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Pushkarnema curajae gen. et sp. nov. (Nodosilineales), a novel filamentous cyanobacterium isolated from a freshwater reservoir of northwest India

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Abstract

During the exploration of cyanobacterial diversity of the semi–arid region of northwest India, a non–heterocytous cyanobacterium was isolated from an aquatic habitat and characterized using a polyphasic approach based on morphology, ecology and sequencing of the 16S rRNA gene. Two strains (AT2016/6; SN2021/02) were isolated and characterized by cells longer than wide, the presence of a thick sheath, and the formation of nodules. 16S rRNA gene sequences revealed that the strains formed a highly supported, distinct clade within the Nodosilineales, ranging from 89.52% to 92.76% similarity to other strains. Secondary structures (the D1–D1′, BoxB, and V3 helices) of the 16S–23 rRNA Internal Transcribed Spacer (ITS) regions of both novel strains exhibited unique sequential differences from phylogenetically related taxa. Based on the combination of morphological characters and well–supported phylogenetic data, we describe a new genus and species: *Pushkarnema curajae gen. et sp. nov.* in accordance with the International Code of Nomenclature for Algae, Fungi, and Plants (ICN).

Keywords

Cyanobacteria; Phylogeny; Polyphasic approach; *Pushkarnema curajae*; 16S–23S rRNA ITS; 16S rRNA gene

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Introduction

Arid regions are typically characterized by low precipitation and high temperatures and serve as home to myriad microorganisms, including bacteria, cyanobacteria, and fungi associated with the upper layer of soil (Alsharif *et al*., 2020; Belnap *et al*., 2003; Su *et al*., 2004). Rising temperatures and shifting precipitation patterns are exerting pressure on these regions' already scarce water resources (García–Ruiz *et al*., 2011; Mall *et al*., 2017). These shifting climatic patterns also influence the diversity and ecology of cyanobacteria, which are often prominent (Huertas *et al*., 2011; Stanojković *et al*., 2022).

The order Nodosilineales (cyanobacteria) consists of thin, filamentous taxa often morphologically, indistinguishable from members of the Leptolyngbyales and Oculatellales (Curren *et al*., 2024; Hentschke *et al*., 2024; Strunecký *et al*., 2023). Consisting of three families, the Nodosilineaceae, Cymatolegaceae, and Persinemataceae, this order contains more than 40 genera (Strunecký *et al*., 2023). Subsequently, numerous *Leptolyngybya*–like lineages has been described as novel genera in this family (e.g., Heidari *et al*., 2018; Mai *et al*., 2018; Strunecký *et al*., 2023). Most taxa are described as simple trichomes, with cells ca. 1–3.5 µm wide and parietal thylakoids. Strains have been isolated from marine water, saline–alkaline lakes, freshwater lakes, and arid soils, indicating great ecological range (Dadheech *et al*., 2012; Hentschke *et al*., 2024; Perkerson III *et al*., 2011; Zhou *et al*., 2018). While cyanobacteria have been extensively studied in tropical and subtropical areas of India, their diversity in arid and semi–arid locations has received very little attention. *Desertifilum tharense* was the first cyanobacterium

described from the biological desert crust of the Thar desert using a polyphasic approach (Dadheech *et al*., 2012). Subsequent investigations have described numerous new taxa (Chakraborty *et al*., 2018, 2021; Kumar *et al*., 2022; Pal *et al*., 2024), indicating great potential novel biodiversity.

The present research investigates unexplored cyanobacterial communities that inhabit semi–arid regions of Rajasthan, India. Two thin, filamentous, non–heterocytous cyanobacterial strains were isolated from a freshwater lake located in a semi–arid region of Rajasthan. Based on a polyphasic approach, a new cyanobacterial taxon is proposed under the International Code of Nomenclature for Algae, Fungi, and Plants (ICN).

Material and Methods

Sampling site: Planktonic samples were collected in August 2016 and September 2021 from Pushkar Sarovar (26°29'14"N, 74°33'15"E), a non–natural "Sacred Lake" situated in one of the oldest cities in India, Pushkar, in the district of Ajmer (Rajasthan) (Fig. 1a–c). The lake has a surface area of 22 ha and a maximum depth of 10 m. The region experiences semi–arid climatic conditions with dry, hot summers and cool winters. The physicochemical characteristics such as pH, TDS, salinity, and conductivity were recorded (Table S1) at the sampling site by multi–parameter PCSTestrTM35 (Eutech instrument, Oakton, Singapore). The collected samples were immediately transported to the laboratory for further investigations.

Isolation and Morphological characterization: To isolate strains, water was centrifuged and resulting pellets thoroughly washed before re–suspension into 30 mL culture tubes containing BG–11 medium (Rippka *et al*., 1979). Culture tubes were kept under white fluorescent lights following a 14:10 h light–dark cycle at 28±2 °C. Clonal cultures were established by picking single filaments using a micropipette and transferring them into fresh culture tubes. The two isolated strains were originally designated as AT2016/6 and SN2021/02. Morphological characters (e.g., cell size and shape, trichome morphology, sheath, motility, calyptra, etc.) were recorded using a Axio Lab Al, Carl Zeiss (Goettingen, Germany) microscope. Furthermore, the Differential Interference Contrast (DIC) microphotographs were taken with Leica Confocal Inverted Microscope (DMi8, Germany) available in Central Instrumentation Facility of Central University of Rajasthan, India. The average values of the cell length and width were calculated from more than 100 individuals at ×1000 magnification. Electron microscopic images were taken using Field Emission Scanning Electron Microscope (FE–SEM), Apreo 2S LoVac model, Czech Republic and the samples were prepared following the protocol described by Chakraborty *et al*. (2019). Initial identification of isolates was performed according to standard references (Komárek & Anagnostidis, 2007).

