Morphological and molecular characterization of *Stigonema jureiensis* sp. nov. (Nostocales, Cyanobacteria) from the Atlantic Rainforest, São Paulo, Brazil

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Abstract: Tropical regions are considered hotspots of biodiversity, and the genus *Stigonema* has been underexplored in all world, mainly due to the difficulties of culturing. In this study, we sequenced the 16S rRNA gene of a natural *Stigonema* population from the tropical Atlantic Rainforest (São Paulo, Brazil) and describe *S. jureiensis* sp. nov. based on phylogenetic and morphological data. For the molecular analysis, the genomic DNA was isolated from aliquots of the population, which were previously manually cleaned under a microscope, using a point–tapered Pasteur pipette to remove other organisms and debris. For morphological analysis, 30 individuals were analyzed and photographed using microscope. Both maximum likelihood and Bayesian phylogenies support *S. jureiensis* as a new species, and morphological analysis revealed the presence of a special type of mosaic ornate sheaths.

Key words: biodiversity, microorganism, polyphasic approach, taxonomy, terrestrial habitats, tropical environments

Introduction

The nostocalean genus *Stigonema* Bornet et Flahault is abundantly distributed worldwide, but phylogenetic studies on it are scarce compared to other cyanobacterial genera. This is mainly due to difficulties on isolating and culturing this taxon, and consequently, until recently, taxonomic studies on *Stigonema* were based solely on morphology. In a study on the diversity of true–branched Cyanobacteria from the Brazilian Atlantic Rainforest, Ferreira et al. (2013) published the first combined molecular and morphological analysis of a *Stigonema* population (*S. ocellatum* Bornet et Flahault). Using the glass–capillary isolation technique, Mareš et al. (2015) were able to sequence the 16S rRNA gene of several morphologically characterized *Stigonema* presenting a robust 16S rRNA gene phylogeny for the genus. In the same year, Song et al. (2015) described *S. dinghuense* Song et Li based on both morphological and molecular data. These two latter studies support the monophyly of

the genus *Stigonema*.

After these initial publications, a large number of 16S rRNA gene sequences of *Stigonema* were deposited in public DNA sequence databases (GenBank). Thus, the current situation of the genus is: (1) there are 164 *Stigonema* 16S rRNA gene sequences in GenBank; (2) most of them are not associated to a published manuscript, thus lack morphological data; (3) there is a lack of information on 16S–23S rRNA Internal Transcribed Spacer (ITS) sequences and secondary structures; (4) as with other cyanobacteria, the infrageneric phylogenetic relationships are uncertain; and (5) there are few *Stigonema* strains in culture collections, making the access to the 16S rRNA gene of these populations difficult. Thus, most of the taxonomic studies of *Stigonema* are restricted to morphological data presented in classic works, such as Bornet & Flahault (1889), Gardner (1927), Frémy (1930), Geitler (1933), and Komárek (2013).

The genus *Stigonema* is abundant in the Brazilian Atlantic Rainforest, being studied by Silva & Sant'Anna

(1988), who described *S. gracile* Silva et Sant'Anna from Ilha do Cardoso (Atlantic Rainforest) and SILVA & SANT'ANNA (1996), who revised the genus for São Paulo State, Brazil. The most recent study on *Stigonema* from the Atlantic Rainforest described three new species and presented four other morphotypes commonly found in the biome (SANT'ANNA et al. 2013). Although there has been a large diversity of *Stigonema* reported for the Atlantic Rainforest, the genus is still poorly studied in this biome, with all papers, except for FERREIRA et al. (2013), restricted to morphological analysis.

In this study, we sequenced the 16S rRNA gene from a natural population of *Stigonema* collected in the Atlantic Rainforest (São Paulo State, Brazil). The population presented a unique type of mosaic–like sheath and based on both phylogenetic and morphological data, we describe and propose the new species, *Stigonema jureiensis* sp. nov.

