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Phylogenetic placement of *Dapisostemon* gen. nov. and *Streptostemon*, two tropical heterocytous genera (Cyanobacteria)

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Abstract

Two species of heterocytous cyanobacteria from the Mata Atlântica of Southeastern Brazil were studied intensively to determine if they were congeneric or belong to different genera. Additionally, their affinity to established genera in the Tolypothricaceae such as *Tolypothrix* Bornet & Flahault, *Hassallia* Bornet & Flahault and *Spirirestis* Flechtner & Johansen was investigated. *Dapisostemon apicaliramis* gen. et sp. nov. isolated from a wooden bridge in a mangrove swamp was found to be basal to a clade containing Tolypothricaceae, Nostocaceae, and Aphanizomenonaceae (but with weak support) by means of 16S rRNA gene sequence analyses. Moreover, *D. apicaliramis* had a 16S-23S ITS region similar in length, sequence, and secondary structure to the members of the terrestrial Tolypothricaceae. *Streptostemon* Sant'Anna *et al.* was placed in the Microchaetaceae (now the Tolypothricaceae) at the time of its description based on unsequenced natural material, but in this study it was found to be even more phylogenetically distant from that clade than *Dapisostemon* based on 16S rRNA gene sequence. It is in an unsupported and unresolved basal position in the Nostocales. It also presents a unique 16S-23S ITS region. The higher level evolutionary relationships of both genera are uncertain at this time, but neither genus can remain in the Microchaetaceae or Tolypothricaceae as the Nostocales undergo future revision. We conclude that *Dapisostemon* is sufficiently unique and separate that it requires a new family, the Dapisostemonaceae. This study further demonstrates that the cyanobacteria of tropical regions often represent novel taxa in unstudied lineages and that the study of tropical cyanobacteria will yield new insights in the biodiversity of this ecologically important group of photosynthetic microorganisms.

Key words: *Dapisostemon apicaliramis, Streptostemon lutescens,* Microchaetaceae, Mata Atlântica, Tolypothricaceae, 16S-23S ITS secondary structure, biodiversity, genetic diversity

Introduction

In tropical and subtropical forests, terrestrial cyanobacteria, along with other organisms, compose extensive biofilms, which grow on a wide variety of substrates such as wood, soil and rocks (Büdel 2002, Gorbushina 2007, Sant'Anna *et al.* 2013). Ecologically these biofilms play important roles in ecosystems, especially as carbon sinks, decomposers, and sources of soil fertility, mainly due to the nitrogen (N_2) fixation activity of cyanobacteria and phosphorus mobilization by mycorrhizae (Vitouzek *et al.* 2002, Belnap *et al.* 2003). However, our knowledge of these microrganisms, particulary cyanobacteria, is still poor for tropical and subtropical forests. Only recently studies showed that cyanobacteria in biofilms perform lower rates of nitrogen fixation in old-growth forests than in disturbed environments under regeneration (Barron *et al.* 2011, Pons *et al.* 2006, Gehring *et al.* 2005). Even less is known in terms of biodiversity. As a result, these communities are still misunderstood and often neglected in ecological studies.

We embarked on an extensive study of cyanobacterial diversity in Mata Atlântica (Atlantic Rainforest), one of the

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most important biodiversity hotspots in the world which covers both tropical and subtropical areas along the Brazilian coast (Rizzini 1997, Myers *et al.* 2000). Due to anthropogenic activities, today it retains only 7% of its original coverage (Rizzini 1997). Nonetheless, the fragmented remnants are almost totally encompassed by protected areas, from which multiple new cyanobacterial taxa (genera, species) have been described during the last decade (Fiore *et al.* 2007, Lemes-da-Silva 2010, Sant'Anna *et al.* 2013).

Among the new taxa discovered, fasciculate filamentous forms have received special attention, as evidenced by the two new genera and seven species of fasciculate Nostocales already described for this biome based on morphological data alone. In addition, the genus *Brasilonema* Fiore *et al.* (2007: 794) was described based on both morphological and molecular data (Fiore *et al.* 2007). The genus *Streptostemon* was described by Sant'Anna *et al.* (2010: 220) for microchaetacean-like cyanobacteria with fasciculate filaments that lack branching.

Recently, we have isolated, characterized and sequenced another apparent member morphologically belonging to either the Microchaetaceae or Tolypothricaceae similar to *Streptostemon* but differing in key characters. This new species differs from *Streptostemon* in that it has false branching and is less fasciculate. However, morphological the two genera resemble each other more closely than compared to other genera, and molecular evidence is required to determine whether or not they are congeneric. Before our putative new genus and species could be described, it was necessary to make a comparative analysis with the morphologically close *Streptostemon*, including sequencing of the 16S ribosomal gene for both taxa in order to perform phylogenetic analysis, as well as characterization of the 16S-23S ITS region.

In this study, we sequenced the 16S rRNA gene and the 16S-23S ITS of the isolates of the putative new genus from the Mata Atlântica and an uncultured population of *Streptostemon lutescens*. The purpose of this paper is to establish the phylogenetic position of both species, demonstrate that they comprise different genera, and distinguish them from other cyanobacterial genera. The new taxon is described as *Dapisostemon apicaliramis gen. et sp. nov.*, and will be referred to under this epithet in the remainder of the paper. Recently, a proposal was made to erect the Tolypothricaceae for most genera that formerly were in the Microchaetaceae (Hauer *et al.* 2014). In the remainder of the paper we will refer to this family level clade as the Tolypothricaceae.

Material and methods

Sampling and morphological analysis

The studied populations of the new genus *Dapisostemon* were collected by scraping biofilms from terrestrial habitats in the subtropical area of Mata Atlântica (State Park of Ilha do Cardoso - 25º04'12"S, 47º55'27"W). One population (CCIBt 3318) was isolated from a unispecific biofilm growing on a wood bridge in a mangrove and the other population (CCIBt 3536) grew in culture from an inoculated sample collected on a riverside rock, but it could not be found in natural material.

Both strains were maintained in liquid BG11 nitrogen-free medium (Ripka *et al.* 1979) under 14:10 hr (light:dark) cycle with white fluorescent light (30 µmol photons m^{-2} s^{-1}) and 23 (±2) ºC, and after the study deposited in the Culture Collection of the Institute of Botany, São Paulo, Brazil. The studied environmental populations of *S. lutescens* were collected also from biofilms growing abundantly on wood, rocks or epiphytic on bryophytes in areas of Mata Atlântica: Nucleus Santa Virginia (23º20'16"S, 45º09'01"W), Ecological Station Jureia-Itatins (24 24'36"S, 47 01'15"W) and State Park of Ilha do Cardoso (25º04'12"S, 47º55'27"W), but even after many attempts could not be isolated.

After sampling, dry material was kept in paper bags and parts of the samples were rehydrated for morphological analysis in the laboratory. At least, 30 individuals of each studied population were evaluated and photographed using a Zeiss Axioplan 2 photomicroscope equipped with a Zeiss Axiocam MRc digital camera. Samples were also preserved in formaldehyde (4%) and deposited in the Herbarium of the Institute of Botany (SP).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was isolated from liquid cultures of the cyanobacterial strains CCIBt 3318, CCIBt 3536 and from the unispecific environmental sample of *S. lutescens* using MOBIO Ultraclean DNA Isolation Kit. Specially for *S. lutescens*, sample preparation for DNA extraction was conducted by manual separation and cleaning of filaments from debris, using tapered Pasteur micropipetes under a steromicroscope, and confirming unialgal nature of the product under a compound microscope.

