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Laura Gonzalez-Resendiz

Jeffrey R. Johansen

Hilda Leon-Tejera

Leon Sanchez

Marcia Morales

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A BRIDGE TOO FAR IN NAMING SPECIES: A TOTAL EVIDENCE APPROACH DOES NOT SUPPORT RECOGNITION OF FOUR SPECIES IN *DESERTIFILUM* (CYANOBACTERIA)[1]

Laura González-Resendiz

Departamento de Procesos y Tecnología, Universidad Autónoma Metropolitana-Cuajimalpa, Cuajimalpa, Av. Vasco de Quiroga 4871, C.P. 05300, Ciudad de México, México

Jeffrey R. Johansen

Department of Biology, John Carroll University, University Heights, Ohio 44118, USA
Department of Botany, Faculty of Science, University of South Bohemia, Branišovská 31, České Budějovice 370 05, Czech Republic

Hilda León-Tejera

Departamento de Biología Comparada, Facultad de Ciencias, Universidad Nacional Autónoma de México (UNAM), Ciudad de México, México

León Sánchez

Doctorado en Biotecnología, Universidad Autónoma Metropolitana-Iztapalapa, México, México

Claudia Segal-Kischinevzky, Viviana Escobar-Sánchez

Departamento de Biología Celular, Facultad de Ciencias, Universidad Nacional Autónoma de México (UNAM), Ciudad de México, México

and Marcia Morales[2]

Departamento de Procesos y Tecnología, Universidad Autónoma Metropolitana-Cuajimalpa, Cuajimalpa, Av. Vasco de Quiroga 4871, C.P. 05300, Ciudad de México, México

A population of *Desertifilum* (Cyanobacteria, Oscillatoriales) from an oligotrophic desertic biotope was isolated and characterized using a polyphasic approach including molecular, morphological, and ecological information. The population was initially assumed to be a new species based on ecological and biogeographic separation from other existing species, however, phylogenetic analyses based on sequences of the 16S rRNA gene and 16S–23S ITS region, placed this strain clearly within the type species, *Desertifilum tharense*. Comparative analysis of morphology, 16S rRNA gene similarity, 16S–23S ITS secondary structure, and percent dissimilarity of the ITS regions for all characterized strains supports placing the six *Desertifilum* strains (designated as PD2001/TDC17, UAM-C/S02, CHAB7200, NapGTcm17, IPPAS B-1220, and PMC 872.14) into *D. tharense*. The recognition of *Desertifilum salkalinema* and *Desertifilum dzianense* is not supported, although our analysis does support continued recognition of *Desertifilum fontinale*. Pragmatic criteria for recognition of closely related species are proposed based on this study and others, and more rigorous review of future taxonomic papers is recommended.

Key index words: **16S rRNA gene; 16S–23S ITS; bacterial species concepts; *Desertifilum*; monophyletic species concept; α-level taxonomy**

***Abbreviations*: BI, Bayesian inference; CCB, Cuatro Cienegas basin; FCME, Phycological Herbarium of the Science Faculty at Universidad Nacional Autónoma de México; GTR+I+G, general time reversible model with proportion of invariable sites and gamma distribution; ML, maximum likelihood; MP, maximum parsimony; TBR, Tree Bisection and Reconnection; UNAM, Universidad Nacional Autónoma de México**

Filamentous cyanobacteria have undergone an extensive taxonomic revision during the last two decades (Boyer et al. 2002, Siegesmund et al. 2008, Engene et al. 2010, Komárek et al. 2014, Mai et al. 2018). The increase in use of 16S rRNA gene sequence data in combination with the characterization of the secondary structure of ribosomal internal transcribed spacer regions (16S–23S ITS) has been found to be important in delimitation of genera

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[2]Author for correspondence: e-mail mmorales@correo.cua.uam.mx.
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and species (Perkerson et al. 2011, Johansen et al. 2014, Osorio-Santos et al. 2014, Pietrasiak et al. 2014). Additionally, for cyanobacterial taxonomy, it is necessary to incorporate diagnostic criteria such as morphological, physiological, or ecological data, and to compare all significant criteria for taxon recognition or delimitation, using a total evidence approach commonly called the polyphasic approach in cyanobacterial studies. With this method in the last decade, key works in the nonheterocytous filamentous cyanobacteria such as those of Siegesmund et al. (2008), Johansen et al. (2011), Dadheech et al. (2012), Osorio-Santos et al. (2014), Strunecký et al. (2011, 2013, 2014, 2017), Hašler et al. (2017), Mai et al. (2018), and many others, have contributed to increasing our knowledge of evolutionary and taxonomic diversity within the cyanobacteria.

The simple filamentous genus *Desertifilum* was originally described from India's Thar desert crusts with *D. tharense* as the type species (Dadheech et al. 2012). Since then, three additional species have been described: *Desertifilum fontinale* from a warm spring in Kenya (Dadheech et al. 2014), *Desertifilum salkalinema* from an alkaline pool in Zhejiang Province, China (Cai et al. 2017), and *Desertifilum dzianense* from a crater lake in Mayotte in the Madagascar Channel of the Indian Ocean (Cellamare et al. 2018).

Desertifilum species have not been recorded previously from México. However, we found a green mat of this genus intermixed with *Scenedesmus obtusiusculus* in a microbial mat collected from Poza Churince, a spring-fed pond in the Cuatro Cienegas basin (CCB), a system of springs, streams, and ponds located in the middle of the Chihuahuan Desert in the state of Coahuila in north-central Mexico. This environment is considered a stressful habitat, due to the lack of nutrients and high solar irradiance (Elser et al. 2005, Bonilla-Rosso et al. 2012, Prieto-Barajas et al. 2018). Previous work at CCB reported filamentous cyanobacteria as often the most abundant organisms within microbial mats and microbialites observed in the system (Domínguez-Escobar et al. 2011, Bonilla-Rosso et al. 2012). Despite their ecological and physiological significance in this stressful habitat, at present little is known about the taxonomic identity of mat-forming filamentous cyanobacteria present in this ecosystem (Bonilla-Rosso et al. 2012).

We and many other recent researchers have been part of an effort to describe new species of cyanobacteria, including cryptic taxa that are morphologically similar and phylogenetically close. We assumed at first that our isolate might be new to science because its ecology was very different from that of the four described species and it was on a separate continent. However, we have studied the phylogeny, ecology, and morphology of the Churince *Desertifilum* population and found it is very similar to the type species *D. tharense*, as well as to *D. salkalinema*, *D. dzianense*, and other *Desertifilum* isolates from Greece (NapGTcm17) and Mongolia (IPPAS B-1220). In a comparative analysis of all sequenced strains and published species in this genus, we conclude that the genus is over-speciated. We present evidence of this instance of over-reaching taxonomic exuberance in the present paper, along with recommendations for future α-level taxonomic work on cyanobacteria.

MATERIALS AND METHODS

Culture isolation. The Mexican strain was isolated from a microbial mat, collected from Poza Churince, a spring pond in Cuatro Cienegas basin. Strain Churince UAM-C/S02 was isolated from agar-solidified BG11 medium enrichment plates (Rippka et al. 1979). The plates were incubated at 25°C in a 12:12 h, light:dark regime in a culture chamber, and isolates were later maintained in the same growth conditions.

