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Diversity of the genus *Brasilonema* (Nostocales, Cyanobacteria) in plant nurseries of central Florida (USA) with the description of three new species: *B. fioreae* sp. nov., *B. santannae* sp. nov. and *B. wernerae* sp. nov.

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Abstract: Florida is a diverse region that supports abundant cyanobacterial diversity, especially in terrestrial environments. To exploit this environment for cyanobacterial diversity, several greenhouses from central Florida were sampled to identify common nuisance and contaminating algae. Most of the algae observed were mat forming, covering nursery pots, plants, and equipment which were macro– and microscopically morphologically analogous to *Brasilonema*. Although macroscopic thallus morphology was similar among the samples, microscopic morphological characteristics such as size, color, and sheath formation were disparate. To uncover the cryptic diversity, mats were processed for species isolation, culture, and molecular taxonomic identification. A total of eleven *Brasilonema* strains were isolated into culture and systematically identified using 16S rRNA and 16S–23S rRNA ITS sequences. Based on morphology and molecular data, five species of *Brasilonema* were found and three are new to science: *B. fioreae*, *B. santannae,* and *B. wernerae.*

Key words: Scytonemataceae, 16S rRNA, 16S–23S rRNA ITS, subtropical, aerophytic, terrestrial, greenhouse, diversity

Introduction

Cyanobacteria have adapted to most habitats on the planet and are amongst the most abundant and geographically widespread groups of phototrophic bacteria (Whitton & Potts 2012). These microorganisms occur ubiquitously in aquatic habitats but are also an ecologically important component of terrestrial and aerophytic environments, where they grow on various substrates such as wood, soil, and rocks as biofilms, mats and within biological soil crusts (Gama Jr. et al. 2014; Miscoe et al. 2016; Pessi et al. 2016; Pietrasiak et al. 2019). Such communities have vital roles in the ecosystem where they sequester carbon, consolidate soil particles, and provide important resources for soil fertility. Yet, the diversity of terrestrial and aerophytic cyanobacteria remains relatively understudied when compared to aquatic habitats, though in recent years there has been an increase in newly described cyanobacterial taxa from these environments using polyphasic methods (e.g., Fiore et al. 2007; Komárek et al. 2014; Miscoe et al. 2016; Pietrasiak et al. 2019). The genus *Brasilonema* Fiore et al. (2007: 794) is an aerophytic and subaerophytic nostocalean genus of cyanobacteria found in subtropical and tropical habitats growing in phytotelmata, epiphytic on plants and tree bark, and on stones, rocks and wet walls (Fiore et al. 2007; Aguiar et al. 2008; Sant'Anna et al. 2011). The genus was originally isolated and described from (sub) tropical Brazil being separated from *Scytonema* (Fiore et al. 2007). Since then, several species have been described from natural (sub)tropical environments and greenhouses (see Fiore et al. 2007; Aguiar et al. 2008; Sant'Anna et al. 2011; Vaccarino & Johansen 2012; Becerra–Absalón et al. 2013; Rodarte et al. 2014; Villanueva et al. 2018, 2019; Montoya et al., 2019; Romanenko et al. 2020).

Brasilonema is characterized by macroscopic filaments with a wide range of coloration – typically dark green, violet, or dark brown – abundant false branching, colorless sheaths, and are commonly epiphytic. This genus is within the family Scytonemataceae and also characterized by the presence of heterocytes and cell division in a single plane. Moreover, *Brasilonema* can be distinguished from other taxa by the presence of erect,

fasciculate thalli, presence of vacuole–like spaces in the cytoplasm center, and subaerophytic habitat.

Combining classical morphology and modern molecular scrutiny (polyphasic approach) is a powerful tool to delimitate cyanobacteria, especially those with cryptic tendencies, and is essential in describing cyanobacterial taxa (Komárek 2016; Wilmotte et al. 2017; Dvořák et al. 2018). During a study on the diversity and management of nuisance algae and cyanobacteria in greenhouses of central Florida, several *Brasilonema*–like species were observed. In order to shed light on this diversity, a morphological and molecular assessment was carried out on these specimens. Using light microscopy, 16S rRNA gene and 16S–23S rRNA internal transcribed spacer (ITS) phylogenies and pairwise distances, and 16S–23S rRNA ITS secondary structures, we found five morphologically and phylogenetically distinct species of *Brasilonema* with three being new to science: *B. fioreae* D.E. Berthold, M. Barbosa, Lefler et Laughinghouse sp. nov., *B. octagenarum* Aguiar et al., *B. santannae* D.E. Berthold, M. Barbosa, Lefler et Laughinghouse sp. nov., *B. tolantongensis* Becerra–Absalón et Montejano, and *B. wernerae* D.E. Berthold, M. Barbosa, Lefler et Laughinghouse sp. nov.

Materials and Methods

Isolation, culturing and morphological analyses. Field samples were collected in March 2018 in several greenhouses and nursey production areas (walls, floors, and containers) with nuisance cyanobacteria located at the Mid–Florida Research and Education Center (MREC; 28°38'14.9"N, 81°32'54.8"W), University of Florida/IFAS in Apopka, FL (USA). Samples were taken from observable algal growth on grounds, walls, and production containers (Fig. 1). Cyanobacteria were isolated using micromanipulation onto solid BG–11 media (3% Agar), cultured using BG–11 liquid media and incubated at 25 °C on a 12:12–h light:dark cycle under cool white (6500K) fluorescent lighting (100 $\mu Es^{-1}.cm^{-2}$). Cyanobacterial isolates were treated with cycloheximide (Sigma; 300 $\mu g.ml^{-1}$) to remove contaminating eukaryotes.

Morphological assessment was carried out by measuring filament and trichome diameter, thallus and sheath morphology and coloration, cell content and color, length and width of heterocytes, branching, and hormogonia development. Cyanobacteria were measured and imaged using either an Epifluorescent microscope (Amscope, XYL–606) or an Olympus (BX51) differential interference contrast (DIC) microscope with accompanying camera and imaging software. Cyanobacterial isolates are maintained live in the BLCC (Ft. Lauderdale Research and Education Center, University of Florida /IFAS, Davie, FL, USA) and in the BCCM/ULC (Université de Liège, Liège, Belgium), while type material (metabolically inactivate), both dried and preserved (4% formaldehyde), of novel species are deposited in the US National Herbarium (National Museum of Natural History, Smithsonian Institution, Washington DC, USA). Information on isolation source, date, location coordinates, and accession numbers of cyanobacterial strains are summarized in Supplementary Table 1.

Molecular Analyses. Approximately 100 mg of fresh unicyanobacterial cultures were used for DNA extraction using a DNeasy PowerSoil Kit (Qiagen, USA). The 16S rRNA gene and the 16S–23S rRNA internal transcribed spacer (ITS) region were amplified by polymerase chain reaction (PCR) on a ProFlex (Applied Biosystems) thermocycler. Primers 359F and 1487R were used for the 16S rRNA gene and primers 1337F and 23S30R for the 16S–23S rRNA ITS region (Wilmotte et al. 1993; Nübel et al. 1997). PCR protocols were followed according to Berthold et al. (2020). Both genes were amplified using a GoTaq® PCR Core Systems kit (Promega, USA). Amplified DNA was purified using QIAquick PCR Purification Kit (Qiagen, USA) and visualized on a 1% agarose gel. The 16S rRNA amplified products were then sequenced using Sanger Sequencing (Eurofins, KY, USA).

PCR products of the 16S–23S rRNA ITS region were cloned using TOP10 chemically competent cells (TOPO–TA, Invitrogen) following manufacturer protocols. Cloned plasmids were extracted from clone libraries using a PureLink Quick Plasmid Miniprep Kit (Invitrogen). Plasmids containing inserts were sent for sequencing using Sanger sequencing (BigDye Terminator v3.1 (Applied Biosystems), Eurofins, KY, USA). Sequences for both the 16S rRNA gene and 16S–23S rRNA ITS region are deposited in GenBank (National Center for Biotechnology Information, NCBI) and found in Supplementary Table S1. The software MEGA (v10.1.7; Kumar et al. 2018) was used to edit and proofread sequences.

Phylogenetic Analyses. Phylogenetic analyses were based on a ~1040 bp fragment of the 16S rRNA gene. Closely related taxa chosen for analysis were identified using BLAST (Basic Local Alignment Tool, NCBI) and previous literature on the genus, including species of the genera *Calothrix*, *Rivularia*, and *Scytonema*, as well as additional Nostocales with *Gloeobacter violaceous* PCC7421 (NR074282) as an outgroup. A total of 70 sequences were used in the final analysis, consisting of 59 OTUs retrieved from GenBank, including *G. violaceous*, and our 11 isolated strains. The initial alignment was constructed using MUSCLE on MEGA (v10.1.17) (Edgar 2004), and the sequences were manually proofread and edited according to conserved regions. The best–fit substitution model was determined using jModelTest in MEGA (Stetcher et al. 2020). Maximum likelihood (ML) analysis was carried out using the model K2+G+I with 1,000 bootstrap resampling replicates using MEGA. Bayesian analysis (BS) was conducted with MrBayes using 1.0×10^6 generation, a 0.25 burn–in rate, and resampling every 100 generations, through the CIPRES network (v.3.3) (Guindon & Gascuel 2003; Ronquist & Huelsenbeck 2003; Miller et al. 2010).

The 16S–23 rRNA ITS region (~ 590–880 bp) phylogeny was constructed following the previously discussed methods above for Bayesian analysis; the ML analysis was carried out using HYK+G model with 1000 bootstraps using our 11 isolates and 5 available 16S–23S rRNA ITS sequences within the genus *Brasilonema*. The phylogenetic tree was constructed using operons with and without tRNA. Secondary structures of the 16S–23S rRNA ITS region were determined for our isolates and compared to previously published sequences. The secondary structures were delineated, distinguishing operons with and without tRNA based on the presence of conserved tRNA bases and folded using Mfold web server (Zuker 2003). The folded structures were downloaded from the web server and edited with Adobe Illustrator (ver. 24.1.1).

