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Khargia gen. nov., a new genus of simple trichal Cyanobacteria from the Persian Gulf

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Abstract: Information regarding the diversity of Cyanobacteria in many parts of the world is still minimal. One example of a region that has not yet been widely studied is south–western Asia, including the region of the Persian Gulf. A culture–dependent study of cyanobacterial diversity in a rainwater basin on Kharg Island enabled the isolation of a novel taxon, previously unnamed, from a simple trichal cyanobacterium. Further comparisons showed the existence of closely related strains/taxa from other parts of the world, namely the strains CCALA 945 isolated from South Italy and "*Leptolyngbya india"* from India. Herein, we have thus described the new genus *Khargia* and the new species *Khargia iranica.* Other strains, isolated by other authors, were included in the *Khargia* genus as additional species: *Khargia italica* and *Khargia indica*. The recognition of the new genus was based on morphological evaluations (identification by both light and electron microscopy), the phylogenetic analyses based on the 16S rRNA gene, and species delimitation based on Automatic Barcode Gap Definition (ABGD), Generalized Mixed Yule Coalescent (GMYC), bayesian version of Poisson Tree Processes (bPTP), and the secondary structure of 16S–23S rRNA ITS region of the studied strains.

Key words: species delimitation, cyanobacteria, ITS secondary structure, polyphasic taxonomy, Persian Gulf

Introduction

Cyanobacteria are widely distributed microorganisms and represent a fundamental part of most ecosystems, including soil and aquatic habitats. They can even live in extreme environments such as saline lakes, thermal springs with temperatures above 60 °C, glaciers (Whitton & Potts 2007), and areas with high natural radioactivity (Heidari et al. 2018). Despite their significance in terms of global species richness and human utilization, the state of knowledge regarding their diversity is far from complete (Nazir et al. 2019).

The traditional classification system of Cyanobacteria based on morphological criteria does not take into account the existence of cryptic taxa (Hašler et al. 2012; Johansen & Casamatta 2005 and many others). The use of molecular phylogeny has become a powerful tool in modern taxonomy to elucidate the evolutionary patterns of Cyanobacteria (Giovannoni et al. 1988; Turner et al. 1999; Wilmotte & Herdman 2001). The 16S rRNA gene is a commonly used molecular marker for identifying prokaryotes (Komárek et al. 2014; Palinska & Surosz 2014), especially at the generic level in taxonomic studies. It also serves as a reference for taxonomic assignments of sequences obtained in metagenomic analyses (Konstantinidis & Rosselló–Móra 2015; Song et al. 2014). Moreover, the use of 16S–23S rRNA internal transcribed spacer (ITS) secondary structures is considered a practical tool to uncover new species in cyanobacteria (Boyer et al. 2001; Casamatta et al. 2006; Johansen et al. 2011; Kilgore et al. 2018). Nevertheless, many taxa within several cyanobacterial orders are difficult to identify, and further study is required to adequately understand their relationships.

Species delimitation techniques (tree–based and non–tree–based approaches) have been developed in

addition to the methods indicated above for determining species boundaries and identifying new species. They are used to distinguish taxa that are genetically but not morphologically distinct (Gevers et al. 2005, 2006; Giarla et al. 2014; Staley 2006; Xu et al. 2015, 2017 and many others). These methods vary from one another in many respects. Automatic Barcode Gap Discovery (ABGD) is one of the most computationally well–organized approaches among the commonly used methods. To use this method, an intraspecific distance threshold must be specified a priori, based on genetic distances calculated from a single locus instead of an explicit species concept (Puillandre et al. 2012). The GMYC method similarly computed data from a single locus and requires an ultrametric gene tree. bPTP demands the same estimate of the gene tree as GMYC, but branch lengths are proportional to the amount of genetic change rather than to time. This algorithm tends to do better than GMYC when interspecific distances are small (Zhang et al. 2013). Indeed, it is documented that species delimitation methods can effectively bridge the gap between the traditional taxonomy and molecular approaches (Dvořák et al. 2018; Rasouli–Dogaheh et al. 2022).

A significant part of cyanobacterial diversity is classified within the order Synechococcales. Currently, the order contains 106 genera (Hauer T. & Komárek 2022), however, some of these are not valid due to nomenclatural issues (97 genera are valid). The order consists of coccoid and simple trichal taxa, which share an ultrastructural feature (i.e., the parietal arrangement of their thylakoids). Other morphological features that can be used for practical determinations are very few, especially in certain trichal types, as observed in the family Leptolyngbyaceae. This family is characterized by trichomes (usually less than 3 µm wide and enclosed in a thin inconspicuous sheath), and cells with a more–or–less homogeneous content (Komárek & Anagnostidis 2005). Recently, more frequent applications of molecular methods, facilitated by these methods' increasing affordability and usually combined with ecological data, have enabled the discovery and description of many new taxa (e.g. Becerra–Absalon et al. 2018; Mai et al. 2018).

Recent contributions to the research on the diversity of Cyanobacteria in Iran (e.g. Etemadi–Khah et al. 2017; Heidari et al. 2018) were based on combinations of morphological characters and molecular data. These studies introduced several new genera and species, including taxa classified as Synechococcales. Such taxa are found in various ecosystems, including thermal springs and terrestrial habitats. However, the data on species richness of Synechococcales in the Persian Gulf are scarce and often scattered in morphological studies (Dulić et al. 2017; Noroozi et al. 2017). For example, Noroozi et al. (2017) published a study on several taxa from Kharg Island, which is located in the NE part of the Persian Gulf. However, the isolated strains were not described as new taxa.

Here, we aimed at (1) a thorough study of strain Kh.T2 isolated earlier by Noroozi et al. (2017), which represents a novel, simple trichal taxon, and its closed related taxa within Synechococcales order, using combining morphological characters and molecular data, (2) estimating species boundary based on multiple approaches, comprising species delimitation analyses and secondary structures of the 16S–23S ITS region, (3) and reclassify previously known species and determining new species within the new monophyletic genus.

Fig. 1. Map of Kharg Island, in the Persian Gulf.

