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TAXONOMIC RESOLUTION OF THE GENUS *CYANOTHECE* (CHROOCOCCALES, CYANOBACTERIA), WITH A TREATMENT ON *GLOEOTHECE* AND THREE NEW GENERA, *CROCOSPHAERA*, *RIPPKAEA*, AND *ZEHRIA*[1]

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The systematics of single-celled cyanobacteria represents a major challenge due to morphological convergence and application of various taxonomic concepts. The genus *Cyanothece* is one of the most problematic cases, as the name has been applied to oval-shaped coccoid cyanobacteria lacking sheaths with little regard to their phylogenetic position and details of morphology and ultrastructure. Hereby we analyze an extensive set of complementary genetic and phenotypic evidence to disentangle the relationships among these cyanobacteria. We provide diagnostic characters to separate the known genera *Cyanothece*, *Gloeothece*, and *Aphanothece*, and provide a valid description for *Crocosphaera* gen. nov. We describe two new genera, *Rippkaea* and *Zehria*, to characterize two distinct phylogenetic lineages outside the previously known genera. We further describe 13 new species in total including *Cyanothece svehlovae*, *Gloeothece aequatorialis*,

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***G. aurea*, *G. bryophila*, *G. citriformis*, *G. reniformis*, *Gloeothece tonkinensis*, *G. verrucosa*, *Crocosphaera watsonii*, *C. subtropica*, *C. chwakensis*, *Rippkaea orientalis*, and *Zehria floridana* to recognize the intrageneric diversity as rendered by polyphasic analysis. We discuss the close relationship of free-living cyanobacteria from the *Crocosphaera* lineage to nitrogen-fixing endosymbionts of marine algae. The current study includes several experimental strains (*Crocosphaera* and "*Cyanothece*") important for the study of diazotrophy and the global oceanic nitrogen cycle, and provides evidence suggesting ancestral N_2-fixing capability in the chroococcalean lineage.**

Key index words: **16S rRNA; coccoid cyanobacteria; diazotrophy; endosymbiosis; morphology; multilocus analysis; rRNA ITS region; ultrastructure**

***Abbreviations*: ACOI, Coimbra Collection of Algae; ATCC, American Type Culture Collection; CCALA, Culture Collection of Autotrophic Organisms of the Institute of Botany, Czech Academy of Sciences; CCAP, Culture Collection of Algae and Protozoa; LM, light microscope; PCC, Pasteur Culture Collection; SAG, Culture Collection of Algae at the University of Göttingen, Germany; WH, Woods Hole Oceanographic Institution**

The taxonomic system of cyanobacteria has been in the process of undergoing extensive revision in the past two decades (Komárek et al. 2014). Phylogenetic analysis of molecular data, particularly 16S rRNA sequence data, has demonstrated that many genera are polyphyletic when old morphological concepts are applied. The recent trend in taxonomy in cyanobacteria is to split existing genera into multiple, more narrowly defined genera so that genera are monophyletic. At the lowest taxonomic level, the phylogenetic species concept has been widely accepted as the most suitable framework for definition of cyanobacterial species (Johansen and Casamatta 2005, Osorio-Santos et al. 2014, Bohunická et al. 2015a). Moreover, gathering all available informative data on morphology, cell ultrastructure, ecology, physiology, or biochemical traits is recommended as the best practice for identification of established taxa. This methodology, also called the "polyphasic approach" or "total evidence approach" (Johansen and Casamatta 2005, Dvořák et al. 2015), has become the gold standard in cyanobacterial taxonomy.

The polyphasic approach has recently been applied, in particular, to several important groups of heterocytous cyanobacteria (Fiore et al. 2007, Komárek and Zapomělová 2007, 2008, Řeháková et al. 2007, Wacklin et al. 2009, Zapomělová et al. 2009, 2012, Hrouzek et al. 2013, Johansen et al. 2014, Kaštovský et al. 2014, Bohunická et al. 2015a, Berrendero-Gomez et al. 2016, Saraf et al. 2018) and numerous non-heterocytous filamentous types (Siegesmund et al. 2008, Strunecký et al. 2011, 2013, 2014, Casamatta et al. 2012, Engene et al. 2012, 2013, Johansen et al. 2014, Mühlsteinová et al. 2014a,b, 2018, Osorio-Santos et al. 2014, Heidari et al. 2018). The coccoid cyanobacteria fall into five major groups, the Gloeobacterales, the Synechococcales, the Pleurocapsales, the Chroococcidiopsidales, and the Chroococcales (Komárek et al. 2014, Mareš 2018). Some representative sequences of coccoid taxa in these five orders have been obtained, but taxonomic revisions and descriptions of new genera in these groups lag behind the progress made in the filamentous forms.

The non-baeocyte producing coccoids with fasciculated thylakoids mostly belong to the Chroococcales (sensu Komárek et al. 2014), which are probably the least studied major group of cyanobacteria. The members of this order remain taxonomically neglected and unclear, with the possible exception of the toxic bloom-forming genus *Microcystis*, which has received a higher level of attention due to its importance to human health (Harke et al. 2016). Chroococcales are often difficult to isolate into culture, have confusingly similar cellular morphologies, and typically lose their mucilage and colonial characteristics in culture, and this has likely contributed to misidentification of both species and genera represented in the culture collections of the world. Only a few studies have dealt with the modern taxonomy of genera such as *Chroococcus* (Kováčik et al. 2011), *Halothece* (Margheri et al. 2008), *Aphanothece* (Komárek et al. 2011), *Cyanobacterium*, *Synechocystis*, and *Geminocystis* (Korelusová et al. 2009). Recently, a new chroococcalean genus *Chalicogloea* was described from a cave habitat in Spain (Roldán et al. 2013). Other important and abundant, predominantly subaeropyhtic coccoid genera such as *Gloeothece*, *Gloeocapsa*, and *Cyanothece* have been little studied using the methods of molecular taxonomy (Nelissen et al. 1995, Castenholz 2001, Komárek et al. 2004, Ohki et al. 2008), and data enabling a comprehensive phylogenetic evaluation are still lacking.

For *Gloeothece*, a newly conserved generitype, *Gloeothece fuscolutea*, has recently been established by us (Mareš et al. 2013c) to avoid confusing the genus with the superficially similar, but primitive and thylakoid-lacking *Gloeobacter* (Mareš et al. 2013a,b). In the current concept, *Gloeothece* includes aquatic, subaerophytic, and aerophytic coccoid cyanobacteria with relatively wide (over ~2–3 μm) oval to cylindrical cells, forming gelatinous, sometimes colored colonies, in which the cells are irregularly distributed and possess individual mucilaginous envelopes. The molecular data available for a group of morphologically similar Chroococcales (*Gloeothece*, *Cyanothece*, *Gloeocapsa*, and *Aphanothece*) do not support the current assignment of names for the strains that are a source of these data (Castenholz et al. 2001, Ohki et al. 2008), and clarification of these

genera and the families containing them is sorely needed if a phylogenetically correct taxonomy is to be achieved.

Cyanothece, a particularly polyphyletic assemblage of strains, is in need of revision. As previously suggested by Komárek and Cepák (1998), the type species *C. aeruginosa* is characterized by its conspicuous cell ultrastructure, with widened peripheral thylakoids, continuing through the whole cell, in which they form a reticulate structure. Also, based on two 16S rRNA gene sequences, the genus seems to hold a phylogenetic position close to filamentous cyanobacteria of the Gomontiellaceae and *Komvophoron* (Komárek et al. 2004, Bohunická et al. 2015b). *Cyanothece aeruginosa* is a freshwater to subaerophytic species, typically dwelling in somewhat acidic habitats. However, numerous isolates of simple oval- to rod-shaped unicellular cyanobacteria without distinct mucilaginous envelopes have been classified in *Cyanothece* (Castenholz 2001, Ohki et al. 2008, Shih et al. 2013), using the "form-genus" concept, although they are probably unrelated to *C. aeruginosa* (Komárek et al. 2004) by phylogeny, ultrastructure, or ecology. Many of them are marine coastal diazotrophic cyanobacteria (Ohki et al. 2008, Park et al. 2014), which raise the question of their possible relationship to the well-known marine nitrogen-fixing coccoid species *Crocosphaera watsonii* nom. nudum et nom. inval. (Zehr et al. 2001).

A clear taxonomic delimitation of *Cyanothece*, *Gloeothece*, and *Crocosphaera* gen. nov. gains further importance when we consider the use of certain isolates of these cyanobacteria in experimental studies. Strains of these three genera have been extensively used in characterization of diazotrophic metabolism (Zehr et al. 2007, Compaore and Stal 2010, Pereira et al. 2011a, Wang et al. 2011, Masuda et al. 2013, Zhang et al. 2014), heavy metal bioremediation (Micheletti et al. 2008, Pereira et al. 2011b), and extracellular polysaccharide production (Pereira et al. 2009, Ohki et al. 2014). Several isolates designated as *Cyanothece* sp. and *C. watsonii* were subjected to whole genome sequencing and phylogenomic studies (Zehr et al. 2007, Welsh et al. 2008, Bench et al. 2011, Shih et al. 2013).

The main goal of our study was a polyphasic revision of the coccal cyanobacterial genera *Cyanothece*, *Gloeothece*, and *Crocosphaera* gen. nov. using modern taxonomic techniques. Several complementary methods, including morphological observation, TEM, phylogenetic analysis of the 16S rRNA gene and two additional housekeeping loci (*rpo*C1 and *rbc*LX), ITS (16S-23S ITS) secondary structure prediction, and assessment of the source habitat type, were applied for in-depth analyses of available strains. The results allowed us to define these genera and the species sequenced thus far in these genera. We herein formally describe the well-characterized *Crocosphaera* nom. nudum et nom. inval., which has been the subject of over 200 published papers (Guiry and Guiry 2018). We describe two new, morphologically cryptic genera, *Rippkaea* and *Zehria*, as part of our revision, as well as a number of new species in our target genera. Some strains incorrectly Gloeothece assigned to these genera are identified as a result of our study, but they are located well outside of the Chroococcales, are poorly characterized, and will require taxonomic correction in future studies.

MATERIALS AND METHODS

Strains and cultivation. Five new strains of *Gloeothece* were isolated from subaerophytic microbial communities in: Palenque, Mexico in 2007 (stone surface); Hawaii, Island of Oahu, Old Pali Highway in 2008; Hawaii (HA4964-KK2 from Thurston Lava Tube, Hawaii Volcanoes National Park, Hawaii in 2010; and two artificial waterfalls in tropical glasshouses of the botanical gardens in Teplice and Liberec, the Czech Republic in 2009–2010. After initial LM observation, portions of the material dominated by *Gloeothece* were spread on agar plates and purified by sequential inoculation to fresh media every 1–4 weeks. Additional strains of *Gloeothece* and *Cyanothece* were purchased from available public culture collections (Pasteur Culture Collection (PCC), Coimbra Collection of Algae (ACOI), Culture Collection of Algae (SAG), Culture Collection of Algae and Protozoa (CCAP), American Type Culture Collection (ATCC), Woods Hole Oceanographic Institution (WH). A list of all strains analyzed is given in Supplemental Materials (Table S1 in the Supporting Information). The cyanobacteria were cultivated in liquid and agar-solidified BG11 medium (Rippka et al. 1979) at 20°C and 16:8 h light:dark cycle. For preparation of herbarium-type specimens of the newly described taxa, biomass from the respective strains was harvested, gently dried on filter paper, and deposited in the CBFS Herbarium (Thiers 2019) at the University of South Bohemia, České Budějovice, Czech Republic under CBFS accession numbers (Table S1). Reference strains were deposited in the public Culture Collection of Autotrophic Organisms (CCALA) in Třeboň, Czech Republic (Table S1).