Results

Taxonomic Descriptions
Class: Cyanophyceae
Order: Nostocales
Family: Scytonemataceae

***Brasilonema fioreae* D.E. Berthold, M. Barbosa, Lefler et Laughinghouse sp. nov. (Fig. 2)**
Description: Thallus dense, erect, forest green in color, with heteropolar trichomes. Filaments with sheath, fasciculate, straight or bent, slightly tapering, with Scytonemataceae–type false branching. Filaments 15.7–22.9 µm wide (19.5 ± 2.1 µm). Sheath thin, thicker near branching, hyaline, 3.2 ± 1.2 µm wide. Trichomes cylindrical, straight, slightly constricted at cross–walls, 12.4–20.2 µm wide (16.4 ± 1.8 µm). Cells somewhat isodiametric, light green to dark grey or orange in color, granulated, highly vacuolated, 12.4–20.2 µm wide × 5.1–10.1 µm long. Apical cells rounded, calyptra not present. Heterocytes common, intercalary, square and sometimes flattened, 12.1–18.3 µm wide × 5.3–11.7 µm long. Akinetes not present. Reproduction by motile and aerosolizing hormogonia.

Holotype: US227704 (US National Herbarium, Smithsonian Institution, Washington, DC, USA; dried material in a metabolically inactive state from reference strain BLCC–T72).
Type locality: Growing in greenhouses attached to walls, rocks, and planters, Apopka, FL, USA; 28°38'18.8"N, 81°32'53.9"W.
Habitat: Subaerophytic and terrestrial.
Etymology: "fioreae" (L.) is an epithet that honors Dr. Marli de Fátima Fiore for her dedication and work on cyanobacterial systematics, genetics, and bioactive compounds.
Reference strain: BLCC–T72 (University of Florida, Davie, FL, USA) and ULC548 (BCCM, University of Liège, Liège, Belgium).
Materials analyzed: strains BLCC–T72, BLCC–T73, BLCC–T83, and BLCC–T86.
GenBank accession numbers: 16S rRNA: MT396512, MT396513, MT396514 and MT396516; 16S–23S rRNA ITS: MT396523, MT396524, MT396525 and MT396527.

***Brasilonema santannae* D.E. Berthold, M. Barbosa, Lefler et Laughinghouse sp. nov. (Fig. 3)**
Description: Thallus dense, erect, green to dark grey in color. Filaments often fasciculate, straight or bent, slightly tapering, with Scytonemataceae–type false–branching, 19.2–26.1 µm wide (22.9 ± 2.2 µm). Sheath thin, hyaline to yellow brown, often encrusted, 4.7 ± 1.3 µm wide. Trichomes cylindrical, straight to flexuous, slightly constricted at cross–walls, 13.0–21.4 µm wide (18.2 ± 2.4 µm). Cells isodiametric, sometimes wider than long, green to reddish brown in color, granulated, highly vacuolated, 13.0–21.4 µm wide × 5.9–13.4 µm long. Apical cells are rounded and blunt, calyptra present. Heterocytes common, intercalary, square and sometimes elongated, 20.3–26.1 µm wide × 12.4–18.2 µm long. Akinetes not present. Hormogonia rare or not present.

Holotype: US227696 (US National Herbarium, Smithsonian Institution, Washington, DC, USA; dried material in a metabolically inactive state from reference strain BLCC–T43).
Type locality: Attached to walls, rocks and planters in greenhouses, Apopka, FL, USA (28°38'18.8"N, 81°32'53.9"W).
Habitat: Subaerophytic and terrestrial.
Etymology: "santannae" (L.) is an epithet that honors Dr. Celia Leite Sant'Anna for her dedication and work in cyanobacterial systematics and taxonomy.
Reference strain: BLCC–T43 (University of Florida, Davie, FL, USA) and ULC544 (BCCM, University of Liège, Liège, Belgium).
Materials analyzed: strains BLCC–T43 and BLCC–T64.
GenBank accession numbers: 16S rRNA: MT396506 and MT396507; 16S–23S rRNA ITS: MT396517 and MT396518.

***Brasilonema wernerae* D.E. Berthold, M. Barbosa, Lefler et Laughinghouse sp. nov. (Fig. 4)**
Description: Thallus dense, erect, composed of heteropolar trichomes, olive green in color. Filaments often fasciculate, straight or bent, slightly tapering, with Scytonemataceae–type false–branching, 24.1–29.9 µm wide (26.6 ± 2.1 µm). Sheath occasionally thick, extends past filament, hyaline, 4.5 ± 1.5 µm wide. Trichomes cylindrical, straight, and slightly constricted at cross–walls, 19–26.7 µm wide (22.6 ± 2.6 µm). Cells isodiametric, sometimes wider than long, light brown to reddish brown in color, granulated, highly vacuolated, 19.0–26.7 µm wide × 7.6–14.4 µm long. Apical cells are rounded, calyptra is present. Heterocytes are common, intercalary, square and elongated, 18.6–26.5 µm wide × 6.3–17.6 µm long. Akinetes not present. Reproduction by hormogonia.

Holotype: US227697 (US National Herbarium, Smithsonian Institution, Washington, DC, USA; dried material in a metabolically inactive state from reference strain BLCC–T49).
Type locality: Growing within and outside of greenhouses attached to walls, rocks, and planters, Apopka, FL, USA (28°38'18.8"N, 81°32'53.9"W).
Habitat: Subaerophytic and terrestrial.
Etymology: "wernerae" (L.) is an epithet that honors Dr. Vera Regina Werner for her dedication and work on the study of cyanobacterial systematics and taxonomy.
Reference strain: BLCC–T49 (University of Florida, Davie, FL, USA) and ULC573 (BCCM, University of Liège, Liège, Belgium).
Materials analyzed: strain BLCC–T49.
GenBank accession numbers: 16S rRNA: MT396508; 16S–23S rRNA ITS: MT396519.

Table 1. Comparison of generic and specific morphological characteristics of several representative species within the genus *Brasilonema*. (–) denotes no description was available.

Characteristics	***B. fioreae* sp. nov.**	***B. santannae* sp. nov.**	***B. wernerae* sp. nov.**	***B. bromeliae***	***B. lichenoides***	***B. tolatongensis***	***B. sennae***
Filament Width (mean ± std) (µm)	15.7–22.9 19.5 ± 2.1	19.2–26.1 22.9 ± 2.2	24.1–29.9 26.6 ± 2.1	15–17	8–12	17–25	10–20
Trichome Width (mean ± std) (µm)	12.4–20.2 16.4 ± 1.8	13.0–21.4 18.2 ± 2.4	19.0–26.7 22.6 ± 2.6	14.4–16.8	–	12.5–20	6–12.5
Cell Length (mean ± std) (µm)	5.1–10.1 7.2 ± 1.4	5.9–13.4 9.1 ± 2.3	7.6–14.4 10.2 ± 2.2	1.8–16	2–4	5.3–18.7	–
Heterocytes (µm) (width x length)	12.1–18.3 × 5.3–11.7	20.3–26.1 × 12.4–18.2	18.6–26.5 × 6.3–17.6	15–16.8 × 4–19	–	12.5–16.5 × 7–15.5	10.2–11.2 × 6.8–15.4
Sheath Morphology	Thin; thicker near branching.	Thin	Thin	Thin	Thin	Thin	Thin; slightly lamellated
Sheath Color	Hyaline	Hyaline	Hyaline	Hyaline, rarely yellow–brown	Brown–orange; Hyaline	Hyaline	Yellow–brown
Cell Color	Light green to dark grey or orange	Green to reddish–brown	Light brown; reddish brown	Greyish blue or brownish, olive–green, or violet	Green to blue green	Violet or brown	Olive green or blue–green
Thallus Color	Forest green	Green to dark grey	Olive green	Blackish–green or blackish–violet	–	Blackish–violet	Brownish or blackish–green
Thallus Form	Dense erect heteropolar trichomes	Dense erect heteropolar trichomes	Dense erect heteropolar trichomes	Free fascicles	–	Mats of prostrate fascicles	Regular erect fascicles
Heterocyte Shape	Square, sometimes flattened	Square, sometimes elongated	Square and elongated	Rounded or cylindrical	Flattened, round or hemispherical	Discoid	Cylindrical
False Branching	Scytonemataceae type	Scytonemataceae type	Scytonemataceae type	Rare	Abundant single and geminate	Rare	–
Tapering	Slight	Slight	Slight	Not attenuated toward end	Rarely heteropolar	Not attenuated	Not attenuated
Trichome	Large vacuoles; slightly constricted at cross walls	Large vacuoles; slightly constricted at cross walls	Large vacuoles; slightly constricted at cross walls	Cylindrical; Slightly constricted at cross walls	Straight, bent, or undulated; constricted at cross walls	Slightly constricted	–
Cell Shape	Almost isodiametric; wider than long	Almost isodiametric; wider than long	Almost isodiametric; wider than long	Cylindrical	Discoid	–	–
Reference	This study	This study	This study	Fiore et al. 2007	Villanueva et al. 2018	Becerra–Absalón et al. 2013	Santa'Anna et al. 2011