MATERIAL AND METHODS

A *Stigonema* population was collected in August 2011 by scraping biofilms growing on an igneous rocks in the tropical Ecological Station Juréia–Itatins (24º24'36''S and 47º01'15''W) area of the Brazilian Atlantic Rainforest. The material was stored and dried using paper bags, and later in the laboratory the dried material was rehydrated for 20 hours in distilled water for morphological analysis. At least 30 individuals were analyzed and photographed using a Zeiss Axioplan 2 light microscope, equipped with a Zeiss Axiocam MRc digital camera. These images were compared to known *Stigonema* species in KOMÁREK (2013). A subsample was preserved in formaldehyde (4%) and deposited in the Herbarium of the Institute of Botany São Paulo, Brazil (SP), under the number SP 427311. Subsequently to the morphological inspection, for the 16S rRNA gene analysis, the natural population was cleaned using a point–tapered Pasteur pipette under a microscope to remove organisms and debris attached to the filaments. Afterwards, 50 individuals were transferred and washed sequentially into five new droplets of sterile distilled water. Total genomic DNA was extracted from these individuals using a DNeasy UltraClean Microbial Kit (Qiagen) and the 16S rRNA gene was amplified using the 27F and 1494Rc PCR primers (NEILAN et al. 1997). For amplification and sequencing of our material, we followed the methods in FIORE et al. (2007) and HENTSCHKE et al. (2016). Phylogeny was inferred based on Maximum Likelihood (ML) and Bayesian (BA) trees, which were constructed in several steps (HENTSCHKE et al. 2016). To position our species in the Nostocales tree, first we aligned our sequence with sequences of *Brasilonema* Fiore et al., *Calothrix* Bornet et Flahault, *Chlorogloeopsis* Mitra et Pandey, *Coleodesmium* Geitler, *Dapisostemon* Hentschke et al., *Hassallia* Bornet et Flahault, *Iphinoe* Lamprinou et Pantazidou, *Microchaete* Bornet et Flahault, *Petalonema* Correns, *Rivularia* Bornet et Flahault, *Scytonema* Bornet et Flahault, and *Tolypothrix* Bornet et Flahault. Then, we added all *Stigonema* sequences used in the phylogeny of MAREŠ et al. (2015), plus other related sequences retrieved from GenBank using BLAST (total of 170 OTUs with 752 informative sites) (tree not shown). To obtain trees with a greater number of informative sites, higher bootstrap support, and to eliminate polytomies, we excluded all identical

sequences and all sequences with less than 1297 nucleotides length. In order to study our populations at the species level, we constructed trees using only those *Stigonema* sequences associated to articles with morphological data. To do this, we searched GenBank for the publications of all 164 *Stigonema* sequences available on 08 March 2019. Then, we selected the published sequences, excluded the clones, and performed the alignment for phylogenetic analysis.

For all generated trees, the sequences were aligned using ClustalW and the GTR+G+I evolutionary model was selected by running Modeltest in MEGA 7.0 (KUMAR et al. 2016). The robustness of ML trees was estimated by bootstrap, using 1000 replications in MEGA 7.0 (KUMAR et al. 2016). The Bayesian trees were constructed in two independent runs, with four chains each for 5×10^5 generations, using Mr. Bayes (RONQUIST & HUEKSENBECK 2003). The outgroups used were *Gloeobacter violaceus* AF132791, *Chroococcidiopsis* sp. FR798923 and *Chroococcidiopsis* sp. AB039005.

A similarity matrix (p–distance) was also generated to compare *Stigonema jureiensis* with the closest phylogenetically related taxa. The 16S rRNA gene sequence of *S. jureiensis* was deposited in GenBank (NCBI) under the accession Uncultured *Stigonema* JUR147 (MK471331).

RESULTS

While studying the cyanobacterial flora of tropical and subtropical areas of the Atlantic Rainforest (São Paulo, Brazil), an interesting new terrestrial stigonematacean taxon was observed. The taxon fit very well into the morphological circumscription of *Stigonema*, featuring true branches (T–type) and multiseriate trichomes. However, it differed from all the currently described species of *Stigonema*, due to unique mosaic–like sheaths that had not been previously described for the genus (Figs 1–7). Thus, we propose a new species, *Stigonema jureiensis* sp. nov., supported by morphological and molecular data.