Morphology and ultrastructure. Morphology of all strains was documented by LM using an Olympus BX-51 microscope equipped with Nomarski DIC optics (up to 1,000× magnification), DP-71 digital camera, and QuickPhoto Micro 3.0 image analysis software for photographic documentation and measurements.

For TEM studies, biological material of cyanobacteria was fixed with 6% glutaraldehyde and kept at room temperature. Samples were washed with 0.05 M phosphate buffer (pH 7.2) and postfixed with 2% osmium tetroxide in the same buffer at room temperature for 2 h, then repeatedly washed with 0.05 M phosphate buffer. Finally, cells were dehydrated with a graded isopropanol series and embedded in Spurr's resin (Spurr 1969) using propylene oxide as an intermediate stage. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Jeol JEN 1010 transmission electron microscope at 80 kV.

For atomic force microscopy (AFM) studies, washed cyanobacterial cells were fixed for 12 h at 4°C in 10 mM PBS containing 2.5% glutaraldehyde, rinsed three times in 50 mM PBS for 30 min in total, and embedded in hot agar (1%). Agar cubes (1 mm side) were dehydrated with a graded acetone series, followed by embedding into epoxy medium (Epoxy–Embedding Kit, Fluka Analytical, Munich, Germany) and let to cure at 48°C for 48 h and for 24 h at 60°C. Semithin sections (2–3 μm thick) were cut with an Ultramicrotome MT-X (EMME 3, Milan, Italy) using a

diamond knife and deposited onto glass slides. Some of the thin sections were then subjected to etching of the epoxy resin for 1 min with sodium methoxide (obtained by dissolving NaOH pellets into methanol until saturation) to better expose the inner structure of the cell by removing the excess embedding medium. After careful rinsing in distilled water, the samples were gently dried under a nitrogen flush and analyzed by AFM. As described in Tiribilli et al. 2005, imaging was performed in air, at room temperature, with an AFM (Pico SPM, Veeco, NY, USA) equipped with a "large scanner" of 100 μM maximum range. Images (256 × 256 or 512 × 512 pixels) were acquired in an acoustic AC mode. An adequate feedback gain was set and a typical scanning speed of 2 lines · s^{-1} or lower was adopted during the acquisition. The AFM was equipped with a NSG01 silicon probe (NT-MDT, Moscow, Russia) with 160 kHz nominal resonant frequency and a typical tip radius of less than 10 nm.

DNA extraction, amplification, and sequencing. The biomass was dried for 48 h over silica gel and pulverized in a Mixer Mill MM200 (Retsch, Haan, Germany) laboratory mill with tungsten carbide beads (3 min, 30 · s^{-1}). Total genomic DNA was isolated following a modified xanthogenate-SDS buffer extraction protocol with addition of 3% polyvinylpolypyrrolidone (PVPP) and polyethylene glycol (PEG)-$MgCl_2$ precipitation (Yilmaz et al. 2009). A section of the rRNA operon containing the partial 16S rRNA gene and the ITS region was amplified with primers 16S378F and 23S30R (Taton et al. 2003). Ten ng of template DNA was mixed with 6 pmol of each primer in a commercial PCR mix with Taq polymerase (Plain PP Master Mix, Top Bio, Czech Republic) to give a final volume of 25 μL and amplified with an initial denaturation step (5 min at 95°C), 38 cycles of denaturation (1 min at 94°C), primer annealing (45 s at 55°C), and elongation (2 min at 72°C), and final elongation for 10 min at 72°C. The PCR product was cloned using the standard pGEM®-T Easy Vector System (Promega Corp., Madison, WI, USA) according to supplier instructions. The plasmid containing the required insert was purified from the bacterial culture using Zyppy Plasmid Miniprep kit (Zymo Research Corp., Irvine, CA, USA). The clones were commercially sequenced using primers T7promoter and SP6r, which are included in the cloning vector, and an internal primer Cyano6r (Mareš et al. 2013a). Multiple clones were sequenced for the rRNA 16S and ITS fragments to obtain the sequences of diverse rRNA operon copies within the individual genomes. The *rpo*C1 gene fragment was amplified using primers rpc/MF and rpc/CR-1 following the protocol by Seo and Yokota (2003): initial denaturation for 5 min at 94°C, 35 cycles of 1 min denaturation at 94°C, primer annealing for 1 min at 52°C, elongation for 2 min at 72°C, and final elongation step for 10 min at 72°C. The *rbc*LX genomic region was amplified using primers CW and CX following the protocol by Rudi et al. (1998): initial denaturation for 4 min at 94°C, 2 cycles of 30 s denaturation at 94°C, primer annealing for 30 s at 40°C, elongation for 2 min at 72°C, followed by 38 cycles differing only by 55°C primer annealing temperature, and final elongation step for 10 min at 72°C. The *nif*D gene fragment was amplified using primers nifD552-F and nifD861-R following the protocol by Roeselers et al. (2007): initial denaturation for 5 min at 95°C, 35–38 cycles of 1 min denaturation at 95°C, primer annealing for 1 min at 52°C, elongation for 1 min at 72°C, and final elongation step for 10 min at 72°C. The PCR products of *rpo*C1, *rbc*LX, and *nif*D were directly sequenced by commercial companies, using the same primers that were used for PCR. All primers are listed in Table S2 in the Supporting Information. Sequences were deposited in GenBank under the listed accession numbers (Table S1), *nif*D accession numbers are MH678556–MH678564.

Phylogenetic analysis. Additional sequences of the rRNA operons, *rpo*C1 and *rbc*LX, were mined, using BLAST searches, from the whole genome database for cyanobacteria available in GenBank and from published studies on related taxa. The high amount of available rRNA data on cyanobacteria allowed us to include a greater number of relevant taxa (135 sequences in total) into the 16S rRNA analysis in comparison to the reconstruction based on all three loci together (95 sequences), although the strain selection was kept as similar as possible in both types of analyses to enable comparison. The sequences were aligned using MAFFT v. 7 (Katoh and Standley 2013) and manually corrected. For the analysis of the 16S rRNA gene data alone, a region of alignment that was complete in the majority of the collected sequences was used. It spanned 1,346 positions after ambiguous gap sites were removed. For the combined analysis of all three loci, an 863 bp long region of partial *rpo*C1 gene and 635 bp of the *rbc*LX region alignment were merged with an aligned 16S rRNA gene (1,141 bp) from corresponding strains into a final concatenated alignment of 2,639 bp. Prior to concatenation, the alignments of the three individual loci were analyzed using RAxML 8.2.4 (Stamatakis 2006) to produce a fast maximum-likelihood (ML) phylogeny for each of them (GTR + I + G substitution model, 1,000 bootstrap pseudoreplications). The phylogenetic trees recovered were manually cross-checked for incongruence to avoid major conflicting signals in the final concatenated data matrix. The best substitution model for ML-based analyses was chosen using jModelTest 2 software (Darriba et al. 2012) using the Akaike information criterion. The best models selected for the individual loci were: GTR + I + G (16S rRNA gene), TVMef + I+ G (*rbc*LX), and TIM3 + I + G (*rpo*C1). Since the latter two submodels are neither available in MrBayes nor RaxML, the closest but more general model GTR + I + G was applied in all analyses. The alignment was partitioned to individual loci in the multilocus analyses, and separate substitution models were applied to each partition. 16S rRNA and multilocus phylogenetic analyses were conducted employing Bayesian inference (BI) in MrBayes 3.2.6 (Ronquist et al. 2012), ML analysis in RAxML 8.2.4 (Stamatakis 2006), and neighbor-joining (NJ) analysis in SeaView 4.4 (Gouy et al. 2010). Bayesian inference employed Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analyses with seven heated chains and one cold chain in each of the two independent runs that were processed for 1–2 million generations, sampling each 100 generations, until the average standard deviation of split frequencies was lower than 0.01, and the potential scale-reduction factor (PSRF) of all parameters reached a value between 1.00 and 1.01. Burn-in of 25% generations allowed stabilization of the likelihood value, and a 50% majority-rule consensus tree with posterior probabilities of branches was constructed. The neighbor-joining (NJ) analysis was calculated using the BioNJ algorithm (Gascuel 1997) and Jukes-Cantor distance model. One thousand bootstrap pseudoreplications were run to evaluate the relative branch support in ML and NJ analyses. Sequences of the *nif*D gene fragment were analyzed using BLAST to confirm their homology to cyanobacterial *nif*D; however, they were not used in phylogenetic reconstruction as they are present only in several clades of coccoid cyanobacteria. Phylogenetic trees were drawn and edited using FigTree v. 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). The CIPRES (Miller et al. 2010) supercomputing facility was used for calculation of ML and BI trees.

For *Gloeothece* species, sequences of the 16S-23S ITS region of the operon containing the $tRNA^{Ile}$ gene were aligned using secondary structure (15 sequences), two phylogenetic analyses were run, both scoring indels in the analysis. The first analysis consisted of a heuristic search in Phylogenetic Analysis Using Parsimony (PAUP; Swofford 2002) utilizing

parsimony as the optimality criterion, with indels scored as a fifth base, multrees=yes, branch-swapping algorithm=TBR, gapmode=newstate, steepest descent=no, and nreps = 10,000. This analysis was unrooted. Bootstrap support was based on running 10,000 replicates. For the second analysis, indels in the alignment were scored (1 = nt, 0 = indel), creating a partitioned data set with DNA (365 characters) and standard data (131 characters). An unrooted phylogenetic tree was then obtained with a Bayesian analysis in MrBayes using STOPRULE=YES (STOPVAL = 0.005), discarding the first 25% of samples as burn-in, choosing NST=MIXED, and applying the GTR-Gamma evolutionary model. Average standard deviation of split frequencies was 0.0045 and the average PSRF for this analysis was 1.002. Posterior probabilities from this analysis were then mapped on to the parsimony tree, and the figure was placed as an inset in the 16S rRNA tree created for all taxa of interest.

ITS secondary structure prediction. The hypothetical secondary structures of conserved helices (D1-D1′, BoxB, and V3) in the 16S-23S ITS region were estimated using Mfold (Zuker 2003). In the case of the D1-D1′ helices, a comparative analysis of the structures was employed and corrections made to the helices through use of the force command in Mfold to preserve the highly conserved unilateral bulge near the terminus of the 3′ strand. For those strains for which sequence is available for the 23S-5S ITS region (primarily draft genomes), the presence and structure of the V3 helix could be checked and confirmed, as the 5′ portions in the 16S-23S ITS of the D4 and D5 helices flanking the V3 bind with the 3′ portions of these helices in the 23S-5S ITS. All structures were redrawn in Adobe Illustrator CS5.1.