Molecular characterization: Genomic DNA was extracted from the freshly grown culture with the NucleoSpin Plant II DNA isolation kit (Germany) following the manufacturer's protocol. Further, the 16S rRNA gene of both isolates were amplified using cyanobacteria specific forward primer CYA106F (Nubel *et al*., 1997) and reverse primer 1492R (Lane, 1991). The amplification of 16S–23S ITS region was carried out using forward primer 322F and reverse primer 340R (Iteman *et al*., 2000). The PCR reaction was set in 25 µL aliquots containing 2.5 µL of 10X PCR buffer (Qiagen, Hilden, Germany), 0.625 µL of 20 mM dinucleotide triphosphates (Qiagen) in an equimolar ratio, 0.5 µL of 10 mM primers, 0.15 µL Taq DNA polymerase enzyme (5 U/ml from Qiagen) and 2 µL of template DNA. A total of 35 cycles were performed in C1000 Touch™ Thermal Cycler, Bio–Rad. The cycling conditions for the 16S rRNA gene and ITS region were as follows: Hot start at 96 °C for 30 s, denaturation at 94 °C for 45 s, annealing at 57 °C for 45 s, extension at 72 °C for 2 min 15 s followed by a final extension at 72 °C for 5 min. Amplified fragments were visualized on 0.8% agarose gel and stained with SYBR–Safe dye. Single–band was obtained from amplification of 16S rRNA gene of both strains and amplified products were purified with HiPurA® PCR Product purification kit using the manufacturer's protocol and sent to Eurofins Genomics, India, for commercial sequencing. However, two bands were detected in amplified products of 16S–23S ITS gene of the both strains, which were manually cut from agarose gel and further processed with QIAquick gel extraction kit, Qiagen (Germany). Subsequently, both 16S–23S ITS products were re–amplified, and purified using aforementioned procedure and sent to Eurofins Genomics, India, for commercial sequencing. The sequences of 16S rRNA gene and 16S–23S ITS gene were deposited in GenBank (National Centre for Biotechnology Information, NCBI) under the accession numbers PQ484143 (AT2016/6), PQ847012 (SN2021/02) and PQ484144, PQ484148 (AT2016/6); PQ859331, PQ859332 (SN2021/02), respectively.

Phylogenetic analysis: A BLASTn search of the 16S rRNA gene sequences of the studied strains were carried out to look for the close relatives and to check the percentage similarity (>900 bp) with sequences available in GenBank. The sequences of type species from filamentous genera representing all nine orders (Strunecký *et al*., 2023) were retrieved from NCBI database and added for phylogenetic analysis. In addition, few unclassified and uncultured strain sequences similar to studied strains were also included. The sequences were aligned by the MUSCLE algorithm in MEGA (Molecular Evolutionary Genetics Analysis) v11 software. Afterwards, ambiguous portion of the sequences was trimmed manually, and finally 1281 nucleotide long fragment was considered suitable for further analysis. The phylogenetic tree for the 16S rRNA gene was constructed by Bayesian inference (BI), Maximum likelihood (ML), and Neighbour–joining (NJ) methods. The BI analysis was performed in Mr. Bayes v.3.2.7 (Ronquist *et al*., 2012) and for ML and NJ, MEGA v11 software was used. The nucleotide substitution model was predicted using jModel test (Darriba *et al*., 2012) and T3+G+I was found best fit based on the Akaike Information Criterion (AIC). The Bayesian tree constructed in two independent runs, with four chains each up to 5×10^6 generations, burn–in fraction set to 0.25 and sample frequency at 1000. The analysis was continued until the standard deviation of split frequencies <0.01 was achieved. The robustness of ML & NJ phylogenetic tree was estimated by 1000 bootstrap replications. Subsequently, these trees were visualized in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). A similarity (*p*–distance) matrix of 16S rRNA gene sequences of studied strains with related taxa was generated in MEGA11.

The sequences of the 16S–23S rRNA ITS region of phylogenetically close strains were retrieved from GenBank and aligned to look for sequential differences in the conserved and variable regions. Secondary structures of D1–D1′, BoxB, and V3 regions of the reference strain were predicted with RNA structure v6.4 (http://rna.urmc.rochester.edu/RNAstructure.html) and redrawn in Inkscape v1.3.2 software.

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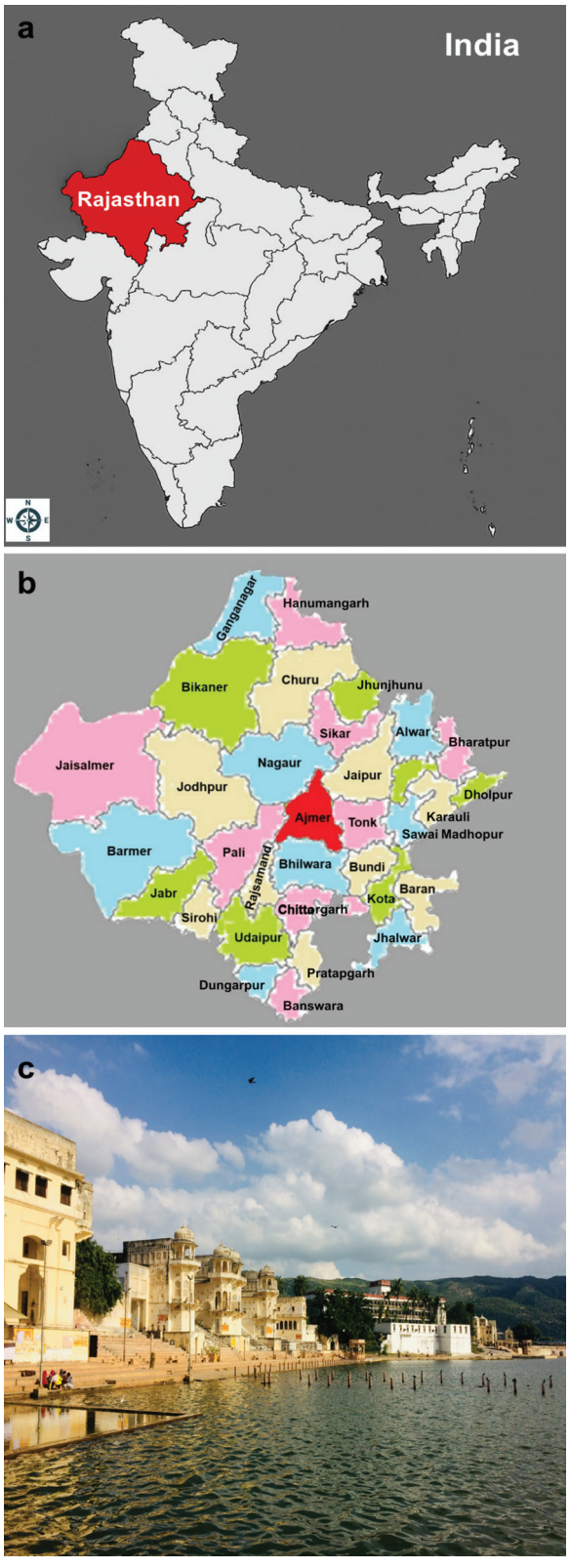

Fig. 1. Map showing sample collection site located in Pushkar, Rajasthan, India and the Pushkar Lake. **(a)** Map showing state Rajasthan situated in northwest county of India. **(b)** Map showing location of Ajmer district in the state Rajasthan (red color). **(c)** A view of sampling habitat, Pushkar Lake at Pushkar, Ajmer.