The first step of the 16S rRNA gene phylogeny (total of 170 OTUs with 752 informative sites) confirmed that our proposed new species was placed in the strongly supported *Stigonema* clade (ML = 100%, BA = 1) (tree not shown). In the second step, the ML and BA trees (63 OTUs with 1325 informative sites) showed similar topology, presenting two main clades with strong phylogenetic support (Fig. 8). In the basal position of Nostocales, the cluster containing *Brasilonema, Chlorogloeopsis, Iphinoe, Rivularia,* and *Scytonema* (ML = 32%, BA = 0.99) is sister to the cluster of *Coleodesmium, Dapisostemon*, *Hassallia,* and *Stigonema* (ML = 99%, BA = 1.0). Although *Stigonema* is monophyletic (ML = 99%, BA = 1.0), the relationships among its species are unresolved. In the *Stigonema* clade, the uni– and multiseriate species are intermixed (Fig. 8). Furthermore, some infrageneric clades were common by different species with strong phylogenetic support.These were the cases of *S. spectabile* KT867140 (multiseriate) and *S. elegans* KT867212 (uniseriate), which are in the same clade with ML support of 100% and BA of 1.0; the clade of *S. cornutum* KT867200 and *S. turfaceum* KT867159

(ML = 96%, BA = 1.0); *S. cornutum* KT867201 and *S. lechangense* KT867217 (ML = 98%, BA = 1.0); and *S. intermedium* KT867215 and *S. fragile* KT867160 (ML = 88%, BA = 1.0). It is also important to highlight that strains of *S. anomalum* Blanchard*, S. cornutum* Gardner*, S. elegans* Gardner*, S. hormoides* Bornet et Flahault, *S. ocellatum* Bornet et Flahault*,* and *S. panniforme* Bornet et Flahault were found in more than one clade. In this tree, *S. jureiensis* is a unique branch separated from other *Stigonema* species.

In order to connect morphological and phylogenetic data, the third step of the ML and BA trees (Fig 9) were built only with strains that contained published morphological data. In these trees, *S. jureiensis* formed again a unique branch separated from other *Stigonema* species, and it was sister to *S. hormoides* and *S. tomentosum* Hieronymus (ML = 85%, BA = 0.98). The similarity (p–distance) values between *S. jureiensis* and these strains range from 97% with S. *hormoides* KM047007 to 99.5% with *S. tomentosum* KM047014.

Species Description
Stigonema jureiensis G. S. Hentschke et C. L. Sant'Anna sp. nov. (Figs 1–7)
Filaments forming a felt–like thallus, erect, more or less parallel, tortuous 23–33 µm diam., sometimes slightly tapered from the base towards the ends, intensely branched. Branches identical to the main filament, bending toward the apex. Sheaths massive, hyaline, yellowish, mosaic–like sculptured with irregular margins. Trichomes with uni– and multiseriate parts 16–25 µm diam, composed by dark–green compressed or spherical cells, 5–11 µm diameter. Apex rounded, usually uniseriate, slightly tapered or not. Heterocytes lateral, irregulary sphaerical.

Holotype: BRAZIL, São Paulo State, Ecological Station Juréia–Itatins, sampled 08/16/2011by Guilherme S. Hentschke, Watson A. Gama Jr., Camila F. S. Malone and Célia L. Sant'Anna. Fixed sample (SP 427311) deposited in Herbarium of São Paulo State, São Paulo, Brazil.
Type locality: Atlantic Rainforest, Ecological Station Juréia–Itatins (24º24'36"S, 47º01'15"W), São Paulo State, Brazil.
**Reference sequence**: Uncultured *Stigonema* sp. JUR147 (NCBI Accession number: MK471331).
Habitat: On a humid igneous rock in a dense rainforest area. Etymology: The name is after the Ecological Station Juréia–Itatins.

DISCUSSION

Although *Stigonema jureiensis* shares high similarity (p–distance 99.5%) with S. *tomentosum* KM047014, both

Table 1. Morphological comparisons among *S. jureiensis* and related species.