RESULTS

The phylogenetic reconstructions inferred from the 16S rRNA gene alignment and the multilocus alignment provided highly congruent phylogenetic trees, producing a number of well-supported clades of derived (i.e., non-synechococcalean) coccoid cyanobacterial lineages corresponding to the genera *Gloeothece*, *Aphanothece*, *Crocosphaera* gen. nov., *Rippkaea* gen. nov., *Zehria* gen. nov., and *Cyanothece* (Figs. 1 and 2). All these clusters were well supported in both trees with ≥99% branch support values from all methods, only *Gloeothece* had slightly lower (but still high) branch support values in the 16S rRNA gene tree (≥89%). The 16S rRNA p-distances within the proposed genera varied from less than 1% (*Rippkaea* with just two strains) to 7.4% (*Gloeothece*, see Discussion for explanation), while the intergeneric p-distances ranged from 3.7% to 10.5% (Table S3 in the Supporting Information). Additionally, the genera exhibited structural synapomorphies in the secondary structure of the 16S-23S ITS regions, which could be used to characterize the generic lineages (Figs. 3–6). The *Gloeothece* clade was related to a number of sequences of other coccoid types in our trees, including known representatives of *Microcystis*, *Synechocystis*, *Eucapsis*, and *Merismopedia*, and several other taxa. Another large isolated lineage was formed by the genera *Aphanothece*, *Rippkaea*, *Zehria*, and *Crocosphaera* analyzed in the current study (Fig. 1). In the 16S rRNA tree, several cyanobacterial endosymbiont sequences from diatoms and a non-photosynthetic prymnesiophyte endosymbiont candidatus *Atelocyanobacterium thalassa* also clustered in this clade. Sequences of a pair of typical strains of the genus *Cyanothece* (*C. aeruginosa*) grouped in a clade very distant from all former genera, in a derived lineage close to filamentous (Gomontiellaceae), baeocytous (Chroococcidiopsidales), and filamentous (*Coleofasciculus*, *Moorea*) cyanobacteria. Other derived coccoid genera, such as *Cyanobacterium*, *Geminocystis*, *Chroococcus*, *Chamaesiphon*, *Halothece*, and members of the baeocyte-producing order Pleurocapsales, all held phylogenetic positions unrelated to the taxa dealt with in the current study. Specific results for each generic cluster follow.

Genus I–Cyanothece. Most available strains designated as *Cyanothece* sp. in culture collections were found to be highly polyphyletic, splitting into several discrete phylogenetic clades (Fig. 1 and 2). Strains attributed to this genus are found in *Gloeothece*, *Crocosphaera*, *Zehria*, *Rippkaea*, and the Synechococcales (PCC 7425). Two strains (SAG 87.79 and NIVA-CYA 258/1) representing the typical *Cyanothece*, including the type species *C. aeruginosa*, grouped in a distinct isolated clade (branch supports ≥99%) with closest sequenced relatives from the filamentous cyanobacterial family Gomontiellaceae (specifically *Crinalium*; Figs. 1 and 2). These two species had 16S rRNA genetic identities ≤92.3 to all other taxa that were the focus of this study (Table S3), well below the generic cut-off proposed by Yarza et al. (2014) of ≤94.5%. *Cyanothece aeruginosa* was further characterized by its large cell dimensions (over 20 μm), a typical thylakoid pattern with peripheral radial thylakoids expanding into the cell center and forming a complex reticulate structure (Fig. 7) and habitat specificity (cool, oligotrophic, more or less acidic environments). The entire putative 16S-23S ITS secondary structure is shown for the operon containing the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes for the type species of *Cyanothece*, *C. aeruginosa* (Fig. 3a). The ITS region in all *Cyanothece* sensu stricto operons currently available can be differentiated from all other taxa in this study by (i) the presence of an operon with two tRNA genes and the absence of an operon with only one tRNA gene; (ii) a D1-D1′ helix basal clamp that is 5–6 bp long (Fig. 3, b–d), in contrast to the almost universal 4 bp clamp in the other taxa treated in this study (Fig. 4; note the exception of 5 bp clamp in Fig. 4, o, p, and s); (iii) absence of any bases opposite the 3′ unilateral bulge above the basal clamp (Fig. 3, b–d), in comparison to the 1–5 bases present in other taxa (Fig. 4). An additional important difference was in the length of the V3 helix, which was 41–59 nt long, as opposed to all others which were generally much shorter, 8–43 nt, when present (Fig. 6; Table 1).

The last representative of *Cyanothece* sensu lato, PCC 7425, clustered in a distant, weakly supported

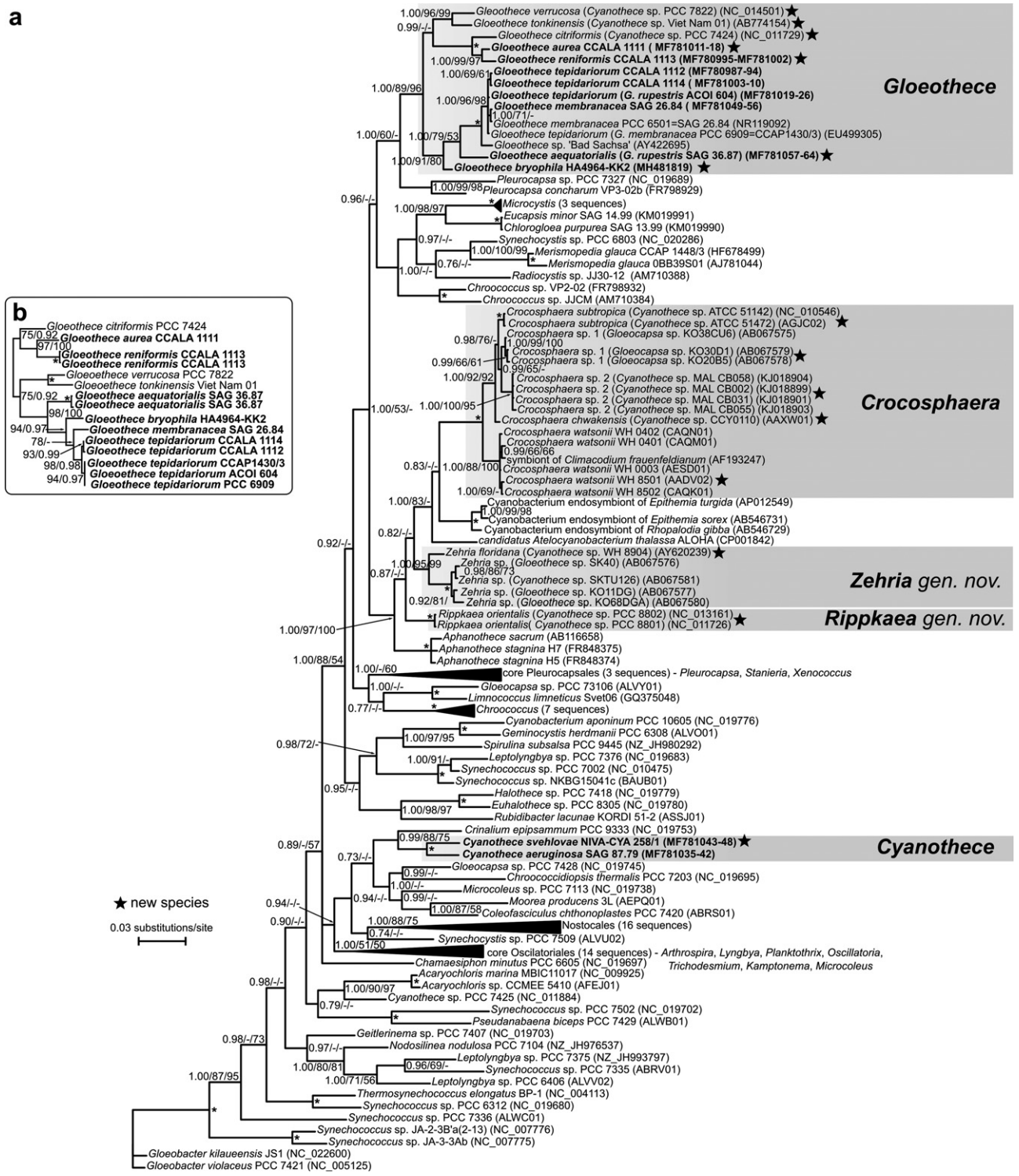

FIG. 1. Phylogenies based on rRNA operon data. (a) Bayesian inference tree based on aligned partial 16S rRNA gene sequences (1,346 positions). Branch support values are given at nodes in this order: BI/ML/NJ; supports ≥99% from all three analyses are indicated by an asterisk. Bold font indicates sequences obtained in this study. Black asterisks indicate new species established in this study. (b) Maximum Parsimony tree of *Gloeothece* based on the 16S-23S ITS region of the operon containing the $tRNA^{Ile}$ gene. Branch support values are given at nodes in this order: MP/BI; supports ≥99% from both analyses are indicated by an asterisk. Bold font indicates sequences obtained in this study.

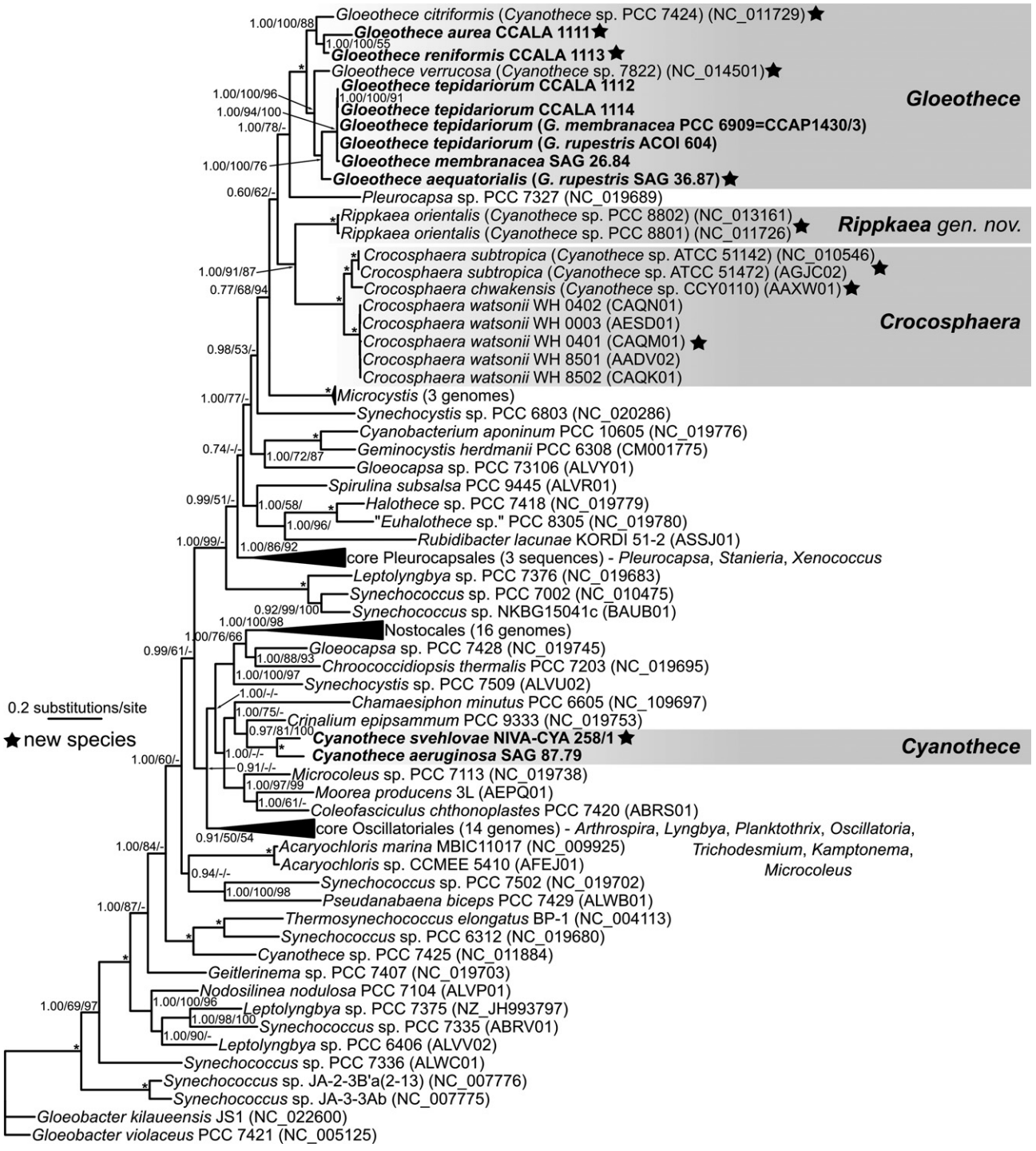

FIG. 2. Bayesian inference phylogeny based on a concatenated alignment of the 16S rRNA gene (1,141 nt), *rpo*C1 gene (863 nt), and *rbc*LX gene (635 nt). Branch support values are given at nodes in this order: BI/ML/NJ; supports ≥99% from all three analyses are indicated by an asterisk. Bold font indicates sequences obtained in this study. Black asterisks indicate new species established in this study.

clade of simple synechococcalean cyanobacteria (near to *Acaryochloris*). Parietal thylakoid structure was documented in this strain by Porta et al. (2000), further indication that it belongs in the Synechococcales. Taxonomic revision of this group is not included in our study, but it clearly is excluded from *Cyanothece* sensu stricto and all other genera treated in this study.