Almost the complete 16S rRNA gene plus the 16S-23S ITS region were amplified by PCR primers 27F1 (Neilan

et al. 1997) and 23S30R (Lepère *et al.*, 2000) in a Techne TC-412 thermocycler (Bibby Scientific). The reaction contained 10 ng of genomic DNA, 0.5 µM of each primer, 200 µM of dNTPs, 2.0 mM of $MgCl_2$, l × PCR buffer and 1.5 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), in a final volume of 25 µL. The PCR cycle had initial denaturation at 94 ºC for 5 min, followed by 10 cycles of 94 ºC for 45 s, 57 ºC for 45 s, 72 ºC for 2 min, another 25 cycles of 94 ºC for 45 s, 57 ºC for 45 s, 72 ºC for 2 min and a final extension step at 72 °C for 7 min. The resulting PCR product was cloned into a pGEM®-T Easy Vector System (Promega, Madison, WI, USA) according to the supplier's manual, cloned by heat-shock in *E. coli* DH5α cells and plated for blue-white selection (Sambrook & Russel 2001). After growth, recombinant plasmids were extracted from white colonies by the alkaline lysis method (Birnboim & Doly 1979). One cloned fragment was sequenced for *Streptostemon*, whereas two were sequenced for *Dapisostemon*. The cloned gene fragments were sequenced using "Big Dye Terminator" v. 3.0 (Applied Biosystems) with the plasmid primers T7 and M13 and the internal primers 357F/357R, 704F/704R and 1114F/1114R (Lane 1991). The cycle sequencing reaction was performed as follow: 25 cycles of 95 ºC for 20 s, 50 ºC for 15 s and 60 ºC for 1 m. DNA was precipitated using 2 µL of sodium acetate buffer (1.5 M sodium acetate - pH 9.0 and 250 mM EDTA- pH 8.0) and 60 µL of 100% ethanol. The tubes were centrifuged at 4 ºC for 15 min at 12,000 × *g* and the supernatants were discarded. The DNA pellets were washed with 150 µL of 70% ethanol, centrifuged for 5 min and the supernatants removed. The pellets were air-dried overnight in the dark and at room temperature. The purified pellets were resuspended in HiDi formamide (Applied Biosystems), and the sample placed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequenced fragments were assembled into contigs using the software package Phred/Phrap/Consed (Philip Green, Univ. of Washington, Seattle, USA) and only bases with a quality >20 were considered.

Phylogenetic analyses

The 16S rRNA gene sequences obtained in this study and reference sequences retrieved from NCBI GenBank were aligned manually and used to generate the phylogenetic trees. We chose a manual alignment to better reflect the secondary structure of the 16S rRNA molecule. The trees were reconstructed using the Maximum Parsimony (MP) method implemented by the MEGA version 5.0 program package (Tamura *et al.* 2011) with 1000 bootstrap replicates and Bayesian (BA) criteria using MrBayes 3.2.2 (Ronquist & Huelsenbeck 2003) in two independent runs, with eight chains each, for 20×10^6 generations. In both cases, the best-fitting evolutionary model GTR+G+I was determined by TOPALI 2.5.

To determine the position of our strains in the first round of phylogenetic analysis, MP and BA trees were constructed with 466 OTU´s (operational taxonomic units) represented by Nostocales sequences (data not shown). Then, we chose the more related taxa and constructed smaller trees based on 210 OTU´s. The MP tree from this analysis appears in the primary text (Fig. 1), while the Bayesian Analysis appears in the supplemental figures (Fig. S1). A final MP tree with 427 OTU's (different taxon sampling than the first tree) was run to maximize taxon sampling, a strategy recommended by Goertzen and Theriot (2003), and this tree is also given in supplementary materials (Fig. S2). A similarity matrix (p-distance) was also generated based on 16S rRNA gene sequence.

For the analysis of the 16S-23S ITS region, we compared the lengths of this region and the secondary structures of our strains, with related taxa based on our 16S rRNA trees. The secondary structures of helices D1-D1´, Box B, V2 and V3 were determined separately using Mfold version 2.3 (Zuker 2003), with folding temperature set at 20ºC, and illustrated using Adobe Illustrator in Adobe CS5.

The 16S rRNA gene and the 16S-23S ITS sequences of the cyanobacterial strains *D. apicaliramis* CCIBt 3318 and *D. apicaliramis* CCIBt 3536 isolated in this study, and of the *S. lutescens* natural population were deposited in the NCBI GenBank database under accession numbers KJ566947, KJ566945, and KJ566946, respectively.

Results

Dapisostemon was found growing on a wooden bridge above a mangrove swamp in the Mata Atlântica in subtropical southeastern Brazil. Morphologically these populations are similar to *Streptostemon* and *Hassallia* Bornet & Flahault (1886-1888: 175), but differ in many aspects (Table 1). The thallus consisted of a mat of upright heteropolar filaments that were commonly apically branched but not often arranged in fascicles, with yellowish sheaths and isodiametric cells. The fascicles, when present, lacked the well-developed lanceolate structure of the fascicles of *Streptostemon.* While *Hassallia* has false branching and upright branches, it does not form the carpet-like layer seen in *Dapisostemon,*

and has discoid cells. Hence, the natural populations and isolates of *Dapisostemon* could not be placed with confidence in any previously described genus.

The studied populations of *Streptostemon* were identified based on nine samples and were in agreement with the original description of the genus (Sant'Anna *et al.* 2010), although they presented more intercalary heterocytes than previously described. We characterized the populations and succeeded in sequencing one of them.

