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ORIGINAL ARTICLE

A new species of *Scytonema* isolated from Bilaspur, Chhattisgarh, India using the polyphasic approach

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Abstract A false branching cyanobacterium (strain 1F-PS) isolated from a fresh water body of Bilaspur (Chhattisgarh, India) is described here as a new species of the polyphyletic genus *Scytonema*. Morphological, ecological, molecular and phylogenetic evidence validated the strain as a new species. Observations of the filaments in different phases of growth, different levels of microscopic studies, the presence of a textured thin sheath throughout the length of the trichome, differences in the shape and dimensions of the vegetative cells and heterocytes and ecological attributes show that the strain differed from the rest of the closely related species. Sequencing of the 16S rRNA gene resulted in 99% similarity with *Scytonema bilaspurensis* and 97.07% sequence similarity with *Scytonema hofmannii* PCC 7110, while *rbcl* and *psb*A sequences showed 99 and 97% similarities with *S. bilaspurensis*, respectively. Phylogenetic assessments indicated a large phylogenetic distance and separate clustering of the strain 1F-PS for all the molecular markers.

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Introduction

Cyanobacterial taxonomy is a challenging field of study that is in a phase of revolution, with increasing description of new taxa using a combination of morphological, ecological and molecular data. The inherent problems of culturing, variations among the natural and laboratory grown cultures and an extremely intricate morphology, are all challenges that have made the study of cyanobacteria interesting but very complex. Taxonomic studies in the last decades (Anagnostidis and Komárek 1985, 1988, 1990; Komárek and Anagnostidis 1986, 1989; Büdel and Kauff 2012; Komárek et al. 2014) have indeed helped in solving the puzzling relationships to some level. The recent Süßwasserflora series on the cyanoprokaryotes (cyanobacteria) has recommended accommodating both morphological and genotypic data in congruence, along with more revisionary work (Komárek 2013).

The genus *Scytonema* is typically characterized by filaments irregularly coiled, or in fascicles, creeping or erect, which may be free, in colonies, or have tendency to form clusters, but very rarely forming layers (biofilms or strata) on the substrate. Usually the filaments have an envelope with firm colorless or maybe colored sheath. Some other features of this genus include the appearance of filaments or thallus usually in the form of clusters, or prostrate with entangled filaments and cells mostly green, olive green, blue green or yellowish (Komárek 2013). The type species is *Scytonema hofmannii*, and till date about 320 species have been described in this genus. Komárek et al. (2014) have placed the genus *Scytonema* in the family

Scytonemataceae of the order Nostocales. As per the recent Süßwasserflora series, this group has been demarked as a species-rich group of false branching cyanobacteria. Previous studies (Fiore et al. 2007; Aguiar et al. 2008; Sant'anna et al. 2011; Vaccarino and Johansen 2011, 2012; Becerra-Absalón et al. 2013; Komárek 2013) have proved that the genera *Scytonema* and *Brasilonema* form a monophyletic group, based on an analysis of 16S rRNA data.

The present work, based on the freshwater strain 1F-PS, has been a challenge because of the huge diversity in the genus *Scytonema* and the close taxonomic affinity of this strain with a recently described strain, i.e., *S. bilaspurensis* (Singh et al. 2016). In spite of their closeness in habitat and ecology, comprehensive studies made it clear that the 1F-PS strain is different from *S. bilaspurensis*.

Materials and methods

Sampling

The sampling was performed in the Bilaspur district of Chhattisgarh State, India. Water samples were collected from a freshwater body and assessed for crucial physico-chemical characteristics such as temperature, pH, total dissolved oxygen, salinity, total dissolved solids and conductivity. Immediately after collection, samples were studied through microscopy in order to assess their overall level of purity and the presence/absence of other cyanobacteria. A thorough purification of the cultures was performed using 1.0% agar plates alternating with suspension sub-culturing, repeating this process four times in order to establish the proper axenicity of the culture. The strain was grown in 150-ml basal medium (BG 11_o medium) (Rippka et al. 1979). The pH of the medium was adjusted to 7.2, and the culture was maintained in culture room under an illumination of approximately 50–55 $\mu Em^{-2}s^{-1}$ with a photoperiod of 14/10 h light/dark cycle at 28 ± 2 °C.

Phenotypic analysis

Microscopy of the strain in different stages of growth was performed using a Nikon YS100 microscope (Nikon, Japan), and microphotographs of the cyanobacteria were taken using an Olympus BX53 microscope (Olympus Corporation, Japan) fitted with a ProgRes C5 camera (Jenoptik, USA). For better resolution and taxonomic clarity, the shape of apical cells, shape, size, orientation and other miscellaneous features of vegetative filaments, vegetative cells and heterocytes were visualized and observed. Fifty to hundred measurements were taken for each parameter. The pattern of false branching was also examined.