All other sequenced strains attributed to *Cyanothece* in the past differ from *C. aeruginosa* and

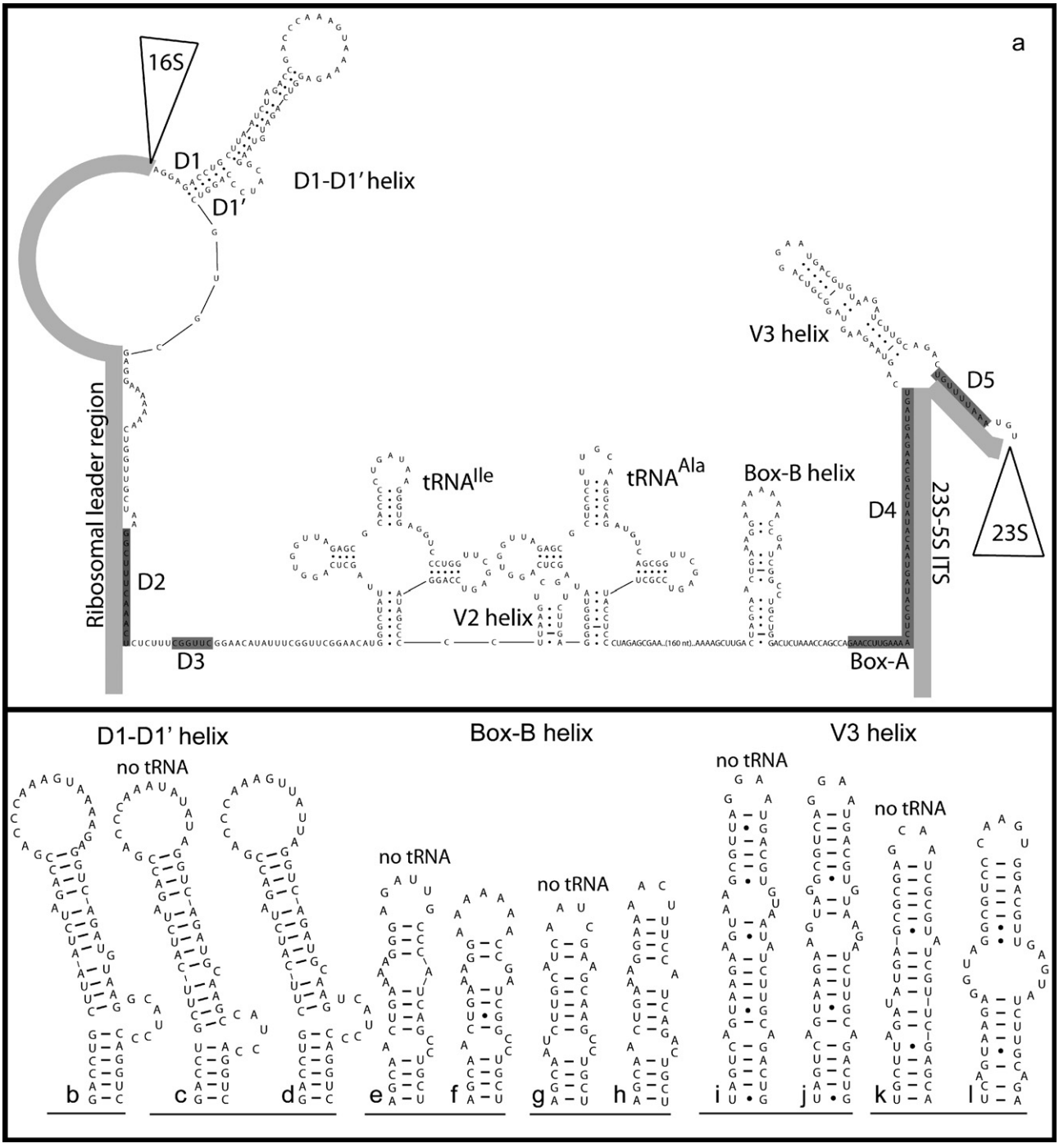

FIG. 3. Secondary structure of the 16S-23S ITS region for *Cyanothece* sensu stricto. (a) Overview of the entire region in *C. aeruginosa* SAG 87.79 for operon with both tRNA genes. The sequences for the leader region and 23S-5S ITS are required to know exact structure of the D2, D4, and D5 helices, so structures shown for these three helices are based on other taxa for which these regions are available. In taxa without both tRNA genes, the V2 helix is not present. Terminology for conserved domains follows Iteman et al. (2000). (b, e, f, i and j) *C. aeruginosa* SAG 87.79; (c, d, g, h, k and l) *C. svehlovae* NIVA-CYA 258-1. The D1-D1′ helix for *C. aeruginosa* SAG 87.79 was the same for operons. Structures from operons lacking tRNA genes are labeled, all others are from operons with both tRNA genes.

C. svehlovae sp. nov. by much smaller size, packets of thylakoids irregularly arranged in the cytoplasm, low sequence identity in the 16S rDNA, and critical characteristics of the 16S-23S ITS region. Diazotrophy is unconfirmed in this genus, although the *nif*D gene was sequenced from *C. svehlovae*. The *nif*D gene could not be recovered from *C. aeruginosa*. Strains attributed to this genus that fall outside

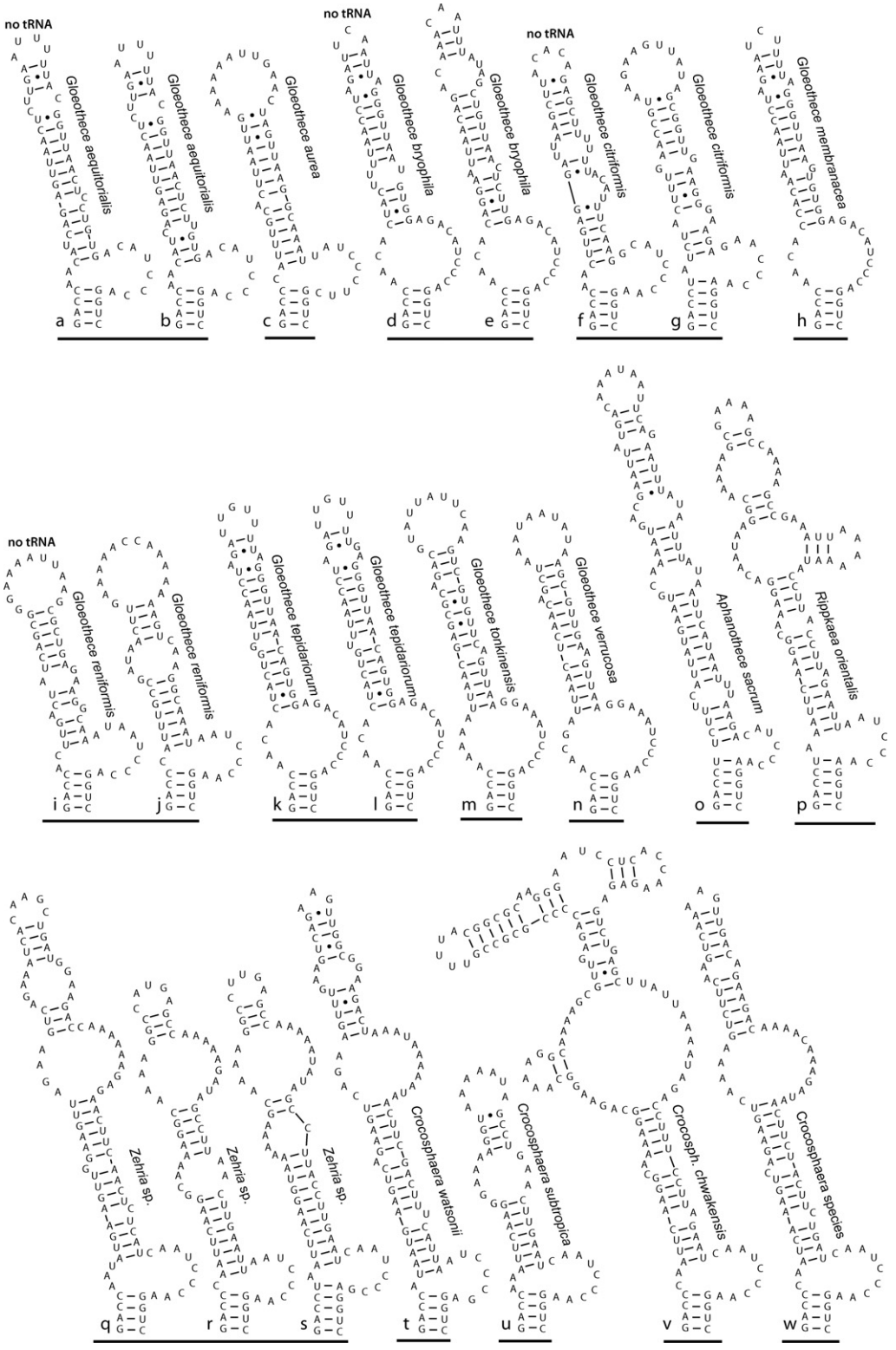

FIG. 4. Secondary structure of the D1-D1′ helices in taxa treated in this work. Structures are from operons with a $tRNA^{Ile}$ gene unless otherwise indicated in figure. (a and b) *Gloeothece aequatorialis* SAG 36.87; (c) *G. aurea* CCALA 1111; (d and e) *G. bryophila* HA4964-KK2; (f and g) *G. citriformis* PCC7424; (h) *G. membranacea* SAG 26.84; (i and j) *G. reniformis* CCALA 1113; (k) *G. tepidariorum* CCAP 1430/3 (=PCC6909) and ACOI 604; (l) *G. tepidariorum* CCALA 1112; (m) *G. tonkinensis* Viet Nam 01; (n) *G. verrucosa* PCC7822; (o) *Aphanothece sacrum* AB116658; (p) *Rippkaea orientalis* PCC 8801 and PCC 8802; (q) *Zehria* sp. SKTU126; (r) *Zehria* sp. SK40; (s) *Zehria* sp. K011; (t) *Crocosphaera watsonii* WH0003, WH0005, WH0402, WH8501, and WH8502; (u) *C. subtropica* ATCC51142 and ATCC51472; (v) *C. chwakensis* CCY0110; (w) *Crocosphaera* sp. KO20B5 and KO38CU6.