PCR amplification and sequencing of Churince strain. Genomic DNA was extracted using the UltraClean® Microbial DNA Isolation kit (MoBio Lab Inc, Carlsbad, CA, USA). A partial region of the 16S rRNA gene and 16S–23S ITS region was amplified using primers 27F after Neilan et al. (1997), and VRF1 after Wilmotte et al. (1993). PCR amplification was performed with the conditions used in González-Resendiz et al. (2018a). PCR products around 2.2 kb were gel purified using the Zymoclean® Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA), quantified and cloned with the CloneJET PCR Cloning Kit (ThermoFisher Scientific, Waltham, MA, USA). Three clones with long PCR products were sequenced at the Biotechnology Institute, UNAM sequencing facility using an Applied Biosystems (Foster City, CA, USA) model 3130xl Genetic Analyzer. Sequences were obtained, assembled, and corrected into 2,027 bp fragments using Bioedit software version 7.0.9.0 (Hall 1999). Only one ribosomal operon was evident in all *Desertifilum* strains, containing both $tRNA^{Ile}$ and $tRNA^{Ala}$ genes in the 16S–23S ITS region.

Phylogenetic analyses. A total of 102 sequences were chosen for analysis, including the consensus sequence newly obtained by this work and of the other species of this genus, as well as representative sequences of the main groups of oscillatorian cyanobacteria available in GenBank. *Gloeobacter violaceus* NR074282 and FR798924 was chosen as outgroup. Multiple sequence alignment was performed in MAFFT (Katoh et al. 2002) and minor changes were done manually with PhyDE® version 0.9971 (Müller et al. 2010).

Bayesian inference (BI), maximum likelihood (ML), and maximum parsimony (MP) analyses were performed using partial 16S rRNA gene sequences containing a maximum of 1,192 characters, which contained the closest relatives from GenBank. BI analysis was conducted with MrBayes XSDE V3.2.6 (Ronquist et al. 2012) through the CIPRES Science Gateway (Miller et al. 2011), applying the GTR+G+I model of nucleotide substitutions; a total of 50 million generations were run. The maximum parsimony analysis (MP) was inferred in Mega version 6 (Tamura et al. 2013) and maximum likelihood analysis (ML) in PhyML 3.0 (Guindon et al. 2010), bootstrapping with 1,000 replicates was conducted for both of the latter analyses. Phylogenies were visualized in FigTree version 1.4.2 and post-edited in Adobe Illustrator CS5 version 15.1.1. For an uncollapsed tree, see supplemental materials (Fig. S1 in the Supporting Information).

To resolve the relationships of the strains within *Desertifilum*, an alignment of the ITS regions was made using a combination of ClustalW (EMBL-EBI 2013, Cambridgeshire, UK) and manual alignment utilizing secondary structure of conserved domains. A heuristic search was conducted in PAUP

(Swofford 2003) utilizing parsimony as the optimality criterion, with multrees = yes, branch-swapping algorithm = TBR, gapmode = newstate, steepest descent = no, and nreps = 10,000. Bootstrap support was based on running 10,000 replicates. The same alignment was modified to add a scoring of the indels in the ITS (1 = base present, 0 = base absent), and the mixed data alignment was then analyzed with MrBayes. Two runs of eight Markov chains were executed for 122 thousand generations with default parameters, sampling every 100 generations to achieve a final average standard deviation of split frequencies below 0.01. Posterior probabilities from the Bayesian analysis and bootstrap values from the first two analyses were mapped on to the Bayesian tree.

The range of 16S rRNA gene divergence values among sequences was calculated using uncorrected "p" distances in Mega V6. Percent dissimilarity among aligned 16S–23S ITS regions was calculated as 100 × p-distance. The secondary structures of the 16S–23S ITS region were determined using Mfold version 2.3 (Zuker 2003), adjusted by comparative analysis of the secondary structures within *Desertifilum* and with related taxa in the topology of the tree based on 16S rRNA. We selected all available representatives of *Desertifilum*. The full structure of the 3′ end of the 16S–23S ITS was estimated using the 23S-5S ITS region from strain IPPAS B-1220, so that the V3 and D5 helices could be determined . ITS figures and phylogenetic analyses were illustrated and manually corrected in Adobe Illustrator CS5 version 15.1.1.

Morphology. Several samples containing *Desertifilum* were collected at Poza Churince and the *Desertifilum* mats were maintained in culture media so that natural material could be analyzed microscopically in the weeks following collection. A single strain was isolated from the enrichment samples and also analyzed microscopically. Dry and formalin (4%) preserved samples and semi-permanent slides were prepared from the strain and deposited in the FCME collection (Phycological Herbarium of the Science Faculty at Universidad Nacional Autónoma de México). Micrographs were acquired with an Olympus DP12 digital camera adapted to an Olympus CX51 microscope (DIC and bright-field). Morphological measurements (20–30 per character) were obtained from micrographs using SigmaScan© automated image analysis software (Jandel Scientific, Sausalito, CA, USA). Transmission electronic microscope (TEM) images were obtained in a JEOL model JEM-1200 EXII microscope at the Institute of Cellular Physiology UNAM following the protocol described in González-Resendiz et al. (2013). Morphological description and identification was done in accordance with new and traditional reference works (Komárek and Anagnostidis 2005, Komárek and Johansen 2015), along with additional studies that describe populations of oscillatorian species (Dadheech et al. 2012, 2014, Cai et al. 2017, Hašler et al. 2017, Strunecký et al. 2017, Cellamare et al. 2018).

Morphological characteristics for six sequenced strains of *Desertifilum* apart from the Mexican strain were compiled from the publications in which they were described or treated (Dadheech et al. 2012, 2014, Bravakos et al. 2016, Cai et al. 2017, Sinetova et al. 2017, Cellamare et al. 2018). In some instances, the descriptions given were incomplete. Additional measurements were made from the figures provided to give a fuller understanding of the morphology of all strains so that comparisons could be made.

RESULTS

Genetic analyses of 16S rRNA gene sequence data. Our population clearly belongs to the *Desertifilum* lineage (Fig. 1). The phylogenetic tree resulting from the 16S rRNA gene analysis showed that the eight sequences of *Desertifilum* available in GenBank were placed in a well-supported clade. The phylogenetic analysis supports three different hypotheses: (i) there are three taxa, *D. tharense*, *D. fontinale*, and *D. dzianense*, (ii) there are two taxa, *D. tharense* (includes *D. fontinale*) and *D. dzianense*, and (iii) there is only one taxon, *D. tharense*. The sister taxon to *Desertifilum* is *Jacksonvillea*, and the two genera comprise the family Desertifilaceae (Hašler et al. 2017). Phylogenies based on the 16S rRNA gene have been found in the past to give ambiguous determinations with regards to species (Johansen et al. 2011, Perkerson et al. 2011, Vázquez-Martínez et al. 2018), so further evidence must be consulted to determine which hypothesis is most supported. The phylogenetic analysis does unequivocally support collapsing *D. salkalinema* into *D. tharense*, but even this conclusion needs to be tested with further evidence.

Genetic identities based on 16S rRNA gene sequence were all high in *Desertifilum* strains (Table 1). Currently, if genetic identities between two strains/populations of a genus of prokaryotes is <98.7%, it is considered strong evidence that the two strains/populations are separate species (Yarza et al. 2014). However, if genetic identity is above this cutoff, it should not be used as evidence that the two strains are the same species. By this criterion, *D. fontinale* should be considered a separate species from *D. dzianense* since genetic identity between the two taxa is only 98.4%. The dilemma with this criterion is that both *D. dzianense* and *D. fontinale* have >98.7% genetic identity with all other strains (*D. tharense* and *D. salkalinema*). Based on this criterion, we can conclude that more than one species exists (rejecting the third hypothesis based on the phylogenetic analysis), but we cannot conclude whether we have two, three, or more species. However, the 100% genetic identity of *D. salkalinema* and two strains of *D. tharense* (including the reference strain upon which the holotype is based) strongly suggests that *D. salkalinema* should be subsumed into *D. tharense*.