	S. mamilosum Bornet et Flahault (Komárek 2013)	**S. tomentosum Hieronymus (Komárek 2013)**	**S. jureiensis sp. nov.**
Thallus	Sessile, pillow–like or forming filamentous clusters. Dark brown or greenish brown	Felt–like, tufty.	Felt–like
Filaments	Multiseriate, heteropolar, richly branched. Up to 65(90) µm diameter.	Uniseriate (in some parts 2 cells), creeping, branched, 14–28(38) µm diameter.	Uni to multiseriate, erect, +–parallel, tortuous, 23–33 µm diameter., sometimes slightly tapered from the base towards the ends, intensely branched.
Branches	With perpendicular hormogonia.	Erect, uniseriate, commonly in fascicles, with the same diameter of the main filament or slightly narrower.	Branches identical to the main filament, bending toward the apex.
Cells	Sphaerical or irregulary sphaerical	Barrel–shaped, often with individual envelopes.	Compressed or sphaerical cells.
Heterocytes	Lateral	Intercalary	Lateral
Sheaths	Colourless or yellow, lamellated.	Narrow, lamellated, yellow–brown.	Sheaths abundant, hyaline or yellowish, mosaic–like sculptured with irregular margins.
Habitat	Aquatic, submersed, benthic, on stony substrates.	Subaerophytic on wet wood and rocks.	Aerophitic, on an igneous rock in the Atlantic rainforest.
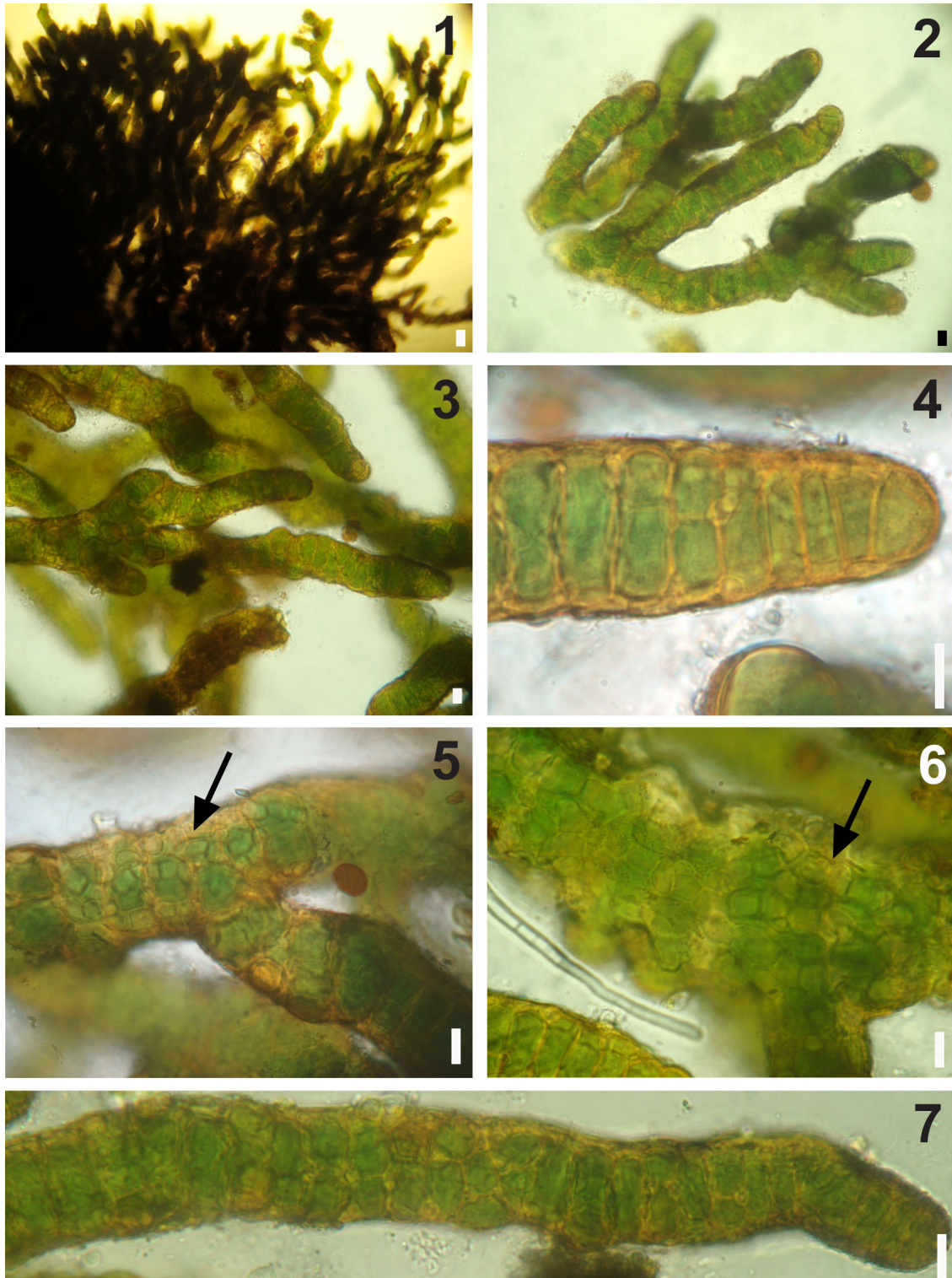
Figs 1–7. *Stigonema jureiensis*: (1–3) General view of the thallus; (4) Detail of rounded filament end; (5) Details of a branch tapered at the base and the mosaic–like sheaths; (6) Mosaic–like sheaths; (7) Filament with uni– and polyseriate parts. Scale bars 50 µm (1), 10 µm (2–7). Arrows indicate the details of mosaic–like sheaths.