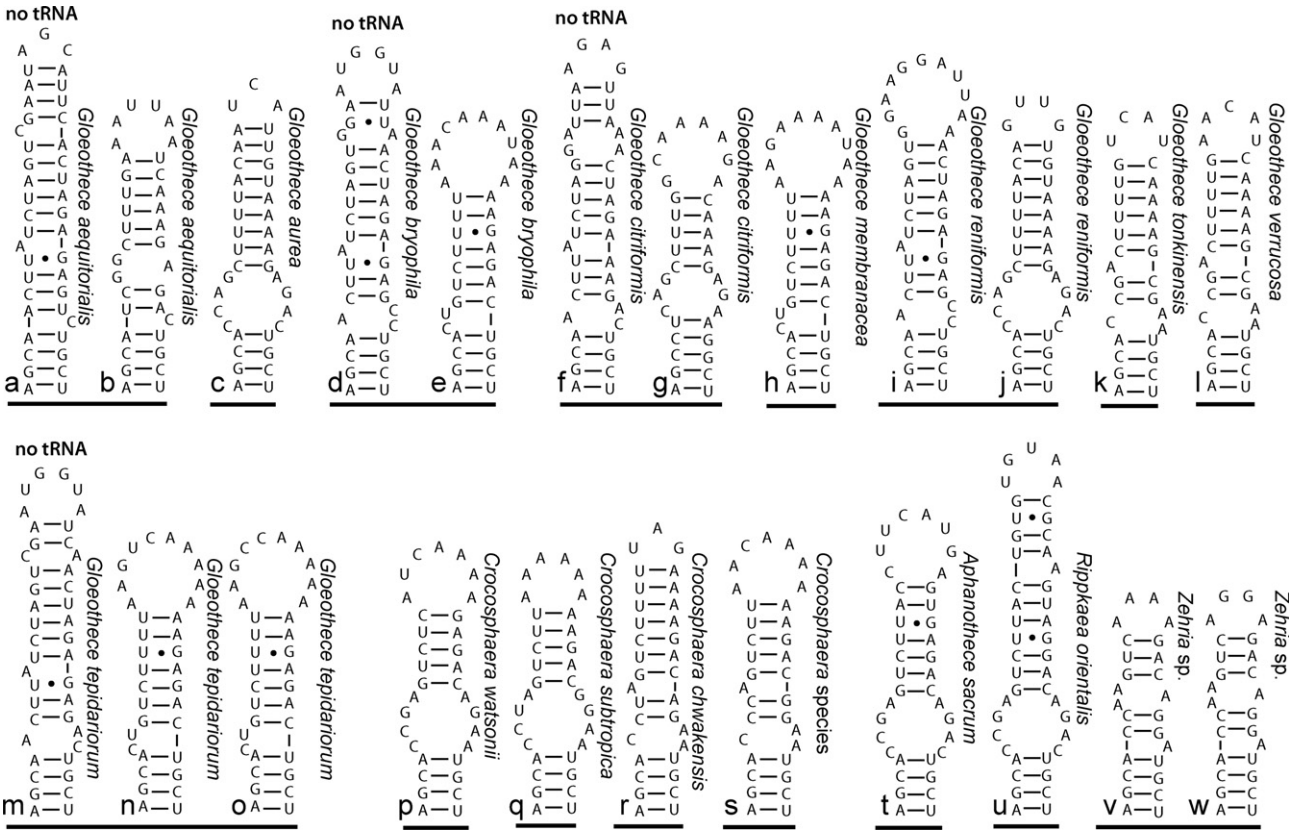

FIG. 5. Secondary structure of the BoxB helices in taxa treated in this work. Structures are from operons with tRNA gene(s) unless otherwise indicated in figure. (a and b) *Gloeothece aequatorialis* SAG 36.87; (c) *G. aurea* CCALA 1111; (d and e) *G. bryophila* HA4964-KK2; (f and g) *G. citriformis* PCC7424; (h) *G. membranacea* SAG 26.84; (i and j) *G. reniformis* CCALA 1113; (k) *G. tonkinensis* Viet Nam 01; (l) *G. verrucosa* PCC7822; (m) *G. tepidariorum* CCAP 1430/3 (=PCC6909), ACOI 604, 20, 25; (n) *G. tepidariorum* CCAP1430/3 (=PCC6909) and ACOI 604; (o) *G. tepidariorum* CCALA 1112 and CCALA 1114; (p) *Crocosphaera watsonii* WH0003, WH0005, WH0402, WH8501, and WH8502; (q) *C. subtropica* ATCC51142 and ATCC51472; (r) *C. chwakensis* CCY0110; (s) *Crocosphaera* sp. KO20B5 and KO38CU6; (t) *Aphanothece sacrum* AB116658; (u) *Rippkaea orientalis* PCC 8801 and PCC 8802; (v) *Zehria* sp. SKTU126 and SK40; (w) *Zehria* sp. K011.

of *Cyanothece* sensu stricto must be recognized as belonging to other genera if any evolutionary significance is to be accorded to cyanobacterial classification.

Genus II – Gloeothece. In both 16S rRNA and multilocus phylogenies, a well-supported clade emerged, which included the majority of the known *Gloeothece* isolates, together with "*Cyanothece*" sp. strains PCC 7822, PCC 7424, and Viet Nam 01 (Figs. 1 and 2). All these strains exhibited a number of common morphological features: oval to cylindrical unicells dividing by transverse binary fission, surrounded by individual multilayered mucilaginous envelopes; and irregular colonies forming a gelatinous mass on the substrate surface. A single exception to this was the morphology of "*Cyanothece*" sp. PCC 7424 which lacked firm mucilaginous envelopes, and a small portion of the cells had an unusual lemon-like shape. The thylakoid arrangement, as documented by TEM analysis (Figs. 8, c, d and h; 9, f and g; 10, d, e and h; 11, i and j; 12), was found to be highly uniform inside the whole *Gloeothece* clade, forming wavy fascicles of parallel thylakoids, irregularly spreading throughout the cell. The thylakoid clusters tended to be more abundant at the cell periphery, particularly in strains that possessed frequent storage granules in the cell center (Fig. 12a). This arrangement was well displayed in a close to native state using AFM analysis of *G. tepidariorum* CCALA 1114 (Fig. 12b). We were successful in amplifying and sequencing the *nif*D gene in eight *Gloeothece* strains (CCALA 1111–1114, SAG 26.84, CCAP1430/3, ACOI 604, SAG 36.87).

Gloeothece had some genus-wide characteristics in the secondary structures of the conserved domains of the ITS region. Like all other diazotrophic taxa treated in this study, they possessed two operons, one with the $tRNA^{Ile}$ gene, the other with no tRNA genes. *Cyanothece* differed from the other diazotrophic lineages in that many *Cyanothece* species had 4–5 nt opposite the 3′ unilateral bulge above the basal clamp (Fig. 4, d, e, h, and k–n), an occurrence very rare in cyanobacteria sequenced thus far. Furthermore, the D1-D1′ helices in this genus were

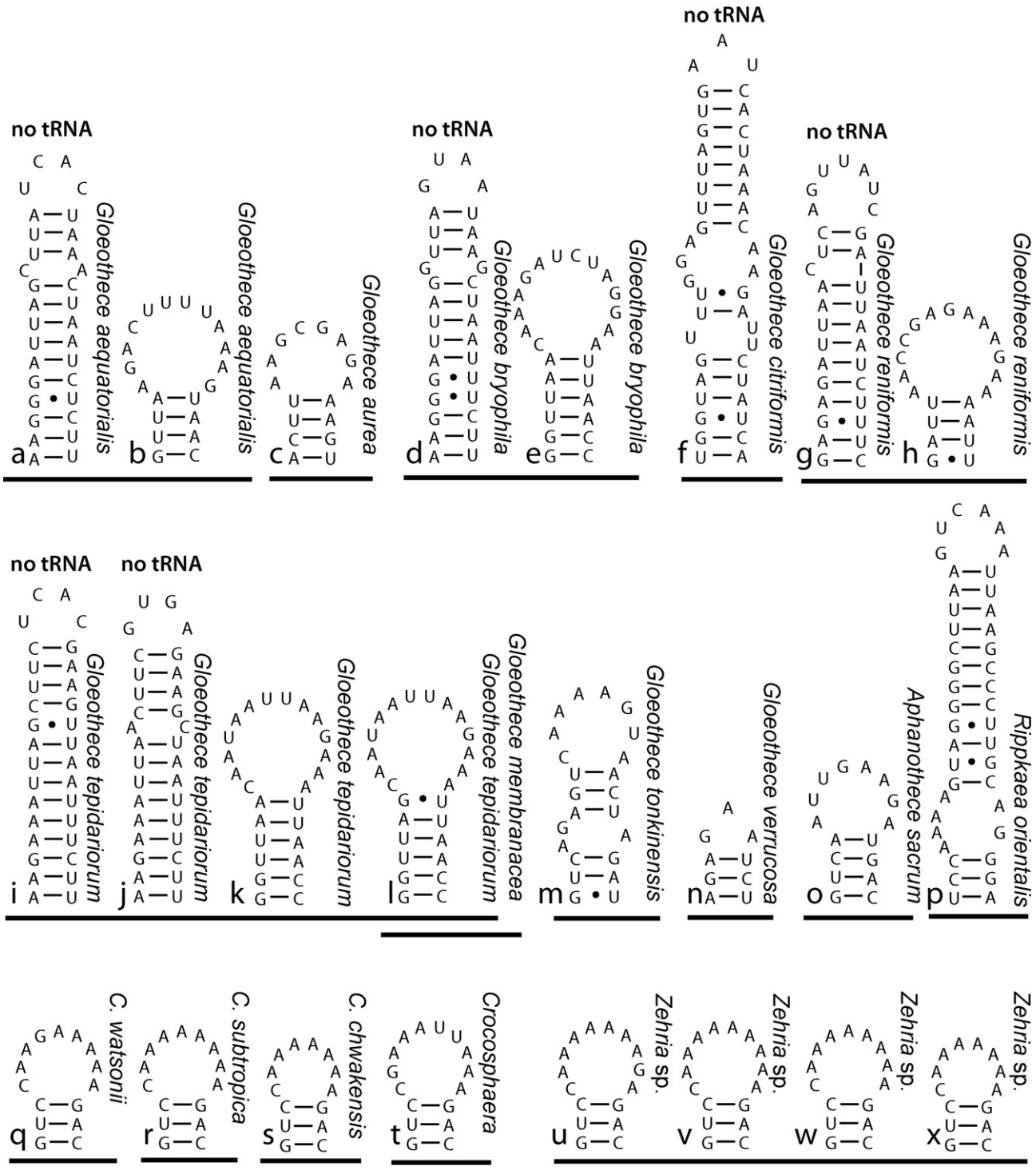

FIG. 6. Secondary structure of the V3 helices in taxa treated in this work. Structures are from operons with tRNA gene(s) unless otherwise indicated in figure. (a and b) *Gloeothece aequatorialis* SAG 36.87; (c) *G. aurea* CCALA 1111; (d and e) *G. bryophila* HA4964-KK2; (f) *G. citriformis* PCC7424; (g) *G. tepidariorum* CCAP 1430/3 (=PCC6909) and ACOI 604; (h) *G. tepidariorum* CCALA 1112, CCALA 1114; (i) *G. tepidariorum* CCALA 1112 and CCALA 1114; (j) *G. tepidariorum* CCAP1430/3 (=PCC6909) and ACOI 604, and *G. membranacea* SAG 26.84; (k and l) *G. reniformis* CCALA 1113; (m) *G. tonkinensis* Viet Nam 01; (n) *G. verrucosa* PCC7822; (o) *Aphanothece sacrum* AB116658; (p) *Rippkaea orientalis* PCC 8801 and PCC 8802; (q) *Crocosphaera watsonii* WH0003, WH0005, WH0402, WH8501, and WH8502; (r) *C. subtropica* ATCC51142 and ATCC51472; (s) *C. chwakensis* CCY0110; (t) *Crocosphaera* sp. KO20B5 and KO38CU6; (u) *Zehria* sp. KO11; (v) *Zehria* sp. KO68; (w) *Zehria* sp. SK40; (x) *Zehria* sp. SKT126.