Table 2. Similarity matrix of 25 strains comparing the 16S rRNA gene sequence data from select *Brasilonema* taxa and sister taxa.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1. *Brasilonema fioreae* BLCC-T72																								
2. *B. fioreae* BLCC-T73	100.00																							
3. *B. fioreae* BLCC-T83	99.81	99.81																						
4. *B. fioreae* BLCC-T86	99.71	99.71	99.71																					
5. *B. santannae* BLCC-T43	97.75	97.75	97.95	97.65																				
6. *B. santannae* BLCC-T64	97.95	97.95	97.95	97.85	99.81																			
7. *B. wernerae* BLCC-T49	97.75	97.75	97.75	97.65	97.15	97.35																		
8. *B. octagenarum* BLCC-T71	99.20	99.20	99.10	98.90	97.99	97.99	97.89																	
9. *B. octagenarum* BLCC-T74	99.03	99.03	98.83	98.74	97.65	97.85	97.95	99.80																
10. *B. tolantongensis* BLCC-T51	98.15	98.15	98.35	98.05	98.34	98.15	98.45	98.50	98.15															
11. *B. tolantongensis* BLCC-T61	98.05	98.05	98.05	97.95	97.85	98.05	98.35	98.20	98.05	99.52														
12. *B. angustatum* HA4187MV1	98.54	98.54	98.54	98.44	97.56	97.75	97.95	98.70	98.54	98.25	98.15													
13. *B. bromeliae* SPC951	96.95	96.95	96.95	96.85	98.44	98.64	97.06	97.18	97.05	97.76	97.65	97.75												
14. *B. geniculatum* HWSC1	98.64	98.64	98.64	98.54	97.85	98.05	97.95	98.80	98.74	97.95	97.85	98.35	97.25											
15. *B. lichenoides* CDV2	99.32	99.32	99.32	99.23	98.05	98.25	97.75	98.90	98.74	98.15	98.05	98.54	97.25	98.93										
16. *B. octagenarum* HA4186-MV1	99.32	99.32	99.13	99.03	97.95	98.15	98.25	99.70	99.52	98.64	98.54	99.03	97.36	98.93	99.13									
17. *B. roberti-lamyi los manantiales*	99.13	99.13	99.13	99.03	98.24	98.44	97.75	99.00	98.83	98.15	98.05	98.54	97.45	99.13	99.42	99.13								
18. *B. sennae* CENA114	97.35	97.35	97.35	97.25	98.83	99.03	97.65	97.58	97.65	98.15	98.05	97.95	99.42	97.75	97.65	97.75	97.84							
19. *B. terrestre* CENA116	97.35	97.35	97.25	97.15	97.45	97.65	97.45	97.89	97.75	97.75	97.65	98.15	97.84	97.35	97.25	97.85	97.25	97.84						
20. *B. tolantongensis tolantongo*	98.17	98.17	98.18	98.07	97.97	98.17	98.99	98.33	98.38	99.70	99.60	98.28	97.77	98.48	98.18	98.69	98.17	98.18	97.87					
21. *Brasilonema* sp. CENA361	98.83	98.83	98.73	98.64	97.55	97.75	98.04	99.30	99.32	98.25	98.14	98.64	96.95	98.64	98.74	99.42	98.73	97.35	97.75	98.28				
22. *Mastigocladopsis repens* MORA	94.54	94.54	94.30	94.17	94.31	94.54	94.41	95.10	94.66	94.42	94.29	94.54	94.41	94.65	94.30	94.78	94.41	94.53	94.53	94.53	94.19			
23. *Scytonema hofmanni* PCC7110	92.66	92.66	92.67	92.53	93.85	94.10	92.41	92.95	92.80	92.68	92.53	92.55	93.19	92.92	92.66	92.80	92.78	93.46	92.94	92.80	92.67	91.89		
24. *Symphyonema* sp. 1517	94.68	94.68	94.45	94.33	94.92	95.14	94.11	95.10	94.80	93.67	93.55	94.90	94.91	94.79	94.45	94.91	94.55	95.02	94.91	93.78	94.24	96.64	92.96	
25. *Symphyonemopsis* sp. VAPOR1	96.70	96.70	96.59	96.48	96.49	96.71	96.04	96.71	96.70	96.16	96.04	96.70	96.59	96.59	96.59	96.59	96.70	96.59	96.81	96.27	96.48	94.18	92.54	94.46

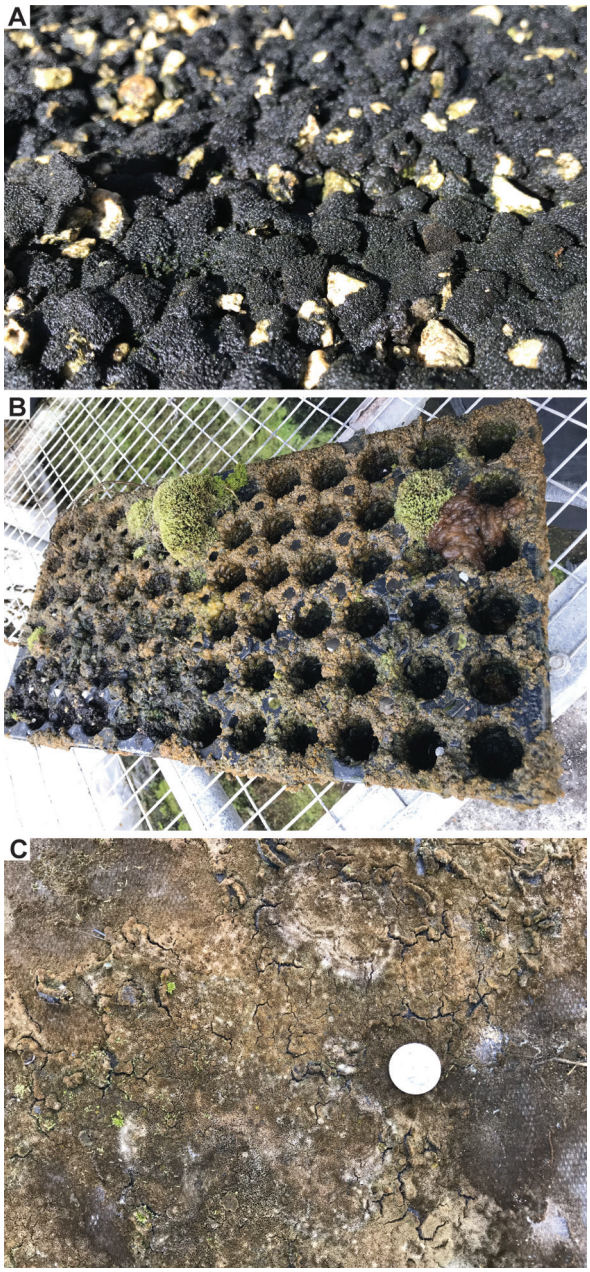

Fig. 1. Field images of mixed algal mats composed of *Brasilonema* and other cyanobacteria and algae growing on various greenhouse surfaces including: (A) indoor gravel; (B) plastic nursery pot containers; and (C) uncovered tarp material. American quarter (diameter= 19.05mm) is shown for size.

***Brasilonema tolantongensis* Becerra–Absalón et Montejano (Fig. 5)**

Comment: Our material fits the original morphological description of this species in Becerra–Absalón et al. (2013) and clusters together with *B. tolantongensis* in the phylogenetic tree.

Thallus dense, erect, composed of heteropolar trichomes, gold to dark brown in color. Filaments often fasciculate straight or bent, slightly tapering, with Scytonemataceae–type false branching, 18.7–28.1 µm wide (22.8 ± 2.8 µm). Sheath thin, extends past filament, thicker near branching, hyaline, 5.1 ± 1.7 µm wide. Trichomes cylindrical, straight, and slightly constricted at cross–walls, 13.7–23.3 µm wide (17.6 ± 2.2 µm). Cells somewhat isodiametric, reddish green to brown in color, granulated, highly vacuolated, 13.7–23.3 µm wide × 4.4–12.8 µm long. Apical cells rounded, calyptra present. Heterocytes common, intercalary, square to elongated, sometimes flattened, 18.7–23.1 µm wide × 11.6–13.2 µm long. Akinetes not present. Reproduction by motile and aerosolizing hormogonia.

Material examined: strains BLCC–T51 and BLCC–T61. Dried and preserved examined material: US227698 and US227700.

GenBank accession numbers: 16S rRNA gene: MT396509 and MT396510. 16S–23S rRNA ITS region: MT396520 and MT396521.

***Brasilonema octagenarum* Aguiar, Fiore, Franco, Ventrella, Lorenzi, Vanetti et Alfenas (Fig. 6)**

Comment: Our material fits the original morphological description of this species in Aguiar et al. (2008) and clusters together with *B. octagenarum* in the phylogenetic tree.

Thallus dense, erect, composed of heteropolar trichomes, reddish green to maroon in color. Filaments often in fascicles, straight or bent, slightly tapering, with Tolypothrichaceae–type false–branching, 14.5–24.1 µm wide (18.3 ± 2.4 µm). Sheath thin, extends past filament, thicker near branching, hyaline, 3.4 ± 1.1 µm wide. Trichomes cylindrical, straight, slightly constricted at cross–walls, 12.1–20.9 µm wide (14.9 ± 2.2 µm). Cells isodiametric, light green to dark brown in color, granulated, highly vacuolated, 12.1–20.9 µm wide × 5.8–12 µm long. Apical cells rounded, calyptra present. Heterocytes common, intercalary, square, 12.7–19.8 µm wide × 5.7–9.9 µm long. Akinetes not present. Hormogonia present.

Material examined: strains BLCC–T71 and BLCC–T74. Dried and preserved examined material: US227703 and US227706.

GenBank accession numbers: 16S rRNA gene: MT396511 and MT396515. 16S–23S rRNA ITS region: MT396522 and MT396526.

Morphological and phylogenetic 16S rRNA gene analyses

The eleven isolates presented in this work fit the generic morphological description of *Brasilonema* with macroscopic velvet–like, dense masses of erect thalli, filaments with sheath, and the presence of large vacuoles in the cells (Table 1, Table S2). Furthermore, the isolates were collected from common *Brasilonema* habitats including subaerophytic environments and attached to substrates such as wet soil, gravel, and artificial surfaces. Five distinct species were observed and isolated, three being novel to science based on morphology, the 16S rRNA gene and 16S–23S rRNA ITS region phylogenies and genetic similarities (p–distance), and the 16S–23S ITS secondary structure.