Genetic analyses of 16S–23S ITS sequence data. Based on the draft genome of *Desertifilum* sp. IPPAS B-1220 (=*D. tharense*), a full reconstruction of the estimated secondary structure of the leader, 16S–23S ITS, and 23S-5S ITS was possible (Fig. 2). This secondary structure diagram showed a unique branched structure to the V3 helix, as well as structure of the D5 helix which pairs the 16S–23S ITS with the 23S-5S ITS. The ITS regions of all *Desertifilum* strains were remarkably similar. The conserved domains of five putative *D. tharense* strains were identical in length (no indels), and differed from *D. dzianense* by one additional nucleotide in the D1-D1′ helix. The conserved domains of *D. dzianense* and *D. fontinale* were identical in length in most regions (Table 2). *Desertifilum fontinale* showed the greatest deviation in domain length, particularly in the V2 helix, which

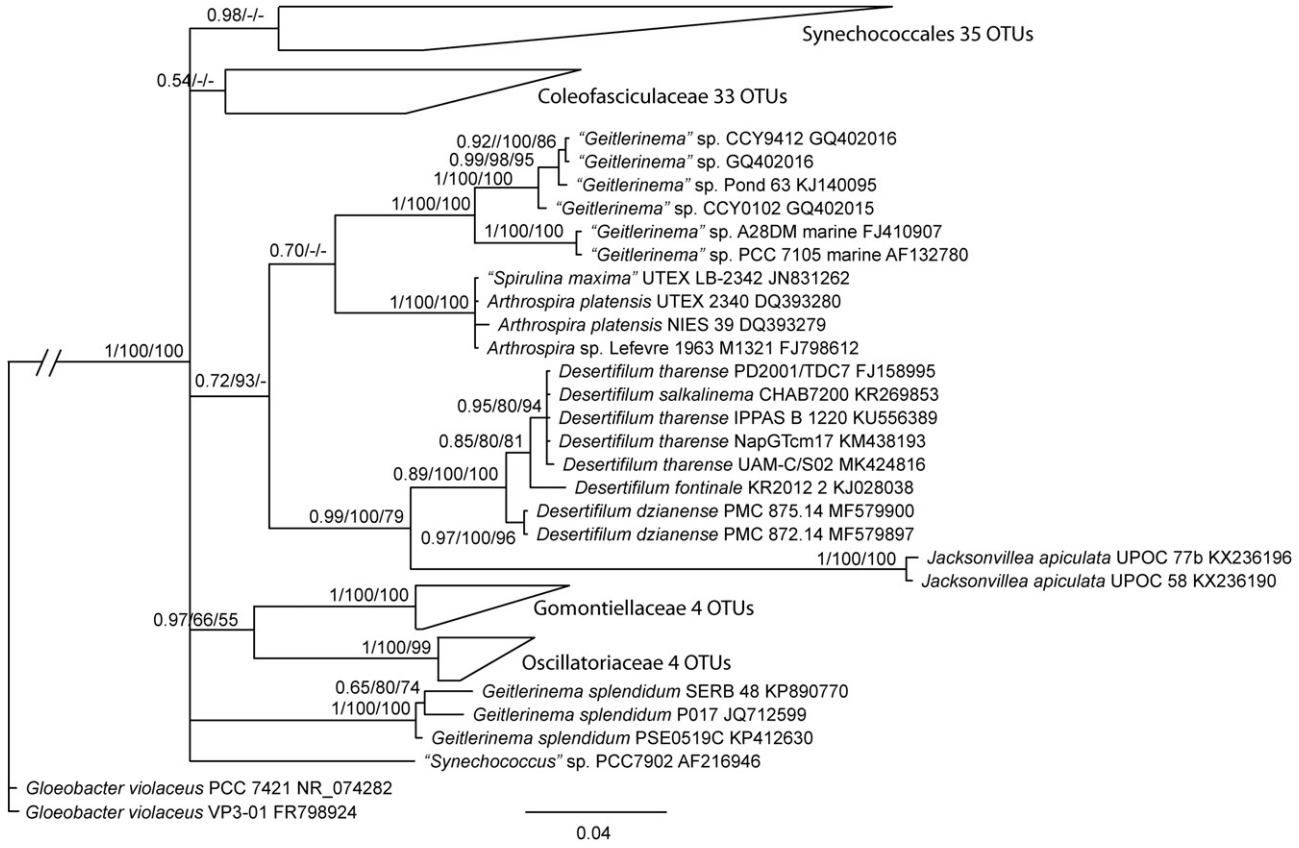

FIG. 1. Bayesian Inference Analysis based on 16S rRNA sequences of 102 sequences, with support values for BI/ML/MP mapped on to the nodes. The cut-off values for bootstrap and probability are 50 and 0.5, respectively, with lower values indicated by "–." Taxa in quotation marks (e.g., "*Geitlerinema*" sp.) are taxa requiring revision.

TABLE 1. Percent genetic identity of strains based on 16S rRNA gene sequence. Identities <98.7% are considered strong evidence for considering compared strains to be in different species, while identities <94.5% are considered to be strong evidence for considering compared strains to be in different genera (Yarza et al. 2014).

	Strain	1	2	3	4	5	6	7	8
1	*Desertifilum tharense* PD2001/TDC7 (Type) FJ158995								
2	*D. salkalinema* CHAB7200 KR269853	100							
3	*D. tharense* NapGTcm17 KM438193	100	100						
4	*D. tharense* UAM-C/S02 MK424816	99.9	99.9	99.9					
5	*D. tharense* IPPAS B/1220 KU556389	100	100	100	99.9				
6	*D. dzianense* PMC 875.14 MF579900	98.9	98.9	98.9	98.8	98.9			
7	*D. fontinale* KR2012/2 KJ028038	98.9	98.9	98.9	98.8	98.9	98.4		
8	*Geitlerinema splendidum* CCALA1004 PSE0519C KP412630	91.1	91.7	91.7	91.6	91.7	90.7	90.9	
9	*Jacksonvillea apiculata* UPOC 77b-2013 KX236194	89.4	89.9	89.9	89.9	89.9	89.4	90.1	88.6

was 21 nucleotides longer than the V2 helices in all other strains.