Molecular and phylogenetic analysis

A 10- to 15-day-old culture was used for DNA isolation and subsequent amplification using the Himedia Ultrasensitive Spin Purification Kit (MB505) with the initial lysis using the AL solution being done for 60 min, and the final lysis with the solution C1 being performed for 30 min. The amplification of the 16S rRNA and *psb*A genes was performed as in Singh et al. (2015b) while the *rbcl* gene was amplified following Singh et al. (2015a).

In the case of the 16S rRNA gene, a clean 1357 bp sequence was obtained and the similarity search was performed using the 'Identify' option of the EzTaxon database (http://www.eztaxon.org, Kim et al. 2012) with validated cyanobacterial strains. Similarity search was also conducted using the NCBI web services with the blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and retrieving only good quality and validated sequences. The *rbcl* and the *psb*A gene sequences were annotated for the coding regions using the NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) and the Expasy Proteomics Server (http://expasy.org). All the sequences were submitted to the NCBI database using the sequence submission tool BankIt. Multiple sequence alignment of the sequences was performed using CLUSTAL X version 1.81 (Thompson et al. 1997). This was followed by manual alignment using DAMBE (Xia 2013). All the phylogenetic analyses were done using the software MEGA version-5 (Tamura et al. 2011). For the reconstruction of the 16S rRNA phylogeny, the model of nucleotide substitution with the lowest Bayesian Information Criterion (BIC) score, the Tamura–Nei model (Tamura and Nei 1993) (T93; BIC score of 55109.942), was selected using the MEGA 5 program. In the case of the *rbcl* gene, the evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter (K2) model (Kimura 1980) with a BIC score of 23,246.646. The *psb*A gene was analyzed by using the Maximum Likelihood method based on the Kimura 2-parameter model (K2 + I) (Kimura 1980) with a BIC score of 5294.106. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 56.3397% sites). Bootstrap values were estimated based on 1000 replications (Felsenstein 1985). The robustness of our phylogenetic findings and confirmation of the tree topology

was achieved by using three different approaches for tree building: neighbor joining (NJ), maximum likelihood (ML) and maximum parsimony (MP) (Fitch 1971; Felsenstein 1981; Saitou and Nei 1987; Tamura et al. 2011).

Results

Morphological analysis

The various morphological features of the strain 1F-PS were studied carefully (Fig. 1), and comparative assessment was made with the closely related strain *Scytonema hofmannii* PCC 7110 and other species of the genus *Scytonema*. Morphological comparisons were also made with some of the other closely related genera like *Petalonema* and *Brasilonema*. Care was taken to make comparative assessments with *S. bilaspurensis*, which our group published recently, so that these closely related species from almost the same locality could be properly distinguished.

Molecular and phylogenetic analysis

The multiple alignments were used with proper care for phylogenetic interpretations (Online Resources 1–3). The analysis of the 16S rRNA gene revealed that the closest relatives of *S. singhii* were *S. bilaspurensis* with 99% similarity and *S. hofmannii* PCC 7110 with 97.07% sequence similarity. In case of the *rbcl* and the *psb*A genes similarity with *S. bilaspurensis* was 99 and 97%, respectively (Table 1).

In the 16S rRNA ML tree (Fig. 2), the strain 1F-PS was found to be most closely related to *S. bilaspurensis,* and sister to their clade was *S. hofmannii* PCC 7110. The bootstrap values were strong at all the nodes, and the tree topology for this marker was similar in all phylogenetic approaches. In the *rbcl* gene ML tree (Fig. 3), the position of *S. singhii, S. bilaspurensis* and *S. hofmannii* PCC 7110 was similar to the 16S rRNA ML tree. The MP and the NJ trees showed a phylogenetic similar to the ML tree. All the *psb*A gene analyses (ML, MP, and NJ) showed the strain 1F-PS (*S. singhii*), sister to *S. bilaspurensis* (Fig. 4).

Discussion

Cyanobacteria are enigmatic microbiological organisms. The severe confusion regarding their taxonomy and morphology has made their study both challenging and confusing. But, the current state of cyanobacterial taxonomy has certainly moved a bit ahead by incorporating genetic data along with studies of morphological architecture.

The genus *Scytonema* is a diverse genus comprising numerous species that exist endemically in tropical and ecologically distinct habitats, such as lateritic soils, dripping rocks and reservoirs with water vegetation. The genus was summarized by Geitler (1932), Desikachary (1959), Starmach (1966), Bourrelly (1970), and Komárek and Anagnostidis (1998); however, modern and recent revisions based on the polyphasic approach have indicated that this genus is much more heterogeneous and evidently polyphyletic in nature (Komárek 2013). Its heterogeneity and polyphyly has been demonstrated by molecular data (Boyer et al. 2002; Berrendero et al. 2008; Zapomělová et al. 2011), and thus, the genus has been divided into other generic units (Bohunická et al. 2012). *Scytonema* sensu stricto is based on the type species *S. hofmannii* C. Agardh ex Bornet et Flahault (1888), which is characterized by cylindrical trichomes along its whole length, more or less quadratic cells in the main trichomes, and also relatively narrow sheaths (Anagnostidis and Komárek 1988).