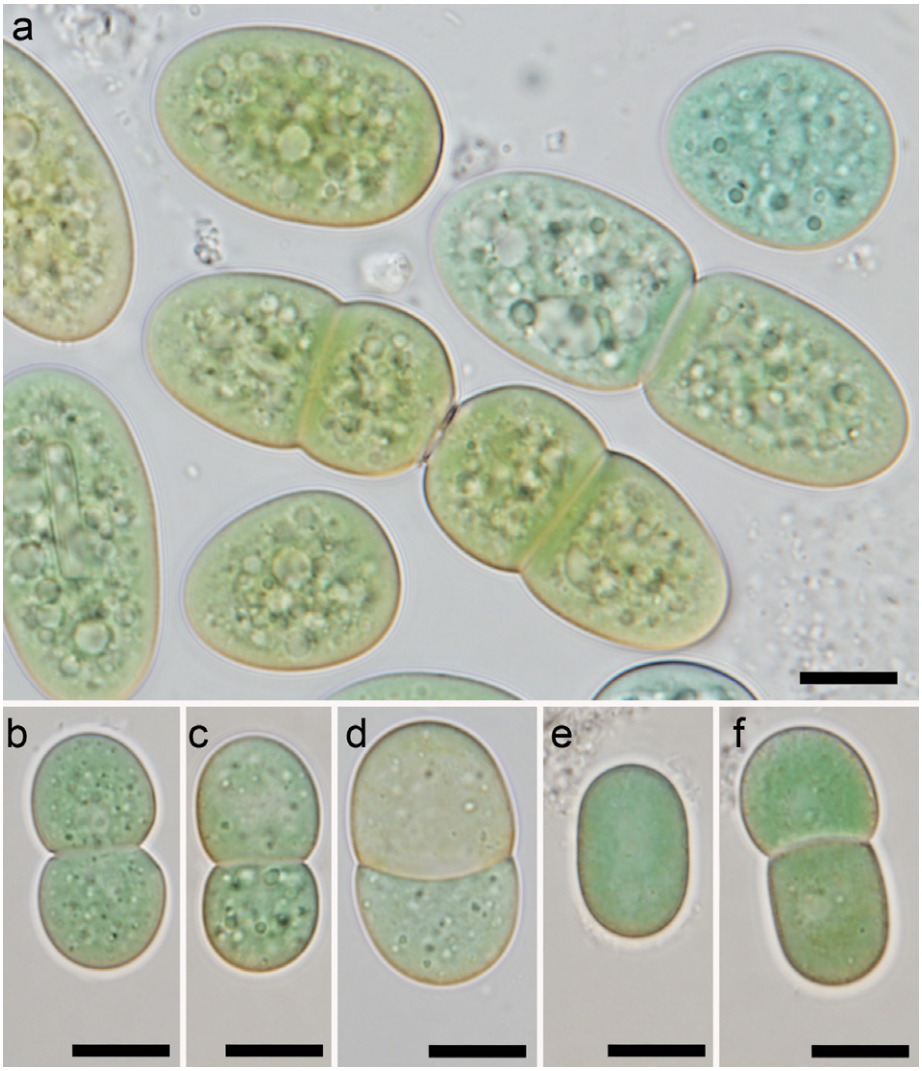

FIG. 7. *Cyanothece* sensu stricto morphology. (a) *C. aeruginosa* SAG 87.79; (b–f) *C. svehlovae* NIVA-CYA 258-1. Scales = 10 μm.

≤64 nt long, compared to *Crocosphaera*, *Zehria*, and *Rippkaea*, in which these helices were ≥73 nt (Table 1).

The *Gloeothece* clade could be further divided into three distinct sublineages (Figs. 1 and 2). The first of them contained strains previously designated as the typical *Gloeothece* species from Europe, *G. membranacea* and *G. tepidariorum*. A summary of observations on their morphology is given in Table 2. The 16S rRNA gene sequences were highly uniform (*P* distance <0.011) within the six strains from this clade: CCAP 1430/3 (=PCC 6909), ACOI 604, CCALA 1112, CALA 1114, SAG 26.84 (=PCC 6501), and "Bad Sachsa" isolate. The available sequences of other housekeeping loci (*rbc*LX, *rpo*C1) were also highly consistent in this clade. SAG 26.84 (=PCC 6501) is designated as *G. membranacea* in the Pasteur Culture Collection and based on morphology and source habitat (freshwater aquatic in temperate climates), we conclude that this is fully consistent with the modern species description for this taxon (Komárek and Anagnostidis 1998). Four strains from this same cluster (CCAP 1430/3 [=PCC 6909], ACOI 604, CCALA 1112, CCALA 1114) exhibited a uniform morphology, slightly different from that of *Gloeothece membranacea*, showing more distinctly delimited and lamellated mucilaginous envelopes (Fig. 9, f and g). These strains (except PCC 6909 with unknown origin) were all isolated from wet walls in tropical greenhouses in European botanical gardens (Table S1). The combined available data were congruent and led to identification of these strains as *G. tepidariorum*. Both species (*G. membranacea* and *G. tepidariorum*) can also be distinguished from other *Gloeothece* species based on differences in secondary structure of the D1-D1′ helix of the 16S-23S ITS region (Fig. 4, h, k and l). Another isolate, *Gloeothece* sp. "Bad Sachsa," lacks morphological data, and it was not further investigated by us. Each of the remaining two strains, SAG 36.87 and HA4964-KK2, exhibits a separate phylogenetic position, unique morphology, and 16S rRNA

TABLE 1. Lengths of conserved domains in the 16S-23S ITS regions of taxa treated in this study.

	Leader	D1-D1′ helix	D2 + Spacer	D3 + Spacer	$tRNA^{Ile}$	V2 + spacer	$tRNA^{Ala}$	Spacer	BoxB helix	Spacer	Box-A	D4	Spacer	V3 helix	Spacer + D5 + Spacer
Cyanothece aeruginosa SAG 87.79	7	60	38	36	74	16	73	182	34	17	11	26	0	51	10
C. svehlovae NIVA-CYA258/1	8	59	37	19	74	19	73	90	34	17	11	26	0	46	17
C. aeruginosa SAG 87.79	7	60	39	174					35	17	11	25	0	59	5
C. svehlovae NIVA-CYA258/1	8	58	33	77					32	17	11	20	0	41	24
Gloeothece aequatorialis SAG36.87	8	60	41	12	74	34			34	24	11	16	4	33	23
G. aurea CCALA 1111	8	59	41	12	74	26			37	17	11	15	3	16	9
G. bryophila HA4964-KK2	8	63	37	16	74	39			34	23	11	19	0	26	1
G. citriformis PCC 7424	8	60	38	16	74	34			34	20	11	35	4	0	0
G. reniformis SAG 26.84	9	59	34	18	74	32			36	22	11	19	0	21	4
G. tepidariorum all strains	8	60	38	16	74	34			34	23	11	16	3	26	0
G. tonkinensis Viet Nam 01	7	64	34	17	74	30			32	22	11	14	1	23	0
G. verrucosa PCC 7822	7	62	37	16	74	23			33	23	11	30	5	8	0
G. bryophila HA4964-KK2	8	60	30	129					42	18	11	18	8	32	26
G. citriformis PCC 7424	8	60	32	145					42	19	11	13	6	43	19
G. reniformis SAG 26.84	8	59	35	145					41	19	11	13	14	32	27
G. tepidariorum all strains	8	60	30	128					42	18	11	13	8	42	21
Crocosphaera watsonii all strains	7	92	36	15	74	29			33	18	11	24	1	15	6
C. subtropica ATCC51472	7	56	38	15	74	33			32	18	11	27	0	15	6
C. chwakensis CCY0110	7	139	37	15	74	31			33	18	11	28	0	14	6
Crocosphaera sp. KO38CU6, KO20B5	7	92	37	18	74	35			33	18	11	29	0	16	6
Rippkaea orientalis PCC8801, PCC 8802	7	94	37	18	74	38			44	21	11	21	7	42	16
Zehria floridana all strains	8	73	35	16	74	34			26	19	11	25	0	16	6

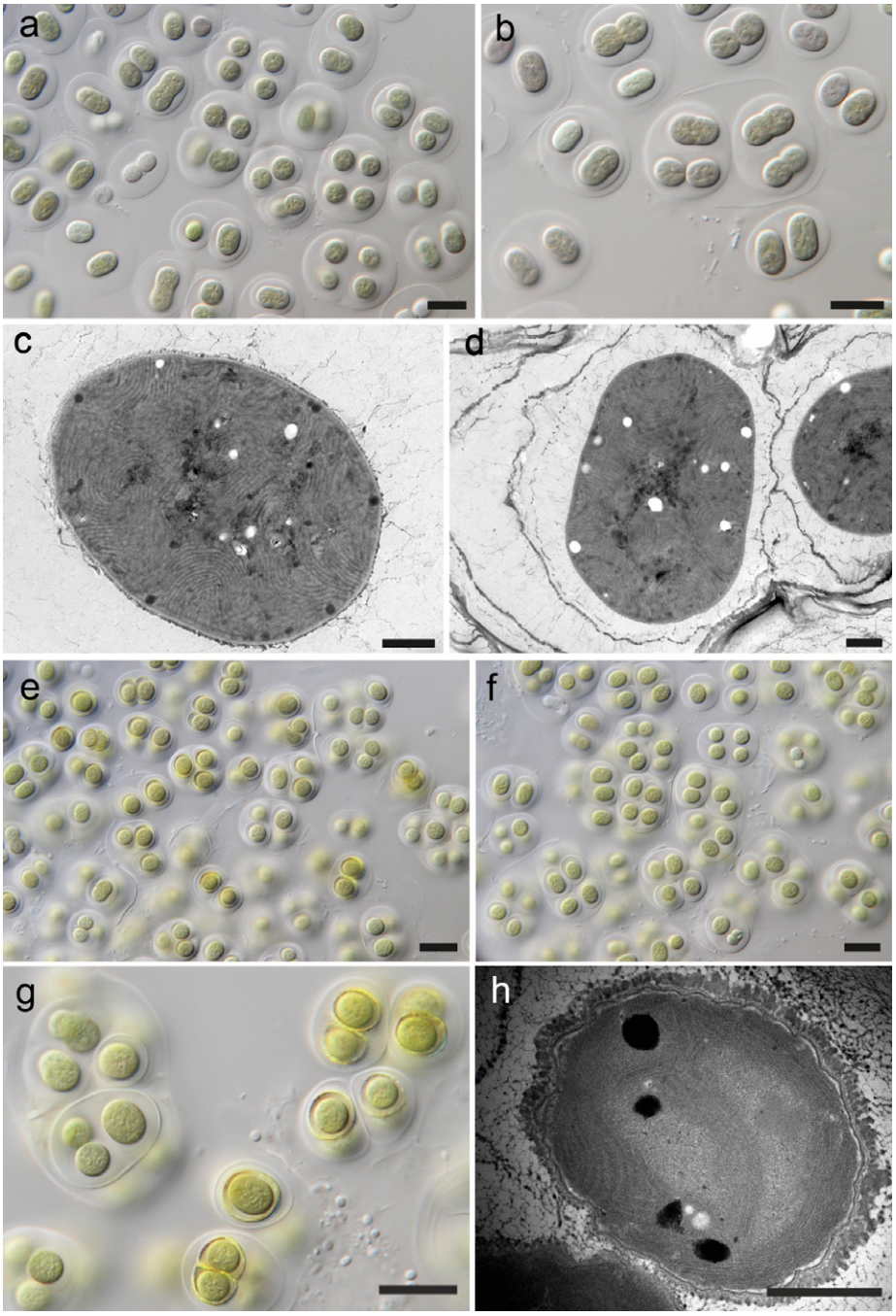

FIG. 8. *Gloethece* morphology and ultrastructure. (a–d) *G. aequatorialis* SAG 36.87, with typically only a single lamellation in the sheath visible in each cell cluster, note the apparent definition of sheath lamellation in TEM (d); (e–h) *G. aurea* CCALA 1111, with firm encapsulation sheath forming around cells (g) when mature. Scales in LM = 10 μm, scales in TEM = 1.0 μm.

and associated ITS sequence. As they were isolated from tropical terrestrial habitats and did not fit the description of any existing morphospecies, we describe them as new species (diagnoses given below).

