseczno, Poland, Psammic in the littoral zone	Sevilleta LTER site, New Mexico, Chihuahuan Desert soil	Soil around thermal spring, Khaksefid, Ramsar, Iran
Special features	Formation of nodules; elaborate and inflated sheaths, older cultures formed fascicles under ambient light	Capable of anaerobic N fixation; inflated sheaths; cells mostly isodiametric, flattened	Capable of anaerobic N fixation; cells mostly isodiametric	Cells typically barrel shaped after cell division, cylindrical in non-dividing trichome	Cells typically barrel shaped to spherical; inflated sheaths	Bluntly conical end cells are unique for this clade	Solitary filaments with thin, colorless sheath, formed nodules seldomyly

Table 4. Morphological and ecological comparisons for novel taxon, *B. variegatus*, and sister genera. Includes type species, *Brasilonema bromelia*

Character	*B. variegatus*	*B. bromelia*	*B. geniculatum*	*B. lichenoides*	*B. robert-lamyii*	*B. angustatum*	*B. octegenarum*
Filament width (μm)	12.5-20	10-21	12.1-20	8-12	12-18	9.8-18	9.8-18.5
Cell length (μm)	2.5-5	1.8-16	3.2-8.6	2-4		3-9	1.5-13.3
Heterocyte shape	Square to flattened, occasionally rectangular	Discoid to cylindrical	Occasionally flattened, typically square	Flattened, round or hemispherical	Discoid to cylindrical	Elongated	Discoid to cylindrical
Heterocyte position	Intercalary, multiple per filament, solitary	Intercalary	Intercalary, single or in pair	Intercalary		Intercalary	Basal or intercalary
Sheath color	Colorless	Colorless to yellowish brown	Purple-brown	Brown-orange to colorless	Colorless	Colorless	Colorless
Cell color	Grass green to grey-green, becoming purple with age	Blue, gray, olive or brownish violet green	Green to blue/green or brownish	Green to blue/green		Brownish or purple grey	Olive green or brownish-violet
False branching	Common	Rare	Abundant, single & germinate	Abundant, single & germinate	Rare, Tolypothricoid	Abundant, single & germinate	Rare, Tolypothricoid, Scytonematoid
Ecology	Periphytic on Ca_2CO_3 stones	Small pools, on wooden substrates	Subaeropyhytic on stone	Endolithic lichen	Aerophytic	Terrestrial, epiphytic	Epiphytic

VITAE

Alyssa D. Garvey

Education

Master of Science in Biology
August 2019
University of North Florida, Jacksonville, Florida

Bachelor of Science in Marine Biology
May 2015
University of New Haven, West Haven, Connecticut

Experience

Science Laboratory Coordinator, Nov. 2015 – Jan. 2017
St. Johns River State College, Orange Park, Florida

Aquatic Phycology Intern, Summer 2015
New York Botanical Garden, Bronx, New York

Presentations

Garvey, A.D., Besuden, A., Casamatta, D.A. (2019, June) Multi-marker metabarcoding as a new approach for monitoring periphytic algal communities in the Florida Springs. Oral presentation at the Phycological Society of America Annual Conference.

Garvey, A.D., Casamatta, D.A. (2018, October) Periphytic algae in a Florida karst spring: establishing a community baseline for future monitoring. Poster presented at the Southeastern Phycological Colloquy.

Garvey, A.D., Brown, A.B., Foss, A.J., Villaneuva, C.D., Casamatta, D.A. (2018, April) *Komarekiella delphikthonos* sp. nov. (Cyanobacteria): an epidermal cyanobacterium implicated in an estuarine bottlenose dolphin (Tursiops truncatus) fatality. Oral presentation given at the Northeast Algal Society Symposium.

Garvey, A.D., Villaneuva, C.D., Casamatta, D.A. (2017, July) Identifying biogeographical patterns within *Brasilonema* (Scytonemataceae, Cyanobacteria) using 16S rRNA and ITS secondary structures. Poster presented at the Phycological Society of America Annual Conference.

Garvey, A.D., Carlile, A.L. (2015, August) Cryptic diversity of algal turf communities isolated from Long Island Sound, Connecticut, USA. Poster presented at the Phycological Society of America Annual Conference.

Garvey, A.D., Carlile, A.L. (2015, August) Identifying cryptic diversity of algal turf communities within Long Island Sound, Connecticut, USA. Oral presentation at the Marine Biology Senior Symposium

Garvey, A.D., Vallei, P., Eisthen, H. (2013, July) Isolation and identification of tetrodotoxin (TTX) producing bacteria on the rough-skinned newt, *Taricha granulosa*. Poster presented at the REU Summer Undergraduate Research Symposium.

Publications

Villanueva, C.D., **Garvey, A.D**., Hasler, P., Dvorak, P., Poulickova, A., Norwish, A.R. & Casamatta, D.A. (2019) Descriptions of *Brasilonema geniculatum* and *Calothrix dumas*

(Nostocales, Cyanobacteria): two new taxa isolated from cemetery tombstones. *Phytotaxa* 387 (1): 001-020.

Grants and Travel Awards

2019. Phycological Society of America Hoshaw Travel Award, $750.
2018. University of North Florida Graduate School, Graduate Research Grant, $600.
2017. Phycological Society of America Hoshaw Travel Award, $500.
2017. University of North Florida Environmental Center, Water Grant "Identifying Periphyton Algal Diversity in Ichetucknee Springs, FL, and their Response to Eutrophic Conditions" $5,000.