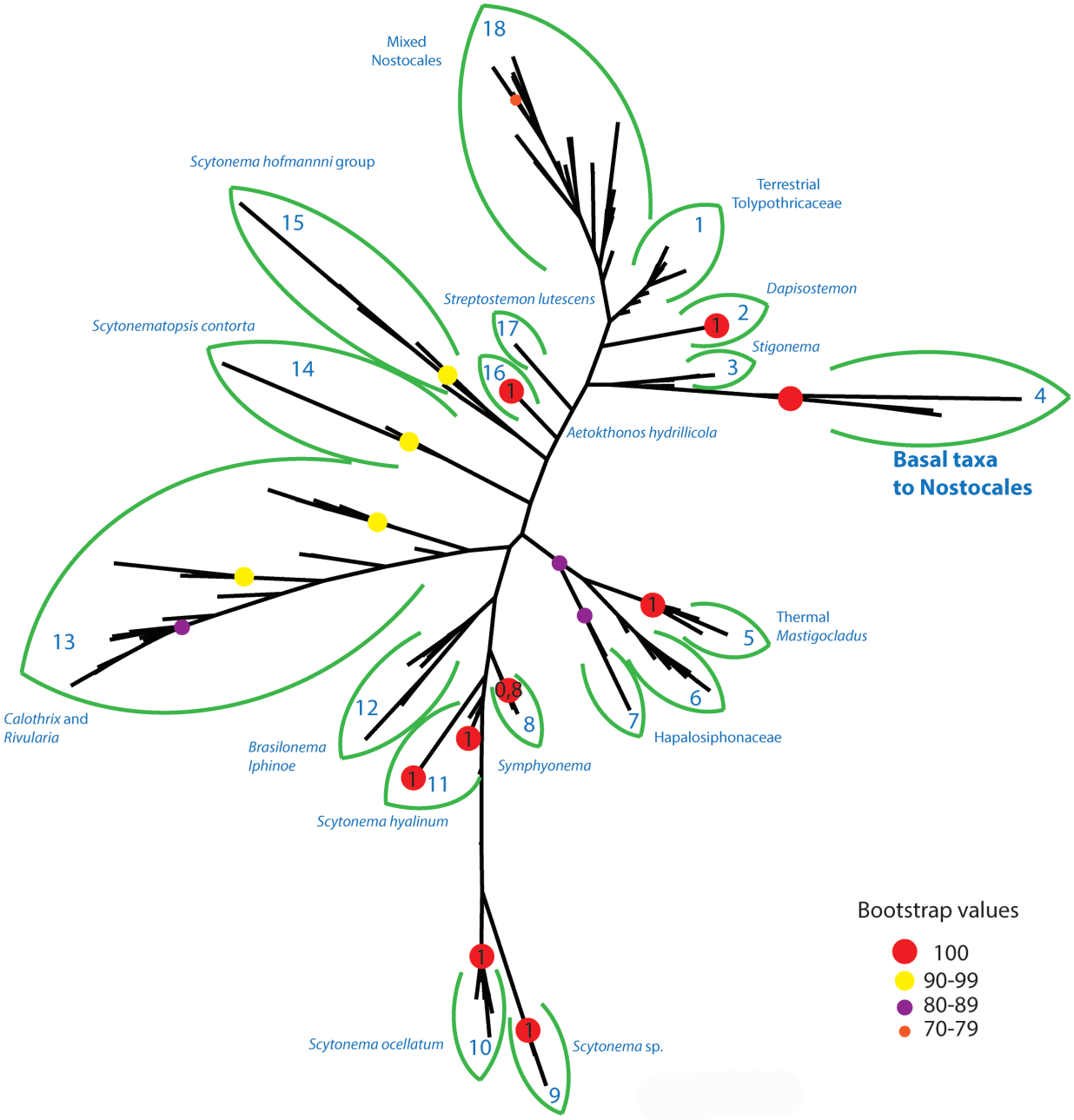

FIGURE 1. Phylogenetic relationships between *Dapisostemon apicaliramis* strains, *Streptostemon lutescens*, and related heterocytous cyanobacteria based on 16S rRNA gene sequences (1223 bp) resulting from Maximum Parsimony (MP) method with 210 OTUs. Bootstrap values above 70 are displayed as colored circles on nodes. The desgnations of strains in each branch are detailed in Table S1 and correspond to the numbers labeling the clades.

The bootstrap support along the backbone of all phylogenetic trees was too low for determining the precise relationship among our strains and other genera. Consequently, for better understanding of the relations between the clades, an unrooted tree is shown (Fig. 1). The calculated similarity values (p-distance) presented in Table 2 indicated that the phylogenetic relationships among the genera of interest and representatives from distinct but neighboring clades (Fig. 1, clades 1, 3, 18). *Dapisostemon* strains were all >99.4% similar to each other, but ≤96.0% similar to all other taxa. Many of these clades represent different families (Tolypothricaceae, Nostocaceae, Aphanizomenonaceae),

with others likely in unnamed family-level groups (Fig. S1). *Streptostemon* is at the base of the clade containing all of these clades, and was dissimilar to all of them (Figs 1, S1, S2; Table 2). A representative from the Nostocaceae clade (Fig. 1 clade 13), was considerably more dissimilar, being ≤92.5% similar to all tested taxa. Based on phylogenetic position and morphological and genetic diversity within the clades we would conclude that 1) *Dapisostemon* must be placed in a separate family or 2) the Tolypothricaceae, Nostocaceae, Aphanizomenonaceae, and Stigonemataceae must be collapsed into a single family to achieve a monophyletic taxon that contains *Dapisostemon*. We find the first of these two options more consistent with taxonomic practice and describe the new family, Dapisostemonaceae, below. The evolutionary position of *Streptostemon* was not stable between analyses (cf. Figs 1, S1, S2), and so we decline to create a family for this genus at this time. However, we can conclude it is not in the same clade/family as *Dapisostemon*, and consequently they must represent separate genera despite morphological similarities.

TABLE 1. Morphological comparison among *Dapisostemon*, *Streptostemon* and related genera.

Genera	Heterocytes	Cells	Thallus	Fascicles	Branching
Dapisostemon	Basal and intercalary	Isodiametric or slightly shorter or longer than wide	Filaments densely entangled in the base, with erect parallel, filaments arranged in a carpet-like layer	Rare, erect, compact, short, truncate (not tapered)	Common, tolypotrichoid, apical, parallel to the main filament
Streptostemon			Filaments loosely entangled in the base, apically fasciculate	Obligate, erect, clearly separated, elongated, frequently tapered	Absent or quite extraordinary scytonematoid or tolypotrichoid
Tolypothrix			Filaments attached basally, free apical ends. Sometimes forming woolly mats	Erect, frequent	Dendroid, tolypotrichoid, rarely geminate
Hassallia		Discoid		Erect or crustaceous, frequent	Dendroid, tolypotrichoid, usually arcuated, rarely geminate
Camptylonemopsis	Mainly intercalary. Isopolar germination of hormogonia	Isodiametric or slightly shorter or longer than wide	Filaments with more or less parallel arrangement	Creeping central parts and commonly with erect ends	Ocasional tolypothrichoid and scytonematoid

The studied populations of *Streptostemon* were identified based on nine samples and were in agreement with the original description of the genus (Sant'Anna *et al.* 2010), although they presented more intercalary heterocytes than previously described. We characterized the populations but succeeded in sequencing only one of them. We provide an expanded description which includes our new populations below so that morphological documentation for the sequence given herein is available.

Phylogenetic analyses using both MP and Bayesian Inference methods produced trees with similar terminal topology, but differing in the backbone (Figs 1, S2). The Bayesian Analysis was considered the less reliable tree because it split the Rivulariaceae into two distant clades, did not place *Chroococcidiopsis* Geitler (1933: 625) taxa at the base of the heterocytous clade, and placed the divergent "Mixed Nostocales" group within the terrestrial Tolypothricaceae. Furthermore, most of the families and genera were in a nine-pronged polytomy. The MP phylogeny with considerably higher taxon sampling (427 OTUs) had the same topology as the MP phylogeny with fewer OTUs (210 OTUs), and so phylogeny was stable within these analyses at the levels of taxon sampling employed in this study. Other trees with taxon sampling excluding distant groups (313 and 282 OTU's, respectively) also showed stability in the position of *Dapisostemon* and *Streptostemon* (data not shown).

The calculated sequence identity values (100*(1-p-distance)) presented in Table 2 for *Dapisostemon* ranged from 93 to 96% identity to representatives of Tolypothricaceae and Nostocaceae. A strain isolated from Kenya in another lab during review of this manuscript was found to be highly similar to *D. apicaliramis* (see *Dapisostemon* sp. Kenya in Table 2), and will likely be the subject of another manuscript by members of that lab. For *Streptostemon* the identity values reached a maximum of 94.5% with *Hassallia* EM2-HA1 (Table 2).