Materials and Methods

Sample collection. Kharg Island, a coral island in the Persian Gulf (29°15'57.5" N, 50°17'57.5" E), is one of the most critical oil–exporting Islands in the region; with more than 95% of Iran's oil production exported from this island (Fig. 1). This rocky limestone island is unique because only a few islands have brackish water within porous limestone. The physico-chemical characteristics of the water sample from the Baghe Pire Mard basin are given in Table 1. The pH is alkaline (8.3), and the NaCl concentration is 163.5 mg.l^{-1}. The sampling was conducted twice between February 2012 and June 2013 in the Baghe Pire Mard basin (29°15'57.5" N, 50°17'57.5" E), which is a rainwater basin with *Phragmites* sp. and other macrophytes on Kharg Island. This basin has no oil contamination unlike other oil–water basins on Kharg Island. Water, sediments, and submerged *Phragmites* stem samples were collected in plastic bottles and bags, and then preserved in a refrigerator at 4 °C until being transferred to the Iranian Biological Resource Center.Samples were then cultured in BBM, BG11, and Chu media (Chu 1942; Nichols 1973; Rippka et al. 1974). Serial dilution with BG11 medium was used to culture the samples in Petri dishes incubated with a light–dark period (16:8 hour) at 25±2 °C and a 2500 to 3000 lux light intensity for two weeks. A single filament of isolated Cyanobacteria was transferred to a separate medium to cultivate the cells for further experiments. A part of each sample was preserved in 37% formaldehyde

for further morphological study. The strain *Leptolyngbya* sp. CCALA 945 was obtained from the CCALA culture collection (Třeboň, Czech Republic).

Morphological Investigation. The morphological characteristics of the strains were analyzed using an Olympus BX 51 light microscope. The width and length of 30 cells were measured to obtain the range of dimensions. For the ultrastructure study, fresh material was preserved with 6% glutaraldehyde, washed with phosphate buffer (0.05 M) at pH of 7.2, dehydrated with an isopropanol series, and fixed in Spurr's resin (Spurr 1969). The observations were done with a JEOL 1010 TEM. The Komárek & Anagnostidis (2005) determination key was used for the basic morphological comparisons with the known genera.

DNA Isolation, PCR Amplification, And Sequencing. The biomass of all strains was dried over silica gel (48 hours) and powdered using 2 mm stainless steel beads and a Mixer Mill MM400 (Retsch, Haan, Germany). The genomic DNA was extracted using an Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The PCR amplification of the 16S rRNA gene and 16S–23S ITS were carried out by using the primers 16S27F (Edwards et al. 1989) and 23S30R (Taton et al. 2003). The PCR reactions include 1 µl of gDNA, 0.6 µl of each primer (10 pmol/µl), and 10 µl of 2× Plain Combi PP Master Mix (1U of hot–start Taq polymerase in the manufacturer›s reaction buffer, 2.5 mM $MgCl_2$, and 0.2 mM of each dNTP; Top–Bio, Prague, Czech Republic), and 7.8 µl sterile water added to make up a final reaction volume of 20 µl. The long PCR amplifications start with an initial denaturation of 5 min. at 94 °C; followed by 45 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 90 s; then a final extension followed by 72 °C for 10 min. The PCR products were analyzed by gel electrophoresis performed at 60 V for 45 min using 1.5% low melting point agarose gel in 1× TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA). The PCR products were cloned into *Escherichia coli* DH5 alpha cells by heat shock transformation using the pGEM–T Easy Vector System (Promega, Madison, WI, USA). Purification of plasmids was performed by a NucleoSpin Plasmid kit (Macherey–Nagel, Düren, Germany). Plasmids of each strain were sent for both forward and reverse Sanger sequencing (Eurofins, Konstanz, Germany) using the plasmid primers SP6 (5'–TAT TTA GGT GAC ACT ATA G–3') and T7 (5'–TAA TAC GAC TCA CTA TAG GG–3') (Nübel et al. 1997). Two plasmids were sequenced on both strands for strain KH.T.2 (*Khargia iranica*); whereas only one plasmid was sequenced for strain CCALA 945 (*Khargia italica*). The sequence data were deposited to the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) under GenBank accession numbers MK839155, MK839156, and MK839154, respectively.

Sequence Analyses. The 16S rRNA sequences of cloned PCR products were assembled and trimmed using BioEdit version 7.2.5 (Hall 1999). Using MAFFT version 7 (Katoh et al. 2019) with default settings. Weobtained the sequences belonging to the order Synechococcales and other related groups based on the most recent cyanobacterial taxonomic system (Komárek et al. 2014) and added them to the sequences with more than 90% similarity (blasting result in National Center for Biotechnology Information (NCBI GenBank)) to our data. Overall, 216 sequences longer than 1000 base pairs (bp) were aligned with our new sequences. (supporting information Table S1). For the dataset, reference sequences of particular taxa cited in CyanoDB 2.0 (Hauer & Komárek 2021) were chosen and marked by an asterisk in Figure 2.

An uncorrected pairwise genetic distance or P–distance method in 16S rRNA was estimated using 1000 bootstraps in MEGA 6 (Tamura et al. 2013), and it was used to calculate the sequence identity with the formula (100*(1–genetic distance).

Phylogenetic analysis. Phylogenetic reconstruction was performed using Bayesian inference (BI) and Maximum Likelihood (ML). The appropriate model (GTR+I+Γ) of sequence evolution was determining using the Bayesian information criterion (BIC) for BI for ML tests in JModelTest 2.1.4 (Darriba et al. 2012). Bayesian inference (BI) analyses were inferred using MrBayes 3.2.6 (Ronquist et al. 2012) and two runs, each with eight Markov chains, were executed for 60 million generations under default parameters. Samples were recorded every 1000 generations (the final average standard deviation of split frequencies was <0.01). Convergences were checked using Tracer version 1.7 (Rambaut et al. 2018). Maximum likelihood (ML) analysis in the IQ–TREE webserver (Trifinopoulos et al. 2016) with 1000 ultrafast bootstrap pseudo–replications was run to evaluate the relative support of the branches (Minh et al. 2013). The first 25% of each sampled tree was discarded as burn–in. The resulting trees were visualized in FigTree version 1.4.4 (Rambaut n.d.). Two sequences of *Gloeobacter violaceus* with accession numbers (FR798924, AF132791) were chosen as outgroups.

Species Delimitation. We applied four species delimitation analyses (Automatic Barcode Gap Definition (ABGD), Generalized Mixed Yule Coalescent (GMYC), Bayesian version of Poisson Tree Processes (bPTP), and the secondary structure of 16S–23S rRNA ITS), to estimate the theoretical species boundaries.

Automatic Barcode Gap Discovery (ABGD). ABGD analysis (available at https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) was run to calculate barcode gaps of pairwise distances distribution for determining the threshold of species level using 16S rRNA dataset. The prior intraspecific divergence values were set between 0.001 and 0.01, and the distance distribution was set to 20 nb bins.

Generalized Mixed Yule Coalescent (GMYC). We constructed an ultrametric tree with the Bayesian analyses using BEAST 1.8.2 with 16S rRNA dataset (Drummond et al. 2012), with an uncorrelated lognormal relaxed molecular clock model. An appropriate substitution model was implemented (BIC) as in Partitionfinder 2.1.1 using the Bayesian information criterion

Table 1. Physiochemical characteristics of water samples from Baghe Pire Mard basin, Kharg Island, Iran.