species are morphologically distinct. *S. tomentosum* is uniseriate and has lamellate sheaths, while *S. jureiensis* is multiseriate and has a unique type of mosaic sculptured sheaths, making the new species morphologically distinct from any other species of the genus (Table 1). This hypothetical genetic relationship was very likely influenced by the relatively short length of *S. tomentosum* KM047014 sequence (1075 bp), and the low number of informative sites in the phylogenetic analysis (965 bp). Also, our phylogenies show that *S. jureiensis* is separated from other species within the *Stigonema* clade. Thus, we propose and describe our novel species following criteria
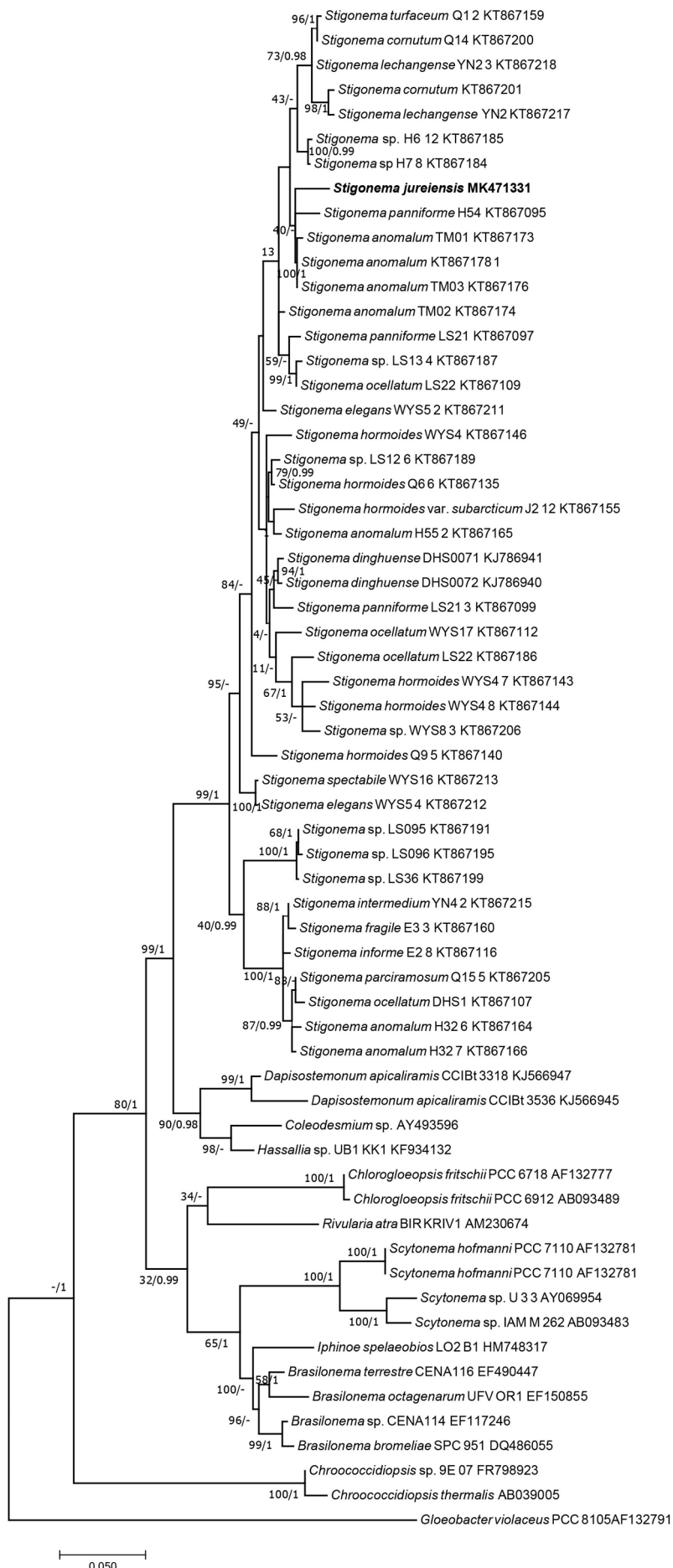