In this study, the strain 1F-PS showed considerable differences from the closely related strains *S. bilaspurensis* and *S. hofmannii* PCC 7110, in terms of morphology and DNA sequence data. The application of various methods like phenotypic characterization through comparative assessments and phylogenetic analyses of 16S rRNA, *rbcl* and *psb*A genetic regions proved that the strain 1F-PS was different from the other closely related species of *Scytonema*.

The phenotypic characterization was very conclusive in differentiating the strain 1F-PS from the closely related taxa. For the comprehensive morphological assessment, we compared the morphology of the strain 1F-PS with *S. bilaspurensis* (Singh et al. 2016), *S. hofmannii* Agardh ex Bornet et Flahault 1887, *S. stuposum* [Kützing] Bornet ex Bornet et Flahault 1887, *Brasilonema* Fiore et al. 2007, *Scytonema sect. Myochrotes* Bornet et Flahault 1887 and *Petalonema* Berkeley ex Correns 1889. The presence of a distinctly textured thin sheath, which was visible throughout the length of the trichome, was an important distinguishing feature which differentiated the strain 1F-PS from the closely related species *S. hofmannii* PCC 7110. It was also evident from the morphology and shape of the cells that the rectangular appearance of the cells with width usually greater than length distinguished the

1F-PS strain from *S. hofmannii* PCC 7110. The prominent constrictions between cells indicated that the strain 1F-PS differed significantly from *S. hofmannii* PCC 7110. The shape of the heterocytes was also different from what was documented by Komárek (2013) for *S. hofmannii*, and the presence of varying shapes of heterocytes within the 1F-PS strain was in fact an interesting feature of the strain 1F-PS. Convincing differences were also found in the size of the vegetative cells and the heterocytes which further validated our hypothesis that the strain 1F-PS was an unknown member of the genus *Scytonema*. Due to similarities in ecology and habitats, we documented the morphological differences of the 1F-PS strain with those of the most closely related strain *S. bilaspurensis* and our assessment indicates that the strains are different in terms of morphology. The strain 1F-PS was found to be much more diverse and different in the shape of the vegetative cells as compared to those of *S. bilaspurensis*. Also, the size of the vegetative cells and the heterocytes was also different. Thus, our comparative morphological analyses showed that there are differences between the strain 1F-PS, *S. bilaspurensis* and *S. hofmanni* PCC 7110. In addition, strain 1F-PS and *S. hofmanni* PCC 7110 were different in the type locality, and the presence of a non-woolly thallus in the strain 1F-PS also indicated ecological differences.

Morphological comparisons were also made with the closely related genera *Petalonema* and *Brasilonema*. It is important to note that *Petalonema* has not been included in a molecular phylogenetic study and that the genus is characterized morphologically by having very wide, lamellated sheaths, which are funnel-like divergent at the ends and whose sheaths are very thick and usually several times wider than the trichomes. *Brasilonema* presents sporadic false branching and cellular contents with dark green, grayish, reddish or even violet colors (though not always).

The close similarities of strain 1F-PS with *S. bilaspurensis* and *S. hofmannii* satisfied one of the primary criteria for species identity indicated by Strackerbrandt and Ebers (2006), who suggested a threshold limit for species identity of 98.7–99%. The polyphyly of *Scytonema* was noticed prominently in the 16S rRNA tree validating earlier reports of Komárek et al. (2013, 2014). According to our molecular results, the strain 1F-PS showed a close pairwise similarity of 99% with *S. bilaspurensis* and 94% with the strain *S. hofmannii* PCC 7110. The phylogenetic placement of the 1F-PS strain in the *rbcl* tree clearly indicated that it is different from the strains *S. bilaspurensis* and *S. hofmannii* PCC 7110. The *psb*A gene sequencing data indicated a 97% similarity with *S. bilaspurensis*, and the phylogenetic analysis showed that the 1F-PS strain and *S. bilaspurensis* differed in branch length.

Overall, the significant morphological, ecological and genetic differences support the recognition of the 1F-PS strain as a novel species of the genus *Scytonema*.