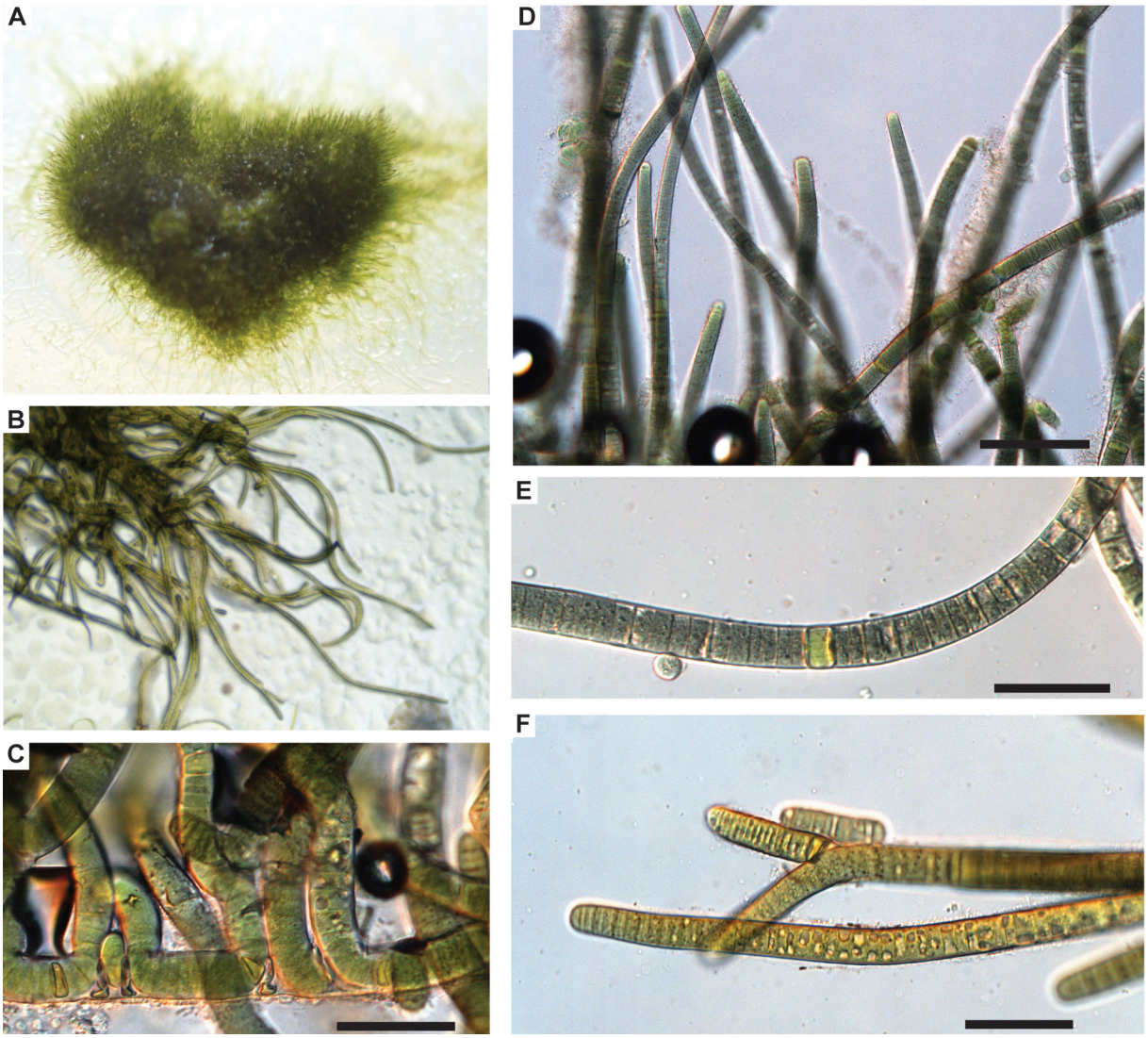

Fig. 2. Images of *Brasilonema fioreae* sp. nov. (reference strain BLCC–T72) showing primary morphological characteristics including: (A–B) macroscopic thallus with densely fasciculate filament growth on agar; (C) Scytonemataceae–type false branching; (D–E) intercalary heterocytes; (F) hyaline sheath formation and isopolar motile hormogonia. Scale bars 100 µm (D), 50 µm (C,E,F).

The 16S rRNA gene phylogenetic analyses clearly showed that our 11 isolates were grouped in the genus *Brasilonema* (Fig. 7; BS: 1.0, ML: 87). The Bayesian analysis produced a tree with the most supported nodes and is presented with ML bootstrap support values added (Fig. 7). Within *Brasilonema*, the eleven isolates represented five distinct species. Of these five, two are established and previously described species including *B. octagenarum* (strains BLCC–T71 and BLCC–T74 (BS: 0.96)) and *B. tolantongensis* (strains BLCC–T51 and BLCC–T61 (BS: 1.0, ML: 94)). Besides these four strains, the remaining seven *Brasilonema* strains formed three novel clades within *Brasilonema* with high support, which are described as the novel species *B. fioreae, B. santannae,* and *B. wernerae. Brasilonema fioreae* is sister to the undescribed *Brasilonema* sp. KEN MK50, *Brasilonema* sp. CR65A, *Brasilonema* sp. BZ HDL 007, and *Brasilonema* sp. CR64B with high support (BS: 0.71). *Brasilonema santannae* was most closely related to *B. sennae* (Komárek) Sant'Anna et Komárek and *B. bromeliae* Fiore et al. with high support (BS: 0.75). *Brasilonema wernerae* is sister to *B. tolantongensis* (BS: 1.0).

Brasilonema fioreae sp. nov. (BLCC–T72, BLCC–T73, BLCC–T83, and BLCC–T86) demonstrated typical characteristics of *Brasilonema*. The species grows attached to substrates and forms dense erect fascicles (Fig. 2A). The trichomes have intercalary heterocytes, possess sheaths that are often hyaline, or colorless, and form vacuole–like structures (Figs. 2D, E). The species has Scytonemataceae–type false branching (Fig. 2C) with isopolar hormogonia that were observed to be motile and aerosolize (Fig. 2F). The extensive formation of vacuole–like structures within the cells of *B. fioreae* are a diacritical marker that distinguishes it from other *Brasilonema* species. Moreover, the larger dimensions of *B. fioreae* compared to other closely related *Brasilonema* taxa further highlights differences between species

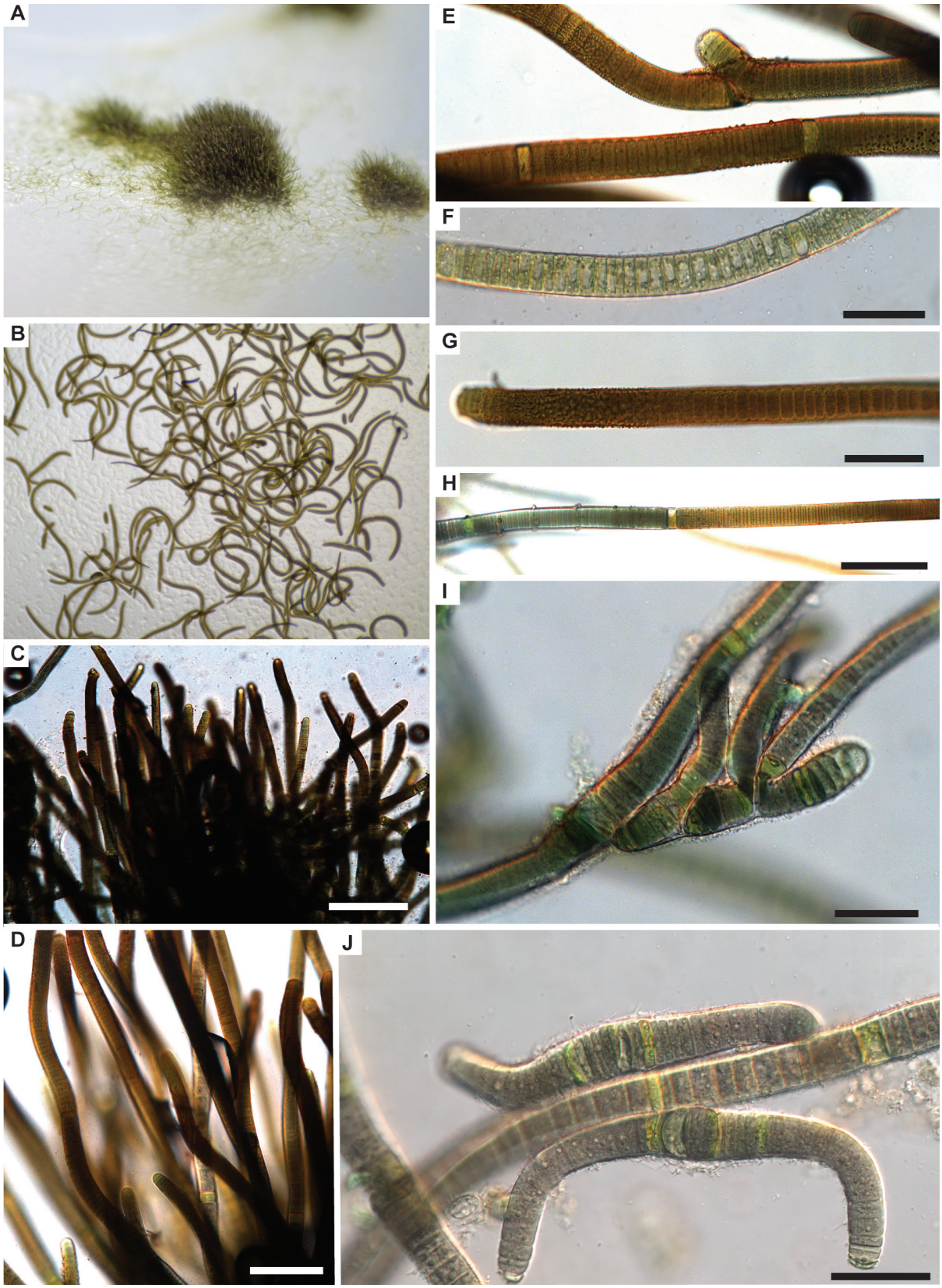

Fig. 3. Images of *Brasilonema santannae* sp. nov. (reference strain BLCC–T43) showing morphological characteristics including: (A–B) thalli growing on solid agar; (C–D) densely packed filaments; (E,H,J) intercalary heterocytes; (F) cell vacuolization; (E,G) extensive crust–like formation on sheath; (I) Scytonemataceae–type false branching; (I,J) hyaline sheath and attached isopolar motile hormogonia. Scale bars 200 µm (C), 100 µm (D), 50 µm (F–J).

Fig. 4. Images of *Brasilonema wernerae* sp. nov. (reference strain BLCC–T49) showing morphological characteristics including: (A–B) thalli growing on solid agar; (C) densely fasciculate filaments; (D) double false branching; (E,G) extensive cell vacuolization; (F) isopolar attached hormogonia; (G) intercalary heterocytes. Scale bars 50 μm (D–G).

(Suppl. Figs 1–3).