Fig. 8. Molecular phylogenetic analysis by Maximum Likelihood method. Bootstrap values and probabilities (ML/BA) are indicated at nodes. The analysis involved 62 nucleotide sequences and there were a total of 1325 informative sites in the final dataset. –=no support.
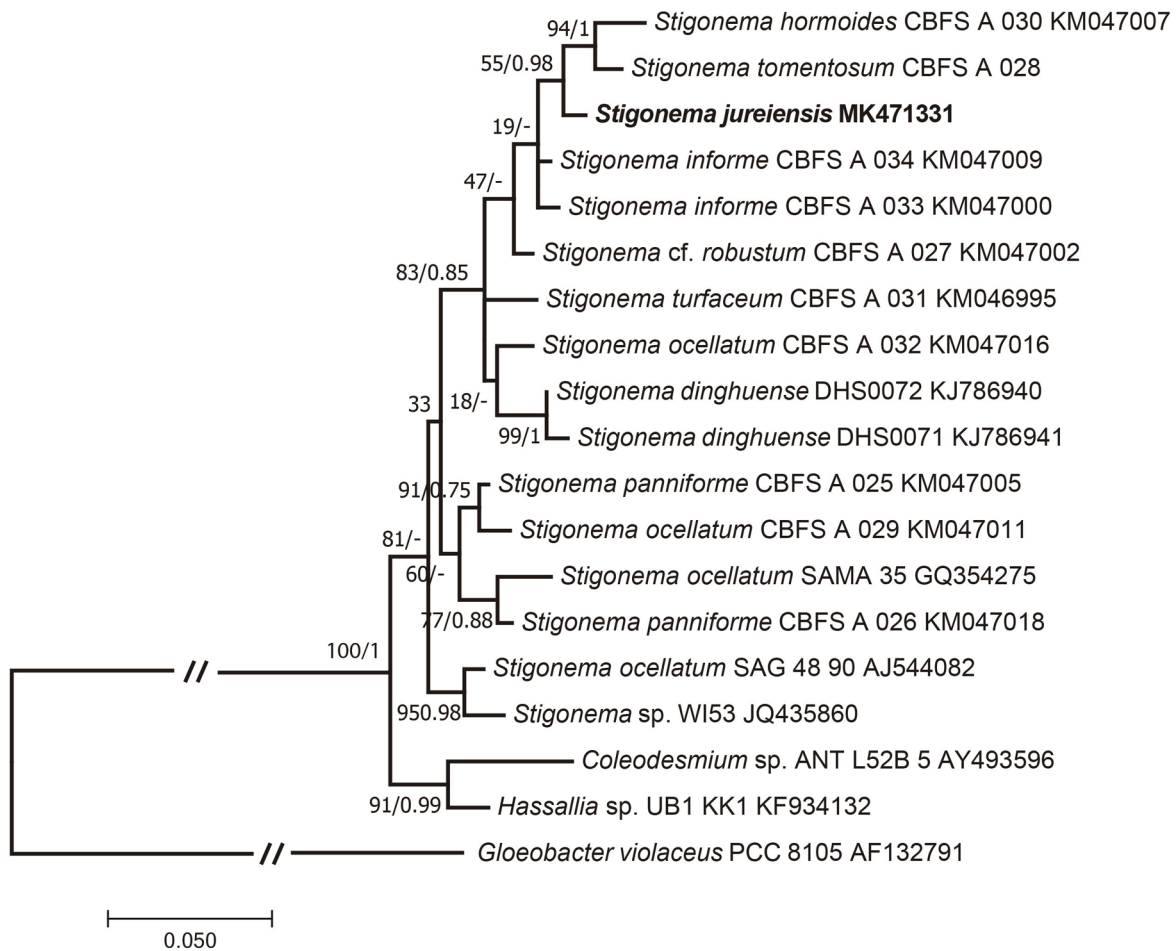
Fig. 9. Molecular Phylogenetic analysis by Maximum Likelihood method. Bootstrap values ML/BA are indicated at nodes. All populations in these trees are morphologically analyzed and published in previous papers. Bootstrap values and probabilities (ML/BA) are indicated at nodes. The analysis involved 19 nucleotide sequences and there were a total of 964 informative sites in the final dataset. –=no support.