The secondary structures of the 16S–23S ITS region were also highly similar among strains. The Box-B helix, Box-A, D4, and D5 were identical in sequence in all strains, and the secondary structures of the Box-B helix and V3 helix were also identical, with the exception of the terminal loop of the V3 helix which had three transversion mutations in *D. dzianense* and one deletion in *D. fontinale*. The D1-D1′ helix and V2 helix, however, did show a number of differences in sequence and minimal difference in structure (Fig. 3). The D1-D1′ helices were highly similar in all strains of *Desertifilum* (Fig. 3, A–G), with *D. fontinale* showing a slightly enlarged bilateral bulge above the bend in the helix near the base (Fig. 3G). The differences in the D1-D1′ helix among the strains do not provide strong evidence for separate species. The V2 helix is generally the most variable helix in the 16S–23S ITS, but was very similar in structure among *D. tharense*, *D. salkalinema*, and *D. dzianense*. The V2 helix in

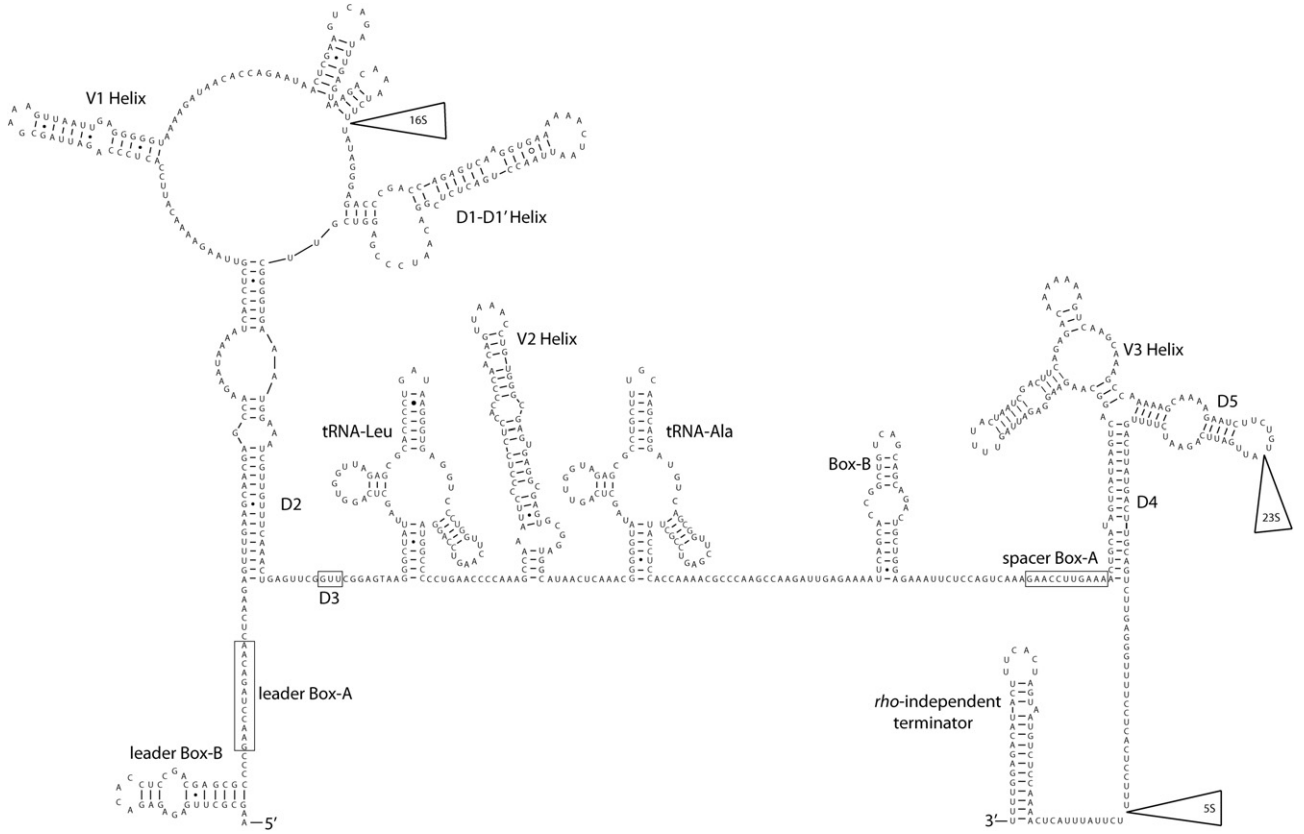

FIG. 2. Secondary structure of all non-rRNA regions of the ribosomal operon for *Desertifilum tharense* IPPAS B-1220, with position of the 16S, 23S, and 5S molecules indicated by triangles. The operon is complete from the leader where transcription starts to the rho-independent hairpin loop which terminates transcription.

D. fontinale, however, was 21 nucleotides longer and considerably different in structure (Fig. 3N). The V2 helix thus provides strong evidence that *D. fontinale* is a species distinct from all others.

A Bayesian Inference analysis of the alignment of the 16S–23S ITS, with bootstrap values from a maximum parsimony analysis (indels counted as fifth base), shows that *Desertifilum fontinale* is clearly phylogenetically distinct from all other species and strains (Fig. 4). *Desertifilum tharense*, *D. salkalinema*, and *D. dzianense* are in a single supported clade, but they do not show reciprocal monophyly, that is, their separation into distinct species is not supported by this evidence.

Differences in p-distance, or the percent equivalent (percent dissimilarity), in aligned ITS regions of members of the same genus has been recently used with consistent effectiveness to separate species (Erwin and Thacker 2008, Osorio-Santos et al. 2014, Pietrasiak et al. 2014, Johansen et al. 2017, Shalygin et al. 2017, González-Resendiz et al. 2018a,b, Mai et al. 2018, Vázquez-Martínez et al. 2018). The primary criterion is to have a discontinuity between percent dissimilarity of populations in the same species (average ~1.0% or less, all pairwise comparisons <3% dissimilarity) and populations representing separate species (>7% dissimilarity). When differences are between 3% and 7% the cutoff is not clear, but the rule of recognizable discontinuity can be used. Based on these criteria, *Desertifilum fontinale* is distinct from all other species/strains, with genetic dissimilarity >3% in all instances (Table 3). The ITS regions of all other strains have an average percent dissimilarity of 1.3%, with maximum percent dissimilarity of 3.11% between *D. dzianense* and one strain of *D. tharense* (Table 3). This last piece of genetic evidence strongly supports recognizing only two species in *Desertifilum*, *D. tharense,* and *D. fontinale*.

The combined genetic evidence is internally consistent with this conclusion. The 16S phylogeny is ambiguous in separating species in this genus, as is the genetic identity based on 16S rRNA sequence data. However, all tests based on the 16S–23S ITS region are in agreement and support recognition of just two species. Furthermore, the molecular evidence supports recognizing *Desertifilum salkalinema* and *D. dzianense* as later synonyms of *D. tharense*.

Morphological analysis. In the morphological comparison, our population (Figs. 5 and 6) and other *Desertifilum tharense* populations (Bravakos et al. 2016, Cai et al. 2017, Sinetova et al. 2017) correspond with the description of *D. tharense* from the Thar desert of India (Table 4). The principal morphological traits such as thallus appearance, filament shape, sheath color, trichome and cell size,

TABLE 2. Comparison of nucleotide lengths of conserved domains in the 16S–23S ITS region for *Desertifilum* strains. Differences in domain length are highlighted in gray. See Figure 2 for pictoral definition of domains.

	Leader	D1-D1′ helix	Spacer + D2	Spacer + D3 + Spacer	$tRNA^{Ile}$	V2 helix with spacers	$tRNA^{Ala}$	Spacer	Box-B helix	Spacer	Box-A	D4 + spacer	V3 helix	Spacer + D5
D. tharense PD2001/TDC17	8	61	34	18	74	77	73	30	33	17	11	19	60	23
D. salkalinema CHAB7200	8	61	34	18	74	77	73	30	33	17	11	19	60	23
D. tharense NapGTcm17	8	61	34	18	74	77	73	30	33	17	11	19	60	23
D. tharense UAM-C/S02	8	61	34	18	74	77	73	30	33	17	11	19	60	23
D. tharense IPPAS B/1220	8	61	34	18	74	77	73	30	33	17	11	19	60	23
D. dzianense PMC 872.14	8	62	34	18	74	77	73	30	33	17	11	19	60	23
D. fontinale KR2012/2	8	62	34	18	74	98	73	31	33	17	11	19	59	23

and apical cell morphology were very similar to those reported by Dadheech et al. (2012). Minor differences in size ranges exist among all strains, but clear morphological separation of *D. salkalinema* and *D. dzianense* from *D. tharense* is not evident. *D. fontinale* is also morphologically similar, but has the notable morphological difference of having cells shorter than wide to isodiametric, whereas all *D. tharense* strains have cells isodiametric to longer than wide. The morphological data are thus consistent with the molecular evidence; *D. tharense* (including *D. salkalinema* and *D. dzianense*) is a different species than *D. fontinale*. All strains of *Desertifilum* appear to be thermotolerant based on reported distribution data.