Petroleum Compounds ($mg.l^{-1}$)	NaCl ($mg.l^{-1}$)	PO_4^{3-} ($mg.l^{-1}$)	Mg^{2+} ($mg.l^{-1}$)	Ca^{2+} ($mg.l^{-1}$)	pH	Temperature (°C)	Sampling area
No detectable	163.5	0.9	185	811	8.3	25	Baghe Pire Mard basin

(Lanfear et al. 2017). We executed the analysis under the constant population size coalescent as the tree prior, and the Ucld mean prior was set to an exponential distribution with mean 10 and initial value 1. Two independent chains for 600 million generations, with sampling every 10000th generation, and the first 25% burn–in trees removed. The outputs were checked for convergence using Tracer version 1.7 (Rambaut et al. 2018), and the consensus tree was generated using TreeAnnotator 1.8.2 (Drummond et al. 2012). The GMYC analysis was done under the single–threshold model. It was implemented in the SPLITS package (Monaghan et al. 2009) in R 3.3.0 (Team 2013) to detect lineages conforming to reputed species.

Bayesian Version of Poisson Tree Processes (bPTP). Within the method, the consensus tree from the resulting MrBayes was used, and the outgroup taxa were discarded from the analysis to develop the delimitation results. The MCMC chain was set for 600,000 generations, thinning set to 100 and burn–in at 0.3. The analysis was conducted on the bPTP webserver available at https://species.h–its.org/ptp.

Secondary Structure of 16S–23S rRNA ITS (ITS Secondary Structure). To resolve and compare the relationships of the strains within the new genus and other clusters, the available sequences of 16S–23S rRNA ITS region including D1–D1′ (from ‹5–GAC to GTC–3›) and Box B (from ‹5–AGC to GCT–3›) helices were supplied on Web Servers for RNA Secondary Structure Prediction (https://rna.urmc.rochester.edu/RNAstructureWeb). We evaluated the similarity of structures and the numbers of base pairs of both helices for all available sequences of 16S–23S rRNA ITS region. ITS secondary structure of the species within the new genus were re–drawn in CorelDRAW Graphics Suite 2018.

Results

Phylogenetic Results

Figure 2 shows the phylogenetic relationships among six families in the order Synechococcales based on the 16S rRNA gene (1049 bp). The phylogenetic tree contains two new distinct lineages (A and B), *Leptolyngbya* sensu stricto, several recently established and revised taxa of *Trichocoleus, Toxifilum, Kaiparowitsia, Tildeniella, Komarkovaea, Pegethrix, Drouetiella, Albertania, Timaviella, Oculatella*, *Thermoleptolyngbya, Geitleribacteron, Arthronema, Tapinothrix, Pantanalinema, Chroakolemma, Onodrimia, Kovacikia, Alkalinema, Planktolyngbya, Phormidesmis, Stenomitos, Scytolyngbya*, *Limnolyngbya*, *Halomicronema, Nodosilinea, Haloleptolyngbya, Romeria, Jaaginema, Limnothrix, Pinocchia, Acaryochloris*, *Aphanocapsa*, *Merismopedia*, and some members of the family Pseudanabaenaceae and other related taxa belong to the orders Oscillatorales and Synechococcales.

Lineage A, named *Khargia*, diverged as a monophyletic group belonging to the family Leptolynbyacaeae and diverged from lineage B with high statistical support values of both posterior probability (PP=1) and bootstrap support (BS=100). Moreover, the sequence identity between sequences of *Khargia* and other similar genera of the family Leptolynbyacaeae ranges between 90.9 and 93.2% (Table 2).

Furthermore, several taxa of Pseudanabaenaceae and Leptolyngbyaceae have fallen outside of those families' clades and resultsshowed that they were polyphyletic in order (Fig. 2).

Species delimitation

The ABGD analysis indicated 112 genetic groups; however, the bPTP analysis revealed 123 species; and 140 species were proposed by GMYC analysis. Furthermore, D1–D1′ and Box B helices of 16S–23S rRNA ITS secondary structure recovered 81 and 83 species, respectively. Nevertheless, it is impossible to record a precise number of species based on the ITS secondary structure because 16S–23S rRNA ITS sequences are not available for all taxa in the NCBI (Fig. 3, Fig. S1).

The phylogenetic tree (Figs 2, 3) revealed that *Khargia* is a monophyletic group that was ranked as single species by ABGD analysis; however, both GMYC and bPTP analyses categorized *Khargia* clade into three distinct species. In other words, the *Khargia* clade can be considered as a new genus with at least three species (1) *Khargia iranica* from Kharg Island, Persian Gulf,

Table 2. 16S rRNA percent similarity among genera groups belonging to Leptolyngbyaceae that are close to the *Khargia* genus both morphologically and genetically.

No.	Genera	1	2	3	4	5	6	7	8	9	10	11	12	13
1	***Khargia***													
2	*Onodrimia*	91.5												
3	*Kovacikia*	93.2	93.5											
4	*Alkalinema*	91.7	92.1	92.5										
5	*Stenomitos*	93.0	92.5	94.7	91.9									
6	*Scytolyngbya*	91.3	91.4	93.5	91.0	93.6								
7	*Limnolyngbya*	91.7	92.3	93.3	91.7	93.6	93.4							
8	*Plectolyngbya*	90.9	89.4	91.5	91.2	90.7	90.8	90.1						
9	*Phormidesmis*	91.2	91.7	92.5	92.8	92.6	91.9	91.6	91.9					
10	*Planktolyngbya*	91.1	90.4	91.8	95.8	90.8	91.4	90.6	91.2	93.1				
11	*Chroakolemma*	92.7	94.6	94.9	93.5	94.5	94.3	93.7	90.4	93.3	92.6			
12	*Pantanalinema*	92.7	92.5	94.0	91.6	94.7	93.0	93.5	89.8	91.0	91.0	94.1		
13	*Leptolyngbya*	91.5	90.0	89.8	93.2	91.3	89.7	89.7	95.2	92.4	91.4	90.4	90.4	

Table 3. Taxon name, morphological description of trichomes and cells, origin, ecology and literature reference of *Khargia* in comparison to morphologically related genera within the Leptolyngbyaceae family, including *Leptolyngbya, Stenomitos, Scytolyngbya, Onodrimia, Limnolyngbya, Alkalinema, Pantanalinema, Chroakolemma, Phormidesmis, Plectolyngbya, Planktolyngbya.*