Results

Pushkarnema* S. Sonam, A.K. Tomer, D.A. Casamatta *et* P.K. Dadheech *gen. nov.

Description: Filaments simple, long, straight, or slightly wavy, unbranched, immotile, sometimes intertwined together like rope. Sheath thick, colorless, rarely 2 trichomes encapsulated in a single sheath. Trichomes isopolar, thin, up to 1.5 µm wide, neither attenuated nor capitate, with or without constriction at cross walls. Cells longer than wide (rarely isodiametric), distinctly visible centroplasma, Reproduction by trichome disintegration without necridic cells.

Type species: *Pushkarnema curajae* Sonam, Tomer, Casamatta *et* Dadheech.

Etymology: *Pushkarnema* a N.L. n. Pushkar, a city in Rajasthan, India; Gr. Nema=thread, n.; *Pushkarnema* a filament from Pushkar Lake. The name of the genus *Pushkarnema* was chosen because it was collected from Pushkar Lake, Rajasthan, India.

Diagnosis: Much longer cells and trichomes with thick sheaths differentiates *Pushkarnema* from similar taxa (e.g., *Nodosilinea* and *Haloleptolyngbya*). In addition, 16S sequence data and notable differences in secondary structure of 16S–23S rRNA ITS motifs separate this taxon.

***Pushkarnema curajae* S. Sonam, A.K. Tomer, D.A. Casamatta *et* P.K. Dadheech *sp. nov.* (Figs 2, 3)**

Description: Thallus blue–green turning yellowish to pale green when old, visible with naked eye. Filaments straight or slightly wavy, long, entwined together forming a mat, unbranched, forming nodules in mature cultures. Sheath thick, diffluent, open, colorless in mature filaments but no or very thin sheaths observed in young filaments. Trichomes isoploar, untampered, and constricted at cross wall. Cells 1.0–1.2 (1.4) µm wide, mostly longer than wide (up to 4×) or rarely isodiametric, cytoplasm dense at the periphery or completely visible centroplasma, refractive granules present either at the cross wall or in the center. Apical cells rounded, no calyptra. Reproduction by immotile hormogonia without necridic cells. It was observed that both studied strains (AT2016/6 and SN2012/02) were morphologically identical.

Holotype: Dry material of the reference strain has been deposited in University of Rajasthan Herbarium (RUBL), Jaipur, Rajasthan, INDIA with accession number RUBL 21798.

Isotype: GCC 202501 (metabolically inactive, cryopreserved material of the reference strain), deposited in Global Collection of Cyanobacteria located at Banaras Hindu University, Varanasi, India.

Type locality: Pushkar Sarovar, Ajmer district, Rajasthan, India (26°29'14"N, 74°33'15"E).

Habitat: Planktonic, Freshwater Lake.

Etymology: The epithet *curajae* refers to the Central

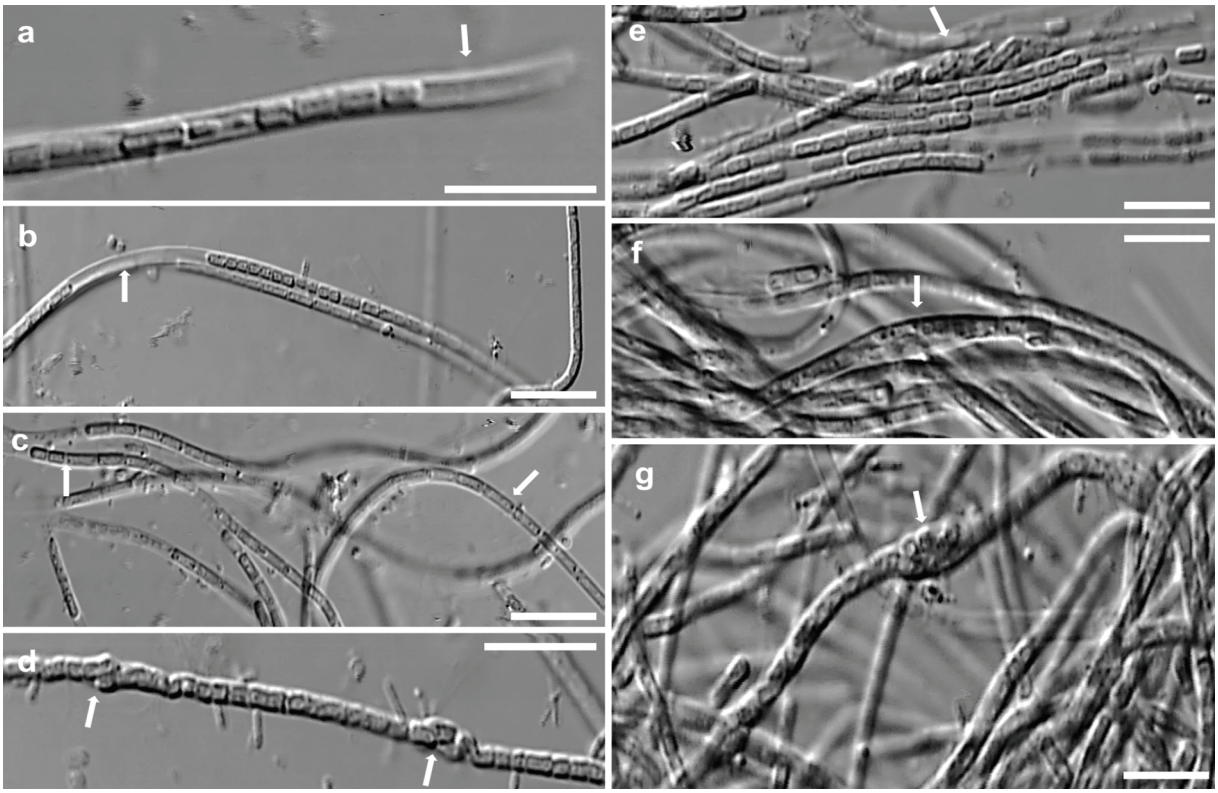

Fig. 2. Differential interference contrast (DIC) microscopic images of *Pushkarnema curajae* gen. nov. **(a, b)** Straight filaments with sheaths. **(c)** Slightly wavy filament (arrows showing granules at cross wall). **(d)** Initiation of nodule formation. **(e, f, g)** Different forms of nodules. Scale bars 10 µm.