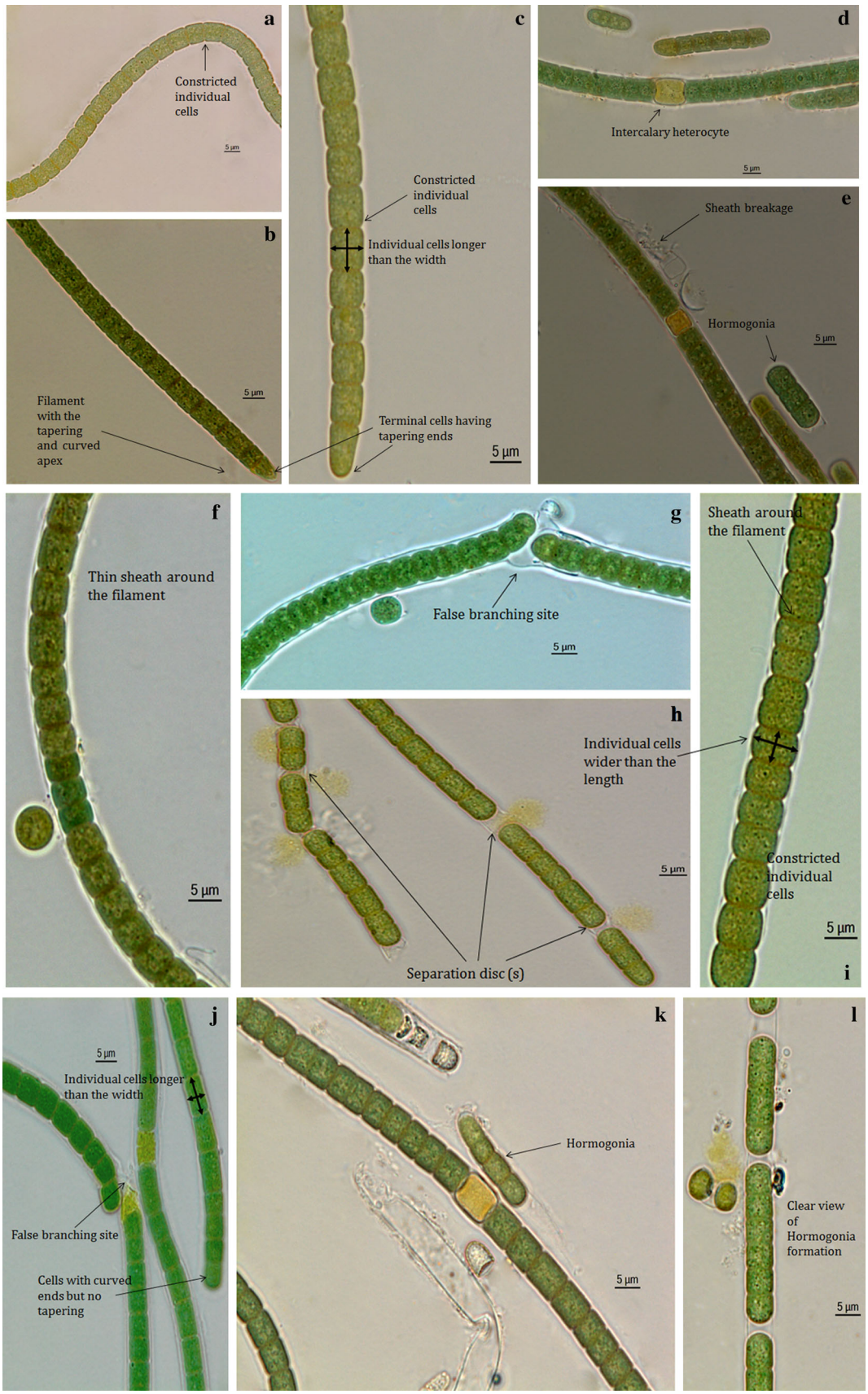
Fig. 1 **a** Trichome showing constricted individual cells. **b** Filament with the tapering and curved apex. **c** Constricted individual cells with tapering terminal cells along with individual cells being longer than wide. **d** Filament with intercalary heterocyte. **e** Sheath breakage sites and hormogonia. **f** Thin filament around the sheath. **g** Development of false branching site. **h** Separation discs and individual cells being wider than long. **i** Filament having sheath around it and constricted individual cells along with individual cells being wider than long. **j** False branching sites; terminal cells with curved ends but no tapering and individual cells longer than wide. **k**, **l** Hormogonia and their formation. **m** False branching sites, separation discs and individual cells longer than wide. **n** Constricted individual cells and terminal cells having tapering ends. **o–v**, **x**, **y** False branching sites. **w** Intercalary heterocyte with two polar nodules

Taxonomic treatment

Scytonema singhii Singh, **sp. nov.**—HOLOTYPE: India, Chhattisgarh, Bilaspur, 22.09°N, 82.15°E, Jan 2015, *S.S. Singh* and *R.A. Minz* (actively growing culture was deposited (as *Scytonema* sp.) at the Microbial Culture Collection (MCC), National Centre for Cell Science (NCCS), Pune, India; cryopreserved culture is maintained at the MCC (Accession Number MCC 2874)) (Fig. 1).