In order to recognize these two species in the future, the following emended descriptions are given for each.

Desertifilum tharense. Thallus a dark green, bright blue-green, or blackish green crust or mat of solitary or entangled filaments. Sheaths thin, colorless, diffluent to firm, often lacking, rarely extending beyond the apical cell. Filaments motile, straight to flexuous, not distinctly constricted at the crosswalls, attenuated at the ends, 1.5–3.9-(5.5) μm wide. Cells cylindrical to barrel shaped, isodiametric to longer than wide, homogenous or with minute granules, 2.4–8.6 μm long. Apical cells bluntly conical or rounded, attenuated when mature, often slightly bent or hooked, sometimes with protuberances.

Notes: Dadheech et al. (2012) in their original description stated that trichomes could reach a diameter of 5.5 μm. No specimens this large were shown in their figures, and this diameter is considerably wider than any specimens observed by us or others.

Dadheech et al. (2012) did not observe the lateral protuberances on the apical cells, but the feature was observed in two other strains and in *Desertifilum fontinale*. We consider this to be an occasionally occurring feature brought on by unknown environmental conditions. It is a distinctive characteristic of this genus, but similar protuberances have been reported in *Oculatella* (Zammit et al. 2012, Osorio-Santos et al. 2014).

Desertifilum fontinale. Thallus a dark blackish green mat of solitary or entangled filaments. Sheath very thin or absent, hyaline, sometimes visibly extending beyond end cell. Filaments motile, straight to flexuous, not or slightly constricted at crosswalls, 2.8–4.1 μm wide. Cells cylindrical, shorter than wide to isodiametric, homogenous or with minute granules, 1.7–4.5 μm long. Apical cells bluntly conical, attenuated, often bifurcated due to a relatively long, lateral protuberance emerging at a wide angle from the trichome axis at the base of the cell.

Notes: In the original description of the species, Dadheech et al. (2014) show trichomes of two distinct morphologies. A few trichomes are 6.2–6.9 μm wide, whereas all others are 2.8–4.1 μm wide. The

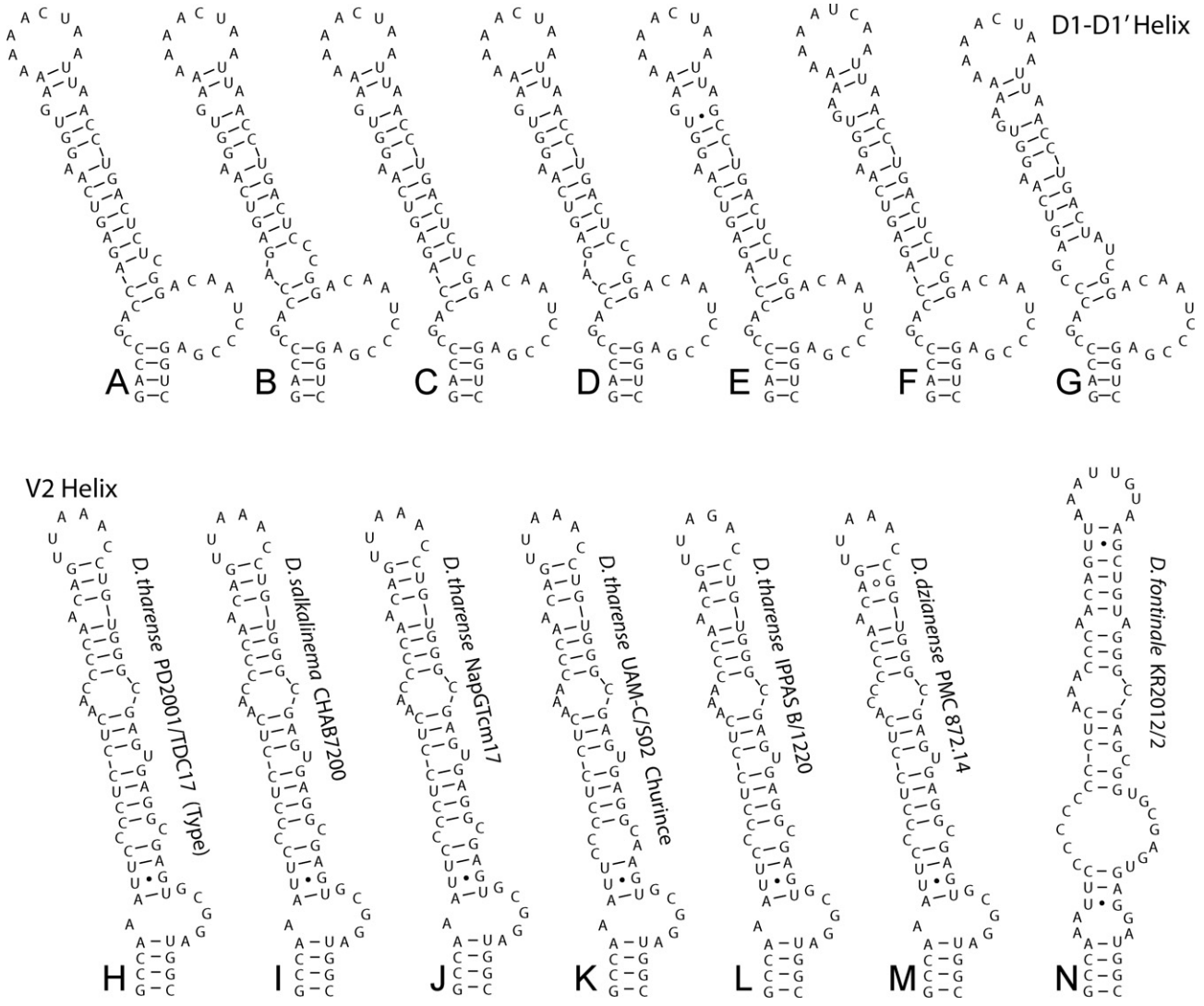

FIG. 3. Secondary structures for the D1–D1′ and V2 helices in the conserved regions of the 16S–23S ITS region for all sequenced *Desertifilum* strains. Strains are labeled in the V2 helix row, with D1-D1′ helices directly above each labeled figure belonging to the same strain.

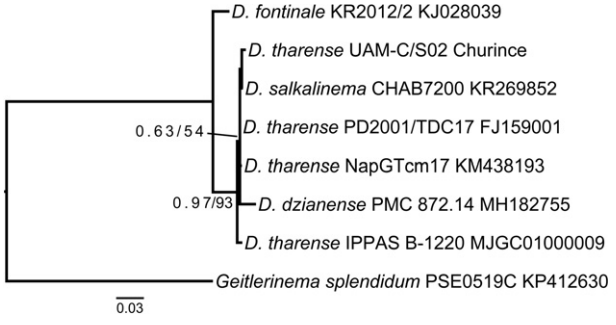

FIG. 4. Bayesian Inference Analysis of aligned ITS regions for all sequenced *Desertifilum* strains, with values of posterior probability indicated at nodes along with bootstrap support values from maximum parsimony analysis.