Taxon name	Trichomes	Cells	Locality	Source	Reference
Khargia iranica	Straight to irregular, variable cell size in trichomes (1.2–1.5 µm width)	Longer than wide, 1.5–5 ×1.0–3.0, terminal cells rounded or conical	Kharg Island, Persian gulf, Iran	Fresh water, epiphyte	This study
Leptolyngbya boryana	Pale blue–green, strongly constricted at the ungranulated cross–walls, (1) 1.3–2 (3) µm width	Isodiametric or shorter /longer than wide, terminal cells rounded	Widespread	Fresh water	Anagnostidis & Komárek 1988
Stenomitos rutilans	Straight or coiled, unconstricted	Longer than wide, 2.8–4.8×0.8–1.2, terminal cells rounded	Kauai island, Hawaii	Basaltic cave walls with mosses	Miscoe & Johansen 2016
Scytolyngbya timoleontis	False branched, , thick sheath, very thin trichome (2.3 µm width)	Longer than wide, 3.5–10.8 ×1.9–2.6, terminal cells rounded	China	Stony wall	Song & Li 2015
Onodrimia javanensis	False Branched, thin trichome (1.9 µm width)	Longer than wide, 1.5 × 8.35, terminal cells rounded or conical	Indonesia	Thermal spring, Submersed bark of tree branches	Jahodářová et al. 2017
Limnolyngbya circumcreta	Spirally to narrowly screw–like coiled, 1.2–2.15 µm width	Quadrate or slightly cylindrical up to 2.15 wide, terminal cells rounded	China	Fresh water	Li & Li 2016
Kovacikia muscicola	Straight, constricted	Slightly longer than wide, 1.0–2.0×1.5–1.7, terminal cells rounded	Kauai island, Hawaii	Basaltic cave walls with mosses	Miscoe et al. 2016
Alkalinema pantanalense	Straight trichome with 1.7–2.2 µm width	Isodiametric or longer than wide, 1.5–4.1 × 1.1–2.2, narrowed or rounded–conical apical cells	Pantanal wetland, Brazil	Saline–Alkaline Lake	Vaz et al. 2015
Pantanalinema rosaneae	Slightly constricted, sheath is hyaline	Isodiametric or wider than length, 1.2–3.1×1.5–3.1, apical cell cylindrical with a rounded apex	Pantanal wetland, Brazil	Saline–Alkaline Lake	Vaz et al. 2015
Chroakolemma opaca	Solitary or intricate, rare single false branched Sheaths firm,	Longer than wide sometimes isodiametric, 1–3.8 ×1.6–6.9. Apical cell cylindrical and rounded	Ampliación La Peña, Hidalgo, Mexico	Aerophytic, in biocrusts, semi–desert soils	Becerra–Absalón & Johansen 2018
Phormidesmis mollis	Solitary or in irregular, Facultative sheaths, distinctly constricted at the cross–walls	Shortly barrel–shaped, 2.5–3 ×4–6, end cells rounded	Belize, Centeral America	Alkaline marshes	Turicchia et al. 2009
Plectolyngbya hodgsonii	Solitary with obligatory false branching (in nature), not or slightly constricted at cross–walls, sheaths very thin	Isodiametric or slightly longer or shorter than wide, 0.8–4 wide, end cells rounded	Larsemann Hills, Antarctica	Periphytic and metaphytic in the lake	Taton et al. 2011
Planktolyngbya limnetica	Cylindrical, isopolar, straight, waved, coiled with 2.8 (4) µm, presence of firm sheaths	Cells cylindrical, usually longer than wide, 1.14–3.15 ×0.65–3.80, end cells rounded or narrowed–rounded	South Scania, Sweden	Freshwater	Komárková–Legnerová & Cronberg 1992

Iran, (2) *Khargia italica* from Ischia Island, Italy, and (3) *Khargia indica* from Lower Gangetic Plain, India.

Additionally, in figure 4, secondary structures of the D1–D1′ and Box B helices of the ITS were compared for the strains CCALA 945 (*Khargia italica*) and KH.T.2 (*Khargia iranica)*. The D1–D1′ helices for both strains (with 51 bp) are similar and most of the helices were conserved in the strains, but they differ by the presence of a base at position 15 from the 5’ end in *Khargia iranica*. The terminal part of the stem contain 12 bp for *Khargia italica* while it has 10 bp for *Khargia iranica* but the terminal loops of helices are similar. In contrast, the Box B helices presented a more variable structure in the strains. Each helix consists of bulges formed by unpaired bases or a side loop and a terminal loop. In addition, the length of both Box B helices (*K. italica* with 44 bp and *K. iranica* with 57 bp) and the numbers of bp of all stems for both strains are different. In the case of *Khargia indica*, there is no sequence of the 16S–23S ITS region available for comparison.

Morphological And Taxonomic Descriptions
Class: Cyanophyceae
Order: Synechococcales
Family: Leptolyngbyaceae

***Khargia* Rasouli–Dogaheh, Noroozi, Khansha et Hauer gen. nov. (Figs 5,6)**
Description: Thallus is usually macroscopic, in mats, occasionally creeping. Filaments straight to bent, occasionally coiled, not branched. Sheath colorless opened at the ends. Trichomes are slightly motile. Cells are cylindrical, usually distinctly longer than wide. Apical cells conical or rounded, no calyptra. Reproduction by trichome disintegration.

Etymology: Name of the genus "*Khargia*" refers to the island where the type species was collected.
Type species: *Khargia iranica* Noroozi, Rasouli–Dogaheh, Khansha et Hauer.

***Khargia iranica* Noroozi, Rasouli–Dogaheh, Khansha et Hauer sp. nov. (Fig. 5 and Fig. 6 C–E)**
Description: The thallus is usually macroscopic, in mats, occasionally creeping, blue–green. Filaments straight to bent, occasionally coiled, and sometimes entangled together with no branching. The sheath is usually colorless, up to 1 µm thick. Trichomes are blue–green to dark green, not constricted to constricted at cross–walls, slightly motile. Cell width 1–3 µm and length 1.5–8 µm; cylindrical, usually longer than wide. End cells conical or rounded, without calyptra, but sometimes with a capitate end. Cells after division shorter, later growing and reaching the size of the original cells before division.

Holotype: a cryopreserved strain IBRC–M 5007 deposited at Iranian Biological Resource Center, Algal culture Collection, Karaj, Alborz province, Iran.
Isotype: CBFS A–96–1 at Herbarium of the University of South Bohemia, České Budějovice, Czech Republic.
Type locality: Iran, Bushehr Province, Kharg Island, Baghe Pire Mard basin (29°15'57.5" N, 50°17'57.5" E). Free–living. Samples were collected in June 2013.
Habitat: Rainwater basin on limestone bedrock, not contaminated with oils, covered with algal growth and macrophytes.
Etymology: Name of species "*Khargia iranica*" refers to the country where the material was collected.
Reference strain: KH.T.2 (reference sequence: 16S rRNA gene + ITS region MK839155).
Materials analyzed: strain KH.T.2.
GenBank accession numbers: MK839155 and MK839156.