Etymology: *Singhii* -in honor of Late. Prof. R.N. Singh, one of the India's finest workers who studied cyanobacteria and was widely respected as one of the stalwarts of the Benares school of algology.

Description: Blue green filaments; sometimes appearing slightly blackish green; false branching prominent; false branching appears solitary; sheath thin, colorless and narrow but perfectly distinct in both texture and color; sometimes may also take a darker shade or brownish color in older filaments; sheath present throughout the length of trichome with constrictions at the cross walls; vegetative cells ranged from 3.56 to 6.82 µm in length and 5.57–6.93 µm in width; vegetative cells are differently shaped with some being longer than wide, some cells with


a
Constricted individual cells
5 µm
b
Filament with the tapering and curved apex
5 µm
c
Constricted individual cells
Individual cells longer than the width
Terminal cells having tapering ends
5 µm
d
Intercalary heterocyte
5 µm
e
Sheath breakage
Hormogonia
5 µm
f
Thin sheath around the filament
5 µm
g
False branching site
5 µm
h
Individual cells wider than the length
5 µm
Separation disc (s)
Sheath around the filament
Constricted individual cells
5 µm
i
j
5 µm
Individual cells longer than the width
False branching site
Cells with curved ends but no tapering
k
Hormogonia
5 µm
l
Clear view of Hormogonia formation
5 µm

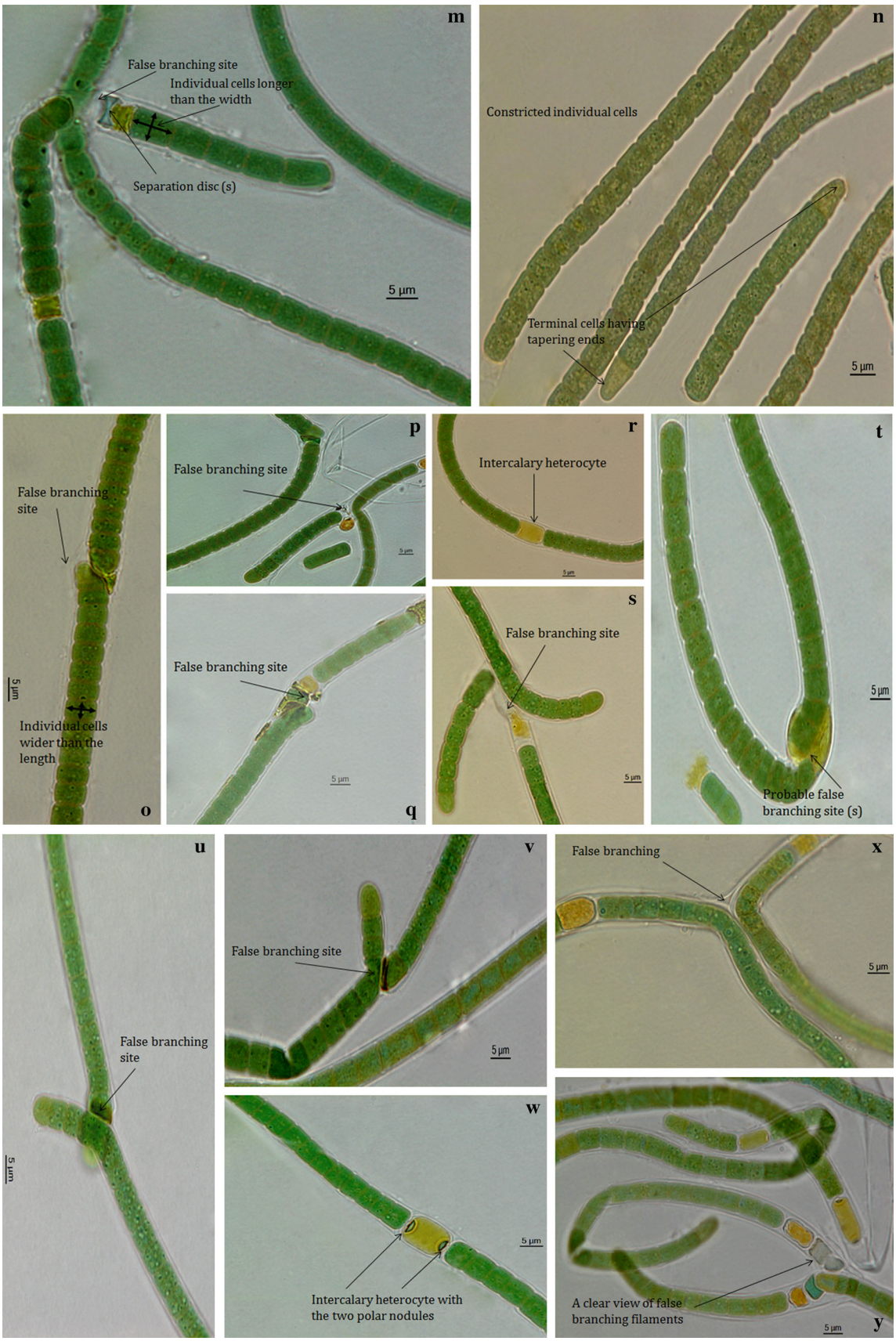


Fig. 1 continued

tapering terminal cells, some being wider than their length and some cells having curved ends but no prominent tapering; curved ends of the terminal cells of a trichome both in the main filaments and the false branches; heterocytes usually solitary; shape varying from square to cylindrical; heterocytes never have rounded ends; ranging

