Phytotaxa 181 (5): 243–260
www.mapress.com/phytotaxa/

Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

http://dx.doi.org/10.11646/phytotaxa.181.5.1

Aetokthonos hydrillicola gen. et sp. nov.: Epiphytic cyanobacteria on invasive aquatic plants implicated in Avian Vacuolar Myelinopathy

SUSAN B. WILDE[1]*, JEFFREY R. JOHANSEN[2,3], H. DAYTON WILDE[4], PENG JIANG[4], BRADLEY A. BARTELME[1] & REBECCA S. HAYNIE[5]

[1]*Warnell School of Forestry and Natural Resources. University of Georgia, Athens, GA 30602.*

[2]*Department of Biology, John Carroll University, University Heights, OH 44118.*

[3]*Department of Botany, Faculty of Science, University of South Bohemia, 31 Branišovská, 370 05 České Budějovice, Czech Republic.*

[4]*Department of Horticulture, University of Georgia, Athens, GA 30602.*

[5]*SePRO Corporation, 11550 North Meridian Street, Suite 600, Carmel, IN 46032.*

**Corresponding author (swilde@uga.edu)*

Abstract

Research into the taxonomy of a novel cyanobacterial epiphyte in locations where birds, most notably Bald eagle and American coots, are dying from a neurologic disease (Avian Vacuolar Myelinopathy—AVM) has been ongoing since 2001. Field investigations revealed that all sites where birds were dying had extensive invasive aquatic vegetation with dense colonies of an unknown cyanobacterial species growing on the underside of leaves. Morphological evaluation indicated that this was a true-branching, heterocystous taxon falling within the former order Stigonematales. However, 16S rRNA gene sequence demonstrated that it did not match closely with any described genus or species. More recent sequence analysis of the 16S rRNA gene and associated ITS region from additional true branching species resulted in a unique phylogenetic placement distant from the other clades of true-branching cyanobacteria. Light, epifluorescent, and transmission and scanning electron micrographs confirm the novel characteristics of this species, which is true-branching form with uniseriate basal filaments. It is encased within a firm sheath and has heterocytes both within the filaments and at the tips of the branches. The species is in a new genus of uncertain family assignment, and is herein named *Aetokthonos hydrillicola gen. et sp. nov.*

Introduction

A fatal disease, Avian Vacuolar Myelinopathy (AVM) killing bald eagles (*Haliaeetus leucocephalus* Linneaus (1766: 123)) and waterfowl in the southeastern U.S. was first documented in DeGray Lake, Arkansas in 1994. During the first three years of monitoring this emerging disease, 70 bald eagles and numerous American coots (*Fulica americana* Gmelin (1789: 704)) were found neurologically impaired or dead at DeGray Lake and nearby Arkansas lakes. Investigations of the mortality events included thorough necropsies and screening tissues for toxins and infectious disease agents. The only consistent finding was the presence of a unique lesion, an intramyelinic edema, most pronounced in the optic tectum and cerebellar tracts within the brains of affected birds in the myelin sheath of the brain (Thomas *et al.* 1998). In the absence of infectious disease agents and anthropogenic toxins that can cause particular type of lesion, researchers began to suspect that the eagles and waterbirds were exposed to a novel biologically produced neurotoxin (Fischer *et al.* 2006).

State and federal biologists from North Carolina, South Carolina, and Georgia tasked with bald eagle recovery were involved in the investigation in Arkansas. The following winter (1998), these biologists noted waterbirds displaying the same clinical signs of neurologic impairment within their home states. Impaired coots were collected during late fall from Woodlake (NC), Thurmond Lake (SC/GA), and Lake Juliette (GA) and histological examinations documented the characteristic AVM brain lesions. Wing clipped sentinel birds (wild American coots and game farm mallards *Anas platyrhynchos* Linnaeus 1758: 125) released at a North Carolina reservoir during a late fall mortality event became neurologically impaired within as few as five days (Rocke *et al.* 2002, Augspurger *et al.* 2003). Mallards housed in an off-site research facility with AVM-afflicted coots did not become neurologically impaired or develop

Accepted by Saúl Blanco Lanza: 25 Aug. 2014; published: 10 Oct. 2014

AVM lesions (Larsen *et al.* 2003). With the expansion of the disease locations, and the knowledge that the exposure to the neurotoxin occurred on site, research efforts shifted to a critical evaluation of all reservoirs where birds were dying from AVM.

Limnological investigations focused on a search for neurotoxins that birds might be exposed to or ingest at these reservoirs. Researchers had not observed any planktonic blooms of known neurotoxin producing cyanobacteria in the reservoirs during the time when bird deaths were occurring in late fall-winter. All of the reservoirs were man-made impoundments and all supported dense, non-native, invasive aquatic plant communities. During an extensive survey of the planktonic, benthic and epiphytic cyanobacteria, a novel species of epiphytic cyanobacteria was noted on hydrilla (*Hydrilla verticillata* (Linnaeus 1782: 416) Royle (1839: 376)) from Lake J. Strom Thurmond (GA/SC). Subsequent investigations confirmed that the previously undescribed cyanobacterial species was growing on invasive aquatic vegetation in all locations where birds were dying from AVM (Wilde *et al.* 2005). DeGray Lake was dominated by non-native Brazilian elodea (*Egeria densa* Planchon (1849: 80)) during 1994–1996, but hydrilla was also present and by 2000 was the dominant submerged aquatic plant in that system. All of the sites where AVM was documented from 1994–2005 had extensive hydrilla, Brazilian elodea, or Eurasian watermilfoil (*Myriophyllum spicatum* Linnaeus (1753: 992)) (Wilde *et al.* 2005). These dense, invasive aquatic macrophyte beds provide ideal substrate for attachment by epiphytic cyanobacteria capable of toxin production and should be considered when evaluating risk of harmful algae in water supply lakes and reservoirs (Wilde *et al*. 2005, Mohamed & Al-Shehri 2010). Feeding trials and field surveys support the hypothesis that birds ingest an unknown neurotoxin produced by this novel epiphytic cyanobacterial species colonizing the aquatic plants (Birrenkott *et al.* 2004, Wilde *et al.* 2005, Wiley *et al.* 2009, Haynie *et al.* 2013). Identification of this cyanobacterial species is critical in order to advance neurotoxin isolation and characterization work that is currently underway. Identifying this species may also afford clues about how to control the species and/or mitigate factors that enhance neurotoxin production.

The undescribed species was a filamentous, heterocytous, true branching cyanobacterium that, according to morphology, was a member of the Stigonematales (Williams *et al.* 2007). The 16S rRNA sequence identity was determined from environmental isolates of this unknown species using DGGE (denaturing gradient gel electrophoresis). The 16S rRNA sequence data were aligned with additional cyanobacteria sequences to determine designations for probe development, to advance understanding of the species' phylogeny, and to lay groundwork for its formal description (Williams *et al.* 2007). Phylogenetic data available at the time supported a sister taxon position to some members of the Stigonematales, this final description expands and completes the genetic and morphological characterization of Williams *et al.* (2007).

This cyanobacterium has since been isolated and characterized in both natural populations and in culture using light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). A longer sequence read in the 16S rRNA gene, phylogenetic analysis with larger taxon sampling, and characterization of sequence and secondary structure of the 16S-23S ITS region demonstrated that this organism belongs to a novel clade in the heterocytous cyanobacteria distant from other lineages historically associated with the Stigonematales. We herein describe it as a new genus and species, *Aetokthonos hydrillicola gen. et sp. nov.*, and will refer to it by this name in the remainder of the paper.

Materials and Methods

Sample collection and culturing:—Aquatic plant samples were acquired from every location where bird deaths from AVM had occurred to check for the presence of the unknown cyanobacterial epiphyte (Table 1, Fig. 1). This included the twelve locations described in Wilde *et al.* (2005) and three more recent locations where eagles or waterbirds were confirmed to have AVM lesions and aquatic plant samples were screened. Four additional sites were considered at risk based on the presence of hydrilla and dense colonization by the epiphyte. In order to evaluate whether the AVM disease was occurring in birds, intensive surveys, sentinel field trials, and laboratory-feeding trials were conducted as previously reported in Wilde *et al.* (2005), Fischer *et al.* (2006), Wiley *et al.* (2007) and Haynie *et al.* (2013). Submerged aquatic plant samples were collected from within one meter of the top of the canopy from all locations during September–December. Plants were placed in 3.8 L plastic bags and screened within 24 hours to evaluate the density of the target species and facilitate successful cultivation. Wet mounts of the plant leaves were visualized under 200x magnification using epifluorescence microscopy on Olympus BX51, X-Cite 120 Flourescence Illumination System EXFO, TRITC/Rhodamine filter (excitation: 505–560 nm, emission 575–655 nm) equipped with a Olympus

DP20 digital camera in order to assess percent coverage on the leaves. Since these colonies are so large that they can be seen with dissecting scope under 50x magnification, we were able to guide the isolation of target colonies from the leaf using microforceps and place them on BG-11 agar plates. Cultures were continually transferred to fresh agar plates to facilitate the development of monocultures of the target cyanobacterium. Agar plates were incubated in varying light intensities from 10–150 µmol photons m^{-2} s^{-1} until the optimal light regime was established. Strength of culture medium varied from 10% to 50% BG-11 to determine the optimal level for culture growth. Cultures were maintained in a growth chamber at 25 ºC and 12:12 h light:dark regime.

Morphological analysis:—Growth form of the cyanobacterial thallus on hydrilla leaves was examined using fluorescence microscopy on a Olympus BX51, X-Cite 120 Flourescence Illumination System EXFO, TRITC/ Rhodamine filter (excitation: 505–560 nm, emission 575–655 nm) equipped with a Olympus DP20 digital camera. Characterization of the thallus, filament morphology, and cellular dimensions was conducted using Olympus BX51 equipped with a Olympus DP20 digital camera. Cellular ultrastructure was studied using transmission electron microscopy (TEM). Cells were fixed with 2 % glutaraldehyde in 0.1 M Na cacodylate buffer, and later post-fixed with 1% osmium tetroxide. The fixed material was dehydrated in an ethanol series (30, 50, 70, 80, 90, 95 and 100%) and embedded in Spurr's resin (Spurr 1969, Electron Microscopy Sciences, Hatfield PA). Ultrathin cross-sections were stained with uranyl acetate and lead citrate and examined in a FEI Technai20 TEM (FEI, Inc., Hillsboro, OR) at 200 kV and documented with an AMT camera. Visualization of the exterior of the thallus (particularly apical cells) was conducted using scanning electron microscope Zeiss 1450EP. Material was fixed in 2% glutaraldehyde in 0.1M Na cacodylate buffer and then post-fixed in 1% OsO4 in Nacacodylate buffer. The samples were then critical point dried (Samdri CPD, Tousimis, Rockville MD) and mounted on aluminum stubs. The material was then sputter coated with gold approx. 15nm thick and imaged on a Zeiss 1450EP SEM (Carl Zeiss SMT, Thornwood NY).