***Khargia italica* Sciuto et Moro sp. nov. (Fig. 6 A–B)**
Description: Expanded mats, creeping on a substrate, blue–green. Filaments straight to bent, sometimes entangled together, not true or false branched: The sheath is usually colorless, inconspicuous to 1 µm thick. Cells divide by binary fission into smaller daughter cells. The latter grow and reach the size of the original cells before division. Trichomes blue–green to dark green, slightly constricted to distinctly constricted at cross–walls, slightly motile. Cell width and length ranging from 1.3–3.3 and 1–1.5 µm respectively; cylindrical, usually longer than wide. Apical cells conical or rounded, without calyptra, never capitate. Reproduction by trichome disintegration in short hormogonia.

Holotype: CBFS A–106–1 at Herbarium of the University of South Bohemia, České Budějovice, Czech Republic.
Type locality: Italy, Island of Ischia (40°44'26" N, 13°56'35" E). Samples were collected in April 2009 (Sciuto et al. 2011).
Habitat: Mats developing at the surface of sedimentary mud in bicarbonate–alkaline thermal springs (Sciuto et al. 2011).
Etymology: Name of the species "*Khargia italica*" refers to the country where the material was collected.
Reference strain: CCALA 945 (reference sequences: 16S rRNA gene + ITS region MK839154).
Materials analyzed: strain CCALA 945.
GenBank accession numbers: MK839154.

***Khargia indica* (Debnath et Bhadury) Rasouli–Dogaheh, Noroozi, Khansha et Hauer comb. nov.**
Basionym: *Leptolyngbya indica* Debnath et Bhadury (Debnath et al. 2017).
Description: Concerning the diagnosis of this species by Debnath et al. (2017), the species was morphologically and ecologically different from other defined soil–dwelling species within the genus *Leptolyngbya*. Its cell width was 0.8–1 µm, cell length was 2 – 2.5 µm, with constrictions at the cross–walls, and a sub–obtuse to an obtuse apical cell. This morphology is similar to species within the newly described genus *Khargia*. Moreover, *K. indica* is closely related to other strains of the genus *Khargia*, and it is distinguished as a separate species based on different species delimitation analyses (Fig. 3).

Discussion

In this study, we looked for the shared morphological and molecular features between the Iranian strain KH.T.2, isolated from a rainwater basin on Kharg Island in the Persian Gulf, and two other similar strains that differed from the known taxa within the family Leptolyngbyaceae. One of these strains was named *Leptolyngbya indica* by Debnath et al. (2017), and the other was proposed as a member of the (to date) not formally established genus *Protolyngbya* (Sciuto et al. 2011). The authors did not formally describe it as the isolation and characterization of more strains belonging to this genus were needed.
A common method for identifying cyanobacteria species based on genetic data sequencing is to employ genetic similarity in order to distinguish the genetic distinctiveness between the species. Yarza et al. (2014) proposed a 94.5% 16S rRNA identity threshold for separation at the

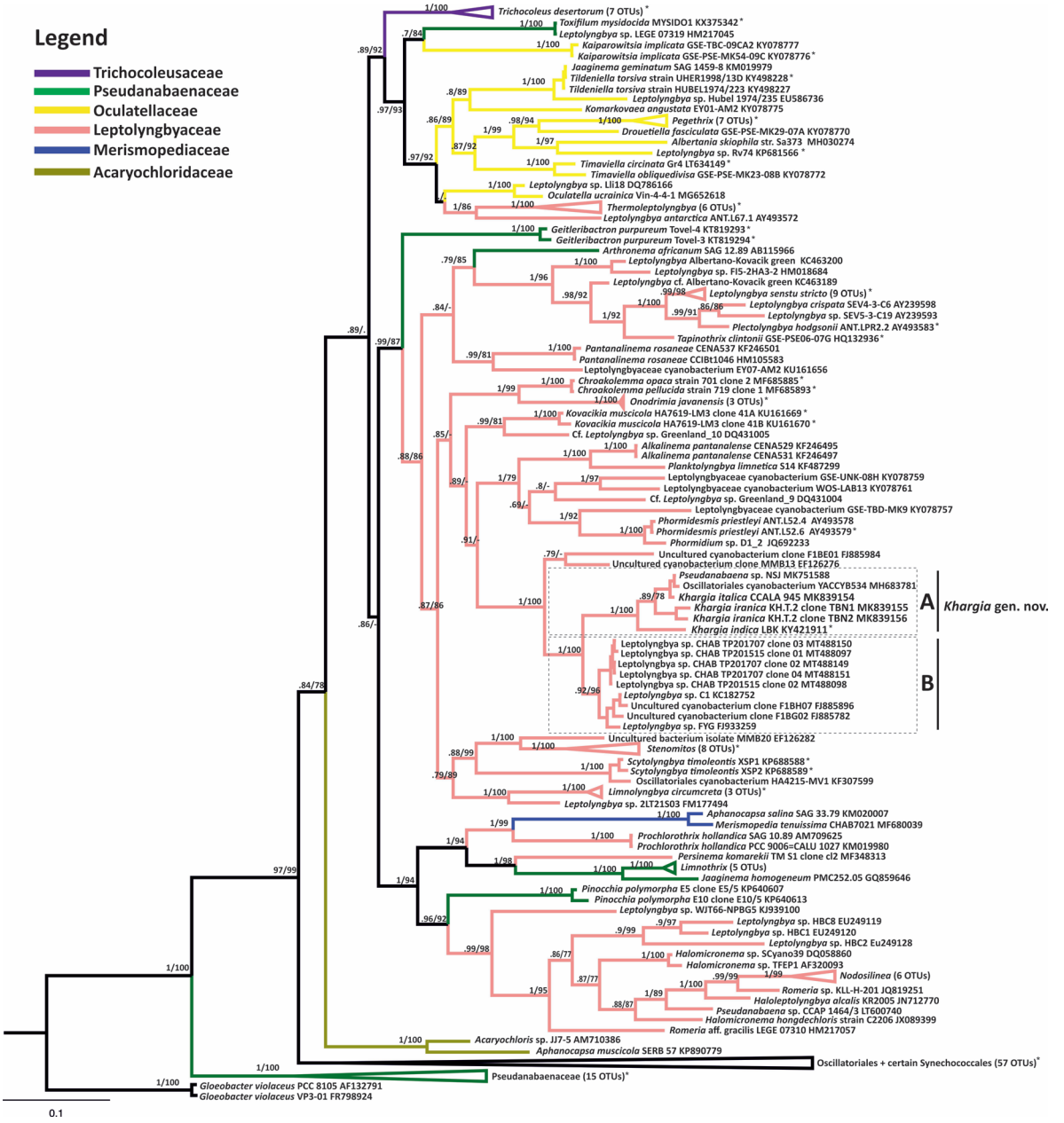

Fig. 2. Phylogenetic analysis of 216 sequences of the 16S rRNA gene showing the position of the newly described taxa (*K. iranica, K. italica,* and *K. indica* in clade A. The phylogenetic tree reconstructed by both ML and BI analyses yielded identical topologies. The support values lower than 0.5 or 50 %, respectively, are shown by a dot, and the ones with a different position contain a dash. The scale bar represents the number of nucleotide substitutions per site. Reference sequences of particular taxa are marked with an asterisk (*). Clade A denotes the clade corresponding to the new genus. With more evidence, Clade B might be considered a separate genus with high support values. The legend indicates the families belong to the order Synechococcales.