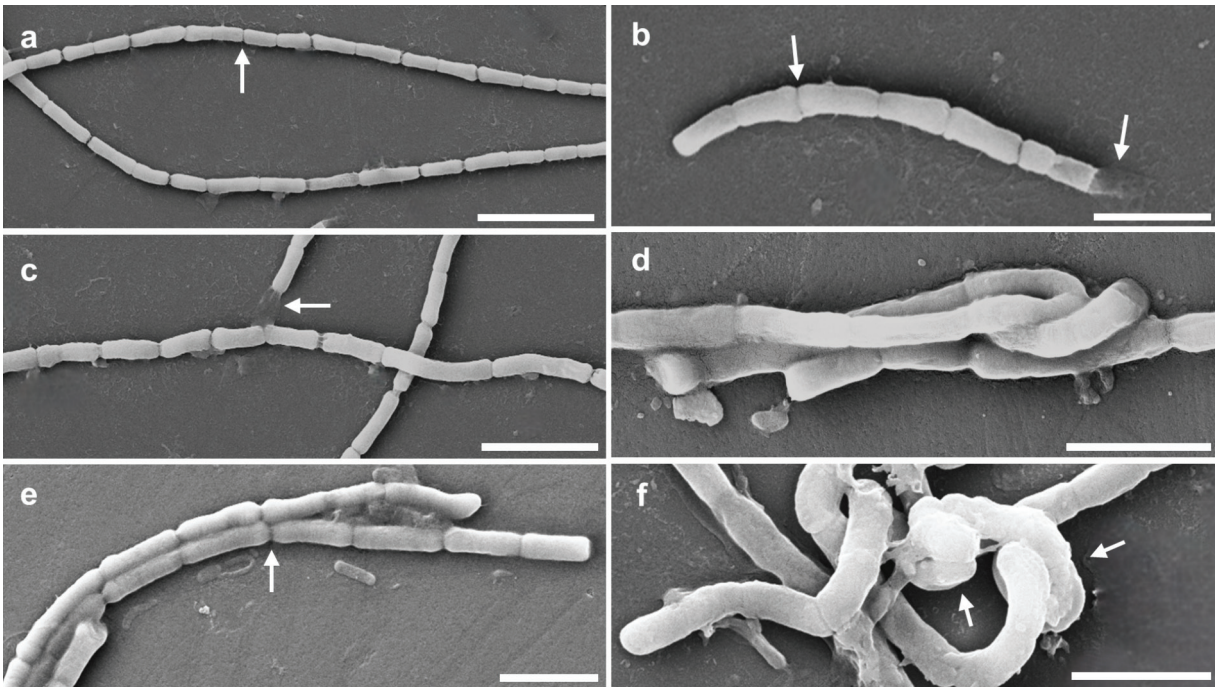

Fig. 3. Field Emission Scanning Electron Microscopic (FE–SEM) images. **(a)** Straight filament (arrow showing sheath and constriction). **(b)** Hormogonia with sheaths and constriction. **(c)** slightly wavy filament with arrowhead indication sheath. **(d)** intertwined filaments. **(e)** filament showing apical cell morphology and constriction. **(f)** filament forming nodule. Scale bars 10 µm.

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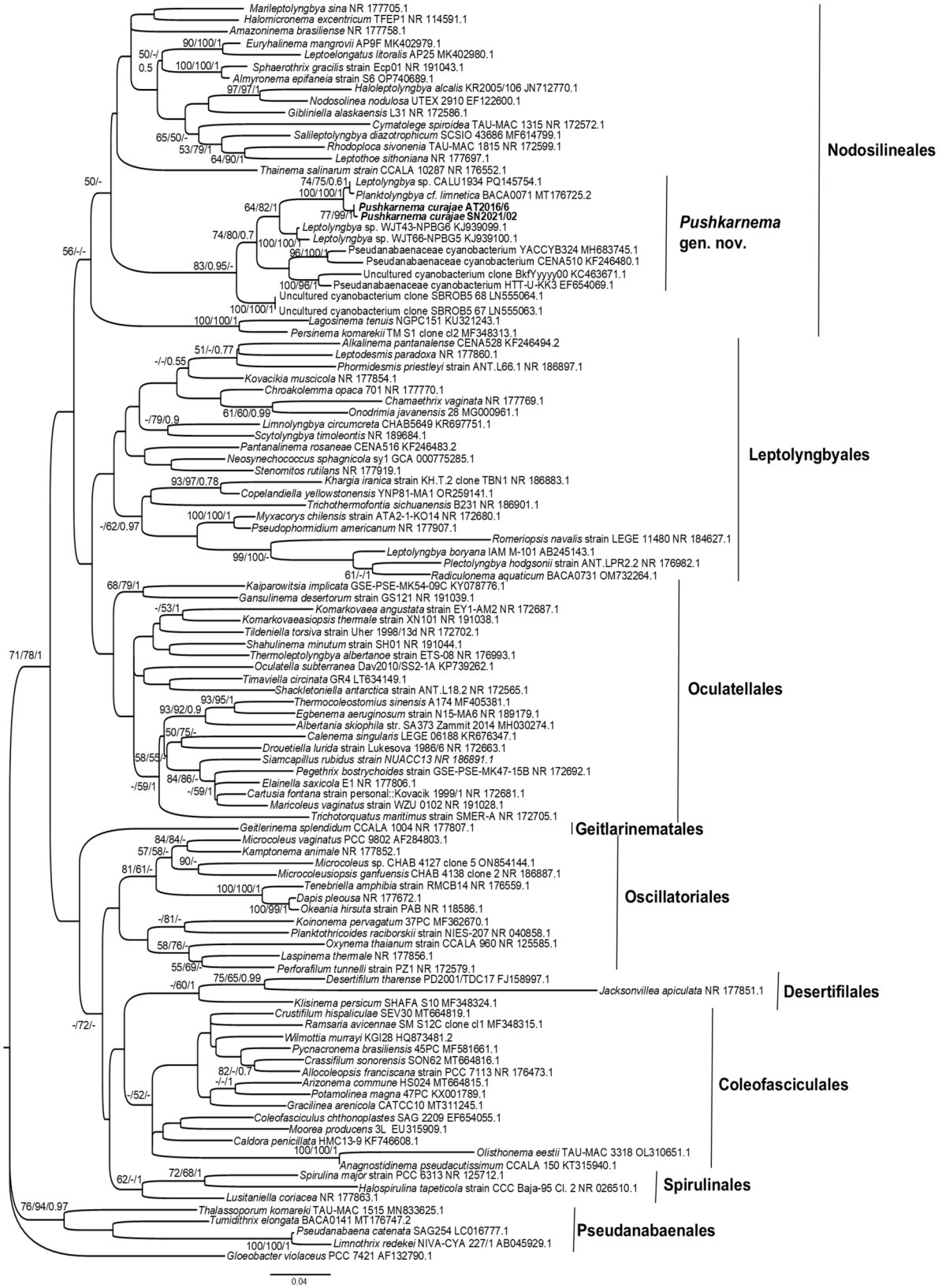