TABLE 2. The 16S rRNA gene sequence similarity between *Dapisostemon apicaliramis, Streptostemon lutescens*, and sequences of related cyanobacterial strains. Groups of strains considered to be unequivocally in the same family based on phylogeny and this similarity analysis are shaded gray.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *Dapisostemon* CCIBt 3318 KJ566947														
2. *Dapisostemon* CCIBt 3536 KJ566945	99.8													
3. *Dapisostemon* sp. Kenya	99.6	99.4												
4. *Hassallia* EM2-HA1 HQ847555	96.0	95.9	95.9											
5. *Spirirestis rafaelensis*SRS6 AF334692	95.4	95.2	95.1	99.5										
6. *Tolypothrix distorta* ACOI731 HG970652	95.2	95.1	95.3	96.6	96.0									
7. *Rexia erecta* CAT4-SG4 KF934181	95.8	95.7	95.5	97.1	96.4	98.0								
8. *Coleodesmium wrangelii* MC-JRJ1 AF334702	95.2	95.0	95.1	97.6	97.2	98.1	97.7							
9. *Nostoc commune* EV1-KK1 AY577536	94.8	94.7	94.6	96.1	95.4	94.5	95.4	95.1						
10. *Fortiea laiensis* HA4221-MV2 HQ847569	94.4	94.4	94.4	95.6	94.9	96.3	95.7	95.6	96.2					
11. *Streptostemon lutescens* KJ566946	94.3	94.2	94.1	94.5	94.0	93.3	93.8	93.8	93.6	93.7				
12. *Cylindrospermum alatosporum* SAG43.79 GQ287650	95.2	95.1	95.1	95.9	95.2	94.9	95.4	94.7	95.5	95.3	94.1			
13. *Nodularia harveyana* Lukesova 18/94 AM711554	94.5	94.5	94.5	95.4	94.6	95.3	96.2	95.7	95.0	94.4	93.3	95.8		
14. *Dolichospermum spiroides* PMC9403 AJ293116	93.2	93.1	93.2	94.1	93.3	94.9	94.9	94.8	94.3	94.1	93.2	94.8	95.9	
15. *Calothrix desertica* PCC 7102 AM230699	92.4	92.3	92.5	92.1	91.3	92.4	93.0	92.1	91.2	92.1	91.7	92.2	92.4	92.4

Analysis of the sequence length of domains and secondary structure of the 16S-23S ITS regions demonstrated that both *Dapisostemon* and *Streptostemon* were distant from each other and other representative Nostocales (Table 3, Figs 2, 3). Most taxa were similar in the lengths of the leader, D1-D1' helix, tRNA genes, Box A, and D4 with spacer (Table 3). The three taxa that were tolypothricoid in morphology (*Dapisostemon, Hassallia, Coleodesmium*) were additionally similar in the length of the D2 and D3 regions (with associated spacer regions). The length of V2 and V3 regions was highly variable, and the structure of the V2 and V3 helices was likewise very variable (Figs 2 g-k, 3 g-l). *Dapisostemon* had a D1-D1' helix similar to both *Hassallia* and *Coleodesmium* Borzi ex Geitler (1942: 154) in the basal and middle portions of the helix, but had a smaller subapical bilateral bulge and a terminal region quite different in sequence (Fig. 2 a-c). The V2, Box B, and V3 helices of *Dapisostemon* were unique to that genus. *Streptostemon* had ITS structures that could be easily distinguished from all other taxa, with the shortest D1-D1' helix as well as the longest V3 helix (Figs 2 e, 3 e). *Scytonema hyalinum* Gardner (1927: 7), the most phylogenetically distant taxon in this group (Fig. 1) had an exceptionally long D1-D1' helix as well as unique V2, Box B, and V3 helices (Figs 2 f, k, 3 f, l). All Nostocaceae have a solitary nucleotide opposite the unilateral bulge in the D1-D1' helix (Lukešová *et al.* 2009), a feature represented in *Nostoc indistinguendum* Řeháková et Johansen in Řeháková *et al.* (2007: 487) (Fig. 2 d).

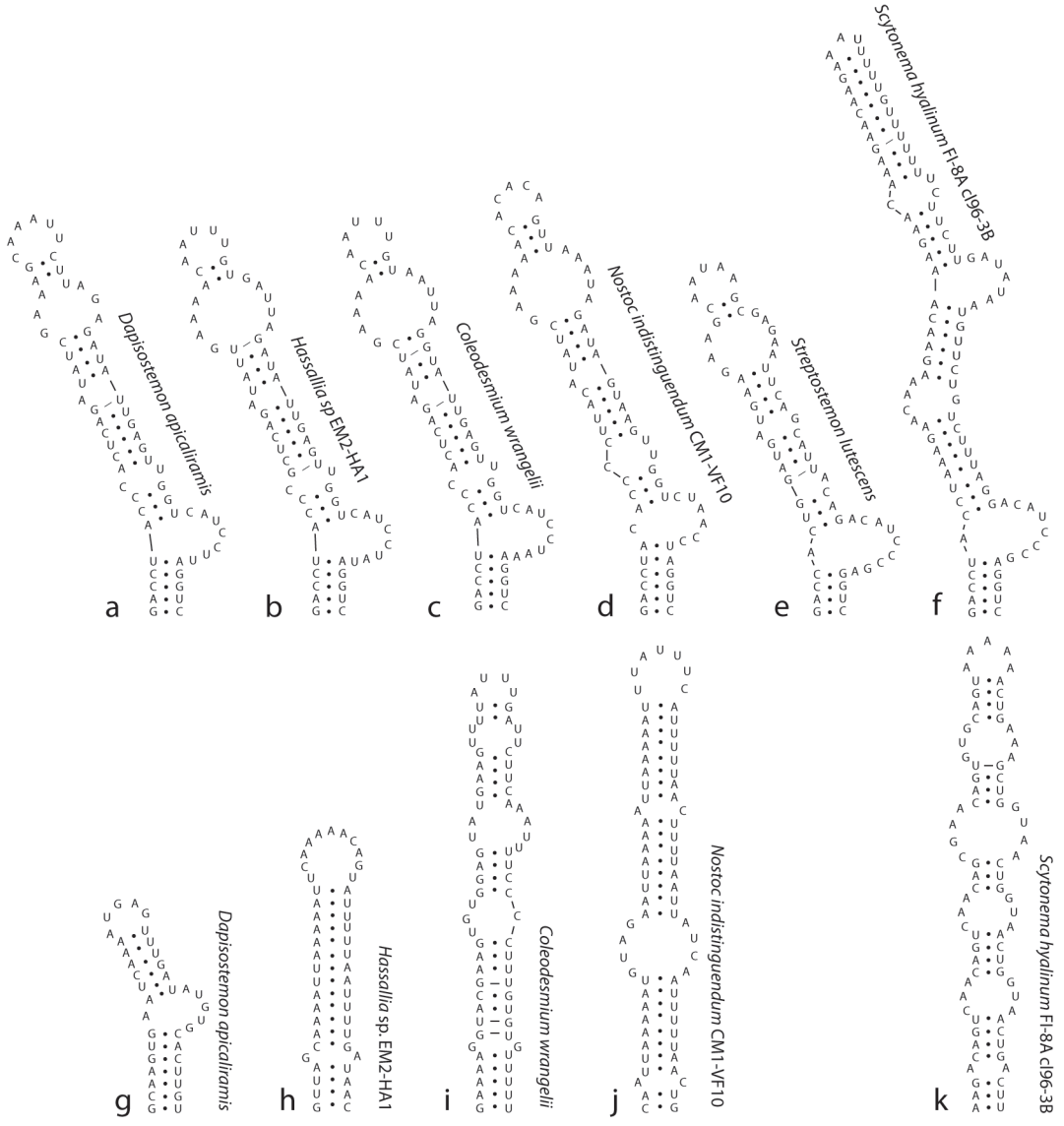

FIGURE 2. Secondary structures in representative Nostocales genera. D1-D1` helix (a-f), V2 helix (g-k). *Hassallia* EM2-HA1 represents the terrestrial Tolypothricaceae, *Coleodesmium wrangelii* represents the aquatic Tolypothricaceae. *Streptostemon lutescens* lacked the tRNA-Ala gene, and consequently had no V2 helix.