Brasilonema santannae sp. nov. (BLCC–T43 and BLCC–T64) also demonstrated typical characters of the genus *Brasilonema*; the species is subaerophytic and forms dense erect fascicles (Fig. 3A). The trichomes possess intercalary heterocytes, have sheaths that are often hyaline, or colorless, and form vacuole–like structures (Figs 3F, H, J). False branching is Scytonemataceae–type (Fig. 3I) and hormogonia are motile and isopolar (Fig. 3J). The morphological feature that distinguishes *B. santannae* from other species in *Brasilonema* are the extensive crust–like formations on the sheath (Figs 3E, G). Moreover, *B. santannae* differs morphologically from its most closely related taxa *B. sennae* and *B. bromeliae* by having larger dimensions of filament width, trichome width, and cell length (Suppl. Figs 1–3).

Brasilonema wernerae sp. nov. (BLCC–T49) was also macroscopically typical to the genus *Brasilonema* with dense erect fascicles and subaerophytic nature (Fig. 4A). The trichomes form intercalary heterocytes, have sheaths that are often hyaline, or colorless, and contain vacuole–like structures (Fig. 4G). False branching is Scytonemataceae–type (Fig. 4D) and hormogonia are isopolar (Fig. 4F). *B. santannae* differs from all other described *Brasilonema* by having much larger filament width, trichome width, and cell length (Suppl. Figs 1–3). Moreover, *B. wernerae* is distinguished from sister taxa by larger and more extensive cell vacuolization.

Besides morphology and 16S rRNA gene phylogeny, we examined the percent similarity of the 16S rRNA gene between our newly described species and 14 of the most closely related taxa from the phylogenetic analysis (Table 2). Results revealed a similarity of ~99.0% for strains BLCC–T43 and BLCC–T64, comprising *B.*

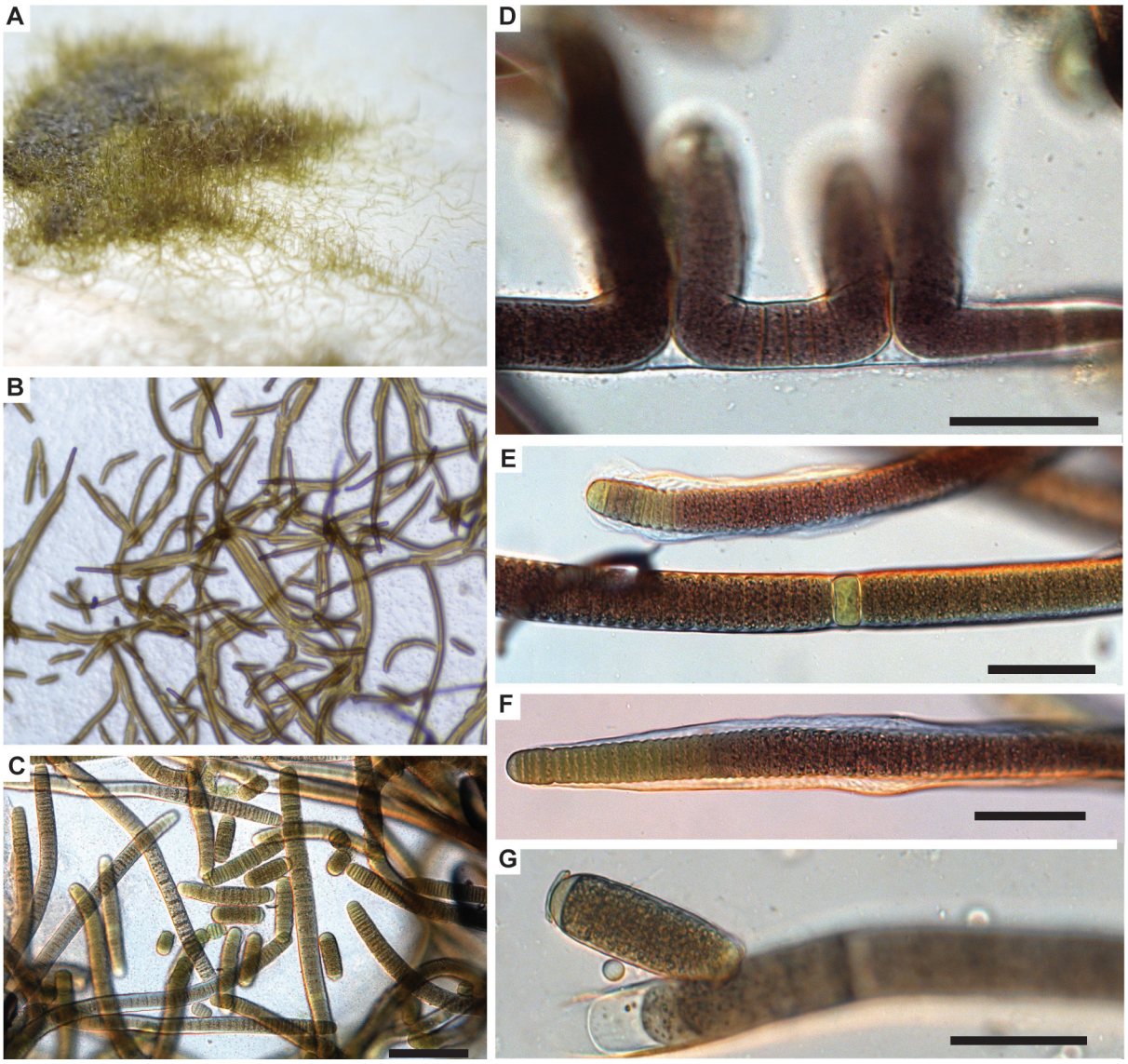

Fig. 5. Images of *Brasilonema tolantongensis* Becerra–Absalón et Montejano (strain BLCC–T51) showing morphological characteristics including: (A–B) thalli growing on solid agar; (C) darkened cell walls and bent, slightly tapering filament ends; (D) double false branching; (E) intercalary heterocytes and cell granulation; (F) thick lamellate, hyaline sheath; (G) attached hormogonia. Scale bars 200 µm (C), 50 µm (D–G).

santannae. In addition, strain BLCC–T49 had similarities lower than 99.0% with related taxa, indicating a unique species and described as *B. wernerae*. Strains of *B. fioreae* (BLCC–T72, BLCC–T73, BLCC–T83, and BLCC–T86) were ~99.0% similar among each other, but also had a similarity ~99.0% with some *B. octagenarum* strains. Our strains of *B. tolantongensis* (BLCC–T51 and BLCC–T61) and *B. octagenarum* (BLCC–T71 and BLCC–T74) were ~99.0% similar to other strains of *B. tolantongensis* and *B. octagenarum*, respectively.

16S–23S rRNA internal transcribed spacer (ITS) phylogeny, p–distance analyses, and secondary structure
Both the 16S–23S rRNA ITS operons with and without tRNAs were recovered from the species examined (Table 3) and a phylogeny was globally constructed. Phylogenetic analyses resulted in ITS sequences with and without tRNAs generally clustering into separate clades. While the clade of sequences containing tRNA was well supported (ML: 79, BS 0.93), those lacking the tRNA sequences formed two unsupported clades (Fig. 8). The 16S–23S rRNA ITS phylogeny of our isolates highly correlated with that suggested by the 16S rRNA gene phylogeny; *B. santannae* formed a single well–supported clade (ML: 100, BS: 1.0), while *B. fioreae* formed two well–supported clades, based on presence or absence of tRNA (ML: 70, BS: 0.93; ML: 99, BS: 0.98; respectively) (Fig. 8). *Brasilonema wernerae* and *B. tolantongensis* BLCC–T61, both possessing tRNA, formed a well–supported clade (ML: 99, BS: 1.00); however, *B. tolantongensis* (BLCC–T51) also produced another subclade alone (ML: 65, BS: –).

We examined the percent dissimilarity of the 16S–23S rRNA ITS region between our newly described