TABLE 3. Percent dissimilarity (100 × uncorrected p-distance) among aligned ITS regions of *Desertifilum* strains.

Strain	1	2	3	4	5	6
1 *D. tharense* PD2001/TDC17 FJ159001						
2 *D. salkalinema* CHAB7200 KR269852	0.18					
3 *D. tharense* NapGTcm17 KM438193	0.18	0.37				
4 *D. tharense* UAM-C/S02 Churince	0.73	0.55	0.91			
5 *D. tharense* IPPAS B-1220 MJGC01000009	0.91	1.10	1.10	1.28		
6 *D. dzianense* PMC 872.14 MH182755	2.20	2.38	2.38	2.56	3.11	
7 *D. fontinale* KR2012/2 KJ028039	3.29	3.47	3.47	3.29	3.47	4.92

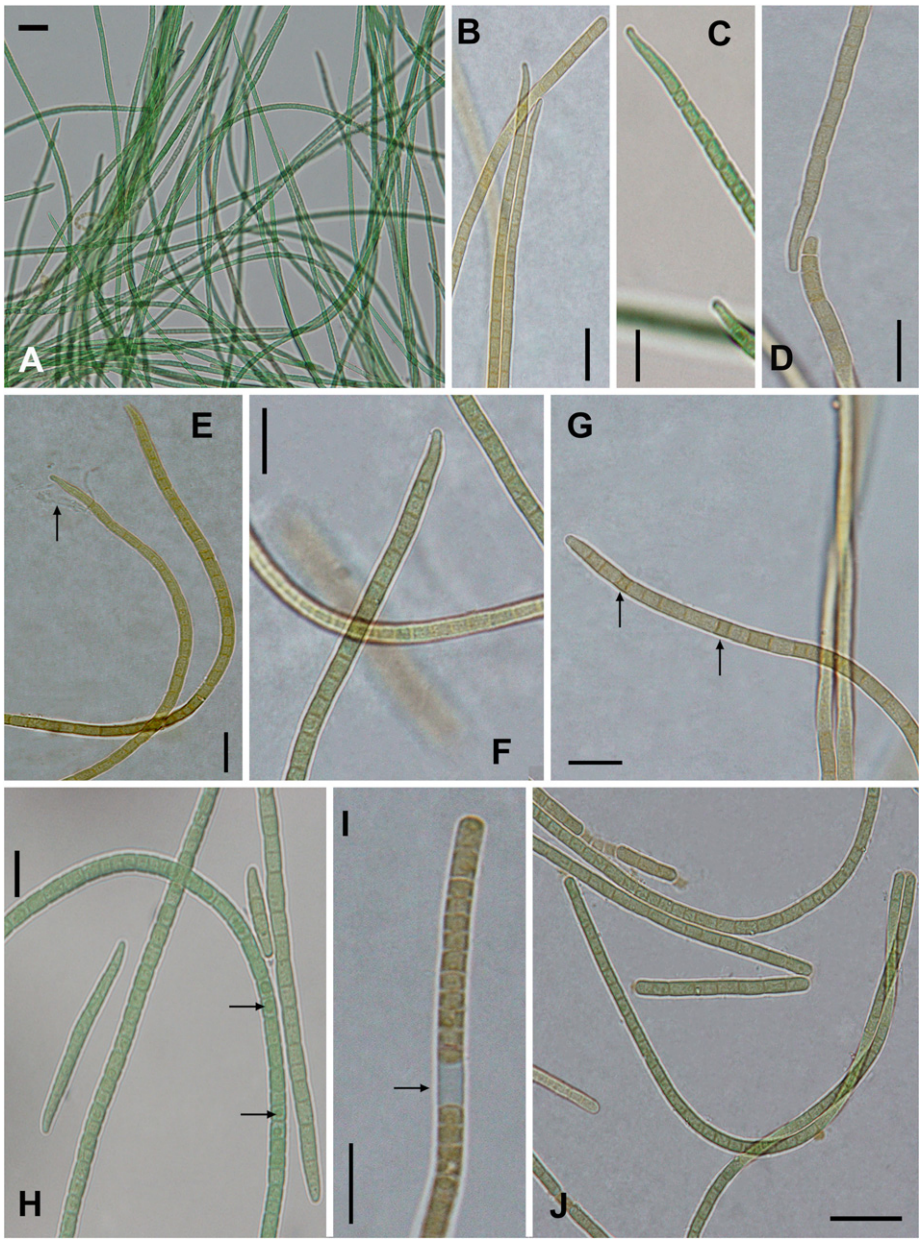

FIG. 5. Morphological traits of *Desertifilum tharense* Poza Churince population. (A) thallus showing parallel arrangement and entangled filaments. (B–D, F) variation in apical cell morphology. (E) funnel-like sheath at end of trichome (arrow). (G) biconcave necridic cells (arrows). (H) isopolar hormogonia, and filaments showing centroplasm (arrow). (I, J) disintegration of trichomes in hormogonia without necridic cells: arrow showing a yellowish sheath. Scale bar (A) = 20 μm, (B–J) = 10 μm. [Color figure can be viewed at wileyonlinelibrary.com]

widened trichomes are not attenuated and do not have elongated apical cells. We consider it likely that these trichomes are a contaminant in the culture, and until independently verified, should be excluded from the description of the species as we have done here.

DISCUSSION

The advent of both transmission electron microscopy and sequence data (particularly 16S rRNA gene and 16S–23S ITS) led to the realization that there were many cyanobacterial genera and species that were comprised of phylogenetically separate lineages that required taxonomic recognition if taxonomy was to accurately reflect evolutionary history (Honda et al. 1999, Robertson et al. 2001). TEM preceded DNA sequencing, but provided insights into cell division and thylakoid structure, which were significant for recognition of higher-level taxonomy and splitting and revision of many existing genera (Anagnostidis and Komárek 1985, 1988,

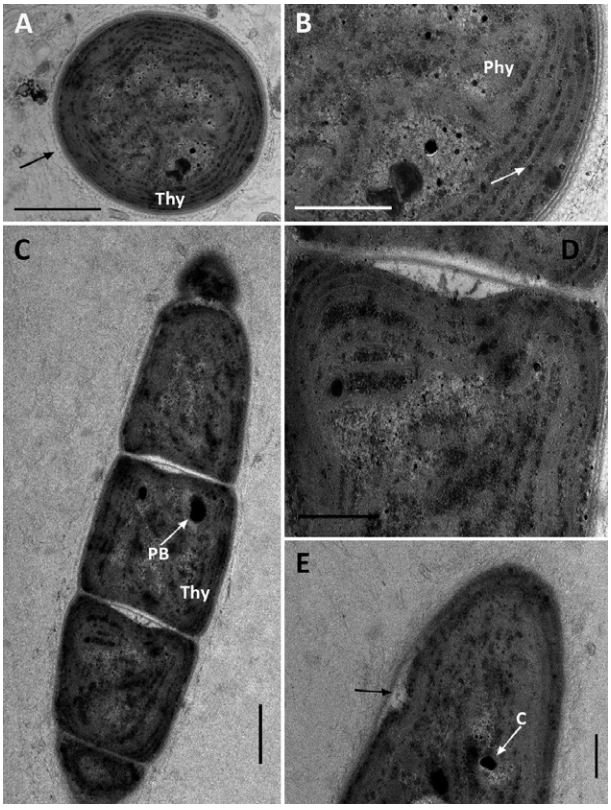

FIG. 6. Ultrastructural traits of *Desertifilum tharense* Churince population. (A, B) transverse section of trichome, (A) details of parietal thylakoid arrangement and diffluent sheath (arrow); (B) detail of phycobilisome in thylakoid membrane. (C–E) Cells in longitudinal section, (C) details of hormogonia and apical cell shape, (D) peripheral thylakoid arrangement; (E) wall constriction in apical cell (arrow). Thy= thylakoids, Phy= phycocyanine body, PB= polyphosphate body, C= carboxysome. Scale bar (A) = 1 μm, (B–E) = 500 nm.