Streptostemon is unusual among the Nostocales sequenced thus far in having an ITS region with only a single tRNA gene ($tRNA^{Ile}$), with most other Nostocales having at least two operons, one with two tRNA genes ($tRNA^{Ile}$ and

tRNA^{Ala}) and one or more with no tRNA genes (Iteman *et al.* 2000, Boyer *et al.* 2001, Flechtner *et al.* 2002, Řeháková *et al.* 2007, Lukešová *et al.* 2009, Vaccarino & Johansen 2011, 2012, Johansen *et al.* 2014). It is interesting to note that *Stigonema* C. Agardh ex Bornet et Flahault (1886-1888: 62) also has a single tRNA gene in the ITS (Table 3). The long spacer between the D3 region and the tRNA^{Ile} is also highly unusual and represents a unique multi-nucleotide insertion. While based on morphology *Streptostemon* is better placed in the Tolypothricaceae than in any other family Sant'Anna *et al.* (2010, as Microchaetaceae), the molecular evidence we have assembled in this study indicates it is in reality distant from all genera in that family.

Dapisostemonaceae G. S. Hentschke, C. L. Sant'Anna *et* J. R. Johansen *fam. nov.*

Thallus forming mats composed of entangled filaments which become erect and parallel, with heteropolar filaments and single false branching, with firm, yellowish sheaths, forming intercalary, bipolar heterocytes, lacking akinetes. Most similar to Tolypothricaceae, but separated phylogenetically from that family.

Type genus: *Dapisostemon.*

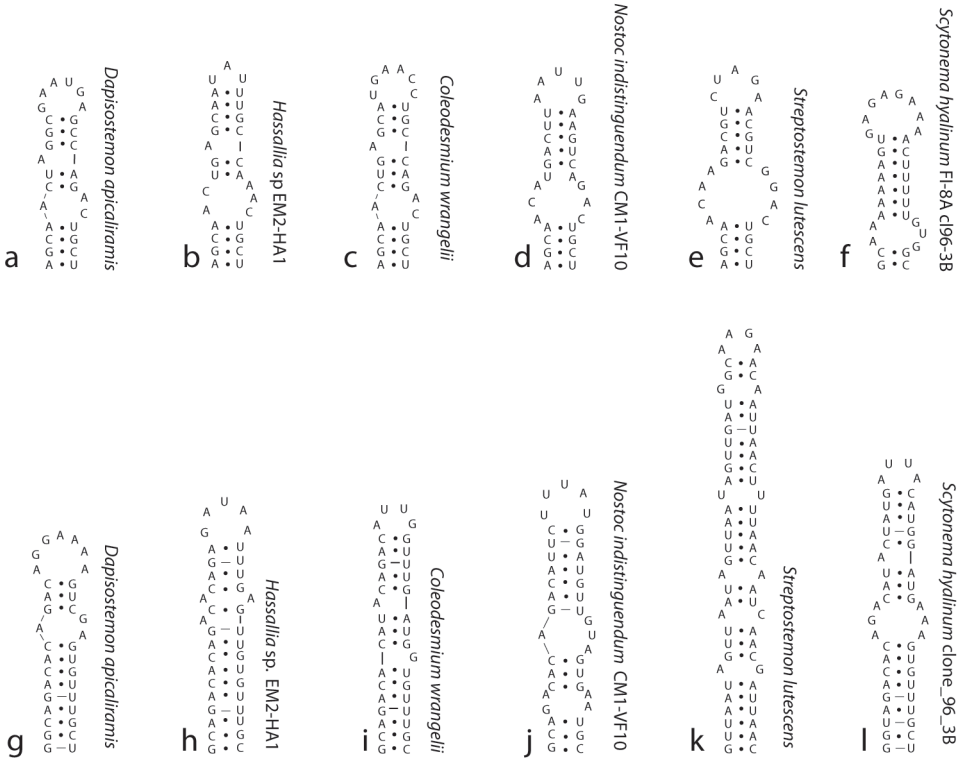

FIGURE 3. Secondary structures in representative Nostocales genera. Box B helix (a-f), V3 helix (g-l). *Hassallia* EM2-HA1 represents the terrestrial Tolypothricaceae, *Coleodesmium wrangelii* represents the aquatic Tolypothricaceae.

Dapisostemon G. S. Hentschke, C. L. Sant'Anna *et* J. R. Johansen *gen. nov.*

Thallus forming mats composed by creeping entangled filaments in basal part, which become erect and parallel/ interwoven toward the apex forming a caespitose *stratum*, 11-14.5 µm wide. Filaments cylindrical, with tolypotrichoid branching, sometimes clustered together in short related fascicles. Trichomes heteropolar, constricted or not at the cross walls, frequently attenuated in the middle, 5.6-9 µm wide. Heterocytes basal and intercalary, 7.3-11.2 µm wide, Cells and heterocytes ± isodiametric or slightly longer or shorter than wide, cells shorter at the apex. Sheaths colorless to brownish, lamellate or homogenous.

Type species: *Dapisostemon apicaliramis*

Etymology: Dapis (Gr.) = carpet, stemon (Gr.) = threads (carpet of threads)

TABLE 3. Nucleotide lengths of the regions of the 16S-23S of comparison strains for *Dapisostemon* and *Streptostemon*, including representatives of the Nostocaceae, Tolypothricaceae and Stigonemataceae, as well as the distant outgroup *Scytonema hyalinum*. *Streptostemon* and *Stigonema* lacked the tRNA-Ala gene, and thus the segment after the tRNA-Ile is Spacer+Box-B+Spacer. The D5 cannot be determined without having the sequence for the 23S-5S ITS region, and so is included within the spacer after the V3 and before the 23S rRNA gene.