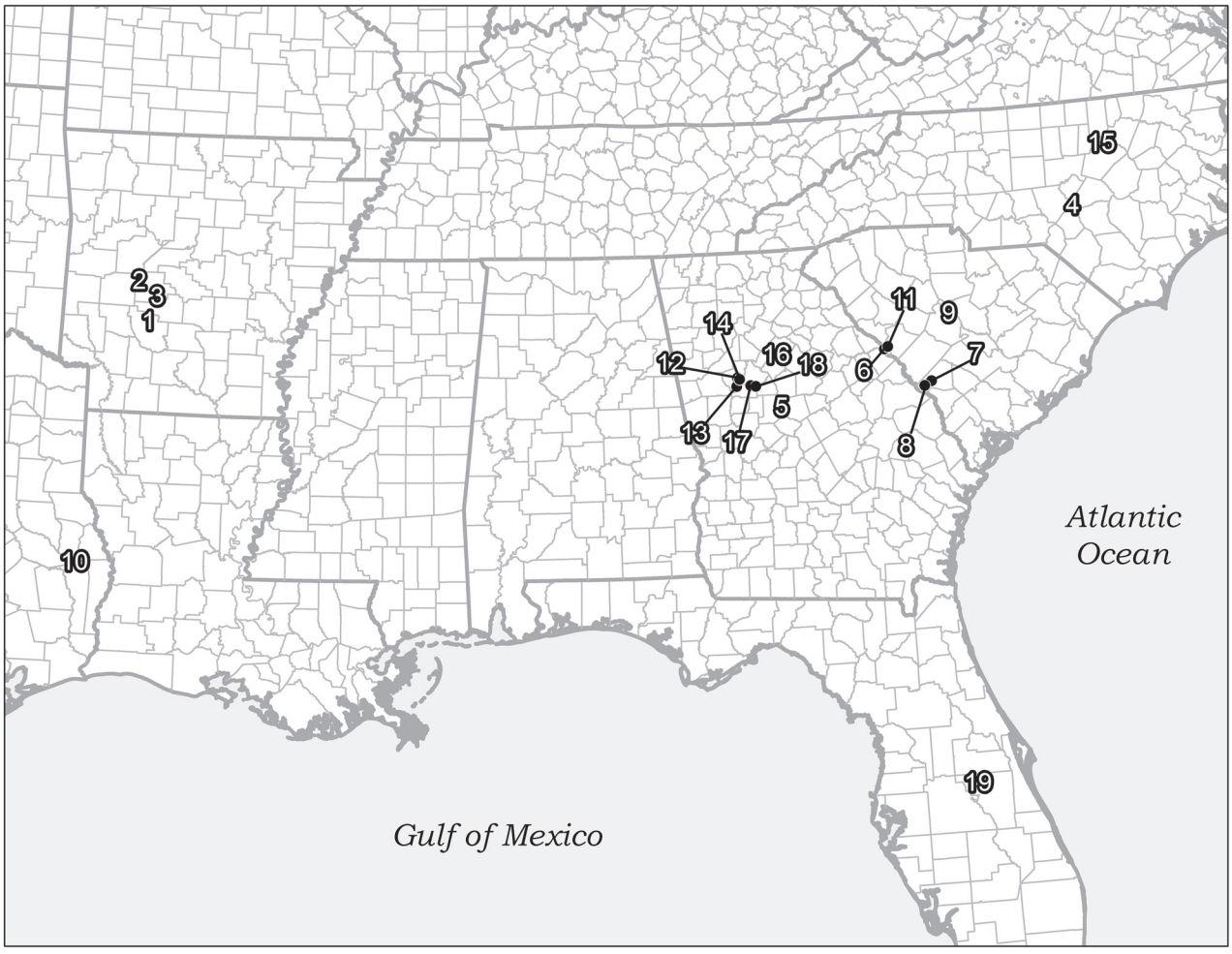

FIGURE 1. Locations (in order of confirmation) across the Southeastern United States where *Aetokthonos hydrillicola*, invasive plants (*Hydrilla*, *Egeria*, *Myriophyllum*), and bird deaths from AVM have been confirmed.

TABLE 1. Reservoir locations where AVM has occurred in order of AVM confirmation. All sites contained invasive plants *Hydrilla*, *Egeria* and/or *Myriophyllum* together with *A. hydrillicola*. Max coverage of is the maximum surface area of the leaf comprising *A. hydrillicola* colonies. Map numbers correspond to locations shown in Fig. 1.

Map	Year	Reservoir	State	Size (hectares)	Plant	Max coverage (%)
1	1994	DeGray	AR	2,260	Egeria/hydrilla	95
2	1996	Quachita	AR	6,561	Hydrilla	95
3	1997	Hamilton	AR	1,189	Milfoil	25
4	1998	Thurmond	SC/GA	11,464	Hydrilla	95
5	1998	Juliette	GA	573	Hydrilla/Egeria/Milfoil	50
6	1998	Woodlake	NC	185	Hydrilla	50
7	1998	Par Pond	SC	432	Milfoil	25
8	1998	L Lake	SC	169	Milfoil/Hydrilla	25
9	1999	Murray	SC	8,320	Hydrilla	25
10	1999	Sam Rayburn	TX	18,752	Hydrilla	10
11	2003	Davis Pond	SC	1	Hydrilla	95
12	2003	Emerald Lake	GA	2	Hydrilla	75
13	2005	Lake Horton	GA	128	Hydrilla	95
14	2005	Smith Reservoir	GA	41	Hydrilla	25
15	2005	Coachman's Trail	NC	3	Hydrilla	95
	2007	Troup County				
16	2007	Lake Varner	GA	135	Hydrilla	95
17	2010	Upper Towaliga	GA	180	Hydrilla	95
18	2011	Longbranch	GA	45	Hydrilla	95
	2011	Morgan County	GA			
19	2012	Lake Tohopekaliga	FL	3,717	Hydrilla	50

Molecular analysis:–DNA was initially extracted from environmental samples using a rapid CTAB buffer DNA isolation technique (Schaefer 1997) and a fragment of the 16S rRNA gene was sequenced using the 1055F forward primer (5'-ATGGCTGTCGTCAGCT-3') and the 1046R reverse primer (5'-ACGGGCGGTGTGTAC-3') as described in Williams *et al.* (2007). This yielded a fragment from nt 1020–1330 in the 16S rRNA gene. Subsequently, additional primers were used to conduct PCR of most of the 16S rRNA gene (from nt 12–1350, see Williams *et al.* 2007). A Cleaved Amplified Polymorphic Sequences (CAPS) assay was designed to increase the specificity of detection of environmental samples. Alignment of 16S rDNA sequences from *A. hydrillicola* and related cyanobacteria found two unique *Rsa*I restriction sites within a 459 bp fragment flanked by the primer binding sites 5'-GTCCGCAGGTGGTATTTCAA-3' (forward) and 5'-CTCCCGAAGGCACTCTTAAC-3' (reverse). *Aetokthonos hydrillicola* could be identified by the presence of a 247 bp band on a 2% agarose gel. Genomic DNA of each strain was isolated using UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.; Carlsbad, CA, USA). A partial region of the 16S rRNA gene (bp 323 to end) and the associated 16S–23S ITS region was amplified using polymerase chain reaction (PCR) with primers 1 and 2 sensu Boyer *et al.* (2001, 2002) yielding a DNA product of 1564 nucleotides. The reactions were prepared in a total reaction volume of 25 µl as described in Lukešová *et al*. (2009). PCR was run in a S1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) set to 35 cycles of: 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 2 min 15 s, followed by 5 min extension at 72 °C and 4 °C hold for an indefinite amount of time. Amplified products were visualized and checked on a 1% agarose/ethidium bromide gel, and subsequently cloned into plasmids using the StrataClone PCR Cloning Kit (La Jolla, CA, USA). Plasmids were isolated from transformed cells using QIAprep Spin Kit (QIAGEN, Carlsbad, CA, USA). The presence of the expected insert was confirmed by plasmid digestion using *Eco*R I. Three purified plasmids with a confirmed insert of expected size were sequenced by Functional Biosciences, Inc. (Madison, WI, USA) using plasmid-anchored primers M13 forward, M13 reverse and internal primers 5, 7, and 8 *sensu* Boyer *et al.* (2001, 2002). Partial sequences were aligned into contigs using Sequencher (v. 4.9, Ann Arbor, MI, USA). Subsequently, a single contig was assembled using the sequence from both first and second PCR amplifications, yielding a consensus contig of 1876 nucleotides.

Phylogenetic Analyses:—To resolve generic identity of our strains, consensus sequences of the 16S rRNA genes

(a partial sequence of 1159–1163 nucleotides) were manually aligned with other sequences available on the NCBI Nucleotide Database (June 2013) using secondary structure to assist in alignment in regions with single-base insertions and deletions. These sequences were chosen using either Blast Search against our sequences, or by their identification with genera in the subclass Nostochophycidae. We especially included all true-branching cyanobacterial taxa in NCBI by searching for them by genus name. In total this alignment of the 16S rRNA gene contained 446 sequences including four *Chroococcidiopsis* sequences including the type *C. thermalis* Geitler (1933: 625) NR102464 as well as *Gloeobacter violaceus* Rippka *et al*. (1974: 435) FR798924 as outgroup taxa. Bayesian analysis was run using the GTR-Γ model. Two runs of eight Markov chains were executed for 30 million generations with default parameters, sampling every 100 generations (the final average standard deviation of split frequencies was lower than 0.02), and the first 25% of sampled trees were discarded as burn-in. This analysis was conducted on the computer cluster MetaCentrum (www.metacentrum.cz), maintained in the Czech Republic. After this analysis, we pared the taxon set down to 318 OTUs and ran the Bayesian Analysis again using 40 million generations. Both trees gave very similar topologies, both with respect to *Aetokthonos* and the other taxa in the analysis. Only the latter tree is shown. We also ran analyses in PAUP v.4.02b (Swofford 1998) to construct the other trees. Trees using parsimony as the optimality criterion in a heuristic search had the following settings: 'multree's = 'no', 'branch-swapping algorithm' = 'TBR', 'gapmode' = 'newstate', 'steepest descent' = 'yes' and 'nreps' = '1000'. Trees using distance as the optimality criterion in a heuristic search had the following settings: 'distance = HKY85', 'multree's = 'no', 'branch-swapping algorithm' = 'TBR', 'gapmode' = 'missing', 'steepest descent' = 'yes' and 'nreps' = '1000'. Bootstrap support was based on running 1000 replicates for both of these analyses. All trees were viewed using FigTree (Rambaut 2007) and subsequently re-drawn using Adobe Illustrator CS5.1. P-distance of 16S sequences was determined in PAUP to reveal similarity among strains of interest. The ITS secondary structures of helices D1–D1', Box-B, and V3 were derived using M-fold (Zuker 2003) and re-drawn in Adobe Illustrator CS5.1 for easy comparison with available structures from other true-branching taxa.