1990, Komárek and Anagnostidis 1986, 1989, Hoffmann et al. 2005). It has been DNA sequencing, phylogeny, and other analyses, however, that has really led to an explosion of new taxa, with over 50 new genera of cyanobacteria being described in the last 20 years and many more species (Komárek et al. 2014). While genera are rather unambiguously defined (a collection of species that forms a monophyletic taxon, see González-Resendiz et al. 2018b), the discussion over what constitutes a species is still a matter of serious debate (Johansen and Casamatta 2005, Komárek 2010, Dvořák et al. 2015). Some researchers feel that phenotypic distinctions should exist between species (Mishler and Theriot 2000, Komárek 2010), while others do not feel this restriction should apply (Johansen and Casamatta 2005, Osorio-Santos et al. 2014). Others would define species based on molecular dissimilarity alone (Wayne et al. 1987, Stackebrandt and Goebel 1994, Herdman and Rippka 2018). Morphologically indistinguishable species (cryptic species) have recently been described based on a combination of molecular and ecophysiological separation (Osorio-Santos et al. 2014, Pietrasiak et al. 2014). We support recognition of narrowly defined species, including cryptic species, and are part of the collective movement to describe new species based on a total evidence approach that at times de-emphasizes morphological separation (Perkerson et al. 2011, Bohunická et al. 2015, Shalygin et al. 2017, Becerra-Absalón et al. 2018, González-Resendiz et al. 2018b, Mai et al. 2018). It is possibly ironic that we are the ones to propose that the partitioning of the cyanobacterial genus *Desertifilum* into four cryptic species has gone too far.

The basis for separation of *Desertifilum salkalinema* from *D. tharense* was that *D. salkalinema* occupied a different ecological niche. *D. salkalinema* showed optimal growth at 1% salinity as opposed to the 0% salinity optimum of *D. tharense*. However, cultures of both taxa grew at 0%–3% salinity, without tolerance for higher salinities (Dadheech et al. 2012, Cai et al. 2017). *Desertifilum salkalinema* was tolerant of a wide range of alkalinities, but *D. tharense* was not tested. Furthermore, *D. tharense* was reported to have gas vesicles, but *D. salkalinema* lacked gas vesicles. However, examination of the TEM micrographs of *D. tharense* shows that what were considered to be gas vesicles by the authors were actually cellular granules. Minor differences in cell dimensions were also reported (Table 4). The cyanobacteria were grown in different culture media under different light regimes. While desert soils are undoubtedly a different ecological niche than alkaline pools, we feel that the evidence for lineage separation is weak. Biotope differences would make good contributing evidence for separation of species if the separation was warranted by the molecular data.

The basis for separation of *Desertifilum dzianense* from the other three described species was stated as sequence differences and secondary structure differences in the D1-D1′, V2, and V3 helices. Comparative analysis was not applied to secondary structure estimation as it was in this work (Figs. 2 and 3), but the authors were correct in their reporting of genetic identity (16S rRNA) and percent dissimilarity (ITS). While they did not state it as a diagnostic feature, they also pointed out that *D. dzianense* lacked aerotopes. They did not point out the differences in ecological niche between desert soil crust and saline-alkaline environment, nor the distinctiveness of the stromatolite formations from which *D. dzianense* was isolated.

A stronger case could have been made in both instances that ecological niche was significant enough as a difference in these two distinctive populations that they should be recognized taxonomically. It is the lack of molecular differentiation that is problematic. There is not strong evidence that these are two separate lineages that have a long history of adaptive radiation into their respective environments. The recognition of these species is in

TABLE 4. Ecological and morphological characteristics of all *Desertifilum* strains. Dimensions of published strains based both on dimensions reported in the papers as well as measurements made from examination of figures in the publications. In some instances, dimensions were expanded from what was reported in descriptions, in particular the ranges (some papers only reported mean sizes).

	Desertifilum tharense						
Taxon	*D. tharense* type (Dadheech et al. 2012)	*D. salkalinemum* (Cai et al. 2017)	*Desertifilum* NapGTcm17 (Bravakos et al. 2016)	*D. tharense* Churince UAM-C/S02	*Desertifilum* IPPAS 1220 (Sinetova et al. 2017)	*Desertifilum dzianense* (Cellamare et al. 2018)	*Desertifilum fontinale* (Dadheech et al. 2014)
Ecology	Thar desert, soil crust, India	Saline alkaline pool, China	Thermal spring of Nea Apolonia, Macedonia	Chihuahuan Desert, microbial mat at Churince, spring pond, México	Warm lake Shar-Nuur, Mongolia	Stromatolites, saline-alkaline crater-lake Dziani Dzaha, Mayotte	Warm spring, Lake Bogoria, Kenya
Thallus	Black and dark-blue crusts. Solitary or entangled filaments	Solitary filaments entangled pale to bright blue-Green	ND	Usually forming thin mats of solitary to entangled filaments. Pale to bright blue-green	Solitary or in loose bundles, intertwined	Dark green or blackish green, forming mats or empty balls in culture, solitary or entangled	Solitary or entangled
Filament description	Straight with curved terminal parts, not constricted or slightly constricted at cross-wall, attenuated at the ends. Motile	Straight to wavy, not or slightly constricted, attenuated at the ends. Motile	Slightly constricted, slightly attenuated and bent at the ends. Motile	Straight to wavy, not or slightly constricted, attenuated at the ends. Motile	Not or slightly constricted, often attenuated at the ends. Motile	Not or slightly constricted, more or less straight or wavy. Motile	Straight to slightly wavy, not or slightly constricted. Motile
Filament width (μm)	2.0–4.0	2.0–3.1 (2.08 ± .80)	1.5–2.1-(3)	1.7–3.9	2.0–3.0–(3.5)	4.5–5.5	2.76–4.14
Sheath	Thin, colorless	Thin, colorless	Thin colorless	Thin, hyaline, firm; sometimes funnel shaped at extremes	Thin colorless	Hyaline, thin and firm	Very thin, hyaline
Cell shape	Cylindrical, isodiametric to elongate, cell content with granules (aerotopes?)	Cylindrical, elongate, cell content homogenous	Elongate	Cylindrical, isodiametric to elongate	Barrel shaped, cylindrical, isodiametric to elongate	Mainly longer than wide (rarely isodiametric)	Isodiametric to shorter than wide.
Cell length/width (μm)	3.3–4.2/2.0–4.0 apical cells 5.6–12 long, narrowing to 1.6 wide	3.3–5.5 (5.14 ± 0.51)/1.9–2.14 (2.08 ± .80)	4–7.3/1.5–3	2.4–8.6/1.7–3.9 apical cells up to 15 long, narrowing to 0.9 wide	2.5–5.4 (3.8 ± 1)/2.5–3.5 (2.8 ± 0.3)	Cells 4–7.7/3.5–4.5 apical cells 8–12.5/2.4–4	1.7–4.5/2.76–4.41 apical cells 5.9–6.2/2–2.8
Apical cell shape	Conical, rounded, attenuated	Slightly elongated, rounded, attenuated or not	Rounded, attenuated	Straight to curved, rarely rounded, slightly hooked or bent, attenuated	Rounded or conical, with extrusions (bifurcated)	Conical-rounded, slightly hooked or bent, with extrusions (bifurcated)	Conical-rounded, with extrusions (bifurcated)

agreement with a position made in Johansen and Casamatta (2005) that any autapomorphy could be sufficient for separation of lineages into different species, including ecological difference. In the present case, we find this difficult to accept because (i) we know of no instance in which two cyanobacterial species share 100% genetic identity, (ii) we know of no instance in which two cyanobacterial species have less than 3% dissimilarity in their 16S–23S ITS sequence, and (iii) the ITS secondary structure differences are minor and similar to intraspecies differences reported in other studies (Řeháková et al. 2007).