Strain	Leader	D1-D1' helix	Spacer+D2	Spacer+D3+spacer	tRNA Ile gene	Spacer+V2+spacer	rRNAAla gene	Spacer+BoxB+spacer	Box A	D4+Spacer	V3+Spacer (with D5)
Dapisostemon apicaliramis CCIBt 3536	8	63	34	16	74	47	73	67	11	22	45
Hassallia sp. EM2-HA1	7	65	33	16	74	59	73	75	11	26	64
Spirirestis rafaelensis WJT-71_NPBG6	7	60	33	16	74	59	73	75	11	23	64
Tolypothrix sp. FI5-MK38	7	66	33	16	74	52	73	74	11	23	48
Coleodesmium wrangelii MC-JRJ1	7	65	32	14	74	73	73	78	11	25	61
Goleter apudmare nom. prov. HA4356-MV2	8	65	33	15	74	80	73	59	11	27	57
Nostoc indistinguendum CM1-VF10	8	67	42	15	74	83	73	75	11	26	67
Stigonema sp. HA04070.00001	7	61	33	21	74	--	--	154	11	37	47
Streptostemon lutescens	8	60	33	63	74	--	--	154	11	49	67
Scytonema hyalinum FI-8A, clone 96-3B	7	97	32	17	74	83	73	153	11	21	71

Dapisostemon apicaliramis G. S. Hentschke, C. L. Sant'Anna *et* J. Komárek *sp. nov.* (Figs. 4, 5)

Thallus forming black mats. Filaments creeping and entangled in the basal part, becoming parallel and erect, occasionally clustered together in closely related fascicles. Fascicles mostly short and truncate, composed by densely arranged parallel/interwoven filaments. Filaments 11-14.5 µm wide, often with short tolypotrichoid branches in the apical portion. Trichomes heteropolar, cylindrical or slightly attenuated in the middle. Cells ± isodiametric, 6-10.8 µm long; 5.6-9 µm wide; shortened at the apex. Cell content homogenous or with vacuole-like structures, pale or dark blue-green. Sheaths brownish-yellow, with parallel or divergent lamella or rarely homogenous. Basal heterocytes hemispherical, isodiametric or cylindrical when intercalary, 5.1-11.7 µm long, 7.3-11.2 µm wide. Hormogonia heteropolar, short (5-10 cells), constricted, dark blue-green.

Holotype: Brazil, State of São Paulo, State Park of Ilha do Cardoso, 06/29/2010, *Watson A. Gama Jr. and Camila F. da S. Malone* (SP 401441) (herbarium preparation of natural material), Herbarium of São Paulo State, São Paulo, Brazil.

Reference strain: CCIBt 3318

Type locality: State Park of Ilha do Cardoso, State of São Paulo, Brazil.

Etymology: apicale (L.) = apically, ramis (L.) = branched

Streptostemon lutescens Sant'Anna *et al.* 2010 (Fig. 6)

Filaments entangled and creeping in the basal part, than joined together in one to several dense, erect and irregularly separated fascicles. Fascicles mostly lanceolate. Filaments 6.7-14 µm µm wide, not branched or rarely with scytonematoid or tolypotrichoid branches. Sheaths hyaline or yellowish, homogenous or with parallel lamella. Tricomes heteropolar, cylindrical or slightly attenuated in the middle. Cells ± isodiametric, 3-12 µm long; 5.5-8.8 µm wide; shortened at the apex. Cell content homogenous, pale or dark blue-green. Basal heterocytes hemispherical, intercalary isodiametric or cylindrical, 4.3-14 µm long, 6.4-11.1 µm wide. Hormogonia mostly heteropolar, containing 7-18 cells, slightly constricted, cells very short with pale blue-green or yellowish granular content. Habitat: Wet rocks, bark of trees, artificial wood substrata or epiphytic on bryophytes.

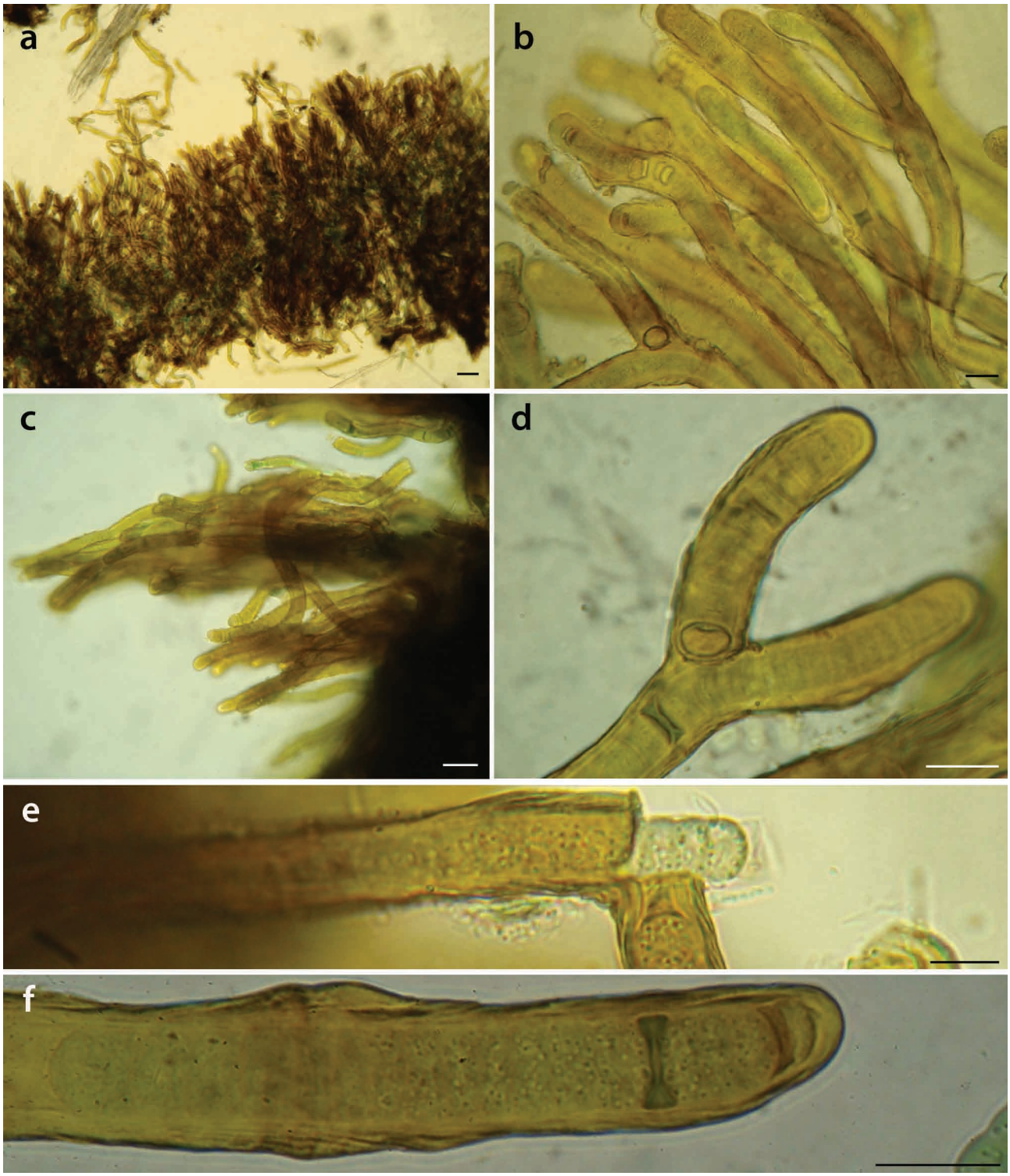

FIGURE 4. *Dapisostemon apicaliramis*. General view of thalli with parallel filaments (a), detail of apices (b), thallus forming fascicle (c), tolypotrichoid branching (d), details of different filaments apices (e, f). Scales: a. 50 µm; b, e, f. 10 µm ; c. 30 µm; d. 10 µm.