Results

Class **Cyanophyceae**
Subclass **Nostocophycideae**
Order **Nostocales**
Family **Incertae sedis**

Aetokthonos Wilde *et* Johansen, *gen. nov.*

Morphologically similar to *Fischerella* (Bornet & Flahault 1887: 100) Gomont (1895: 52), from which it differs by possession of dehiscent apical cap cells at the apices of the branch filaments.

Type species:—*Aetokthonos hydrillicola*

Description:—Main filaments creeping, forming attached irregular disks on the underside of leaves of aquatic plants, more or less radiating from the center of the colony, sometimes biseriate to multiseriate, typically branching mostly to one side. Sheath colorless, homogenous. Trichomes monoseriate to multiseriate, tapering towards filament apices. Cells in main filaments compressed spherical to almost spherical. Cells in branches becoming longer than wide and cylindrical. End cells separated from subterminal cells, forming a dehiscent cap like structure at the end of branches. Heterocytes present, mostly intercalary, rarely lateral. Akinetes present, in masses within the main filaments. Hormogonia released from tips of branches.

Etymology:—*Aetokthonos* (Gr.) = 'Eagle killer'

Aetokthonos hydrillicola Wilde *et* Johansen, ***sp. nov.*** (Figs. 2–4)

Main filaments creeping, forming attached irregular disks on the underside of leaves of aquatic plants, more or less radiating from the center of the colony, sometimes biseriate to multiseriate, typically branching mostly to one side, subtorulose, 9.1–14.0 μm wide. Sheath absent, thin, or firm and slightly widened, colorless, homogenous, sometimes extending beyond tips of branches following hormogonia release. Trichomes mostly monoseriate, biseriate to multiseriate in older parts of colony, tapering abruptly towards ends of main filaments, tapering more gradually in terminal parts of branches, 6.0–8.0 μm wide. Cells in main filaments often with their

own envelopes, compressed spherical to almost spherical, after division semi-quadratic, with minutely granular dull blue-green cell contents, 5.0–10.0 μm long, 10.0–14.0 μm wide. Cells in branches becoming longer than wide and cylindrical, not strongly constricted at the crosswalls, ungranulated, 8.0–10.0 μm long, 9.5–11.0 μm wide. Cytoplasm with irregular thylakoids showing a characteristic pattern with phycobilisomes, with polyphosphate bodies and cyanophycin. End cells separated from subterminal cells, forming a cap like structure at the end of branches (possibly functioning as a hormocyte), typically with reduced thylakoid development, becoming colorless in LM, sometimes remaining attached to branch after separation and consequently appearing to rise mid-trichome. Heterocytes mostly intercalary with two polar nodules, rarely lateral or terminal with a single polar nodule, light tan-colored to almost clear, 7.0–9.0 μm long, 9.0–10.5 μm wide. Akinetes enlarged, with thickened walls, granular, dark yellowish olive in color, in masses within the main filaments, shorter than wide, 10.5–13.0 μm wide. Hormogonia short, few celled, released from tips of branches.

Type:—UNITED STATES. South Carolina, Lake Thurmond (holotype: US Algal collection #217986, Algal Herbarium, Smithsonian National Museum of Natural History, Washington, DC.). Reference strain: CCALA 1050, Culture Collection of Autotrophic Organisms, Třeboň Czech Republic.

Habitat:—Growing on the underside of aquatic plants in subtropical regions.

Etymology:—*hydrillicola* = 'living on *Hydrilla*'.

Observations:—*Aetokthonos hydrillicola* is morphologically and ecologically closest to *Fischerella reptans* Geitler (1933: 629) var. *reptans* and *F. reptans* var. *stigonematoides* (Geitler 1933: 629). Both varieties of *F. reptans* are tropical and occur growing on aquatic plants. They differ in cell size, in particular in having branches that are considerably thinner (4–6 μm wide). Akinetes were not recorded for this species. The apical caps were also not reported. Further study (i.e. molecular sequencing) of these very poorly known taxa must be completed before we know if they belong to *Fischerella* or *Aetokthonos*. Nineteen sites from North Carolina to Arkansas have been confirmed as AVM positive sites and all sites contain invasive aquatic plants (primarily hydrilla) and dense colonies of *A. hydrillicola* (>50% leaf coverage). These colonies appear as dark spots on the underside of the leaf and can be been with the naked eye while still in the field collecting plant material. While *Aetokthonos* colonies are large enough to be seen on the leaf in sunlight, these colonies are quite cryptic under normal light microscopic illumination. Epifluorescent lighting and a rhodamine filter allowed optimal discernment of cyanopigments within the cyanobacterial cells on the surface of the hydrilla leaf. The species assemblage on the hydrilla leaves containing *Aetokthonos* includes both diatoms and additional cyanobacterial species, and obtaining pure cultures was challenging. Our final successful cultures were isolated from Lake Thurmond, GA/SC during October 2012 using 10% BG11 media under low light conditions (< 20 μmol s^{-1} m^{-2}). Colony growth overall was slow in culture, and was markedly higher in the lower nutrient agar (10% BG11). *Aetokthonos hydrillicola* could be identified genetically by a CAPS assay, in which a 247 bp restriction fragment was diagnostic for the species (Fig. 5). In field sample analyses, a fragment of 314 bp was often observed that resulted from the presence of other cyanobacteria or possibly from incomplete *Rsa*I digestion.

***Toxicity*:—**Laboratory feeding trials have been conducted to confirm the presence of the AVM neurotoxin within the hydrilla/*A. hydrillicola* complex. Farm raised mallards and chickens have been used to investigate the potential toxicity of field-collected hydrilla containing the epiphytic cyanobacteria *A. hydrillicola*. Trial duration varied from 12–28 days, and total quantity of the hydrilla material varied, but in all cases, only birds receiving the hydrilla material with the *A. hydrillicola* epiphytes developed the characteristic AVM lesions (Table 2). The hydrilla from control sites (Lake Seminole, Harris Lake, and Lake Marion) did have numerous epiphytic diatoms, chlorophytes and cyanobacterial species, but the novel *A. hydrillicola* species was not present. All of the trials were conducted using hydrilla collected during late fall (October–December) when densities of *A. hydrillicola* are maximal on the hydrilla leaves at AVM sites. Since 2001, we have had the capability to screen for *A. hydrillicola*, and have been able to confirm the presence of dense colonies of this species growing on invasive plants during the AVM mortality events in Arkansas, North Carolina, South Carolina and Georgia (Fig. 1, Table 1).

Phylogeny*:—Aetokthonos* is phylogenetically isolated from all other true-branching clades (Fig. 6, arrowed clades). It clustered with an uncultured bacterium (MIZ23) sequence, which is so similar in sequence (98.2%) that we assume that this sequence belongs to an undescribed and uncharacterized *Aetokthonos* species (Table 3). Of the true branching clades (Stigonemataceae, Hapalosiphonaceae, Chlorogloeopsidaceae, Symphyonemataceae), *Aetokthonos* is closest to strains in the Stigonemataceae (e.g. *Stigonema ocellatum* Thuret ex Bornet & Flahault (1887: 69), *Petalonema* Berkeley ex Correns (1889: 321) in Table 3). However, in none of our numerous phylogenetic analyses did the genus cluster with the Stigonemataceae with any support. It was generally distant, as shown in the Bayesian