Table 1 Similarity of strain 1F-PS with its closest relative *Scytonema bilaspurensis* based on three molecular markers

Serial. no.	Molecular marker	Closest relative	Percentage similarity (%)
1	16S rRNA	*Scytonema bilaspurensis*	99
2	*rbcl*	*Scytonema bilaspurensis*	99
3	*psb*A	*Scytonema bilaspurensis*	87

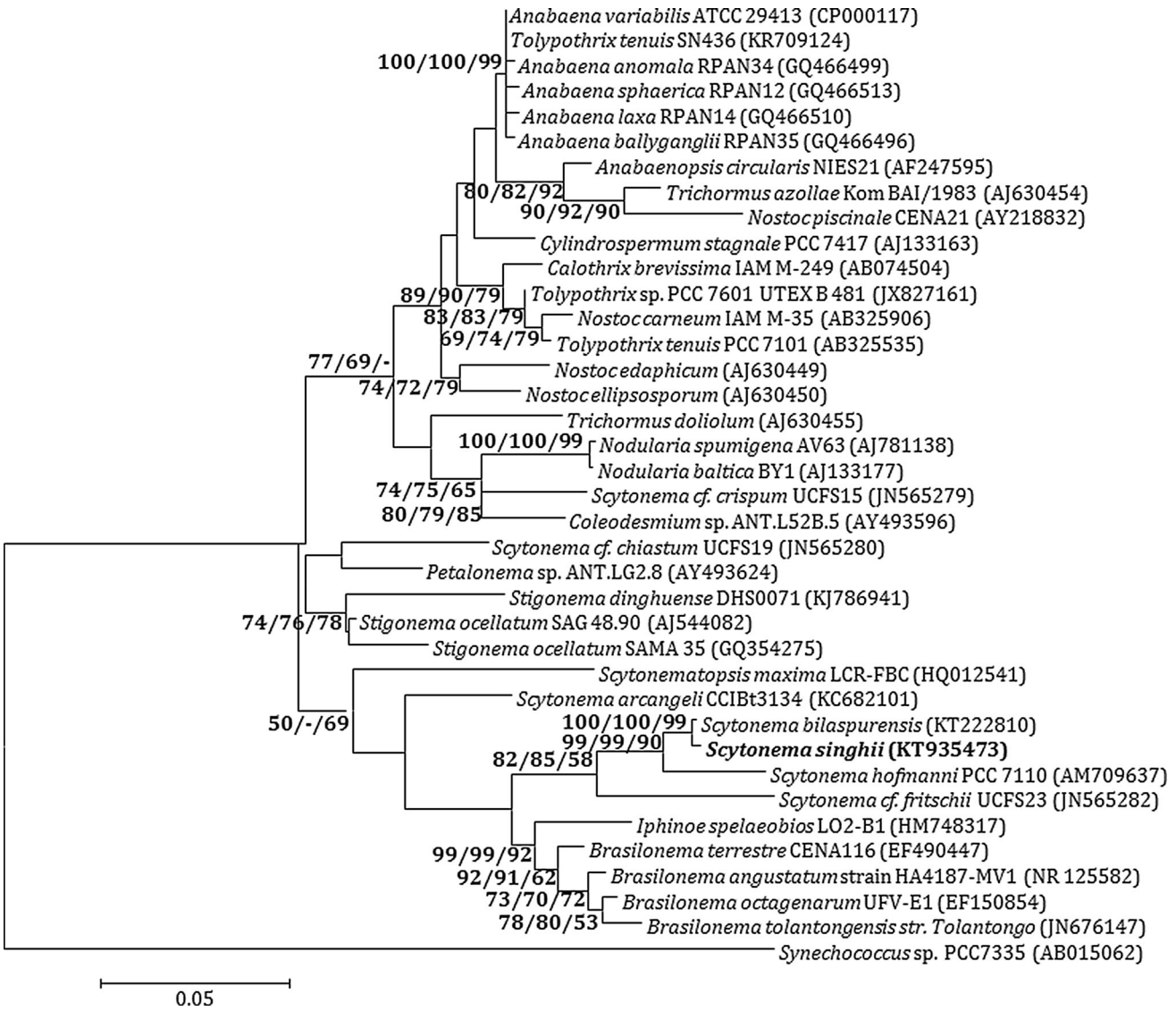


Fig. 2 Maximum likelihood (ML) tree of the strain 1F-PS and related taxa based on the 16S rRNA gene. ML, neighbor joining (NJ) and the maximum parsimony (MP) bootstrap values are indicated at some nodes