genus level. The results of this study showed the range of 16S rRNA identity similarity range of the highest and lowest similarity between *Khargia* lineage and other closely related genera within the family range between 93.2% and 90.9% and 93.2%, respectively (Table 2).

Furthermore, the topology of the phylogenetic tree and different specific analyses showed that clade A includes two other sequences belonging to the new genus: the strain *Pseudanabaena* sp. (MK751588) and Oscillatoriales cyanobacterium (MH683781). They might belong to K. italica, but the lack of data, absence of morphological and ecological features, and the short length of 16S rRNA sequences has precluded any conclusion at this time. Clade B appears as a well–supported clade based on the phylogenetic methods and might be a separate genus, but more evidence and research are needed.

Dvořák et al. (2018) demonstrated that taxonomic databases do not clearly show the diversity in cyanobacteria as many species are ambiguously recognized with a low certain of proper identification. In addition, they discovered more than 2100 species by PTP species

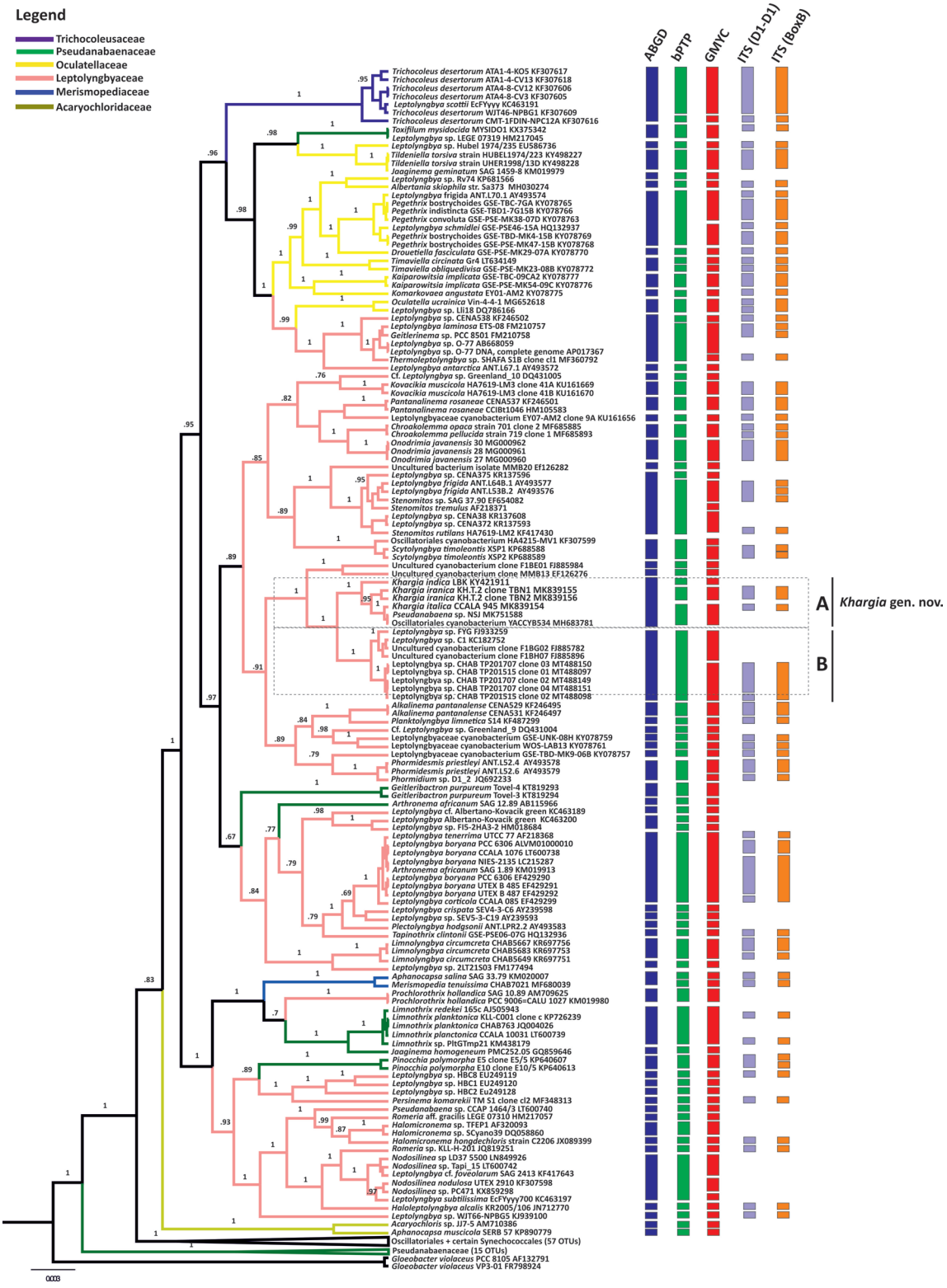

Fig. 3. Phylogenetic tree of 216 sequences of the 16S rRNA gene reconstructed using Beast. The support values illustrate Bayesian posterior probabilities. Each column on the right shows the results of a different species delimitation method (ABGD, bPTP, GMYC and ITS secondary structure), and every single rectangle indicates a separate species. The legend indicates the families belonging to the order Synechococcales (Collapsed clades information shows in supplementary data).

delimitation analysis, that are not identifiable in databases CyanoDB and AlgaeBase (see DVOŘÁK et al. 2018).