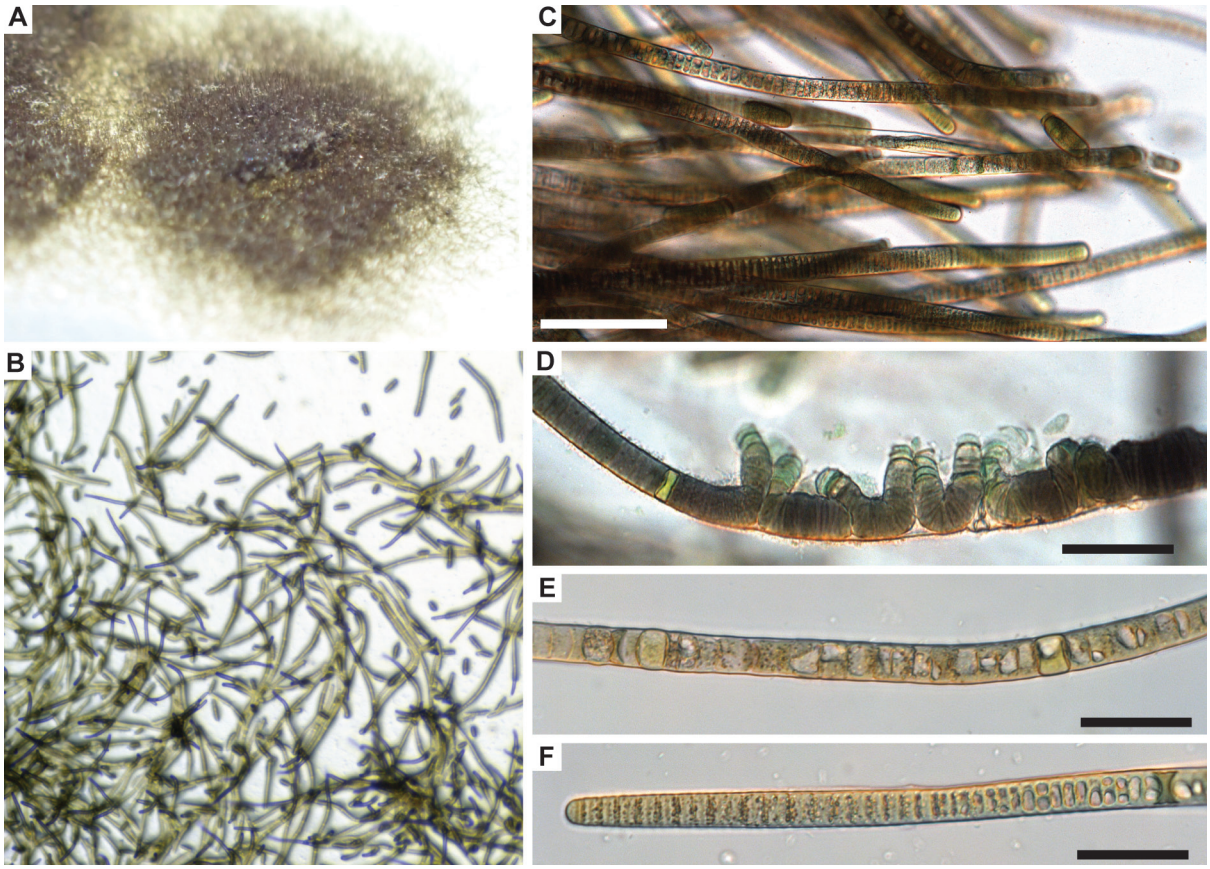

Fig. 6. Microscopic images of *Brasilonema octagenarum* Aguiar, Fiore, Franco, Ventrella, Lorenzi, Vanetti et Alfenas (strain BLCC–T71) showing morphological characteristics including: (A–B) thalli growing on solid agar; (C) fasciculate filaments and hormogonia; (D) Tolypothrichaceae–type false–branching; (E–F) cell vacuolization and intercalary heterocytes. Scale bars 100 µm (C), 50 µm (D–F).

species and five other 16S–23S rRNA ITS sequences from previously described species of *Brasilonema* (Table 4). Genetic dissimilarities resulted in 0.15% dissimilarity in *B. santannae* (BLCC–T43 and BLCC–T64). *Brasilonema wernerae* BLCC–T49 and *B. tolantongensis* BLCC–T61 had a dissimilarity of 0.71%, while *B. fioreae* (BLCC–T72 and BLCC–T83) showed 0.0% dissimilarity. Results also revealed a dissimilarity of greater than 8% between the rest of our strains and a dissimilarity of greater than 10% between all our strains and other species of *Brasilonema*. Secondary structures (D1–D1' helix and the Box–B helix) of the 16S–23S ITS regions were compared between the 11 isolates that belong to five species and other published sequences. The D1–D1' helices displayed nine distinct structures with varying sequences and length between all the strains (Fig. 9). *Brasilonema santannae* (BLCC–T43 and BLCC–T64) demonstrated identical structures and base pairs among isolates (Fig. 9a). *Brasilonema wernerae* BLCC–T49 and *B. tolantongensis* BLCC–T61 shared the same general structure but with different base pairs within the terminal bulb (Figs. 9b, d). *Brasilonema tolantongensis* BLCC–T51 formed a comparatively unique structure (Fig. 9c). *Brasilonema octagenarum* BLCC–T71 formed an identical structure to *B. octagenarum* BLCC–T74 (Fig. 9e, f). *Brasilonema fioreae* BLCC–T73 (Fig. 9h) formed a similar structure to *B. fioreae* (BLCC–T72 and BLCC–T83) (Fig. 9g) but with differing base pairs in the terminal bulb, while *B. fioreae* BLCC–T86 (Fig. 9i) was similar to *B. fioreae* BLCC–T73 but with differing base pairs resulting in two bulges in the stem rather than a single bulb. From the eleven isolates examined, no isolates shared similar D1–D1' structures with other *Brasilonema* species.

The Box–B region revealed 8 structures with similar lengths and configuration but with varying sequences among all the strains (Fig. 10). Strains of *B. santannae* (BLCC–T43 and BLCC–T64) shared identical structures and sequence (Fig. 10a). *Brasilonema wernerae* BLCC–T49 and *B. tolantongensis* BLCC–T61 (Figs. 10b, d) also had the same structure and sequence. *Brasilonema tolantongensis* BLCC–T51 (Fig. 10c) possessed a similar structure to *B. wernerae* BLCC–T49 (Fig. 10b) and *B. tolantongensis* BLCC–T61 (Fig. 10d), but with differing sequences. *Brasilonema octagenarum* BLCC–T71 (Fig. 10e) formed a similar structure to *B. santannae* (Fig. 10a) but base pairs differed. *Brasilonema fioreae* (BLCC–T72 and BLCC–T83; Fig. 10g) was similar to *B. santannae* (BLCC–T43 and BLCC–T64; Fig. 10a) and *Brasilonema octagenarum* BLCC–T71 (Fig. 10e). *Brasilonema fioreae* (BLCC–T73 and BLCC–T86) (Figs. 10h, i) and *B. octagenarum* BLCC–T74 (Fig. 10f) shared similar structures, as well as, but with differing sequences.

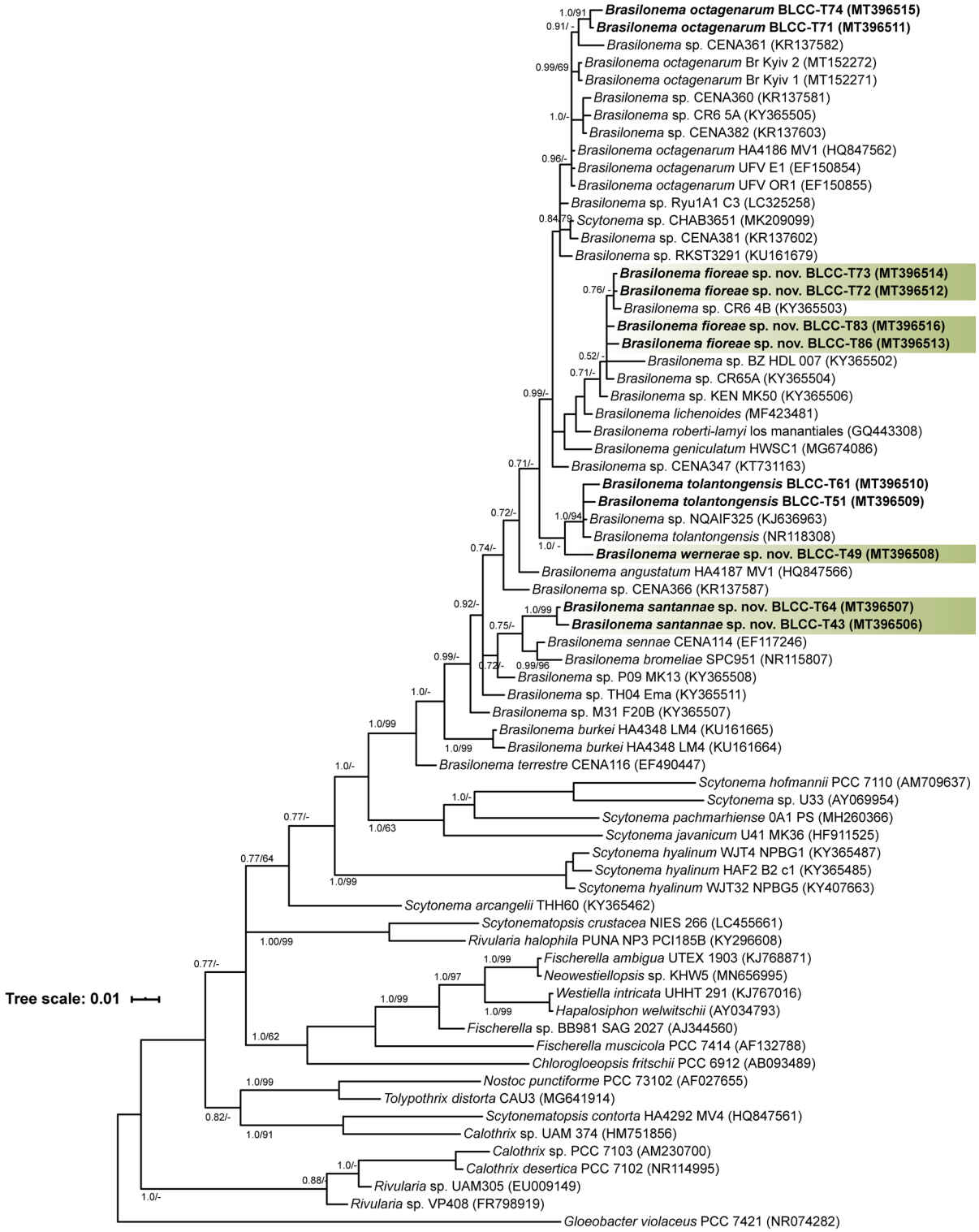

Fig. 7. Bayesian inference phylogenetic tree showing the relationship of the 16S rRNA gene sequence representative of 11 cyanobacterial species isolated in this work and 59 cyanobacterial species including *Gleobacter violaceous* (NR074282) as an outgroup. Maximum likelihood bootstrap support and Bayesian posterior probabilities ≥ 50 and 0.5 are shown at nodes, respectively.