A second cluster (Figs. 1 and 2), sister to the previous one, consisted of two isolates of [*Cyanothece*] sp. (PCC 7822 and Viet Nam 01). Microscopy data obtained for PCC 7822 (isolated from rice fields in India) revealed a morphology corresponding to *Gloeothece*, including lamellated, yellow-brown colored envelopes around cells, and formation of distinct gelatinous colonies (Fig. 11, g and h). *Gloeothece*-like cells arranged in mucilaginous colonies were reported also for the strain Viet Nam 01 (Fig. 11, a–f). Both strains showed a morphology different from any existing species, in particular they had larger cell dimensions (Table 2). Based on total evidence provided by phylogenetic reconstruction, rRNA operon sequence, ITS secondary structure and sequence, and morphology data, we describe two new species of *Gloeothece*, each represented by one of these two strains.

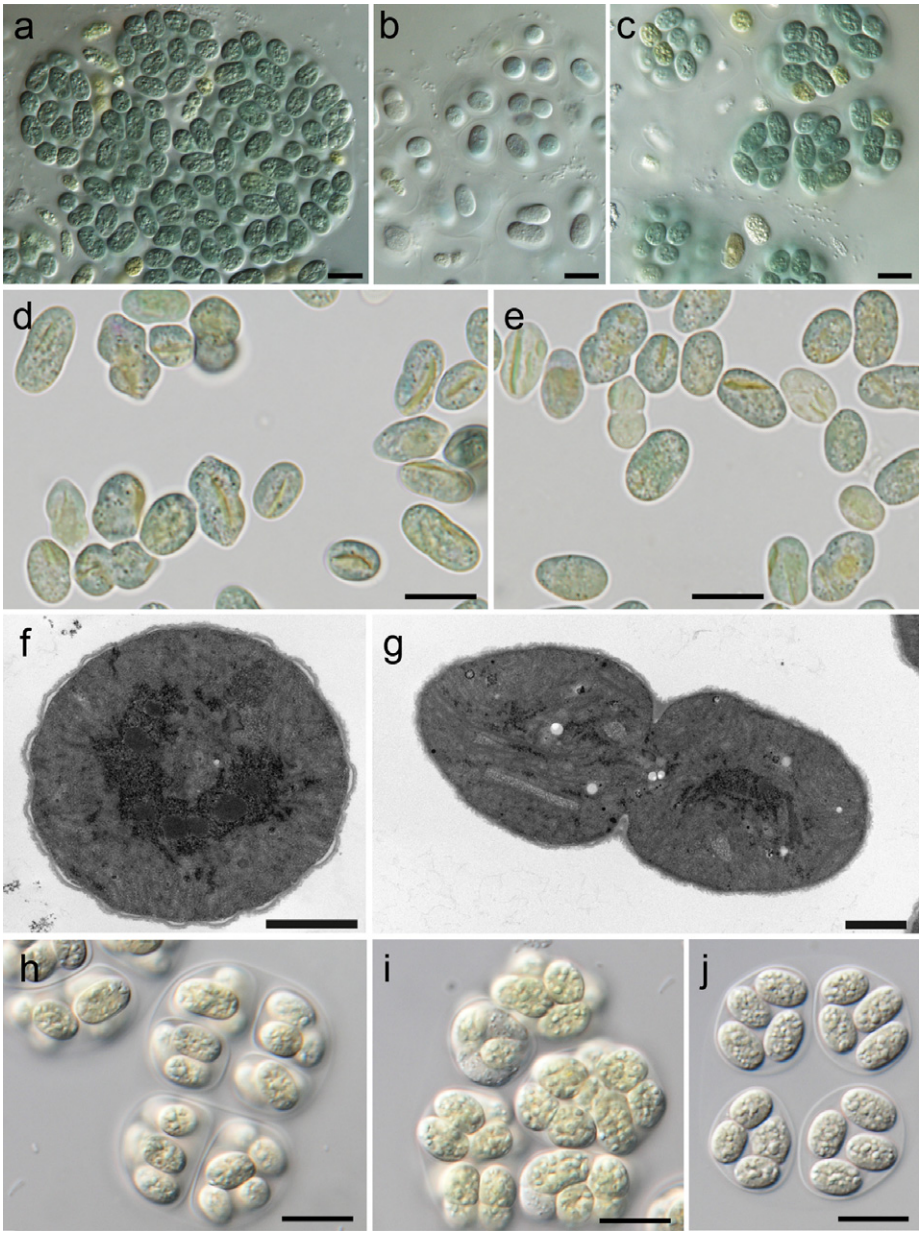

FIG. 9. *Gloethece* morphology and ultrastructure. (a–c) *G. bryophila* HA4964-KK2, showing densely packed colonies lacking lamellate sheaths, stationary phase appearance in b; (d–g) *G. citriformis* PCC 7424, lacking sheath and showing characteristically shaped cells which are drawn out into distally tapered ends in dividing cell pairs; (h–j): *G. membranacea* SAG 26.84. Scales in LM = 10 μm, scales in TEM = 1.0 μm.

The remaining phylogenetic cluster within *Gloeothece* (Figs. 1 and 2) was comprised of two new isolates from Mexico (CCALA 1111) and Hawaii (CCALA 1113) and a sister branch of [*Cyanothece*] sp. PCC 7424. Both CCALA strains shared an ecologically similar source habitat (subaerophytic epilithic in tropical countries), but were isolated from different substrates (the former from limestone, the latter from a volcanic rock) and were geographically distant. Based on 16S rRNA gene and ITS sequence, supported by unique morphological characters (especially cell shapes uncommon in *Gloeothece*; see Figs. 8, e–g; 10b), we erect here two new species represented by these two isolates. The strain PCC 7424 was phylogenetically isolated and did not produce any mucilaginous structures in culture and is also described as a new species.

Genus III – Crocosphaera. The third distinct genus-level lineage recovered in our phylogenetic trees contained several Crocosphaera *watsonii* sequences, with a sister group comprising 10 strains designated as [*Cyanothece*] sp. or [*Gloeocapsa*] sp. (Figs. 1 and 2). In addition to phylogenetic position and low 16S rRNA gene variability (*P*-distance <3.6%), polyphasic characterization of the clade revealed a number of common features: (i) cells are spherical, oval, or elliptical, around 3 μm in diameter; mucilaginous sheaths are absent or simple, without distinct lamellation (Fig. 13, a–h); (ii) thylakoids are arranged in sinuous parallel clusters

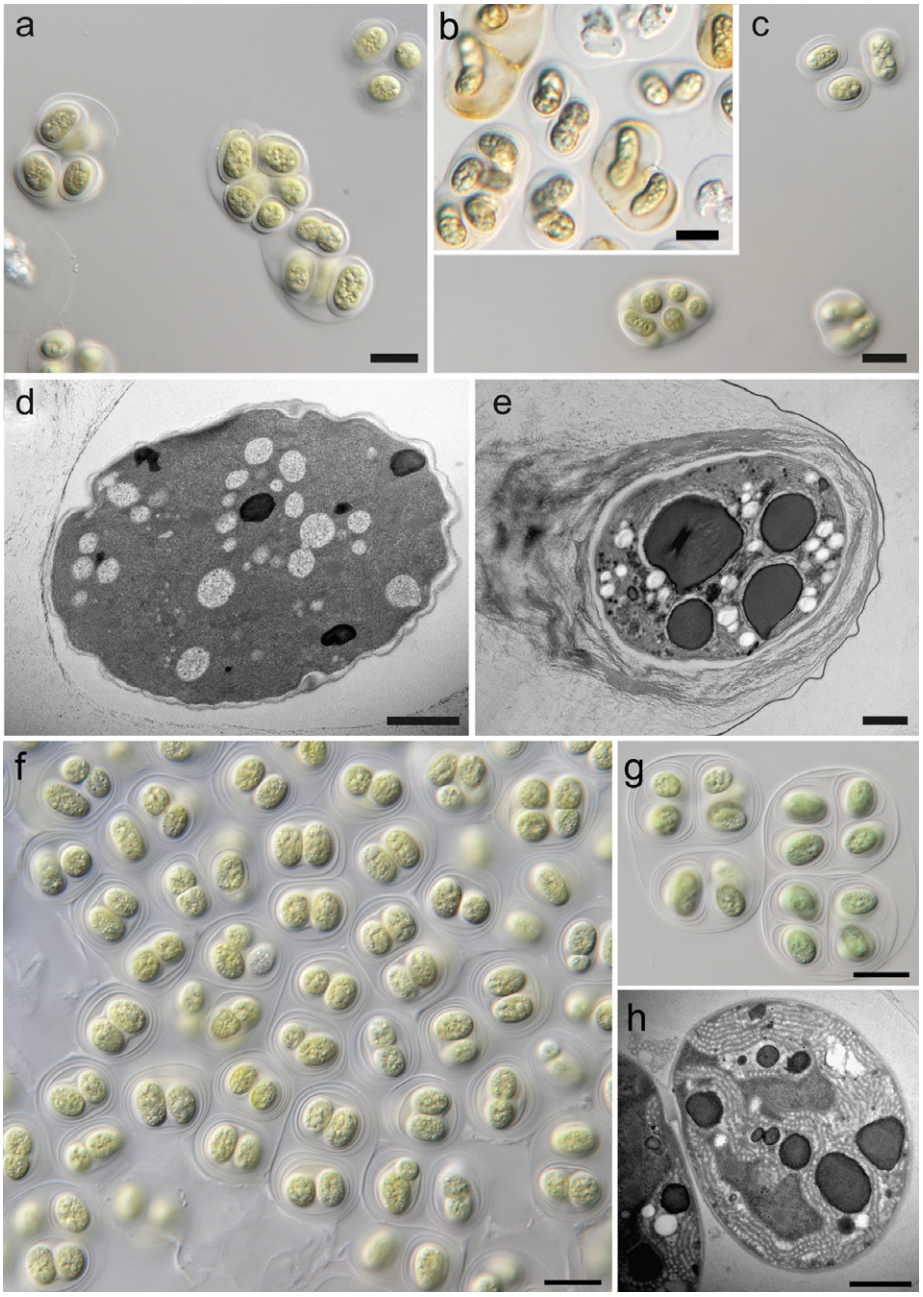

FIG. 10. *Gloethece* morphology and ultrastructure. (a–e) *G. reniformis* CCALA 1113, with characteristic kidney-shaped cells in b; (f) *G. tepidariorum* CCALA 112 and (g and h) *G. tepidariorum* CCALA 1114, with characteristic multiple lamellations in firm sheath. Scales in LM = 10 μm, scales in TEM = 1.0 μm.

spreading throughout the entire cytoplasm (Fig. 13, m and n); (iii) all strains were isolated from marine habitats; (iv) the strains are capable of fixing molecular dinitrogen. The total evidence warrants including these strains in the genus *Crocosphaera*, yielding two new species that are morphologically rather cryptic, but separated based on marked differences in rRNA ITS data and geographical distance of their source habitats. Two additional putative species clusters were resolved based on phylogenetic analysis (Fig. 1). The strains included in these clusters unfortunately were not available upon request, therefore we provisionally designate them as *Crocosphaera* sp. 1 and 2 in the phylogenetic tree. The genus was never described or typified (both a nom. nudum and nom. inval.), thus we provide a valid taxonomic description later in this manuscript.