Differences in morphology, ecology, and physiology could overcome the evidence of genetic identity in species recognition if the differences were more convincingly demonstrated. What is needed is comparison of the reference strains in common garden experiments. *Desertifilum* strains were grown in BG-11, Zarrouk, and Z8 medium under different light regimes, with stage of growth not indicated. If these strains were grown in the same medium under identical conditions, and measured at similar times after transfer into fresh medium, differences in morphology would be more convincing. If all four *Desertifilum* species were tested physiologically using identical conditions, then variable growth in media of different salinities, different alkalinities, and different temperatures could be used as a means to establish phenotypic differences worthy of taxonomic recognition. Furthermore, a similar set of characterizations is needed. Reporting size dimensions as a mean ± SD without giving ranges (Dadheech et al. 2014, Cai et al. 2017, Sinetova et al. 2017) is insufficient for comparative morphology as size dimensions can vary with differences in environmental conditions. Details regarding sheath, apical cell morphology (including dimensions), and length at which cells undergo cell division, could also be consistently reported and could potentially provide greater morphological evidence. We consider it possible that *D. salkalinema* and *D. dzianense* could be separate species, we just find the evidence presented so far incomplete and unconvincing in light of the molecular similarity. The description of these two species with this slight evidence could lead to an unmanageable explosion of species epithets that likely would be rejected by ecologists. If cryptic species are to be described based on ecology alone, the evidence must be more clearly and comprehensively presented. With the high similarity in sequences of both the 16S rRNA gene and associated 16S–23S ITS region, scientists could reasonably argue that these strains represent locally adapted populations that have not been separate lineages for any significant length of time.

Recommendations for recognition of cyanobacterial species. In making a decision to place a novel strain or population in an existing or new species, we recommend that researchers pose several questions to themselves: (i) Does the strain or population fit an existing species in terms of morphology and ecology (including species that have not been sequenced)? New species should not be named if they closely fit described species, even though those species are not sequenced. Taxonomy should not be conducted in a vacuum that excludes the historical literature. For example, *Phormidium californicum* is an exact match to *D. tharense* morphologically, and was found on soils. It is very likely synonymous with *D. tharense*, but was not mentioned by the authors in their manuscript describing the new genus and species, a serious omission. (ii) If the strain is a match to a previously described taxon in morphology and ecology, does molecular evidence indicate that it represents a separate lineage that maintains its identity over space and through time (i.e., fits the definition of an evolutionary species)? Molecular evidence, particularly the changes in ribosomal genes, which are theoretically not subject to directional selection, can be used to establish temporal separation of lineages. (iii) Is the strain worthy of taxonomic recognition, a requirement of the phylogenetic species concept of Mishler and Theriot (2000) and the monophyletic species concept of Johansen and Casamatta (2005)? These are the questions reviewers and subsequent readers will ask, and they should be anticipated.

There are several pragmatic criteria that can be used to convince oneself and others that a strain or population represents a novel lineage worthy of species recognition. If the species does not fit any described taxon, and if its 16S rRNA genetic identity is <98.7% to any named sequenced species, the evidence is strong that it represents a new species. If it has genetic identity >98.7%, other evidence of lineage separation is needed. Differences in secondary structure of conserved domains of the 16S–23S ITS region can be used as autapomorphies, but differences should be supported by other evidence, such as minor morphological differences, major ecological differences, or demonstrated differences in other genes. The most recent pragmatic criterion that has not yet failed to clearly separate species when intraspecies variation is known is the percent dissimilarity of aligned ITS regions in orthologous ribosomal operons (Erwin and Thacker 2008, Osorio-Santos et al. 2014, Pietrasiak et al. 2014, Bohunická et al. 2015, Johansen et al. 2017, Shalygin et al. 2017, Vázquez-Martínez et al. 2018, Becerra-Absalón et al. 2018, González-Resendiz et al. 2018b, Mai et al. 2018, Mareš et al. 2019). Phylogenetic analyses of aligned orthologous ITS regions can also be useful in demonstrating reciprocal monophyly within species (Perkerson et al. 2011, Vázquez-Martínez et al. 2018). However, the existence of multiple ribosomal operons in most genera of cyanobacteria means that care must be taken to obtain orthologous operons so that ITS comparisons are valid. This means that ITS amplicons must

be cloned before sequencing, and multiple clones must be sequenced to obtain as many operons as possible. Draft genomes often do not recover all ITS regions due to problems in assembly, but they can be useful in establishing the number of ribosomal operons in the genome (Johansen et al. 2017).

In addition to the molecular evidence, morphological, physiological, and biochemical evidence is very helpful, but as indicated in this study, all relevant strains must be treated in experiments elucidating these characters as they are all subject to variation due to environmental conditions. Characterizing morphology in natural material, exponential growth phase in culture, and senescent growth phase in culture is highly desirable where possible.

Finally, there have been problems in taxonomic descriptions of new taxa in which nomenclatural rules have not been followed, such that the new taxa are invalid. As a case in point in *Desertifilum*, *D. salkalinema* is invalid due to inappropriate designation of type (Cai et al. 2017). The type locality was designated as the type, rather than a herbarium preparation, preserved preparation, or cryopreserved culture, all of which can serve as valid type materials. Furthermore, the type should be set off in the protologue by saying "typus," "holotypus," or the modern equivalent (type, holotype). Authors of new taxa should read Article 40 of the International Code of Nomenclature for algae, fungi, and plants to ensure that type materials are properly prepared and accessioned (Turland et al. 2018), as problems with type designation are the most common errors made in description of new cyanobacterial taxa.

Recommendations for reviewers of cyanobacterial taxonomic papers. With the plethora of taxonomic papers on cyanobacteria that are coming out with ever faster rapidity in diverse journals, reviewing such papers becomes a difficult task. Certain standards for reviewers could be adopted when evaluating papers that describe new genera and species. We recommend that a more rigorous review process be followed than in some instances in the recent past. Reviewers should ask the following questions during their review: (i) Did the authors show awareness and study of the existing taxonomic literature by reporting the morphologically and ecologically closest taxa and making convincing diagnoses that separate their new taxon from these existing taxa? (ii) Did the authors provide convincing evidence, including molecular separation in the 16S rRNA gene and 16S–23S ITS region (or other genes), that their new taxon is a molecularly distinct lineage? (iii) Did the authors truly use a total evidence approach in which all lines of evidence are used to make taxonomic decisions? (iv) Did the authors provide a description that is detailed enough to evaluate whether it is truly different from existing taxa and will serve to distinguish future similar taxa? (v) Did the authors show awareness of the codes of nomenclature, specify the code under which they are operating, and then follow those nomenclatural rules?

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Uncollapsed tree from Bayesian inference analysis.