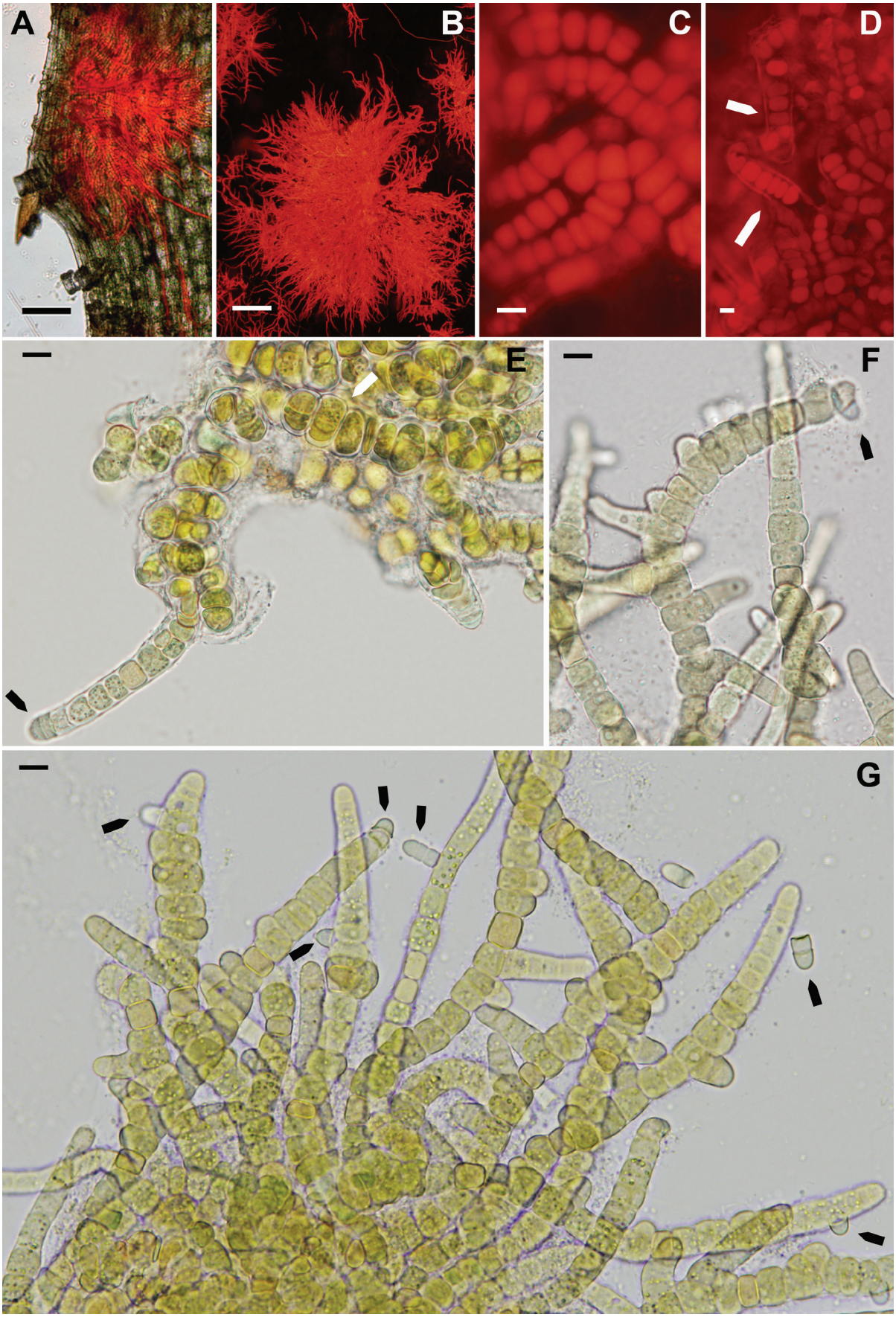

FIGURE 2. Light microscope views of *Aetokthonos hydrillicola*. A. Fluorescence microscopy of *A. hydrillicola* on leaf of *Hydrilla*, scale = 100 µm. B. Fluorescence microscopy of thallus morphology in culture, scale = 100 µm. C–D. Fluorescence microscopy of branching filaments, arrows show hormogonia production in sheath, Scale = 10 µm. E. Thallus showing apical caps (black arrow) and thick-walled enlarged cells possibly functioning as akinetes (white arrow), scale = 10 µm. F–G. Thallus showing typical branching pattern and dehiscent apical cells (hormocytes?) (arrows), scale=10 µm.

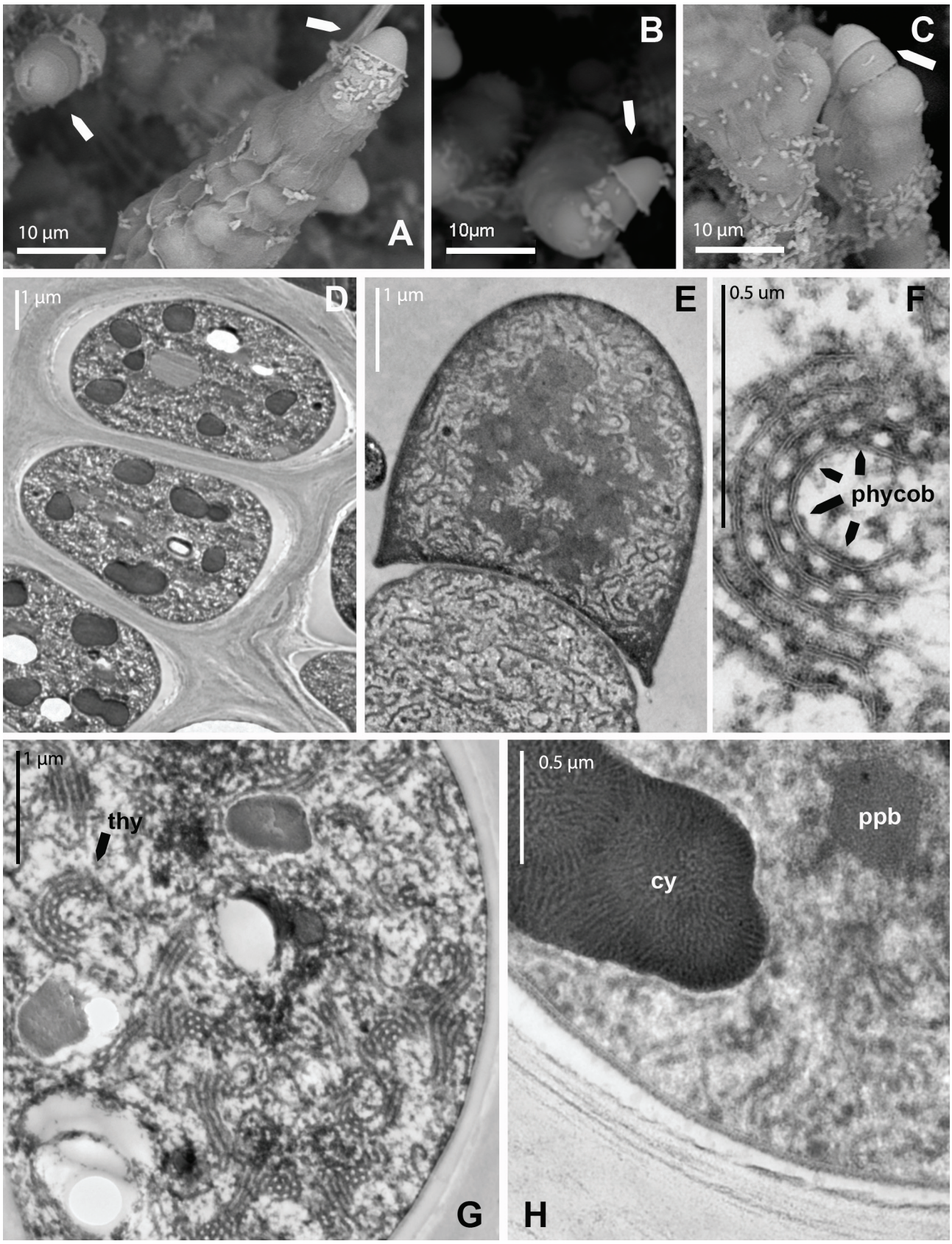

FIGURE 3. Electron Microscope views of *Aetokthonos hydrillicola*. A–C. Scanning electron microscope views showing dehiscent apical caps (white arrows). D. Cells embedded in firm, thick, mucilage. Dark inclusions are likely cyanophycin bodies. E. Dehiscent apical cell showing initiation of dehiscence. F. Four thylakoids with characteristic electron dense and electron light areas, with dark areas associated with thylakoids representing phycobilisomes. G. Cell showing characteristic thylakoids, arrowed thylakoids shown at greater magnitude in Fig. 3F. H. High magnification views of cyanophycin and polyphosphate bodies.

TABLE 2. Summary of feeding trials using hydrilla with and without epiphytic *A. hydrillicola* colonies. Lesion rate varied from 50–100% in the birds fed hydrilla from locations where AVM bird death have occurred and *A. hydrillicola* has been verified. No lesions where found in any birds fed the *control hydrilla where *A. hydrillicola* was not detected.

Publication	Site	Bird	# birds	Hydrilla amount fed	*Aetokthonos* present	AVM +	AVM rate
Birrenkott (2004)	Thurmond	mallards	9	ad libitum	yes	6	67%
Lewis-Weis (2004)	Thurmond	chickens	4	20g/day	yes	3	75%
	**Lake Seminole*		*4*	*20g/day*	*no*	*0*	*none*
Rocke (2005)	Thurmond	mallards	4	ad libitum	yes	2	50%
	**Harris Lake*	*mallards*	*2*	*ad libitum*	*no*	*0*	*none*
Wiley (2007)	Thurmond	mallards	6	500–1200 g/day	yes	6	100%
	**Lake Marion*	*mallards*	*3*	*500–1200 g/day*	*no*	*3*	*none*
Wiley (2007)	Davis Pond	mallards	6	500–1200 g/day	yes	4	67%
	**Lake Marion*	*mallards*	*3*	*500–1200 g/day*	*no*	*0*	*none*
Wiley (2009)	Thurmond, Davis Pond	mallards	3	4 mL, 3x/wk	yes	3	100%
Haynie (2013)	Thurmond	mallards	15	250 g/day	yes	10	67%
	**Lake Marion*	*mallards*	*5*	*250 g/day*	*no*	*0*	*none*
Haynie (2013)	Thurmond	mallards	3	370 g/day	yes	2	67%
Robertson (2012)	Thurmond	chickens	5	25 g/day	yes	5	100%
	**Lake Hatch*	*chickens*	*5*	*25 g/day*	*no*	*0*	*none*

Analysis we present. It appears it is distant from all true-branching forms, and is consequently likely in its own family with *Cylindrospermum*-like strains from the PMC collection (only one shown in Fig. 6). These strains do not belong to *Cylindrospermum sensu stricto* (Fig. 6), which has been studied in some detail (Johansen *et al*. 2014). Morphologically, *Aetokthonos* is completely different than *Cylindrospermum* Kützing ex Bornet et Flahault (1888: 249), and we do not doubt that it is both a separate species and genus from these problematic strains. The position of most of the clades in the Nostocales were not resolved with respect to each other, and *Aetokthonos* was in an unresolved position between the Nostocaceae/Aphanizomenonaceae and the Rivulariaceae. From our phylogenetic analyses, we lack sufficient evidence to make a family-level assignment. It is morphologically most similar to the Hapalosiphonaceae, but very dissimilar in 16S rRNA gene sequence to any members of that family (e.g. *Fischerella*, *Mastigocladus* Cohn ex Kirchner (1898: 81) in Table 3).