Fig. 4. Maximum Likelihood (ML) phylogenetic tree based on 16S rRNA gene sequences. Numbers at the node represent the bootstrap values/ posterior probability scores which are given in the following order: Maximum likelihood/Neighbour–joining/Bayesian inference.

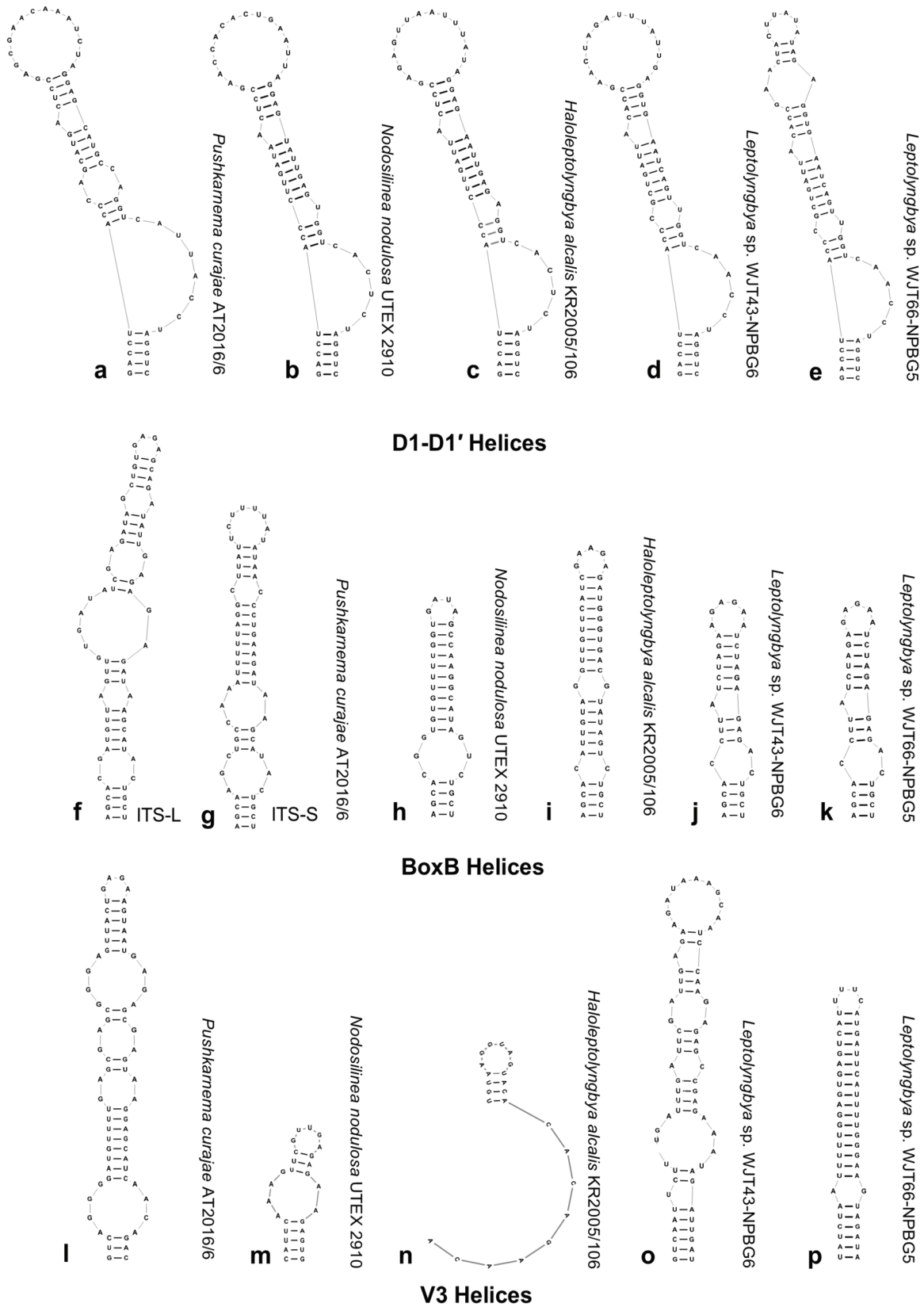

Fig. 5. Secondary structures of conserved helices in the 16S–23S internal transcribed spacer (ITS) of *P. curajae* and other phylogenetically close genera. **(a–e)** Secondary structures of D1–D1′ helices. **(f–k)** Secondary structures of BoxB helices. **(l–p)** Secondary structures of V3 helices.

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University of Rajasthan, where research work was carried out.

Reference strain: AT2016/6.

Diagnosis: Based on the light microscopy, this species resembles other simple, thin filamentous cyanobacteria. Distinct 16S rRNA gene sequence from sister taxa (e.g., *Nodosilinea* and *Haloleptolyngbya*).