In the present study, three main taxonomic approaches were used to evaluate the classification of the genus *Khargia* within the family Leptolyngbyaceae. The first method was conducted using the distance methods (ABGD) and 16S rRNA similarity, based on P–distance. The genetic distance method is one of the most utilized taxonomic

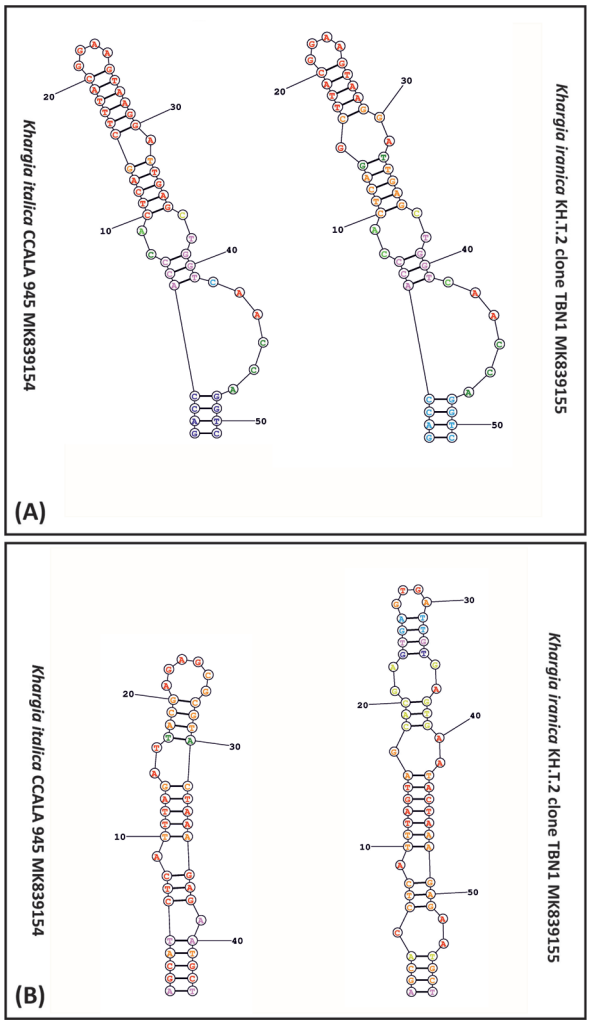

Fig. 4. (A) represents the secondary structure of the D1–D1′, and (B) depicts Box B helices of the ITS region. *Khargia iranica* KH.T.2 (MK8389155), *Khargia italica* CCALA 945 (MK839154).

analyses, primarily to determine species for most of the eukaryotes (Fontaneto et al. 2015; Kekkonen & Hebert 2014; Tang et al. 2014), as well as for prokaryotes such as Cyanobacteria (Eckert et al. 2015). The second approach included the tree–based method (GMYC, bPTP) on the 16S rRNA dataset (Fujisawa & Barraclough 2013; Lorén et al. 2018; Pons et al. 2006; Zhang et al. 2013). In addition, one functional pattern analysis (ITS secondary structure) of the 16S–23S ITS dataset was applied. This analysis represented diagnostic apomorphic characteristics that have congruence with the 16S rRNA gene–based phylogeny in the Cyanobacteria (Boyer et al. 2001; Johansen et al. 2011; Siegesmund et al. 2008).

Generally, most species delimitation methods were reported as supported analyses of candidate species (Barraclough et al. 2009; Košuthová et al. 2020; Lorén et al. 2018; Malavasi et al. 2016; Tang et al. 2014). In this study, the results of species delimitations showed that GMYC could recognize more species than the bPTP and ABGD methods. This result is consistent with previous studies, where researchers discovered more operational taxonomic units using GMYC analysis compared to other species delimitation methods (Esselstyn et al. 2012; Fujisawa & Barraclough 2013; Kekkonen & Hebert 2014; Miralles & Vences 2013; Talavera et al. 2013; Tang et al. 2014; Lorén et al. 2018; Rasouli–Dogaheh et al. 2022). In addition, among the different approaches in this study, the ITS secondary structure was the most sensitive method to separate genetic groups in Cyanobacteria. However, the combination of different species' delimitation methods is more effective for taxonomic workflows (Barraclough et al. 2009; Lorén et al. 2018).

Moreover, ABGD analysis as a distance–based method demonstrated a divergence between the *Khargia* lineage and other lineages (Fig. 3). On the other hand, the phylogenetic tree result showed that *Khargia* diverged from the other lineages with strong support values in both the BI and ML analyses (Figs 2, 3). This is additionally supported following the tree–based analyses in Figure 3 (GMYC, bPTP), and the ITS secondary structure (D1–D1′ and Box B helices) in Figure 3 and 4. *Khargia* is a separate genus within the Leptolyngbyaceae and consists of three species (*Khargia iranica*, *Khargia italica*, and *Khargia indica*) in the clade. However, the description of *Khargia indica* is incomplete due to the lack of the ITS sequences for the proposed taxon. Therefore, we can introduce the *Khargia* lineage as a new genus comprising three species within the family Leptolyngbyaceae because all species' delimitation analyses were congruent with each other in the present study.

Concisely, we had an opportunity to study the Italian strain CCALA 945 (*Khargia italica*) from the CCALA culture collection together with the strain KH.T.2 (*Khargia iranica*) isolated in this study to show that both belong to the genus "*Khargia"*. We propose a new generic name instead of raising the subgenus *Protolyngbya* to the generic level to avoid confusion. Moreover, the new genus contains another species previously classified in the genus *Leptolyngbya* by Debnath et al. (2017).

A morphological and ecological comparison of *Khargia iranica* with 12 similar genera represented by their type species is shown in Table 3. These taxa are almost cryptic, and it is difficult to distinguish them precisely; however, there are some unique, and detailed differences. For instance, *Khargia*, *Leptolyngbya*, *Kovacikia*, *Alkalinema*, *Planktolyngbya*, and *Stenomitos* have straight trichomes and constricted trichomes, while *Stenomitos* has unconstricted trichomes. *Khargia* has no false branchings, although some of the similar genera such as *Onodrimia*, *Scytolyngbya*, *Chroakolemma*, and *Plectolyngbya* have false branchings. *Limnolyngbya* is a different genus with a variable morphology, which contains spiral and screw–like trichomes. *Pantanalinema* has nearly the same cell dimensions as *Khargia*, but it has entangled filaments. In contrast, *Phormidesmis* has more–or–less trichomes, but its cell width is almost two times larger than *Khargia.* Concerning the typical characteristics such as trichomes, cell morphology, and