Discussion

Based on morphological, molecular, and phylogenetic data we discovered a total of five species of *Brasilonema*, including three new species to science within plant nursery and greenhouse areas (Fig. 1). The taxa described in this paper conform to the generic characteristics of *Brasilonema* (Table 1, Table S2). Specifically, phylogenetic analyses of the 16 rRNA gene showed the inclusion of all of the described taxa into the *Brasilonema* clade with high support (BS:1.0; ML: 87). Molecular phylogenetic

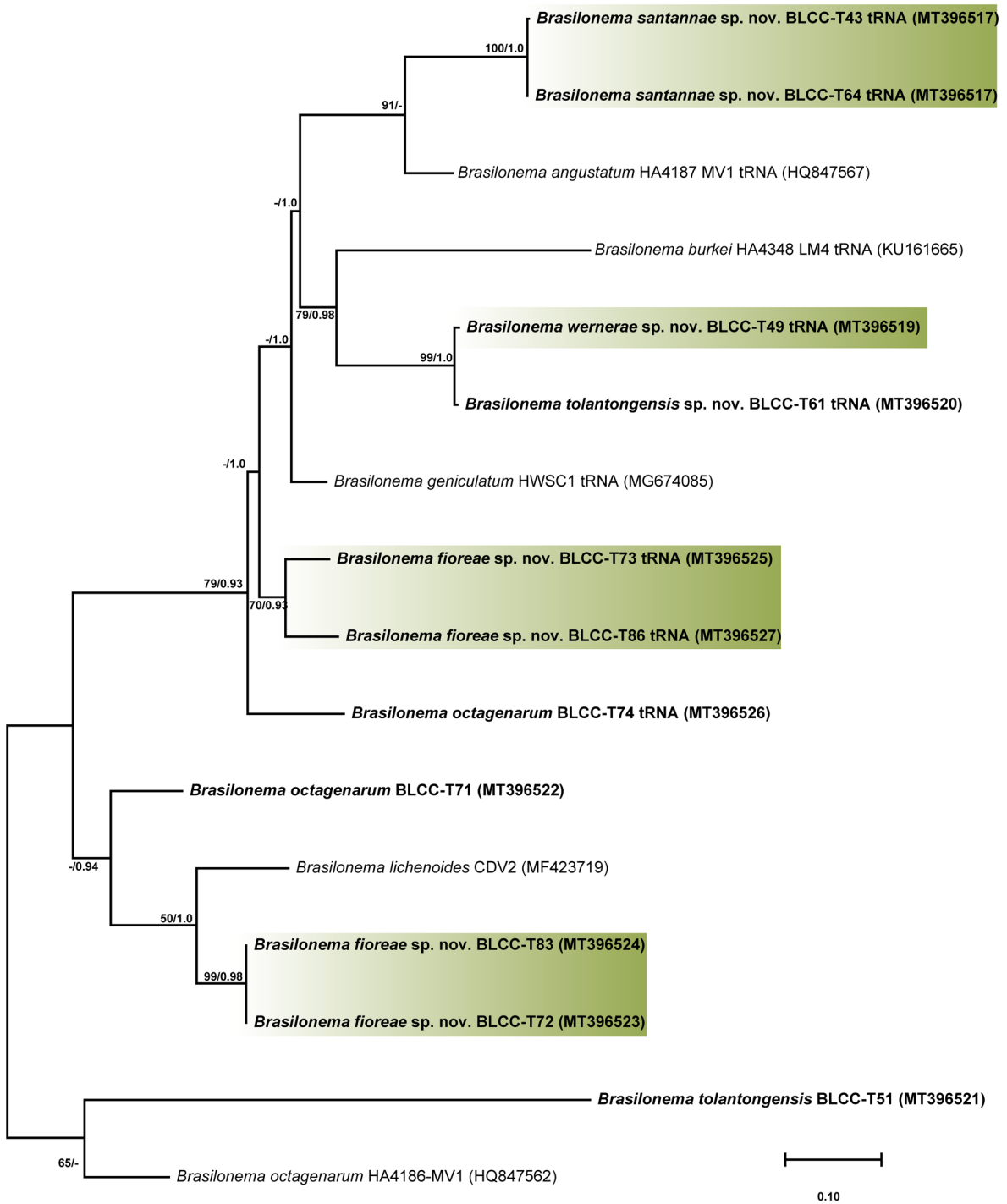

Fig. 8. Bayesian inference and Maximum likelihood phylogenetic relationship of the 16S–23S rRNA gene ITS (internal transcribed spacer) sequence representative of 11 cyanobacterial species isolated in this work and 5 closely related cyanobacterial species. Operons with/without tRNA are included in tree. Maximum likelihood bootstrap support and Bayesian posterior probabilities ≥ 50 and 0.5 are shown at nodes, respectively.

evidence also supports the proposal of three new species, *B. fioreae*, *B. santannae,* and *B. wernerae,* with previously described *Brasilonema* species. To fully appreciate the diversity among the isolates and to clearly delimitate the proposed species, applying the polyphasic method was necessary.

Both the 16S rRNA gene and the 16S–23S rRNA ITS region phylogenies suggest the erection of three new species (Figs 7, 8). The secondary structure of the D1–D1' region of the 16S–23S rRNA ITS region also greatly supported the delimitation of the proposed species based on configuration (Fig. 9). The genetic dissimilarities of sequences of the 16S–23S rRNA ITS region were greater than 10% for the different species, which further supported these novel species (González-Resendiz et al. 2019). Morphological data indicated that critical

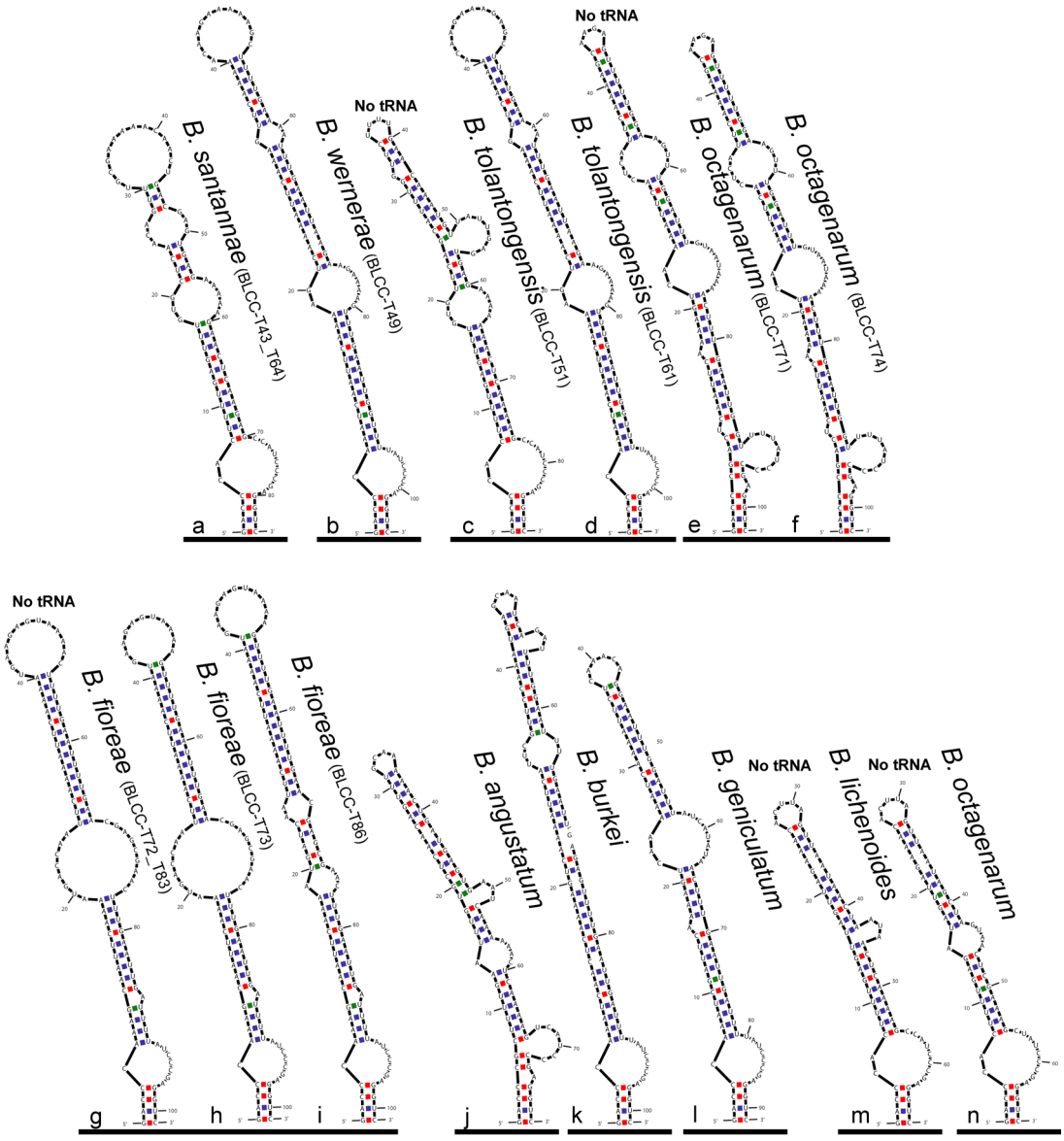

Fig. 9. Secondary structures of the D1–D1' helices from the 16S–23S rRNA ITS regions of 11 isolates and closest relatives from *Brasilonema* for which 16S–23S rRNA ITS sequence data are available: (a) *B. santannae* (BLCC–T43 and BLCCT64); (b) *B. wernerae* (BLCC–T49); (c) *B. tolantongensis* (BLCC–T51) (no tRNA); (d) *B. tolantongensis* (BLCC–T61); (e) *B. octagenarum* (BLCC–T71) (No tRNA); (f) *B. octagenarum* (BLCC–T74); (g) *B. fioreae* (BLCC–T72 and BLCC–T83) (no tRNA); (h) *B. fioreae* (BLCC–T73); (i) *B, fioreae* (BLCC–T86); (j) *B. angustatum*; (k) B. *burkei*; (l) *B. geniculatum*; (m) *B. lichenoides* (no tRNA); (n) *B. octagenarum* (no tRNA).

characteristics of each new species used for delimitation include crust–like texture of the sheath of *B. santannae*, large cell vacuoles of *B. fioreae*, and the relatively large cell dimensions of *B. wernerae*.

From the 16S–23S rRNA ITS phylogeny, we found that *B. wernerae* BLCC–T49 clustered with a sequence of *B. tolantongensis* BLCC–T61 (Fig. 8). It was also observed that the 16S–23S rRNA ITS D1–D1' secondary structure of strains of *B. tolantongensis* (BLCC–T51 and BLCC–T61; Figs 9c, d) and *B. fioreae* (BLCC–T72, BLCC–T83, BLCC–T73, and BLCC–T86; Figs 9g–i) were variable resulting with two different structures each. Together with the little variation observed among the Box B secondary structures (Fig. 10) of *Brasilonema* species, it is evident that multiple lines of evidence are crucial to properly identify these species. Though there was much overlap in molecular and morphological characteristics, the combination of all the molecular and morphological data support the five species found, including the three novel species proposed: *B. fioreae*, *B. santannae* and *B. wernerae*.