Molecular and Phylogenetic analyses

Partial 16S rRNA gene sequences were compared to GenBank sequences using BLASTn search. The closest match was "*Leptolyngbya*" sp. CALU1934 (PQ145754), isolated from the Neva River, St. Petersburg (Russia). *Pushkarnema* formed a separate, highly supported (100% bootstrap value) lineage within a distinct sub–cluster (64/82% bootstrap), which included strains *Planktolyngbya* cf. *limnetica* BACA0071, *Leptolyngbya* sp. WJT43–NPBG6, *Leptolyngbya* sp. WJT66–NPBG5 (Fig. 4). This well–defined cluster fell within the order Nodosilineales, containing *Haloleptolyngbya, Salileptolyngbya, Leptoelongatus,* and *Euryhalinema*.

The partial *p*–distance similarity matrix (1009 nt) revealed that *P. curajae* shared high sequence identity of 99.60% and 99.50% with the ambiguously described strain of "*Leptolyngbya*" sp. CALU1943 and *Planktolyngbya* cf. *limnetica* BACA0071 (MT176725), respectively (Table 1). Comparison of *p*–distance similarities between *P. curajae* and other type species within the Nodosilineales was < 93% (Table S2).

Secondary structure analysis of 16S–23S RNA ITS

Two different size ITS regions were detected in both AT2016/6 and SN2021/02, designated as ITS–L (505 bp, large fragment) and ITS–S (334 bp, small fragment). Both tRNA (tRNA^{Ile} and tRNA^{Ala}) genes were absent in ITS–S fragment. Additionally, some significant differences in

Table 1. Similarity matrix (p–distance) generated using 16S rRNA gene sequences of *Pushkarnema curajae* and its phylogenetically related strains.

	Strain/Genus	1	2	3	4	5	6	7	8	9	10	11	12	13
1	***Pushkarnema curajae* AT2016/6**													
2	***Pushkarnema curajae* SN2021/02**	99.80												
3	*Planktolyngbya cf. limnetica* BACA0071 (MT176725)	99.50	99.31											
4	Leptolyngbyaceae cyanobacterium CALU 1934 (PQ145754)	99.60	99.40	99.70										
5	*Nodosolinea nodulosa* UTEX 2910 (EF122600)	92.37	92.17	92.17	92.16									
6	*Haloleptolyngbya alcalis* KR2005/106 (JN712770)	91.77	91.58	91.58	91.57	94.85								
7	*Leptolyngbya* sp. WJT66–NPBG5 (KJ939100)	96.63	96.43	96.73	96.43	92.57	92.17							
8	*Leptolyngbya* sp. WJT43–NPBG6 (KJ939099)	96.93	96.73	97.03	96.73	92.86	91.48	99.31						
9	Pseudanabaenaceae cyanobacterium HTT–U–KK3 (EF654069)	95.54	95.34	95.34	95.14	92.77	92.77	96.33	96.43					
10	Pseudanabaenaceae cyanobacterium CENA510 (KF246480)	96.13	95.94	95.84	95.83	91.58	92.27	94.85	95.14	95.24				
11	Pseudanabaenaceae cyanobacterium YACCYB324 (MH683745)	95.64	95.44	95.34	95.34	91.28	91.58	95.14	95.24	96.13	97.52			
12	Uncultured cyanobacterium clone BkfYyyyy00 (KC463671)	95.74	95.54	95.54	95.54	92.27	92.67	95.84	96.13	98.51	95.54	95.54		
13	Uncultured Oscillatoriales cyanobacterium clone SBROB5 67 (LN555063)	95.24	95.04	95.04	94.94	92.27	92.07	95.94	96.63	95.44	95.54	95.74	94.95	
14	Uncultured Oscillatoriales cyanobacterium clone SBROB5 68 (LN555064)	95.24	95.04	95.04	94.94	92.27	92.07	95.94	96.63	95.44	95.54	95.74	94.95	100

Table 2. Characteristics of the 16S–23S ITS region for *Pushkarnema curajae* and its related cyanobacterial taxa.

Organism	No. of ITS region	ITS length	$tRNA^{Ile}$	$tRNA^{Ala}$
***Pushkarnema curajae* AT2016/6**	2	ITS–L (505)	+	+
		ITS–S (334)	–	–
Leptolyngbya sp. WJT43–NPBG6 (KJ939099)	1	344	–	–
Leptolyngbya sp. WJT66–NPBG5 (KJ939100)	1	350	–	–
Nodosolinea nodulosa UTEX 2910 (KF307598)	1	506	+	+
Haloleptolyngbya alcalis KR2005/106 (JN712771)	1	521	+	+

size and folding of ITS–L and ITS–S of BoxB structure were noted (Fig. 5f, g).

The secondary structures of the D1–D1′ helix were identical for both strains of *P. curajae* (AT2016/6; SN2021/02). The D1–D1′ helix contained 62 bp; the basal stem of the folding had 5′–GACCU:CUGGA–3′ sequences followed by a unilateral bulge of 8 bp (CAUUACCU) and a unique bilateral bulge of 2 bp (CA:AC) in the central helix. The loop formed at the apex consisted of 15 bp. The D1–D1′ region of the strain differed from phylogenetically related taxa, including type species of *Nodosilinea* and *Haloleptolyngbya*, that had a unilateral bulge of only 6 bp (CACUCU) in the helices. This sequence only differed in *Leptolyngbya* sp. WJT43 and *Leptolyngbya* sp. WJT66 in the middle, with AC bases instead of CU bases (CAACCU). Likewise, the formation of a bilateral bulge in the central helices is absent in both *N. nodulosa* and *H. alcalis,* whereas *Leptolyngbya* sp. WJT43 and *Leptolyngbya* sp. WJT66 had a bilateral bulge of one base (C:U). Furthermore, *Leptolyngbya* sp. WJT66 had an additional asymmetric loop near the apex with 3 bases on the 5′ side and 1 base on the 3′ side (GAA:A), absent in the studied strain. The loop at the apex of the helices had 15–16 bp in related strains except for *Leptolyngbya* sp. WJT66, which had a relatively small loop of 6 bp in total. Thus, the D1–D1′ helix of *P. curajae* was distinct from the helices of *N. nodulosa*, *H. alcalis, Leptolyngbya* sp. WJT43, and *Leptolyngbya* sp. WJT66, as it possessed an additional unique central bilateral bulge and also a unilateral bulge consisting of 8 bp forming the helices (Fig. 5a–e).