***Comparative Analysis of 16S-23S ITS Region*:—**The two clones of the ITS sequenced were identical in sequence, and did not contain any tRNA genes. The members of Nostocales can have up to four ribosomal operons, with 2–3 markedly different ITS regions in these operons sometimes being found (Iteman *et al*. 2000, Boyer *et al*. 2001, Flechtner *et al*. 2002). Typically, the operons more easily recovered are those with two tRNA genes, $tRNA^{Ile}$ and $tRNA^{Ala}$. However, there is some evidence that in some genera the operons containing the tRNA genes may be missing. For example, in all three *Mojavia* Řeháková et Johansen in Řeháková *et al.* (2007: 488) strains sequenced thus far only the operon lacking tRNA genes was recovered. A more systematic effort (such as obtaining whole-genome sequence) will need to be made in both *Aetokthonos* and *Mojavia* to know if there are indeed no ribosomal operons containing tRNA genes in these genera. For purposes of comparison, a representative set of heterocytous taxa were chosen to compare lengths of conserved domains, with the operons lacking tRNA genes generally being chosen for comparison (Table 4). This analysis showed that the length of some conserved domains (leader, D1–D1' helix, Box-B helix, Post Box-B spacer, Box A antiterminator) were similar in length to the same structures in a number of taxa. The D4, V3 helix, and D5 had lengths similar to some taxa in solitary comparisons, but no comparison taxa had the same combination of sequence lengths as *Aetokthonos* (Table 4). The V2 spacer was markedly different in length than all other V2 spacers in operons lacking the tRNA genes (Table 4). The secondary structure for the conserved domains of the 16S-23S ITS region in *Aetokthonos* (D1-D1', BoxB, and V3 helices) were markedly different from those observed in the morphologically similar genus *Fischerella* (Fig. 7). A comparison with published secondary structures for *Nostoc* Vaucher ex Bornet & Flahault (1886: 181), *Mojavia*, *Cylindrospermum*, *Aulosira* Kirchner ex Bornet & Flahault (1886: 256), *Rexia* Casamatta *et al.* (2006: 23) and *Spirirestis* Flechtner & Johansen *in* Flechtner *et al.* (2002: 6) (Casamatta *et al.* 2006, Řeháková *et al.* 2007, Lukešová *et al.* 2009, Johansen *et al.* 2014), as well as other unpublished structures sequenced in our research group but not yet published demonstrated that the three helices in *A.*

hydrillicola are unique among all ITS secondary structures recovered thus far in the heterocytous cyanobacteria. Only one strain, *Cylindrospermum marchicum* (Lemmermann 1905: 148) Lemmermann (1907: 196) CCALA 1001, had a structure identical to that found in *Aetokthonos* (the Box-B helix, see Johansen *et al.* 2014, fig. 3c), but the sequence was different (70% similar), particularly in the terminal portion of the helix. The terminus of the D1–D1' helix was unusual in that only a single C-G base pair formed between the terminal loop and subterminal bilateral bulge (Fig. 7A). Typically, the energetics of rRNA folding does not allow the formation of such a singleton pair, but the program we used (Mfold) gave only this structure for *Aetokthonos*.

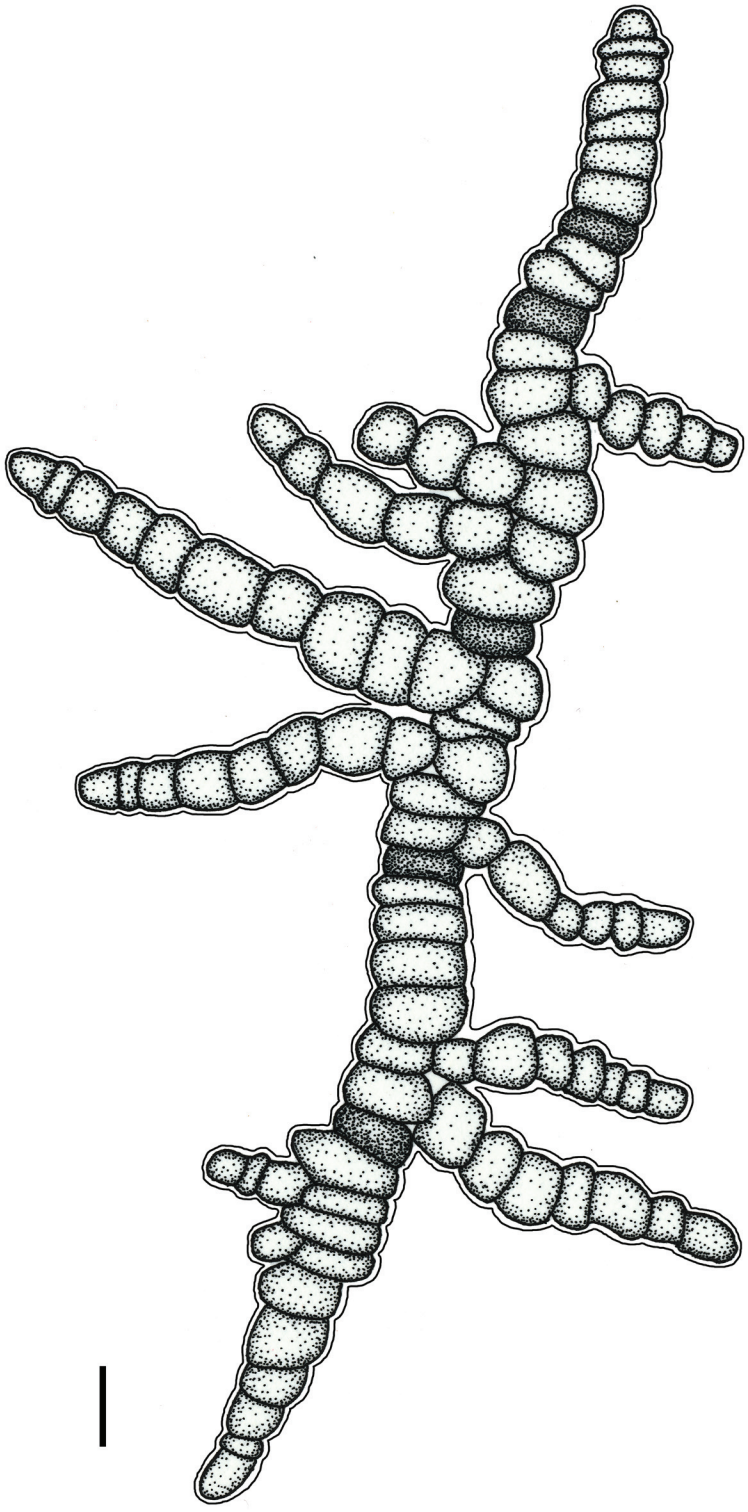

FIGURE 4. Line drawing showing branching pattern (artist: Bradley Bartelme). Scale = 10 μm.

TABLE 3. Percent similarity matrix for *Aetokthonos hydrillicola* and closely related and other relevant taxa, based on S=(1-P-distance) for 16S rRNA gene sequence data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Aetokthonos hydrillicola B3 8C	-																
Aetokthonos MIZ23	98.2	-															
Cylindrospermum PMC186-03	96.2	95.5	-														
*Cylindrospermum musicola*_HA4236-MV2	95.7	95.3	95.7	-													
*Stigonema ocellatum*_SAG 48.90	95.6	95.8	94.4	95.7	-												
Nostoc commune NC1-M2	95.4	94.8	95.1	97.4	95.4	-											
Microchaete tenera ACOI630	95.4	94.3	95.2	96.1	94	98	-										
Cylindrospermum_badium CCALA 1000	95	95.2	95.1	96.5	96.6	96.4	97.9	-									
Calochaete cimrmanii CCALA 1012	95	94.7	94.9	96.4	96.3	97.5	96.9	97.2	-								
Scytonema chiastum UCFS19	95	95.2	93.5	95.2	95.7	94.7	93.8	94.1	94.8	-							
Petalonema ANT.LG2.8	94.8	94.8	93.2	94.7	95.6	94.4	93.9	94.5	94.8	98.8	-						
Petalonema HA4277-MV1	94.7	95.3	93.6	94.8	96.9	95	95.6	95.6	95.8	95.4	95.5	-					
Stigonema HA04070-00001	94.6	95.1	93	95.6	97.4	94.2	94.9	95.4	95.1	95.3	95.1	97.1	-				
Tolypothrix distorta ACOI731	93.9	94.1	93.6	95.5	95.2	95.4	95.7	96	96.2	94	94.2	94.4	94.5	-			
Iphinoe spelaeobios LO2-B1	93.6	93.3	94.5	93.2	93.2	92.3	92.8	93.4	92.9	93.3	93	94.1	92.7	92.3	-		
Symphyonema 1269-1	93.6	93.1	92.7	94.3	94.2	93.6	93.6	93.4	94.1	94.6	94.8	94.4	94.6	93	94.7	-	
Fischerella HA7617-LM2	93.4	93.6	92.6	93.6	94.9	92.8	92.7	94	93.5	94	93.9	94.1	93.2	93.2	94.2	93.7	-
Mastigocladus Kovacik1987/7B	91.9	91.8	91.3	92.5	92.6	92.3	92.6	92.4	92.3	93.1	93.4	93.1	92.7	92.2	92.2	92.9	94.5

TABLE 4. Lengths of conserved and variable domains in the 16S-23S ITS for selected strains. All regions are for operons containing no tRNA genes (like *Aetokthonos*), except *Fischerella*, which only has an operon with both tRNA genes.

	Leader	D1-D1' helix	D2 with spacer	D3 with spacer	$tRNA^{Ile}$ gene	V2 spacer	$tRNA^{Ala}$ gene	Pre-Box-B spacer	Box-B helix	Post-Box-B spacer	Box-A	D4	V3	D5
Aetokthonos hydrillicola	8	63	32	93					30	17	11	21	58	23
Fischerella ATCC43239	8	71	33	17	74	88	73	28	30	17	11	28	67	24
Scytonema HTT-U-KK4	8	65	31	157					30	17	11	20	52	31
Mojavia pulchra JT2-VF2	8	64	31			55			26	18	11	27	103	23
Nostoc commune EV1-KK1	8	63	31			130			33	17	11	27	57	23
Nostoc desertorum CM1-VF14	8	67	32			44			31	17	11	26	39	27
Nostoc indistinguendum CM1-VF10	8	67	32			41			31	17	11	26	39	28
Nodularia PCC 73104	8	65	31			50			31	17	11	23	60	57
Cylindrospermum HA4236-MV2	8	62	30			50			27	17	11	20	39	23
Hassallia CNP1-B1-c09	8	63	31			242			33	17	11	27	39	27
Cyanocohniella HDL9DIL2	7	65	30			55			30	17	11	27	35	22
Entophysalidaceae UFS-A4UI-NPMV4	7	67	33			158			27	17	11	27	34	31
Microcoleus steenstrupii FI-LIZ3	8	65	31			156			30	17	11	21	59	23
Trichocoleus desertorum WJT46-NPBG1	8	63	31			301			35	19	11	21	48	17
Trichocoleus desertorum WJT16-NPBG1	8	62	31			390			35	19	11	21	48	17