This paper is also the first record of *B. tolantongensis* and *B. octagenarum* in the continental USA. The existence of five different species of *Brasilonema* co–occurring in plant nurseries may be attributed to the commercial trade of ornamental plants. Ornamental plant trade among greenhouses and nurseries is widespread (Reichard & White 2001; Bradley et al. 2012) and this trade is evident by the occurrence of *B. tolantongensis* and *B. octagenarum* in Florida, which were originally

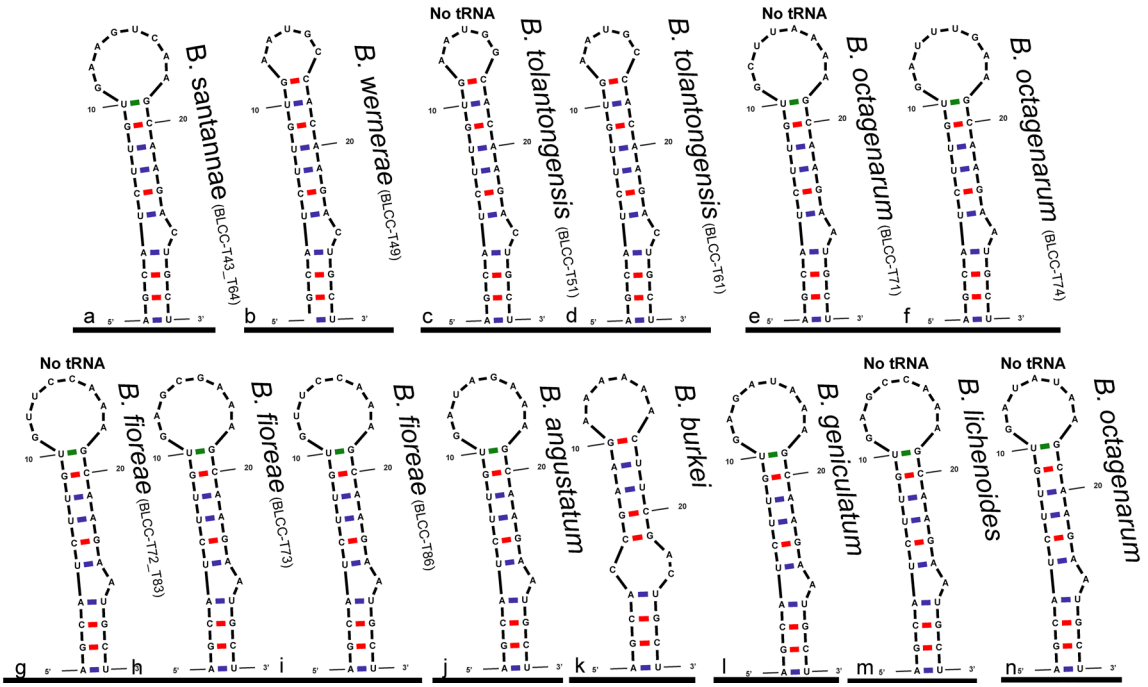

Fig. 10. Secondary structures of the Box–B region from the 16S–23S rRNA ITS regions of 11 isolates and closest relatives from *Brasilonema* for which 16S–23S rRNA ITS sequence data are available: (a) *B. santannae* (BLCC–T43 and BLCCT–64); (b) *B. wernerae* (BLCC–T49); (c) *B. tolantongensis* (BLCC–T51) (no tRNA); (d) *B. tolantongensis* (BLCC–T61); (e) *B. octagenarum* (BLCC–T71) (no tRNA); (f) *B. octagenarum* (BLCC–T74); (g) *B. fioreae* (BLCC–T72 and BLCC–T83) (no tRNA); (h) *B. fioreae* (BLCC–T73); (i) *B, fioreae* (BLCC–T86); (j) *B. angustatum*; (k) B. *burkei*; (l) *B. geniculatum*; (m) *B. lichenoides* (no tRNA); (n) *B. octagenarum* (no tRNA).

described from Mexico and Brazil, respectively, and the occurrence of *Brasilonema* in a tropical greenhouse in Ukraine (Romanenko et al. 2020). Additionally, *B. octagenarum* could potentially be invasive and a potential threat for horticulture as this species has been observed to damage the leaves of *Eucalyptus* plants (Aguiar et al. 2008; Alvarenga et al. 2020). Until now, *B. tolantongensis* had only been described in its type location Hidalgo, Mexico, thus the occurrence in Florida shows that this species is potentially more widespread than originally thought. Furthermore, a strain from Australia, *Brasilonema* sp. NQAIF325 (KJ636963), also clusters with this species, suggesting that *B. tolantongensis* is more widespread in (sub)tropical regions.

Greenhouses used as nurseries for growing tropical and agricultural plants make ideal habitats for the development of subaerophytic cyanobacteria, biofilms, and microbial mats (Aguiar et al. 2008; Laughinghouse et al. 2019; Romanenko et al. 2020; Berthold et al. 2021). Their warm temperature, high moisture, and relatively stable environmental setting provides optimal conditions for the growth of cyanobacteria (including *Brasilonema*) and other algae that can become a nuisance. The high diversity of *Brasilonema* found in the studied greenhouses suggests that these production areas may be a potential 'hotspot' of cyanobacterial diversity. Overall, *Brasilonema* is a genus of cryptic cyanobacteria containing species with much overlap in morphology, molecular phylogeny, and habitat and combined molecular and morphological scrutiny are indispensable in deciphering and describing species.

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Table 3. Lengths of identifiable domains in the 16S–23S rRNA Internal Transcribed Spacer (ITS) regions of *Brasilonema* species.

Strain	Leader	D1-D1' helix	Spacer + D2 + Spacer	D3 + Spacer	tRNA^{Ile}	Spacer+ V2+ Spacer	tRNA^{Ala}	Spacer	Box-B Helix	Space	BoxA	D4 + Spacer	V3 helix to ITS end
B. santannae BLCC-T43	8	83	41	11	74	83	73	129	29	18	11	21	56
B. wernerae BLCC-T49	8	104	41	11	74	80	73	127	28	18	11	21	60
B. tolatongensis BLCC-T51	8	88	38	118	-	-	-	-	28	18	11	21	60
B. tolatongensis BLCC-T61	8	105	41	11	74	80	73	127	28	18	11	21	60
B. octagenarum BLCC-T71	8	102	38	122	-	-	-	-	29	18	11	21	63
B. octagenarum BLCCT-74	8	102	38	11	74	87	73	122	29	18	11	21	63
B. octagenarum HA4186-MV1	8	67	38	140	-	-	-	-	28	18	11	21	61
B. floreae BLCCT-72	8	101	38	139	-	-	-	-	29	18	11	21	63
B. floreae BLCCT-73	8	101	38	11	74	91	73	135	29	18	11	21	63
B. floreae BLCCT-86	8	101	38	11	74	94	73	136	29	18	11	21	63
B. angustatum HA4187-MV1	8	79	38	11	74	61	73	127	29	19	11	21	63
B. burkei HA4348LM4	8	101	38	11	74	80	73	131	27	19	11	21	66
B. geniculatum HWSC1	8	91	38	11	74	78	73	137	29	18	11	21	60

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Table 4. Percent dissimilarity among aligned 16S–23S rRNA Internal Transcribed Spacer (ITS) regions of 16 *Brasilonema* strains.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 *Brasilonema santannae* BLCC-T43 tRNA															
2 *Brasilonema santannae* BLCC-T64 tRNA	0.15														
3 *Brasilonema wernerae* BLCC-T49 tRNA	18.84	18.69													
4 *Brasilonema tolatongensis* BLCC-T51	25.82	25.59	24.36												
5 *Brasilonema tolatongensis* BLCC-T61 tRNA	18.84	18.69	0.71	24.59											
6 *Brasilonema fioreae* BLCC-T73 tRNA	18.84	18.69	17.29	30.16	17.29										
7 *Brasilonema fioreae* BLCC-T83	29.98	29.75	27.1	27.91	26.88	18.7									
8 *Brasilonema fioreae* BLCC-T72	29.98	29.75	27.1	27.91	26.88	18.7	0								
9 *Brasilonema fioreae* BLCC-T86 tRNA	17.88	17.73	17.41	30.84	17.27	8.09	14.53	14.53							
10 *Brasilonema octagenarum* BLCC-T71	29.86	29.63	22.86	29.31	22.42	19.07	13.93	13.93	21.06						
11 *Brasilonema octagenarum* BLCC-T74 tRNA	20.25	20.09	16.47	30.82	16.17	11.52	24.2	24.2	12.26	14.81					
12 *Brasilonema octagenarum* HA4186-MV1	26.73	26.49	26	20.99	25.53	23.91	18.69	18.69	24.19	16.75	24.6				
13 *Brasilonema geniculatum* HWSC1	17.52	17.36	14.42	31.18	14.26	10.55	24.46	24.46	10.23	20.5	13.25	25.13			
14 *Brasilonema angustatum* HA4187-MV1	12.19	12.04	17.03	23.34	17.18	15.15	26.67	26.67	15.45	23.29	17.67	22.3	14.1		
15 *Brasilonema burkei* HA4348-LM4	24.96	24.81	20.54	37.62	20.68	21.3	29.93	29.93	20.47	26.08	22.47	30.86	19.97	24.01	
16 *Brasilonema lichenoides* CDV2	29.65	29.38	33.16	26.4	32.62	28.53	11.4	11.4	25.98	15.91	29.35	16.3	27.03	27.54	37.5

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Supplementary material

The following supplementary material is available for this article:

Table S1. Cyanobacterial strain information including voucher numbers of type material, strain numbers of live cultures, and accession numbers genetic information.

Table S2. Comparison of generic and specific morphological characteristics of several representative species within the genus *Brasilonema*, extended.

Fig. S1. Filament width (µm) boxplot of 11 isolates from this study, grouped into species, and closest relatives from *Brasilonema* for which filament width data was available.

Fig. S2. Trichome width (µm) boxplot of 11 isolates from this study, grouped into species, and closest relatives from *Brasilonema* for which trichome width data was available.

Fig. S3. Cell length (µm) boxplot of 11 isolates from this study, grouped into species, and closest relatives from *Brasilonema* for which cell length data was available.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

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