The BoxB ITS–S consisted of three bilateral bulges: two symmetrical (uniform distribution of nucleotides on the 5′ and 3′ sides of the helix) and one asymmetrical (non–uniform distribution of nucleotides), compared to ITS–L which had a greater number of bulges, including four symmetrical and one asymmetrical. The terminal loop of ITS–S consisted of 8 bp, mostly unpaired pyrimidines (uracil); however, the loop of ITS–L was shorter with only 4 bp and rich in purines. The BoxB region had an extremely long sequence (68 bp) compared to *N. nodulosa* (40 bp), *Leptolyngbya* sp. WJT43 (35), and *Leptolyngbya* sp. WJT66 (36 bp), while *H. alcalis* had 49 bp in the folded structure. The BoxB ITS–L had 5 bilateral bulges: 2 bulges of 1:1 base and 2:2 base, and one bulge in the centre with unequal distribution of bases i.e., 6 bases on the 5′ side and 2 bases on the 3′ side (GUGAUA:GA). Significant variations in the terminal loop of related strains were noted: *P. curajae* had only four bases (GAGA), while *H. alcalis* and *Leptolyngbya* sp. WJT66 had an extra bp, *Leptolyngbya* sp. WJT43 had two, and substitution of G with U in *N. nodulosa* (Fig. 5f–k).

The V3 region of *P. curajae* differed from *N. nodulosa, Leptolyngbya* sp. WJT43, *Leptolyngbya* sp. WJT66, and *H. alcalis* in structure and length (Fig. 5l–p). The V3 helix of the *P. curajae* was 70 bp vs. 27 bp for *N. nodulosa,* 23 in *H. alcalis*, 67 in *Leptolyngbya* sp. WJT43, and 52 in *Leptolyngbya* sp. WJT66 (Fig. 5l–p).

Discussion

Cyanobacterial taxonomy has rapidly advanced in recent years with the description of more than 100 taxa using the polyphasic approach (Casamatta, 2023; Pal *et al*., 2024), with the goal of monophyly at all ranks (Mai *et al*., 2018; Strunecký *et al*., 2023). The taxonomy of *Leptolyngbya*–like genera is particularly challenging due to cryptic diversity, convergent evolution, and a general paucity of morphological features. That noted, numerous new genera have recently been erected from these simple filaments (e.g., *Halospirulina*, *Halomicronema*, *Thainema*, *Euryhalinema*, etc.).

In the present study, we describe a new genus *Pushkarnema* isolated from a freshwater, holy lake (Pushkar Sarovar) from the semi–arid region of Rajasthan, India. Based on the presence of thin trichomes (cells ≤ 1.5 µm wide), distinctly visible thick sheaths, and nodule formation, this taxon falls into the family Nodosilineaceae (Strunecký *et al*., 2023). However, it must be noted that nodule formation is not limited to *Nodosilinea*, but also reported from *Cymatolege spiroidea* (Konstantinou *et al*., 2021), *Kaiparowitsia implicata,* and *Pegethrix bostrychoides* (Mai *et al*., 2018). The ontogeny and

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Table 3. Comparison of lengths (nt) of D1–D1′ helices, BoxB and V3 helix folded structures of *Pushkarnema curajae* and related taxa.

Organism	16S–23S ITS region	Total length	Symmetric bilateral bulge	Asymmetric bilateral bulge	Unilateral basal bulge	Terminal loop
Pushkarnema curajae AT2016/6	D1–D1′ helix	62	4	Ab	8	15
	BoxB	68	12 (4 Bulge)	8	Ab	4
	V3 helix	70	24 (4 Bulge)	Ab	Ab	4
Leptolyngbya sp. WJT43–NPBG6 (KJ939099)	D1–D1′ helix	63	2	Ab	6	16
	BoxB	36	Ab	3	Ab	6
	V3 helix	67	14 (3 Bulge)	Ab	Ab	16
Leptolyngbya sp. WJT66–NPBG5 (KJ939100)	D1–D1′ helix	63	2	4	6	6
	BoxB	35	Ab	3	Ab	5
	V3 helix	52	Ab	6	Ab	5
Nodosolinea nodulosa UTEX 2910 (EF122600)	D1–D1′ helix	62	Ab	Ab	6	16
	BoxB	40	6	Ab	Ab	4
	V3 helix	27	Ab	6	Ab	5
Haloleptolyngbya alcalis KR2005/106 (JN712770)	D1–D1′ helix	61	Ab	Ab	6	15
	BoxB	49	4 (2 Bulges)	Ab	Ab	5
	V3 helix	23	Ab	Ab	9	6

*Ab– Absent

functional role of these nodules are unclear; however, some putative roles include anti–herbivory, buoyancy, and nitrogen fixation (Perkerson III *et al*., 2011; Strunecký *et al*., 2023). It is likely that nodule formation is culturally inducible and thus phenotypically plastic (Komárek *et al*., 2014; Mai *et al*., 2018). These nodules develop under low–light conditions in old cultures where cells divided into two planes and a bulge–like structure forms after the completion of cell division, as reported in *N. nodulosa* (Perkerson III *et al*., 2011).

Morphological differentiation of thin, filamentous cyanobacteria may be challenging. Morphologically, *Pushkarnema* closely resembles *Nodosilinea* and *Haloleptolyngbya*, all three genera have similar apical cell shape and distinctly visible centroplasm or cell content. However, *P. curajae* cells are longer and the filaments possess a thick, diffluent sheath (Fig. 2a, b and Fig. 3b). Likewise, habitat preference, absence of necridic cells, longer cells, and lack of constrictions distinguish *P. curajae* from *H. alcalis* (Table 4). Although other strains, such as *Leptolyngbya* sp. WJT43 and *Leptolyngbya* sp. WJT66, were found to be more closely related during phylogenetic analysis, there is insufficient data for morphological comparison. These two strains have been in numerous papers over the last decades, and to assign them taxonomic standing would be beneficial. However, these strains do not appear to be readily available outside their sequence data and no known images exist, so elevation to a "species" designation is unlikely at this time.

Phylogenetically, *P. curajae* falls into the Nodosilineales, with 16S rRNA gene sequence *p*–distance values ranging from 89.52% to 92.76% (Table S2), this was lower than the currently suggested value of ≤94.5% for distinct genera of bacteria (Yarza *et al*., 2014), although it must be noted that this exact value is subject to some speculation. Cyanobacterial taxa with ≤95% 16S rRNA gene similarity, distinct phylogenetic position, morphological discontinuity, and/or unique ecology should be considered for revision and described as a new genera or species (Komárek *et al*., 2014). The strains of *P. curajae* were well supported by these criteria and formed a separate lineage within the order Nodosilineales, providing substantial evidence for consideration as a novel genus.