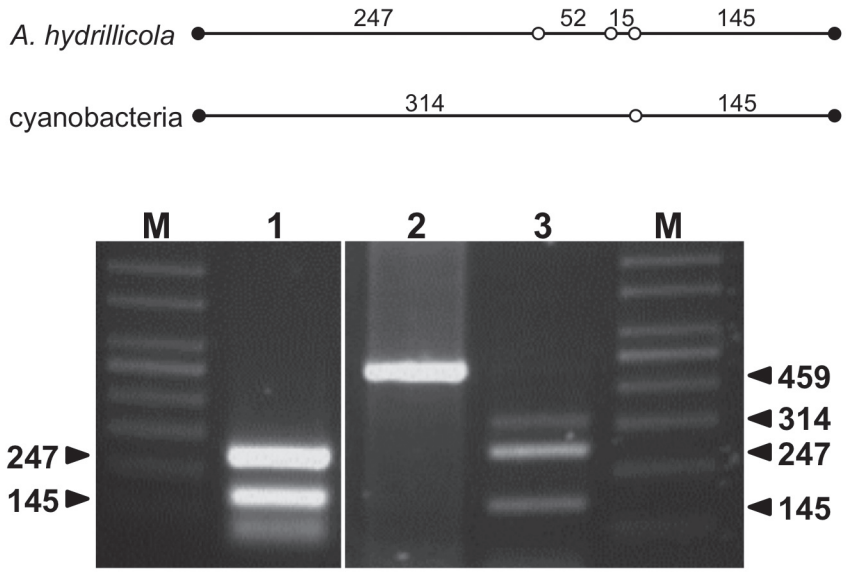

FIGURE 5. CAPS assay for *A. hydrillicola*. *RsaI* digestion of a PCR-amplified fragment of the16S rRNA gene produces a 247 bp sequence that is diagnostic for the species. At top, the sizes of *RsaI* digestion products from *A. hydrillicola* and related cyanobacteria are shown. Lane 1: *RsaI* digestion products of PCR-amplified DNA from an axenic culture of *A. hydrillicola*. Lane 2: PCR product from DNA of a field sample. Lane 3: *RsaI* digestion products of PCR-amplified DNA from a field sample. Arrowheads indicate the size (in base pairs) of the products of PCR and *RsaI* digestion. M = 100 bp DNA markers. Closed circles = PCR primer binding sites. Open circles = *RsaI* restriction sites.

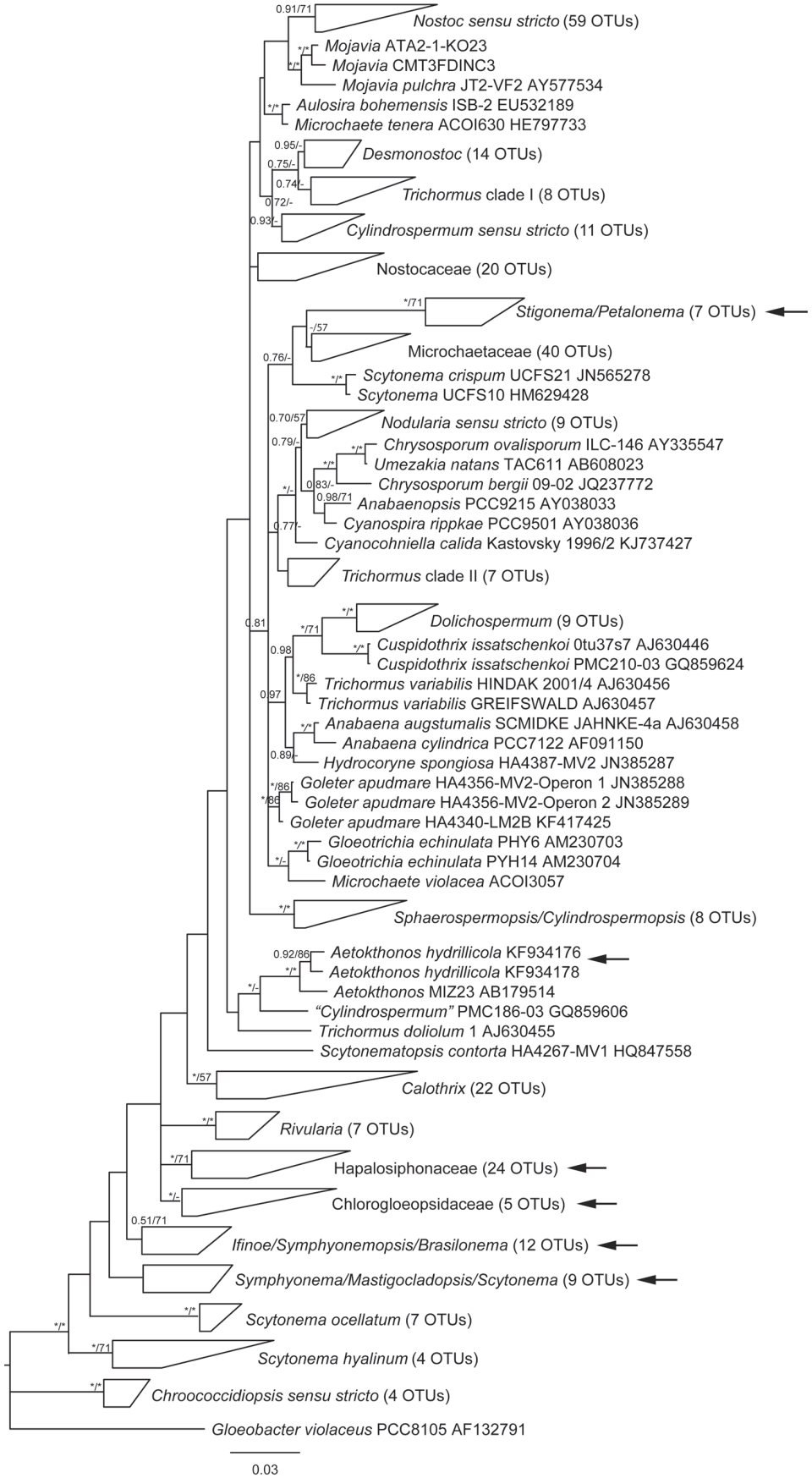

FIGURE 6. Bayesian Analysis based on alignment of 318 OTU's. Posterior probabilities and bootstrap values from a parsimony analysis of the same alignment are shown above or in close proximity to the nodes that are supported at least at the 50% level. Asterisks (*) indicate 1.00 or 100% support, hyphens (-) indicate unsupported node. Nodes with no support from either analysis have no annotation. The six arrows indicate the clades in the tree consisting of true-branching taxa, and show that the former concept of Stigonematales or Section V cannot be supported based on clear phylogenetic evidence.

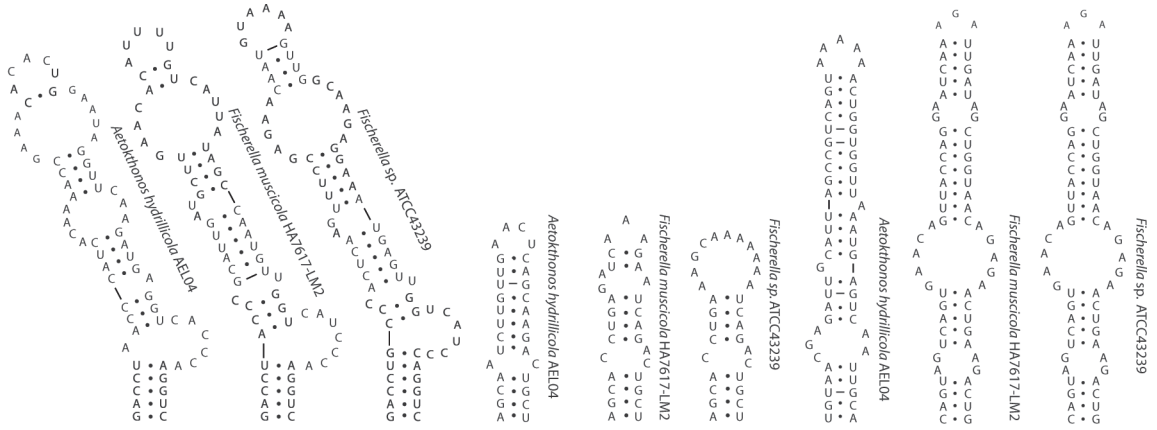

FIGURE 7. Secondary structure of conserved domains in the 16S-23S ITS of true-branching *Aetokthonos* and *Fischerella.* A–C. D1–D1' helix, D–F. Box-B helix, G–I. V3 helix.

Discussion

Aetokthonos hydrillicola is a very unique cyanobacterium. Our work and works cited herein strongly suggests that it is the cause of AVM, and it is entering aquatic food webs through a pathway unique from most documented planktonic cyanobacterial toxins (Chorus 2001, Landsberg 2002, Ferrão-Filho & Kozlowsky-Suzuki 2011). Epiphytic cyanobacteria may be more prevalent than is currently recognized in this cryptic submerged aquatic plant biofilm. Mohamed and Al-Sheri (2010) demonstrated microcystin production in epiphytic *Merismopedia tenuissima* (Lemmermann 1898: 154) and *Leptolyngbya boryana* (Anagnostidis & Komárek 1988: 391) on submerged macrophytes. *Aetokthonos hydrillicola* also has a distinctive phylogeny and while it superficially resembles *Fischerella*, and has a relatively high sequence similarity to *Stigonema ocellatum*, phylogenetic analysis shows it is very distant from these two true-branching taxa as well as all other clades of true-branching taxa. In the Nostocales, sequence similarity among different genera is often high (>98%, see Flechtner *et al.* 2002, Řeháková *et al.* 2007). Consequently, sequence similarities below 97% are fairly clear evidence of genus-level taxon separation. The highest similarity with a strain outside of the genus ("*Cylindrospermum*" PMC-186-03 = 96.2%) is low, and all other taxa were less similar than this. The lengths of many of the ITS domains were different than other taxa, and the secondary structure of the helices was unique. Most true branching taxa occur on rocks or soils, in streaming or stagnant water, or epiphytically on tree bark, or subaerial on diverse substrates (Komárek 2013). The list of taxa reported as epiphytes on aquatic vascular plants is small, and includes only *Hapalosiphon intricatus* West & G.S. West (1894: 271), *H. stuhlmannii* Hieronymus (1895: 10), *Fischerella caucasica* Woronichin (1923: 114), *Fischerella reptans*, *Desmosiphon maculans* Borzì (1907: 371), *D. vivieri* Bourrelly (1957: 590) and *Pulvinularia suecica* Borzì (1916: 575). Of these, the tropical species *F. reptans* is morphologically similar, and we could well imagine that this is a separate species of *Aetokthonos*. *Desmosiphon* has a similar growth habit, but lacks both heterocytes and akinetes, and has limited branching that produces branches parallel to the axis rather than branches perpendicular to the axis (Komárek 2013). The other epiphytic taxa have no morphological similarity to *Aetokthonos*.