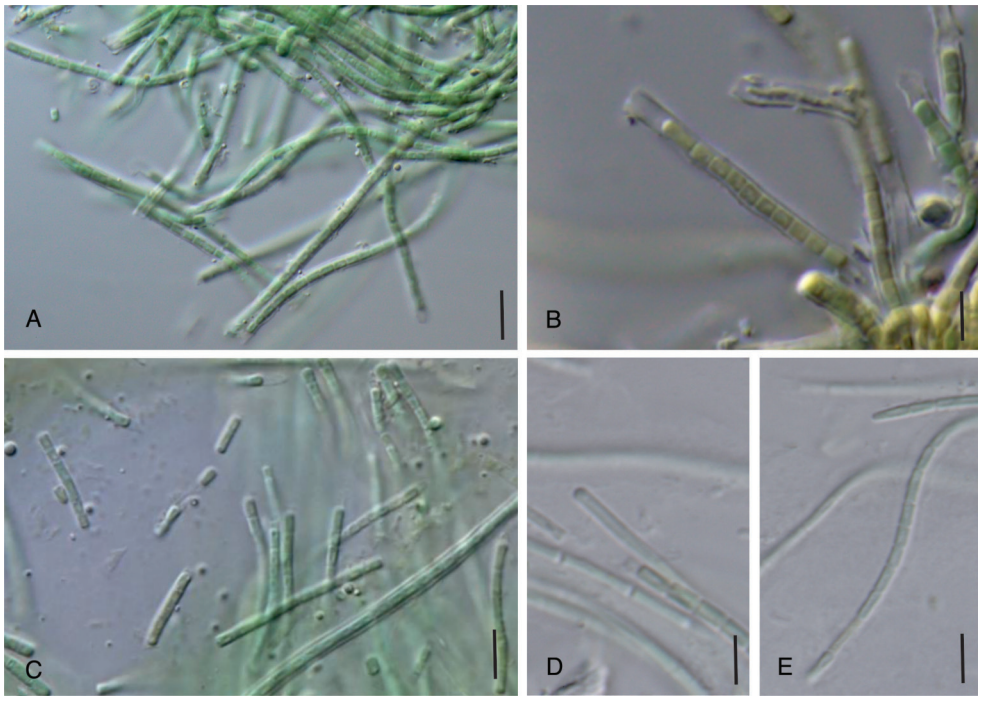

Fig. 5. Light microscope image of *K. italica* (A, B) and *K. iranica* (C, D, E). The cylindrical cells with rounded and conical end cells. Trichome with sheath slightly constricted to constricted at cross–wall. Scale bar 10 µm (A, C) and 5 µm (B, D, E).

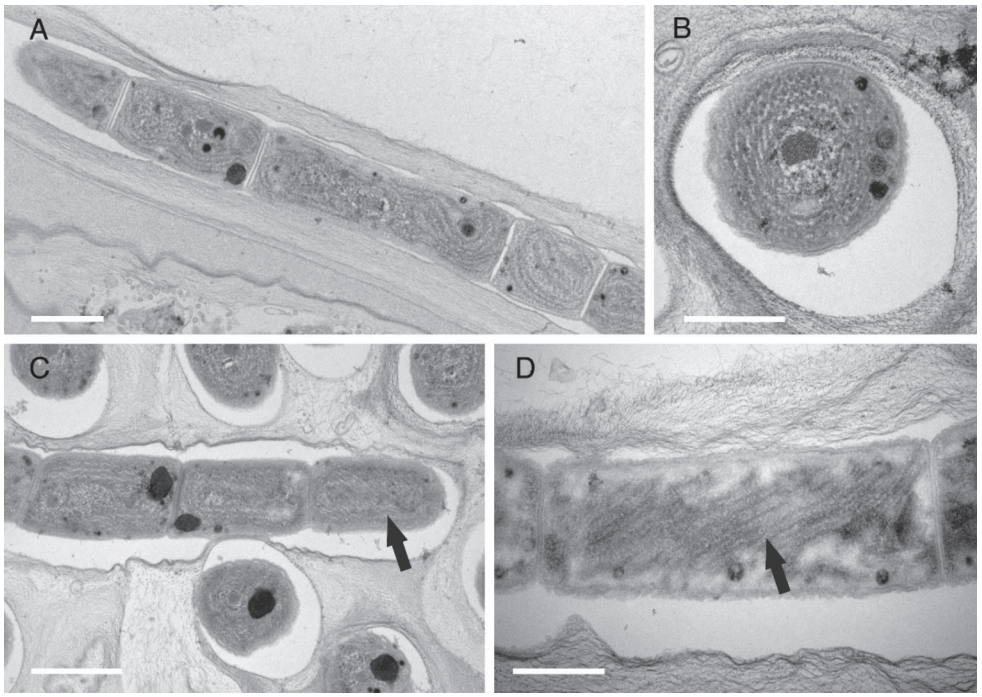

Fig. 6. TEM micrographs of *Khargia iranica*: (A) longitudinal section showing variable cell length along one trichome; (B) cross–section of the trichome; (C, D) longitudinal sections showing screw coiled thylakoids (marked with arrows). Scale bar length 1 µm (A, C), 0.5 µm (B, D).

ecological features (species from freshwater, soil, and extreme habitats), the type species of the new genus, *Khargia iranica*, belongs to the family Leptolyngbyaceae (Komárek et al. 2014; Komárek & Anagnostidis 2005). The mass sequencing of incorrectly determined natural materials and cultures results in a significant number of incorrectly named sequences in major repositories that can, consequently, bias the results in many phylogenetic analyses. To reduce such biases as much as is possible, it is necessary to utilize the reference sequences of particularly well–established and taxonomically valid taxa if they are known and available. It might be difficult for

some authors to recognize such reference sequences. One of several possible methods is using the NCBI Reference Sequence Database (RefSeq; https://www.ncbi.nlm.nih.gov/refseq/). However, it needs to be used with caution since the database also contains data on atypical members of genera (e.g., for the genus *Gloeocapsa* Kützing the RefSeq contains the records of two strains (PCC 7428 and PCC 73106). Still, none represent the type species or any other typical member of the genus). An alternative method is to obtain the accession numbers of reference sequences from a database dedicated to the taxonomy of Cyanobacteria, CyanoDB.cz 2.0 (Hauer T. & Komárek 2022); (http://www.cyanodb.cz), where the information on the taxa is either obtained from protologues or revisions of particular taxa. As many such sequences as possible were used in the phylogenetic analyses of this research. They are marked with an asterisk in Figure 2. We also encourage other authors to use these reference sequences. Such an effort will likely yield more comparable phylogenies.

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Author Contribution
Conceptualization: S.R–D, M.N, J.K, MA.A, R.R, T.H; Data analysis: S.R–D; Investigation: S.R–D., MN, J.K, R.R, T.H; Methodology: S.R–D, J.K, M.MN, T.H; Project administration: M.N; Writing: S.R–D, M.N, J.K, MA.A, T.H.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. Phylogenetic tree of 216 sequences of the 16S rRNA gene reconstructed using Beast. The support values illustrate Bayesian posterior probabilities. Each column on the right shows a different species delimitation method, and every single rectangle indicates a separate species. The legend shows the families belong to the order Synechococcales.
Table S1. Information of sequences within the dataset.

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

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