Studied material: Brazil, State of São Paulo, State Park of Ilha do Cardoso, 06/29/2010, *Watson A. Gama Jr. and Camila F. da S. Malone*, (SP 427336, SP 427501); State Park of Serra do Mar, Nucleus Santa Virgínia, 02/11/2010, *Watson A. Gama Jr. e Ewerton C. Manarin* (SP 427502); Ecological Station Jureia-Itatins, Guilherme S. Hentschke, 08/16/2011, *Watson A. Gama Jr., Camila F. da S. Malone* and *Célia L. Sant'Anna* (SP 427304, SP 427320, SP427505).

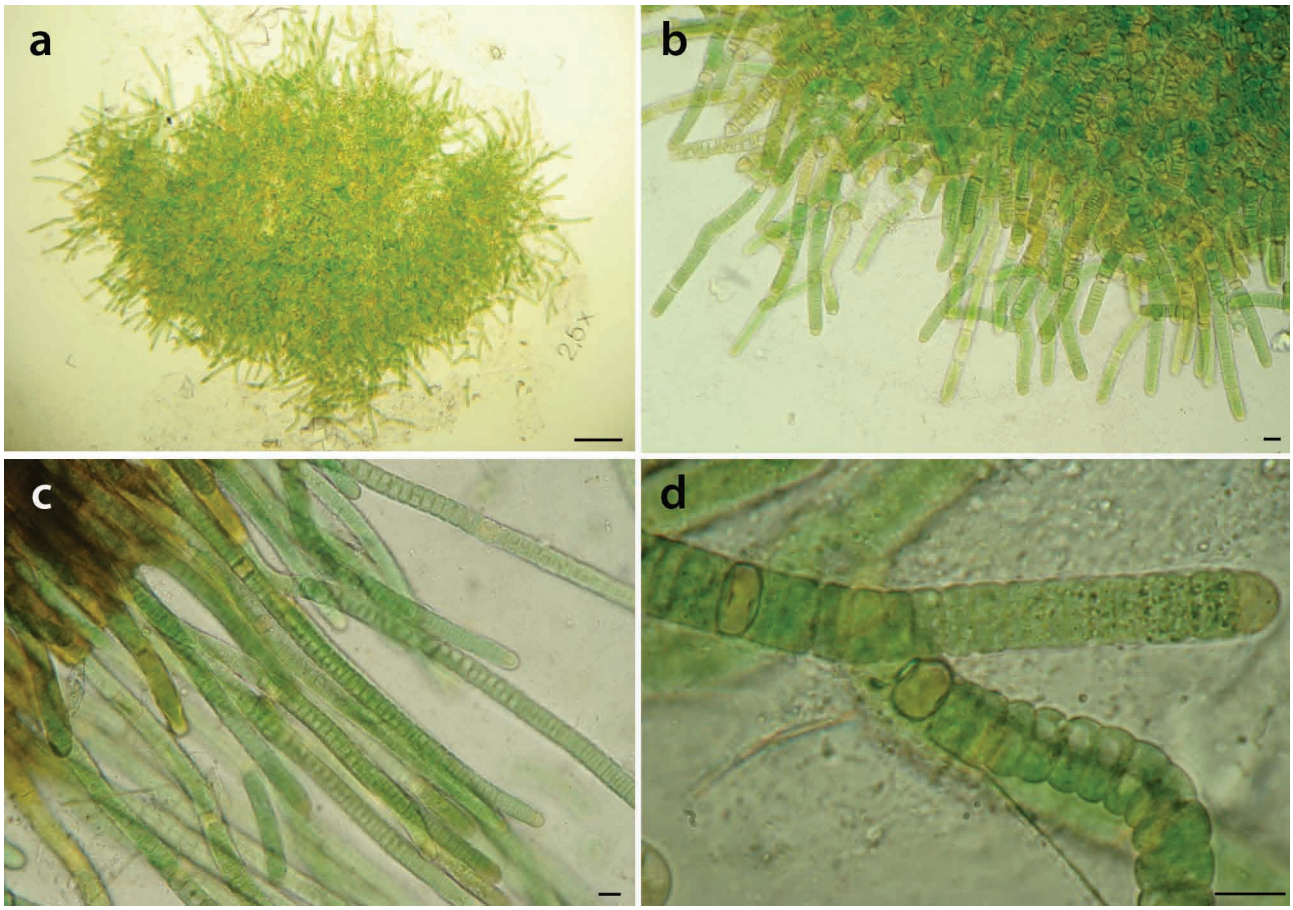

FIGURE 5. Cultured *Dapisostemon apicaliramis*. General view of thalli (a-b), young culture with yellowish, older sheaths (c), tolypothrichoid branching (d). Scales: a. 100 µm; b,c,f. 10 µm.

Discussion

The new genus *Dapisostemon* is morphologically most similar to *Streptostemon* Sant'Anna *et al.*, but differs mainly by the presence of more common tolypothrichoid branching and the parallel arrangement of erect filaments composing a caespitose, but not commonly fasciculated layer. The genus *Streptostemon* features filaments joined in dense erect, tapering fascicles but lacks branching or quite extraordinarily possesses tolypothrichoid or scytonematoid branching. *Dapisostemon* differs also from *Hassallia, Coleodesmium, Tolypothrix* Kützing ex Bornet et Flahault (1886-1888: 118), and *Rexia* Casamatta *et al.* (2006: 23) mainly by the lack of the typical repeated branching of the dendroid filaments observed in these former taxa. The complete morphological comparisons among these genera are detailed in Sant'Anna *et al.* (2010).

Despite the relatively high similarity (96%) among 16S rRNA gene sequences with other genera retrieved from GenBank, the MP and BA phylogenetic trees combined with morphological and 16S-23S ITS data, strongly support the decision to describe a new genus. As already shown for *Spirirestis* Flechtner et Johansen in Flechtner *et al.* (2002: 6) and Komárek (2011), the "95% similarity between strains rule" to aggregate genera cannot be strictly applied for Nostocales and the morphology of populations together with the phylogenetic tree topologies must be considered. This is endorsed based on the high similarity of *Dapisostemon* and *Streptostemon* when compared to *Hassallia*, *Tolypothrix* and also morphologically distinct genera like *Nostoc* Vaucher ex Bornet et Flahault (1886-1888: 181) and *Cylindrospermum* Bornet & Flahault (1886-1888: 249) (Table 2). In fact, within the Nostocaceae, distinct clades with internal morphological congruence, tend to diverge from each other (Fig. 1), even though the similarity among their strains (>95%) would suggest they are congeneric by the old and discredited criterion (Novis & Smissen 2006, Hrouzek *et al.* 2013).