Our work confirms other studies showing that the true-branching taxa are not a monophyletic group. This was first shown definitively by Gugger and Hoffmann (2004), but has been confirmed by others (Vaccarino & Johansen 2012). Our phylogeny shows that the Stigonemataceae are very distant from the other true-branching taxa and that certainly the Stigonematales or Subsection V of the Cyanobacteria in Bergey's Manual (Boone & Castenholz 2001) is not a monophyletic group and should not be recognized taxonomically in the future. The several families affiliated with Hapalosiphonaceae (Fischerellaceae, Nostochopsidaceae, Mastigocladaceae) should probably be placed into a single family, with genera presently unclearly delimited. The Chlorogloeopsidaceae could be recognized as a monophyletic group. The Symphyonemataceae is actually in two clades in our analysis and confounded with the Scytonematacean genera *Brasilonema* Fiore *et al*. 2007: 794, 796 and *Scytonema* Bornet & Flahault, 1886: 85 (Fig. 6). With increased taxon sampling, the need to revise the true-branching taxa becomes ever more clear and imperative. We anticipate these morphology-rich cyanobacteria will receive increased attention in the future.

Current culture isolates now maintained for over nine months reveal that *Aetokthonos hydrillicola* grows at lower light (<10μmol photons m^{-2} s^{-1}), nutrient (10% BG11) and temperature (<10 °C) than co-occurring cyanobacterial species. We

propose that this species becomes the dominant late-season epiphyte due to its ability to survive in dense beds of senescing invasive plants when other epiphytes disappear due to low light, nutrients or temperature. Additionally, the neurotoxin it produces may deter the growth of other species. New hydrilla locations with AVM bird disease and dense colonies of *Aetokthonos hydrillicola* during 2011–2012 add additional confirmation of this species' role in AVM and expanding concern for wildlife.

While numerous toxin-producing planktonic species have long been recognized as a risk for fish, wildlife, and even human health (Chorus 2001, Landsberg 2002, Stewart *et al.* 2011), toxic cyanobacteria associated with invasive aquatic vegetation present a novel threat. Additional research on epiphytic cyanobacterial species should be conducted in order to determine toxin-producing capabilities. There may be environmental cues, including declining water temperature, lake turnover, nutrient release from the hypolimnion, declining day length, light levels, and aquatic plant senescence that enhance neurotoxin production. *Aetokthonos hydrillicola* has currently been detected throughout the southeastern United States. Spread of invasive aquatic vegetation may facilitate the invasion of this cyanobacterium. If these two invasions continue to expand their distribution, it could undermine the recovery of the bald eagle and threaten populations of waterbirds and other birds of prey. Recent feeding studies verified that both grass carp and herbivorous turtles were susceptible to AVM toxin present in a hydrilla/*A. hydrillicola* complex (Haynie *et al.* 2013, Mercurio *et al.* 2014). The spread of invasive aquatic vegetation and the toxicity of *A. hydrillicola* must be evaluated to determine food chain implications. Future research is needed to characterize the toxin to better evaluate and manage vegetation to mitigate the potential ecological impact of invasive aquatic vegetation and *A. hydrillicola* on waterbirds, birds of prey, and other aquatic wildlife.

Acknowledgements

We are thankful for all the graduate students that contributed research on the connection between *A. hydrillicola* and AVM; Anna Birrenkott, Faith Wiley, Jessie Shannon, Michael Hook, James Herrin, Shelley Robertson, Jamie Morgan, Brigette Haram and Wallace Woods. Critical monitoring and experimental field assistance was contributed by Tom Murphy and Charlotte Hope (South Carolina Department of Natural Resources), Jim Ozier (Georgia Department of Natural Resources), Ken Presley (Henry County Water Authority), Ken Boyd and Michael Netherland (Army Corps of Engineers) and Dean Jones (University of Florida). Guidance from Jiri Komárek was valuable for taxonomic placement of *A. hydrillicola*. Electron microscopy was conducted at the Center for Ultrastructure Research at the University of Georgia with the expert assistance and photographic skills of Dr. John Shields. We would like to thank the two anonymous reviewers for Phytotaxa for their constructive evaluation. US Army Corps of Engineers Engineer Research and Development Center, US Fish and Wildlife Service, and Florida Fish and Wildlife Conservation Commission provided funding for this research.

References

Anagnostidis, K. & Komárek, J. (1988) Modern approach to the classification system of cyanophytes. 3. Oscillatoriales. *Archiv für Hydrobiologie, Supplement* 80: 327–472

Augspurger, T., Fischer, J.R., Thomas, N.J., Sileo, L., Brannian, R.E., Miller, K.J.G. & Rocke, T.E. (2003) Vacuolar myelinopathy in waterfowl from a North Carolina impoundment. *Journal of Wildlife Diseases* 39: 412–417. http://dx.doi.org/10.7589/0090-3558-39.2.412

Birrenkott, A., Wilde, S.B., Hains, J.J., Fischer, J.R., Murphy, T.M., Hope, C.P., Parnell, P.G. & Bowerman, W.W. (2004) Establishing a food-chain link between aquatic plant material and avian vacuolar myelinopathy in mallards (*Anas platyrhynchos*) *Journal of Wildlife Diseases* 40: 485–492. http://dx.doi.org/10.7589/0090-3558-40.3.485

Boone, D.R. & Castenholz, R.W. (2001) *Bergey's Manual of Systematic Bacteriology: the Archaea and the deeply branching phototrophic bacteria: Cyanobacteria*. Springer, New York, 722 pp.

Bornet, E. & Flahault, C. (1886–1888) Révision des Nostocacées hétérocysteées. *Annales de Sciences Naturelles*, *Botanique, série 7* 3: 323–381; 4: 343–373; 5: 51–129; 7: 177–262.

Borzì, A. (1907) I generi delle Stigonemacee. *In*: *Atti del Congresso dei naturalisti italiani: promosso dalla Società italiana di scienze naturali, Milano, 15-19 settembre 1906.* Società italiana di scienze naturali, Tipografia degli operai, Milan, pp. 370–375.

Borzì, A. (1916) Studi sulle Mixoficee. II. Stigonemaceae. *Nuovo Giornale Botanico Italiano, series 2* 23: 559–588.

Bourrelly, P. (1957) Une nouvelle espèce de Cyanophycée d'eau douce du genre *Desmosiphon*. *Bulletin du Centre d'Études et de*

Recherches Scientifiques Biarritz 4: 589–593.

Boyer, S., Flechtner,V.R., & Johansen, J.R. (2001) Is the 16S–23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in Cyanobacteria. *Molecular Biology and Evolution* 18: 1057–1069. http://dx.doi.org/10.1093/oxfordjournals.molbev.a003877

Boyer, S.L., Johansen, J.R., Flechtner, V.R. & Howard, G.L. (2002) Phylogeny and genetic variance in terrestrial *Microcoleus* (Cyanophyceae) species based on sequence analysis of the 16S rRNA gene and associated 16S–23S ITS region. *Journal of Phycology* 38: 1222–1235. http://dx.doi.org/10.1046/j.1529-8817.2002.01168.x

Casamatta, D.A., Gomez, S.R. & Johansen J.R. (2006) *Rexia erecta* gen. et sp. nov. and *Capsosira lowei* sp. nov., two newly described cyanobacterial taxa from the Great Smoky Mountains National Park (USA) *Hydrobiologia* 561: 13–26. http://dx.doi.org/10.1007/s10750-005-1602-6

Chorus, I. (2001) *Cyanotoxins: occurrence, causes, consequences*. Springer. New York, 357 pp.

Correns, C. (1889) Ueber Dickenwachsthum durch Intussusception bei einigen Algenmembranen. *Flora (Jena)* 72: 298–347.

Ferrão-Filho, A.D.S. & Kozlowsky-Suzuki, B. (2011) Cyanotoxins: bioaccumulation and effects on aquatic animals. *Marine Drugs* 9: 2729–2772. http://www.dx.doi.org/10.3390/md9122729

Fischer, J.R., Lewis-Weis, L.A., Tate, C.M., Gaydos, J.K., Gerhold, R.W. & Poppenga, R.H. (2006) Avian vacuolar myelinopathy outbreaks at a southeastern reservoir. *Journal of Wildlife Diseases* 42: 501–510. http://dx.doi.org/10.7589/0090-3558-42.3.501

Fiore, M.F., Sant'Anna, C.L., de Paiva Azevedo, M.T., Komarek, J., Kastovsky, Sulek, J. & Lorenzi, A.S. (2007) The cyanobacterial genus Brasilonema, gen. nov., a molecular and phenotypic evaluation. *Journal of Phycology* 43: 789–798 http://dx.doi.org/10.1111/j.1529-8817.2007.00376.x

Flechtner, V.R., Boyer, S.L., Johansen, J.R. & DeNoble, M.L. (2002) *Spirirestis rafaelensis* gen. et sp. nov. (Cyanophyceae), a new cyanobacterial genus from arid soils. *Nova Hedwigia* 74: 1–24. http://dx.doi.org/10.1127/0029-5035/2002/0074-0001

Geitler, L. (1933) Diagnosen neuer Blaualgen von den Sunda-Inseln. *Archiv für Hydrobiologie Supplement* 12: 622–634.

Gmelin, J.F. (1789) *Systema naturæ per regna tria naturæ, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis locis. Tomus 1, Pars III. Editio decimal tertia, aucta, reformata*. G.E. Beer, Leipzig, pp. 501–1032. http://dx.doi.org/10.5962/bhl.title.545

Gomont, M.M. (1895) Note sur le *Scytonema ambiguum* Kütz. *Journal de Botanique* 9: 49–52.

Gugger, M.F. & Hoffmann, L. (2004) Polyphyly of true branching cyanobacteria (Stigonematales) *International Journal of Systematic and Evolutionary Microbiology* 54: 349–357. http://dx.doi.org/10.1099/ijs.0.02744-0

Haynie, R.H., Bowerman, W.W., Williams, S.K. Morrison, J.R. Grizzle, J.R. Fischer, J.R. & Wilde S.B. (2013) Are triploid grass carp suitable for aquatic vegetation management in systems affected by Avian Vacuolar Myelinopathy? *Journal of Aquatic Animal Health* 25: 252–259. http://dx.doi.org/10.1080/08997659.2013.833556

Hieronymus, G. (1895) Unterabtheilung: Schizophyta. Klasse: Schizophyceae (Myxophyceae) *In*: Engler, A. (ed.) *Die Pflanzenwelt Ost-Afrikas und der Nachbargebiete. Hrsg. unter Redaktion von professor Dr. A. Engler. Theil C.* Geographische Verslagshandlung Dietrich Reimer, Berlin, pp. 8–11.