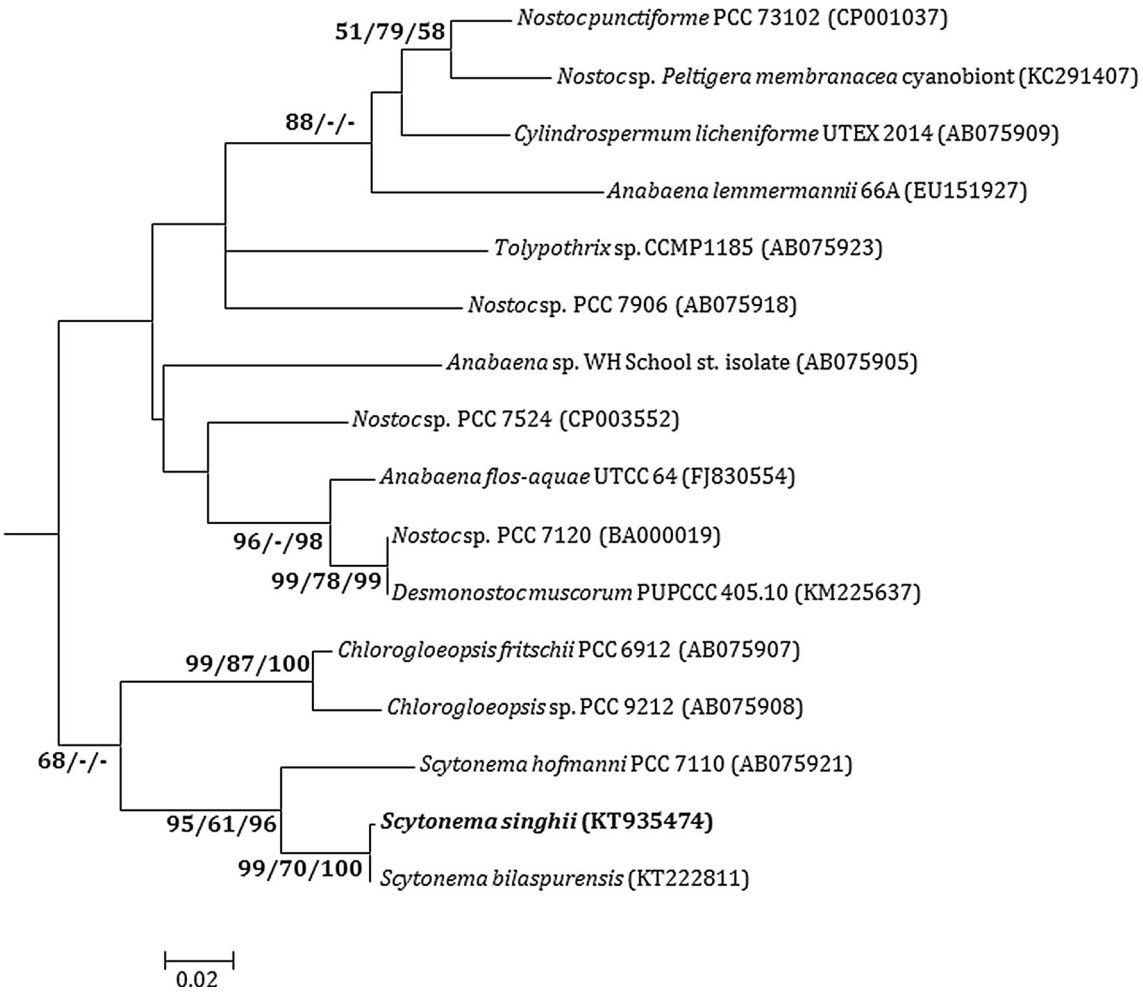


Fig. 3 Maximum likelihood (ML) tree of the strain 1F-PS and related taxa based on the *rbcl* gene. ML, neighbor joining (NJ) and the maximum parsimony (MP) bootstrap values are indicated at some nodes

from 4.77 to 13.05 µm in length and 5.19–6.86 µm in width.

Diagnosis: Differing from other closely related *Scytonema* species in having a thin and narrow but distinctly textured sheath visible throughout the length of the trichome (Fig. 1b–f, h–j, l), individual cells prominently constricted at the cross walls (Fig. 1j). The new species differs significantly from *S. bilaspurensis* in having cells which may be differently shaped with some being longer than wide (Fig. 1c, j, m), some cells with tapering apical cells (Fig. 1b, c, n), some being wider than long (Fig. 1i, o), and some cells having curved ends but no prominent tapering (Fig. 1j), solitary heterocytes with varying shapes from square to cylindrical. *S. singhii* also differs from *S. bilaspurensis* in the size of the vegetative cells and the heterocytes. Genetically, it has close affinity, based on 16S rRNA, *rbcl* and *psb*A genes, with *S. bilaspurensis* and *S. hofmannii* PCC 7110, but the phylogenetic analyses indicate that *S. singhii* is different from those two species.