outlined in Johansen & Casamatta (2005). Our results agree with the first criterion of new species description presented by the authors, which requires evidence of genetic separation to existing species. The mosaic–sculptured sheaths are a strong evidence of genetic separation. Our results also follow the authors' second criterion, with *S. jureiensis* separated from other phylogenetic clades. According to those, the morphological feature of *S. jureiensis* can be used to separate the species, and reinforces the phylogenetic data, strongly indicating that our population is a new species of *Stigonema*. The special kind of sheath in *S. jureienesis* has previously been described in *Brasilonema ornatum* Sant'Anna et al.*, Scytonema santannae* Hentschke et Komárek and *S. stuposum* Bornet et Flahault (Hentschke & Komárek 2014; Sant'Anna et al. 2011) from the Brazilian Atlantic Rainforest, but was never reported for stigonematacean taxa. Up to now, all the cyanobacterial populations with mosaic–sculptured sheaths were found growing with fungal hyphae (Hentschke & Komárek 2014), but we did not observe this for *S. jureiensis*. Probably, this character evolved independently in different lineages as an adaptation to the harsh conditions found in this

environment such as high temperature and herbivory. In addition to the description of *S. jureiensis*, we corroborate previous studies (Mareš et al. 2015; Song et al. 2015) showing that uni– and multiseriate species of *Stigonema* are intermixed in phylogenies. These characters have most likely arisen more than once in the genus *Stigonema*. Our phylogenies also highlight the problem of species delimitation in Cyanobacteria, since the same species is found in more than one clade (i.e. *Stigonema hormoides* and *S. ocellatum*). In this case we agree with mareš et al. (2015) that molecular and ecological divergent populations of these morphospecies will likely be further separated with more molecular data.

We also found that different morphospecies are grouped in monophyletic clades, as for example, the multiseriate *S. spectabile* WYS16 and the uniseriate *S. elegans* WYS54 (ML = 100%, BA = 1.0). Considering the species concept of Mishler & Theriot (2000), adapted by Johansen & Casamatta (2005), which states that species should be "monophyletic clusters of strains or natural populations that are diagnosable by some unique combination of traits, those traits being any combination of morphological, biochemical, molecular, or other

characteristics", we think that in our trees, the clustering of different morphospecies in monophyletic clades is primarily due to the poor resolution of the 16S rRNA gene at the species level and also by the scarce number of available 16S sequences of *Stigonema* populations. Other factors that negatively influence species delimitations in *Stigonema* are that most of the sequences of morphologically studied *Stigonema* populations are short, causing polytomies in phylogenies (Mareš et al. 2015; Song et al. 2015), and that there is a lack of 16S–23S rRNA ITS data. The longer and more useful *Stigonema* sequences in GenBank are from populations that have not yet been published, thus cannot be used in deeper discussions of species delimitations.

Conclusion

In this paper, we propose a new species *Stigonema jureiensis,* and conclude that the delimitations of species of *Stigonema* remain unclear. The current lack of ecological and morphological data from morphotypes which have sequences available in GenBank coupled with the short sequences from those morphologically well–studied, make the discussion at the species level difficult. Also, access to more molecular markers, such as the 16S–23S rRNA ITS is urgently needed to improve the resolution of monophyletic clades in the genus.

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