Phylogenetic analysis showed that *P. curajae* formed a highly supported clade (100% bootstrap) with *P. cf. limnetica* BACA0071 and *Leptolyngbya* sp. CALU1934 (Fig. 4), along with high similarity value (>99%) for 16S rRNA gene sequences (Table 1). We note it possible these two strains represent a species of *Pushkarnema,* as well as the two sister strains "*Leptolyngbya"* (WJT43 and WJT66). The strains described as Pseudanabaenaceae cyanobacterium (HTT–U–KK3, YACCYB324 and CENA510) would also fall within the newly erected *Pushkarnema,* as all four strains shared >95% sequence similarity (Stackebrandt, 2006; Stackebrandt & Goebel, 1994; Yarza *et al*., 2014).

A distinctive characteristic of the studied strains was the presence of two operons: one containing both

Table 4. Morphological characteristics of *Pushkarnema curajae* and phylogenetically closely related type cyanobacterial species.

	Pushkarnema curajae	***Nodosilinea nodulosa***	***Haloleptolyngbya alcalis***
Cell width (μm)	1.0–1.2(1.4)	1.1–1.5	1.2–1.9
Cell length (μm)	1.5–3.5(5.0)	1.2–2.4	1.2–2.1
Cell shape	Longer than wide or rarely isodiametric	Cylindrical, isodiametric to longer than wide	Cylindrical, elongated, or isodiametric
Filaments	Straight or slightly wavy, long	Straight or slightly wavy	Straight, long, in cluster, slightly wavy
Sheath	Thick, diffluent, open, overpassing filament	Thin, colorless	Hyaline, firm, colorless
Constrictions	Distinct	Distinct	Distinct
Necridic cells	Absent	Absent	Present
Apical cells	Rounded	Rounded	Rounded
Nodule formation	In low light	In low light	Absent
Occurrence	Freshwater Lake, Pushkar, India	Marine–South China Sea	Saline alkaline water, soda lake, Kenya

$tRNA^{Ile}$ and $tRNA^{Ala}$ genes, the other lacking both. This is in contrast to the single ITS operon that was found in phylogenetically related taxa (Dadheech *et al*., 2012; Osorio–Santos *et al*., 2014; Perkerson III *et al*., 2011). The ITS folding motifs deviated considerably between the two operons and from closely related genera. *Pushkarnema* is the first known genus in the Nodosilineaceae family with two variants, as multiple operons are mainly present in the heterocytous lineages (Boyer *et al*., 2001; Iteman *et al*., 2000; Řeháková *et al*. 2014). Iteman *et al.* (2000) showed that *Nostoc* PCC 7120 possessed both ITS–L and ITS–S forms which were almost identical besides the regions containing tRNA genes. The ITS–L variant in *P. curajae* contained a D1–D1′ helix, $tRNA^{Ile}$, $tRNA^{Ala}$, BoxB, BoxA, D4, and V3 region, while the tRNAs were absent in ITS–S (Table 2). The sequence of both ITS–L and ITS–S fragments was similar except for Box B, which exhibited variations in sequence length (Table 2) and folding pattern (Fig. 5f, g).

Comparison of ITS rRNA region sequences is increasingly a common tool in phylogenetic analyses, with caveats (e.g., Johansen & Casamatta, 2005; Villanueva *et al*., 2024). Numerous new taxa have been erected using secondary structures of the ITS region (e.g., Chakraborty *et al*., 2019; Genuario *et al*., 2018; Johansen *et al*., 2011; Roy *et al*., 2024). The ITS region of the studied strains was extremely variable in sequence length and number of loops, in contrast to phylogenetically close cyanobacterial taxa (Tables 2, 3). Differences in *p*–distance (percent dissimilarity) >7% in aligned ITS regions of members of the same cyanobacterial genus were recently taken into account with consistent efficacy to differentiate species (Erwin & Thacker, 2008; González–Resendiz *et al*., 2019; Osorio–Santos *et al*., 2014). The *p*–distance dissimilarity value of aligned ITS of *Pushkarnema* with *Leptolyngbya* sp. WJT43 and *Leptolyngbya* sp. WJT66 were 33% and 32%, respectively indicating that these two strains could be distinct species of the genus *Pushkarnema* (Table S3). Caution is warranted in these endeavours, though, as Villanueva *et al.* (2024) point out in their recent work on paralogous vs. orthologous variants. These seemingly rather insignificant differences may actually be quite potent for phylogenetic reconstructions, as evidenced in *Brasilonema* (Villanueva *et al*., 2024) and our BoxB variant. How these differences will manifest in other cyanobacterial lineages, both heterocytous and the non–heterocytous, remains to be explored.

While often overlooked, ecology can provide a rich set of distinguishing features, especially useful for character–poor lineages of cyanobacteria (Dvořák *et al*., 2015). Ecology provides a potential understanding of the diversity, distribution, and adaptation strategies of cyanobacteria (Bertos–Fortis *et al*., 2016; Komárek *et al*., 2006). For instance, *P. curajae* was isolated form freshwater lake, *H. alcalis* from saline–alkaline lake, *Planktolyngbya cf. limnetica* BACA0071 and *Leptolyngbya* sp. CALU 1934 were reported from freshwater bodies, while *Leptolyngbya* sp. WJT43 and *Leptolyngbya* sp. WJT66 inhabited desert soils. The two closest relatives in terms of 16S rRNA gene sequence similarity are from the Azores, an island in the Atlantic Ocean. The next two similar strains, both *Leptolyngbya* strains (WJT43 and 66), are from desert soils in Joshua Tree National Park, USA. Thus, it seems likely that these two strains may constitute a desert–soil dwelling, novel species of *Pushkarnema* in the future if the cultures become available for systematic assessment. The ecological range of cyanobacterial genera is a topic of interesting research. For example, the type of *Nodosilinea* was originally isolated from a marine environment (Li & Brand, 2007),

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while a dozen new species have been erected in the last decade isolated from such disparate habitats as desert soils to freshwater environments.

In conclusion, based on the polyphasic approach that combined morphological characters, molecular and phylogenetic studies, and ecology, we propose a new cyanobacterial taxon, *Pushkarnema curajae* in the order Nodosilineales.

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