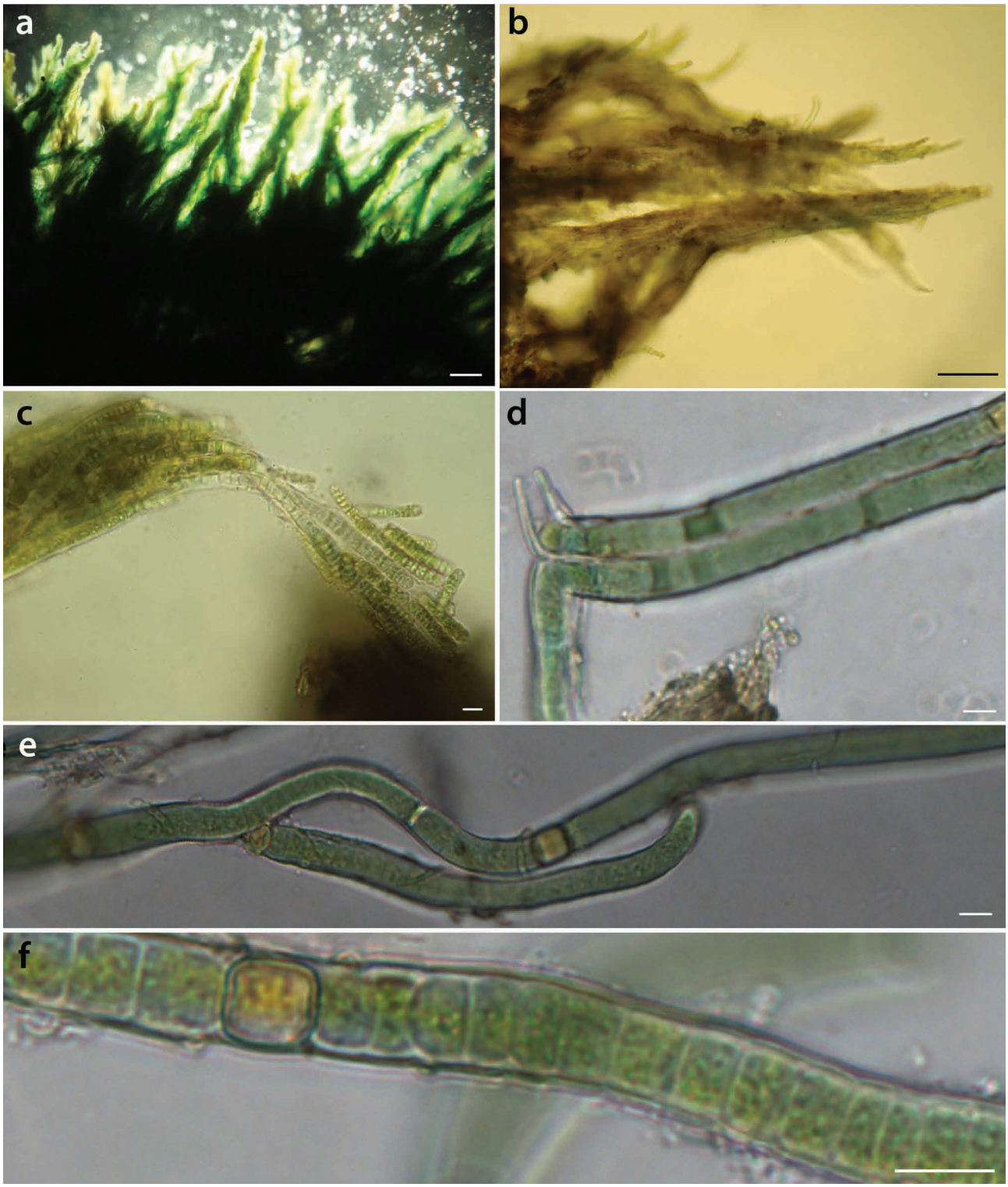

FIGURE 6. *Streptostemon lutescens.* Thalli forming fascicles (a-b), releasing of hormogonia (c), scytonematoid branching (d), tolypotrichoid branching (e), detail of filament with intercalary heterocyte (f). Scales: a, b. 100 µm, c-f.10 µm.

In the previously unsequenced genus *Streptostemon*, although it is confirmed as a distinct and special taxon by 16S rRNA and 16S-23S ITS data, in our analysis the genus has an uncertain phylogenetic position and it is not possible to determine to which family it belongs based on the 16S rRNA phylogeny alone. Relevant to this problem, we found an intermediate morphological condition between the Tolypothricaceae and Scytonemataceae, with *Streptostemon* presenting both intercalary heterocytes and isopolar trichomes (Scytonemataceae characters) and basal heterocytes in heteropolar trichomes (Tolypothricaceae characters). Indeed, in the illustrations for the genus in Sant'Anna *et al.* (2010), intercalary heterocytes are also very common. The phylogenetic relevance of the heterocyte position for determination

of family level was already discussed by Vaccarino & Johansen (2011, 2012), in which the authors described two species of Scytonemataceae (*Scytonematopsis contorta* Vaccarino et Johansen 2011: 151 and *Brasilonema angustatum* Vaccarino et Johansen 2012: 1180), with isopolar hormogonia becoming heteropolar after breakage. Hence, while we believe that the position of heterocytes is important in classification at the family level, more studies must be done to clarify the observed morphological and genetic anomalies more precisely.

The taxonomic placement of *Streptostemon* is further complicated by the fact that the Scytonemataceae are not currently monophyletic (Komárek *et al.* 2013). Our phylogeny shows four clusters of Scytonemataceae, including three putatively being "*Scytonema*". Based on our phylogeny and the finding of others (Vaccarino & Johansen 2011, Johansen *et al.* 2014, Komárek *et al.* 2013) the Scytonemataceae must be revised. *Streptostemon* is unique in the secondary structures of the 16S-23S ITS region, and this consequently fails to align it with existing families. For now it remains in a basal position in all phylogenies and cannot be placed in any family without considerable uncertainty.

The presence of erect fascicles is a remarkable characteristic for both genera presently studied and probably this character was developed independently in many cyanobacterial genera like *Brasilonema*, *Tolypothrix* and *Stigonema* Bornet & Flahault. However their ecological role is not yet understood. The parallel filaments of creeping rope-building cyanobacteria like *Microcoleus* Gomont (1892: 15) and *Hydrocoleum* Gomont (1892: 332) (Oscillatoriales) allows them to colonize physically unstable sedimentary environments (Garcia-Pichel &Wojciechowski 2009), but unlike them, the erect fasciculate types of Nostocales grow commonly on stable substrates like rocks, tree bark or bryophytes (Sant'Anna *et al.* 2010, 2011, 2013). Hypothetically, the erect fasciculate thalli could be an advantage to improve light harvesting into the dense and species-specific diverse biofilms found in forests like the Atlantic Rainforest, or a self-shading strategy to avoid extreme solar radiation.

Finally, based on our sampling and previous data (Sant`Anna *et al.* 2010), the genera *Dapisostemon* and *Streptostemon* are widespread in tropical and subtropical Mata Atlântica from São Paulo State, as other fasciculate types of *Brasilonema* and *Stigonema* (Sant'Anna *et al.* 2011, 2013). Many of these taxa were described recently as a result of the increasing number of taxonomic studies in these areas, meaning that the cyanobacterial diversity is still underestimated due to poor sampling or misidentification of the species based on European types. Considering recent biogeographic studies (Bahl *et al.* 2011) we conclude that the terrestrial cyanobacterial diversity composition in tropical areas is very different from temperate zones and the current tendency of describing these tropical populations as new species using polyphasic characterization is valid. In this paper, we describe the new genus *Dapisostemon* in the new family Dapisostemonaceae, confirm *Streptostemon* as a special genus and highlight the potential of Mata Atlântica as a discovery source of new cyanobacterial taxa, as well as the importance of conservation of this biome in order to preserve its high biodiversity.

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