Iteman, I., Rippka, R., Tandeau De Marsac, N., & Herdman, M. (2000) Comparison of conserved structural and regulatory domains within divergent 16S rRNA–23S rRNA spacer sequences of cyanobacteria. *Microbiology* 146: 1275–1286.

Johansen, J.R., Bohunická, M., Lukešová, A., Hrčková, K., Vaccarino, M.A., & Chesarino, N.M. (2014) Morphological and molecular characterization within 26 strains of the genus Cylindrospermum (Nostocaceae, Cyanobacteria), with descriptions of three new species. *Journal of Phycology* 50: 187–202. http://dx.doi.org/10.1111/jpy.12150

Kirchner, O. (1898) Schizophyceae. *In*: Engler, A. & Prantl. K. (Eds.) *Die natürlichen Pflanzenfamilien nebst ihren Gattungen und wichtigeren Arten*, *insbesondere den Nutzpflanzen*, *unter Mitwirkung zahlreicher hervorragender Fachgelehrten*, *I. Teil*, *Abt. 1a*. Wilhelm Engelmann, Leipzig, pp. 45–92.

Komárek, J. (2013) Cyanoprokaryota 3. Teil/3rd Part: Heterocytous genera. *In*: Büdel, B., Gärtner, G., Krienitz, L. & Schagerl, M. (Eds.) *Süßwasserflora von Mitteleuropa 19/3*. Elsevier GmbH, Spektrum Akademische Verlag, Munich, 1130 pp.

Landsberg, J.H. (2002) The effects of harmful algal blooms on aquatic organisms. *Reviews in Fisheries Sciences* 10: 113–390. http://dx.doi.org/10.1080/20026491051695

Larsen, S.R., Nutter, F.B., Augspurger, T., Rocke, T.E., Thomas, N.J. & Stoskopf, M.K. (2003) Failure to transmit avian vacuolar

myelinopathy to mallard ducks. *Journal of Wildlife Diseases* 39: 707–711.
http://dx.doi.org/10.7589/0090-3558-39.3.707

Lemmermann, E. (1898) Der grosse Waterneverstorfer Binnensee. Eine biologische Studie. *Forschungsberichte aus der Biologischen Station zu Plön* 6: 166–205

Lemmermann, E. (1905) Brandenburgische Algen. III. Neue Formen. *Forschungsberichte aus der Biologischen Station zu Plön* 12: 145–153.

Lemmermann, E. (1907) Algen I (Schizophyceen, Flagellaten, Peridineen) *In*: Band, D. (Ed.) *Kryptogamenflora der Mark Brandenburg und angrenzender Gebiete*. Verlag von Grebrüder Borntraeger, Leipzig, pp. 1–304.

Lewis-Weis, L.A., Fischer, J. & Gerhold, R.W. (2004) Attempts to reproduce vacuolar myelinopathy in domestic swine and chickens. *Journal of Wildlife Diseases* 40: 476–484.
http://dx.doi.org/10.7589/0090-3558-40.3.476

Linnaeus, C. (1753) *Species Plantarum, Vol. 2*. Imprensis Laurentii Salvii, Stockholm, pp. 561–1200.

Linnæus, C. (1758) *Systema naturæ per regna tria naturæ, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Tomus I. Editio decima, reformata.* Salvius, Stockholm, 824 pp.
http://dx.doi.org/10.5962/bhl.title.542

Linnaeus, C. (1766) *Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Tomus I. Editio duodecima, reformata.* Laurentii Salvii, Stockholm, 532 pp.
http://dx.doi.org/10.5962/bhl.title.542

Linnaeus, C. (1782) *Supplementum Plantarum Systematis Vegetabilium*. Orphanotropheum, Brunswick, 467 pp.

Lukešová, A., Johansen, J.R., Martin, M.P. & Casamatta, D.A. (2009) *Aulosira bohemensis* sp. nov.: further phylogenetic uncertainty at the base of the Nostocales (Cyanobacteria) *Phycologia* 48: 118–129.
http://dx.doi.org/10.2216/08-56.1

Mercurio, A.D., Hernandez, S.M., Maerz, J.C., Yabsley, M.J., Ellis, A.E., Coleman, A.L., & Wilde, S.B. (2014) Experimental feeding of *Hydrilla verticillata* colonized by Stigonematales Cyanobacteria induces vacuolar myelinopathy in painted turtles (*Chrysemys picta*) *PloS ONE* 9(4): e93295.
http://dx.doi.org/10.1371/journal.pone.0093295

Mohamed, Z.A. & Al Shehri, A.M. (2010) Microcystin production in epiphytic cyanobacteria on submerged macrophytes. Toxicon 55(7): 1346–1352.
http://dx.doi.org/10.1016/j.toxicon.2010.02.007

Planchon, J.E. (1849) Synopsis specierum *Anacharidis* et *Apalanthes* et des déscriptions de quelques Hydrocharidées nouvelles. *Annales des Sciences Naturelles, Botanique. séries 3* 11: 73–82.

Rambaut, A. (2007) Fig Tree. Available from: http://tree.bio.ed.ac.uk/software/figtree/.

Rippka, R.R., Waterbury, J.J. & Cohen-Bazire, G.G. (1974) A cyanobacterium which lacks thylakoids. *Archives of Microbiology* 100(1): 419.
http://dx.doi.org/10.1007/BF00446333

Robertson, S.M. (2012) *Potential threats of the exotic apple snail Pomacea insularum to aquatic ecosystems in Georgia and Florida*. Doctoral dissertation, University of Georgia, Athens, 65 pp.

Rocke, T.E., Thomas, N.J., Augspurger T. & Miller, K. (2002) Epizootiologic studies of avian vacuolar myelinopathy in waterbirds. *Journal of Wildlife Diseases* 38: 678–684.
http://dx.doi.org/10.7589/0090-3558-38.4.678

Rocke, T.E., Thomas, N.J., Meteyer, C.U., Quist, C.F., Fischer, J.R., Augspurger, T. & Ward, S.E. (2005) Attempts to identify the source of avian vacuolar myelinopathy for waterbirds. *Journal of Wildlife Diseases* 41: 163–170.
http://dx.doi.org/10.7589/0090-3558-41.1.163

Royle, J.F. (1839) *Illustrations of the Botany and other branches of natural history of the Himalayan Mountains, and of the Flora of the Cashmere. Vol. 1*. William H. Allen & Co., London, 472 pp.
http://dx.doi.org/10.5962/bhl.title.449

Řeháková, K., Johansen, J.R., Casamatta, D.A., Xuesong, L. & Vincent. J. (2007) Morphological and molecular characterization of selected desert soil cyanobacteria: Three species new to science including *Mojavia pulchra* gen. et sp. nov. *Phycologia* 46: 481–502.
http://dx.doi.org/10.2216/06-92.1

Schaefer, E.F. (1997) *A DNA assay to detect the toxic dinoflagellate Pfiesteria piscicida and the application of a PCR based probe*. MS Thesis, UNC-Greensboro, Greensboro, 86 pp.

Spurr, A.R. (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* 26: 31–43.
http://dx.doi.org/10.1016/S0022-5320(69)90033-1

Stewart, I., Carmichael, W.W. & Backer, L.C. (2011) Toxic cyanobacteria. *In*: Selendy, J.M.H. (Ed.) *Water and Sanitation-Related Diseases and the Environment*. John Wiley & Sons, New York, pp. 95–110.

Swofford, D.L. (1998) Phylogenetic analysis using parsimony (paup), version 4. Sinauer Associates, Sunderland. Available from: http://paup.csit.fsu.edu.

Thomas, N.J., Meteyer, C.U. & Sileo, L. (1998) Epizootic vacuolar myelinopathy of the central nervous system of Bald Eagles (*Haliaeetus leucocephalus*) and American coots (*Fulica americana*) *Veterinary Pathology* 35: 479–487.
http://dx.doi.org/10.1177/030098589803500602

Vaccarino, M.A. & Johansen, J.R. (2012) *Brasilonema angustatum* sp. nov. (Nostocales), a new filamentous cyanobacterium from the Hawaiian Islands. *Journal of Phycology* 48: 1178–1186.
http://dx.doi.org/10.1111/j.1529-8817.2012.01203.x

West, W. & West, G.S. (1894) On some freshwater algae from the West Indies. *Journal of the Linnean Society of London*, *Botany* 30: 264–280.

Wilde, S.B., Murphy, T.M., Hope, C.P., Habrun, S.K., Kempton, J.W., Birrenkott, A., Wiley, F., Bowerman, W. & Lewitus, A.J. (2005) Avian vacuolar myelinopathy (AVM) linked to exotic aquatic plants and a novel cyanobacterial species. *Environmental Toxicology* 20: 348–353.
http://dx.doi.org/10.1002/tox.20111

Wiley, F.E., Wilde, S.B., Birrenkott, A.H., Williams, S.K., Murphy, T.M., Hope, C.P., Bowerman, W.W. & Fischer, J.R. (2007) Investigation of the link between avian vacuolar myelinopathy and a novel species of cyanobacteria through laboratory feeding trials. *Journal of Wildlife Diseases* 43: 337–344.
http://dx.doi.org/10.7589/0090-3558-43.3.337

Wiley, F.E., Twiner, M.J., Leighfield, T.A. Wilde, S.B., Van Dolah, F.M., Fischer, J.R. & Bowerman, W.W. (2009) An extract of *Hydrilla verticillata* and associated epiphytes induces avian vacuolar myelinopathy in laboratory mallards. *Environmental Toxicology* 24: 362–368.
http://dx.doi.org/10.1002/tox.20424

Williams, S.K., Kempton, J., Wilde, S.B. & Lewitus, A.J. (2007) A novel epiphytic cyanobacterium associated with reservoirs affected by avian vacuolar myelinopathy. *Harmful Algae* 6: 343–353.
http://dx.doi.org/10.1016/j.hal.2006.07.005

Woronichin, N.N. (1923) Algae nonnullae novae e Caucaso. II. *Botanicheskie Materialy Instituta Sporovykh Rastenij Glavnogo Botanicheskogo Sada R.S.F.S.R.* 2(8): 113–128.

Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* 31: 3406–3415.
http://dx.doi.org/10.1093/nar/gkg595