Habitat: Fresh water body; pH 8.2; temperature 32 °C; salinity 4 g/L; total dissolved solids 100 mg/L; dissolved oxygen 70 ppm; conductivity 84.81 µS/cm.

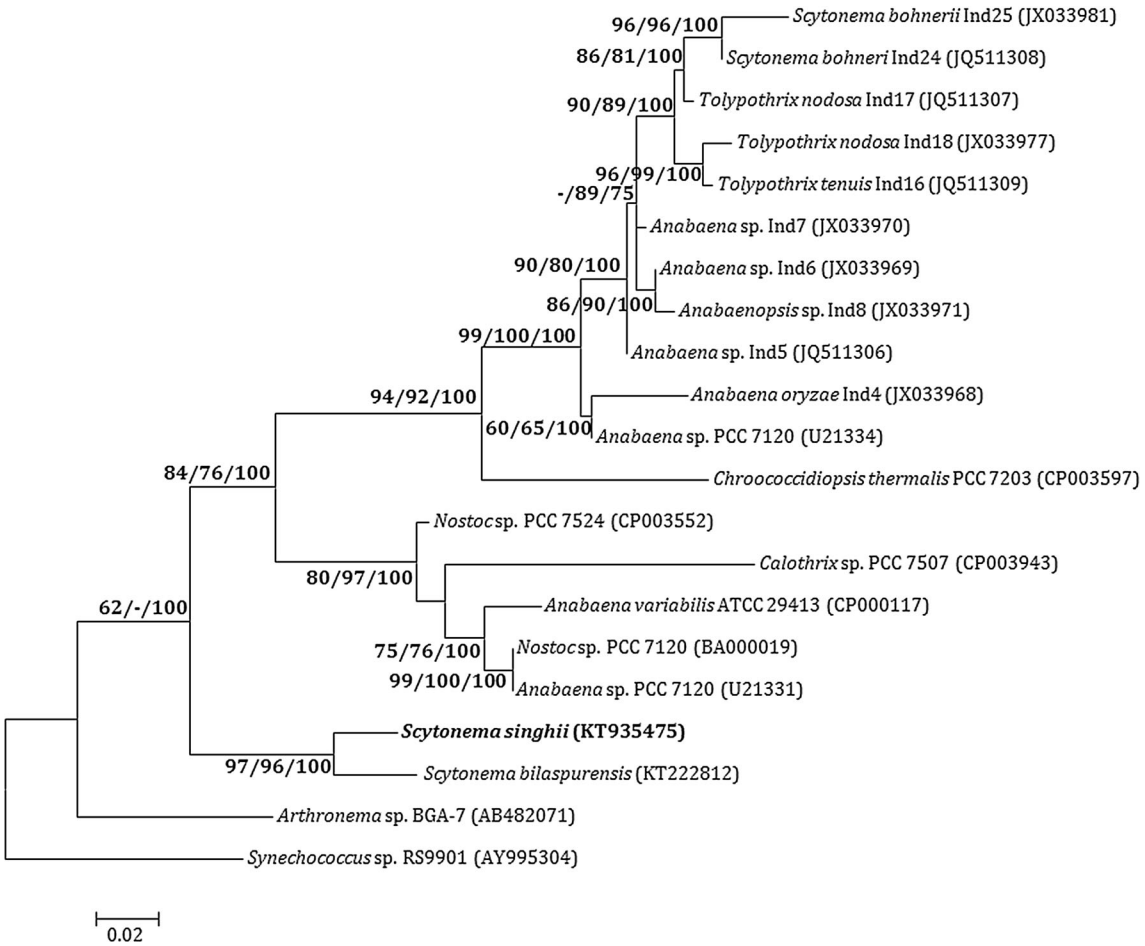


Fig. 4 Maximum likelihood (ML) tree of the strain 1F-PS and related taxa based on the *psb*A gene. ML, neighbor joining (NJ) and the maximum parsimony (MP) bootstrap values are indicated at some nodes

Distribution area: The 1F-PS strain is reported here for the first time in India, but is expected to occur in tropical and subtropical areas worldwide.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Information on Electronic Supplementary Material

Online Resource 1. 16S rRNA alignment file with the strain 1F-PS and all the considered taxa.

Online Resource 2. *rbcl* alignment file with the strain 1F-PS and all the considered taxa.

Online Resource 3. *psb*A alignment file with the strain 1F-PS and all the considered taxa.

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