Genus IV – Zehria. This lineage consists of a number of marine coccoid strains that occupy a sister position to *Crocosphaera* and marine symbiotic cyanobacteria in our 16S rRNA phylogeny (Fig. 1). *Zehria* strains differ from *Rippkaea* by having considerably less mucilage development and a marine distribution. They differ from *Crocosphaera* by having slightly smaller cells. They lack the lamellated sheaths of *Gloeothece* and have slightly elliptical to sometimes almost spherical cells (Fig. 13, i and j). The genus is poorly defined by morphology alone,

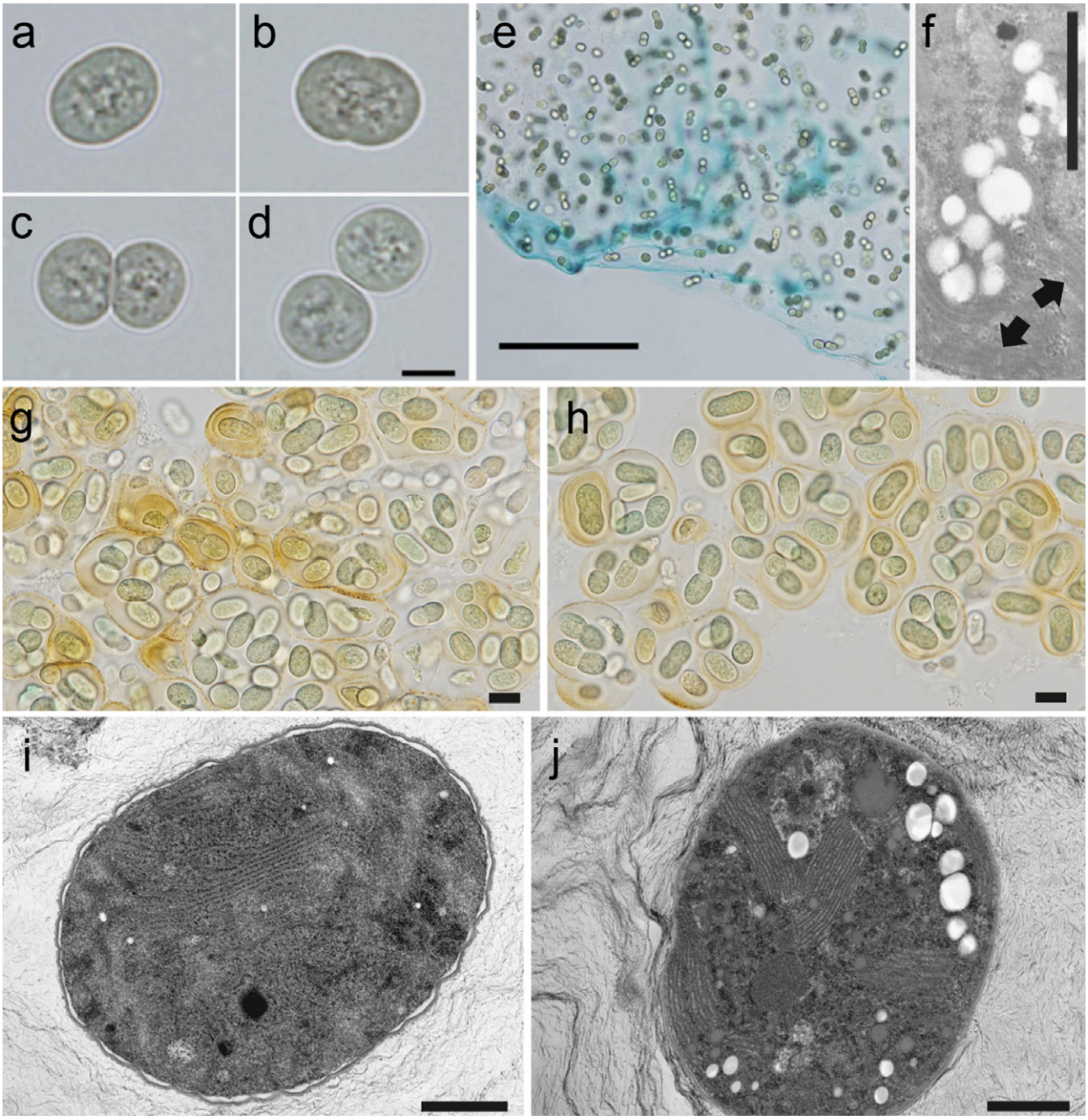

FIG. 11. *Gloethece* morphology and ultrastructure. (a–f) *G. tonkinensis* Viet Nam 01, with diffuse, diffluent mucilage only visible with staining (e), figures reprinted from Ohki et al. (2014) with permission from the publisher (Springer); (g–j) *G. verrucosa* PCC 7822, showing sheath pigmentation accumulating in the outer margins of the colonial sheath. Scales in LM: a = 5 μm, b = 100 μm, g and h = 10 μm; scales in TEM = 1.0 μm.

but is well removed phylogenetically from all other sequenced taxa in this study. *Zehria* additionally has distinctive secondary structures for the 16S-23S ITS region, particularly the short BoxB helix which is distinctive compared to the helices of other coccoid genera in this study (Fig. 5), while the V3 helix highly resembled those we found in both *Aphanothece* and *Crocosphaera* (Fig. 6, o, q–x) While there is a fair amount of genetic variability in the 16S rRNA gene and ITS sequences among strains, we chose to name only a single species in this cryptic genus. More study of the genus is needed, and given its diazotrophic ability, such study may be forthcoming.

Genus V – Rippkaea. The third genus lineage consisted of two [*Cyanothece*] sp. strains (PCC 8801

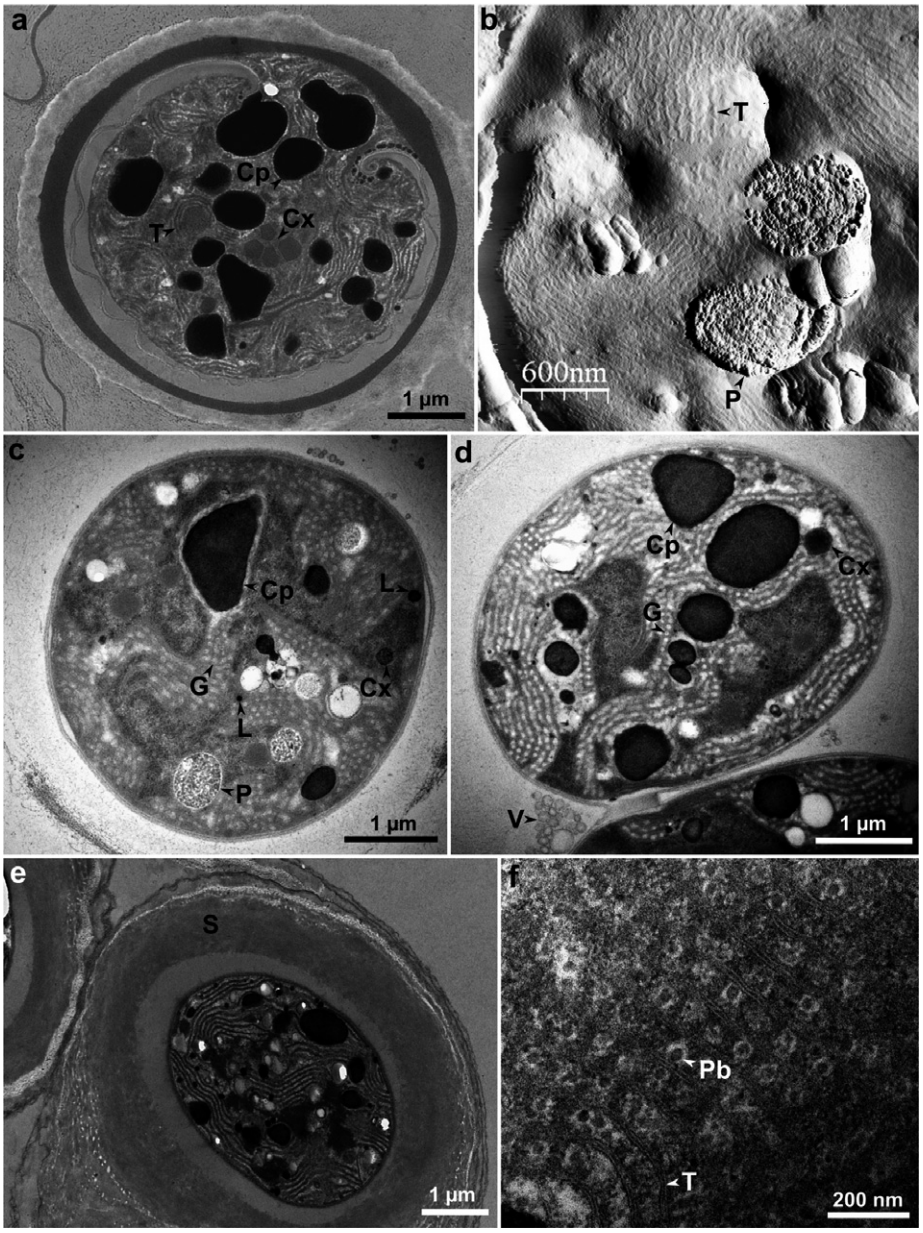

FIG. 12. Ultrastructure of *Gloeothece tepidariorum* CCALA 1114, exemplifying the subcellular morphology of the genus and other genera studied in this work. Irregularly waved fascicles of thylakoids (T) are visible in the cross-section in TEM (a, f) and confirmed in close-to-native state by atomic force microscopy (b). Characteristic inclusions are visible (a–d): Cp – cyanophycin granule; Cx – carboxysome; G – glycogen granule; L – lipid droplet; P – polyphosphate granule. Extracellular vesicles (V) are produced during the new cell wall formation (d). (e) The cells are embedded in mucilaginous sheaths (S). Detail of thylakoid membranes with attached phycobilisomes (Pb) is shown in (f).

and PCC 8802) isolated from rice fields in Taiwan, forming a clade basal to *Crocosphaera* and *Zehria* in the 16S rRNA tree. Another related genus *Aphanothece* was basal to this whole group. There are no multilocus DNA sequence data available for *Aphanothece* and *Zehria* (Fig. 2). Based on polyphasic analyses, these two strains exhibited cell morphology and ultrastructure convergent with that reported for *Aphanothece*: oval to cylindrical unicells dividing by simple binary fission (Fig. 13, k and l), thyalkoids in parallelly arranged fascicles crossing the entire cell (Fig. 13o); absence of clearly delimited and concentrically lamellated mucilaginous envelopes around individual cells or colonies. Unlike *Aphanothece*, the formation of mucilaginous colonies by the two PCC strains was facultative and far less intense. The 16S rRNA gene sequence showed a relatively low genetic identity between *Aphanothece* and *Rippkaea* (95.4%–96.1%) and conserved domains of the ITS region were highly divergent between the two genera (Figs. 4, o and p; 5, t and u; 6, o and p). The separate phylogenetic position combined with the morphology and ITS data led us to describe a new genus and species to accommodate these unique strains.

Taxonomic descriptions/validations. Cyanothece aeruginosa (Nägeli) Komárek (1976: 150)

This is a valid taxon. We report the following description of strain SAG 87.79 to facilitate its comparison to other taxa included in this study. We also designate a specimen prepared from strain SAG 87.79as the neotype for the species.

TABLE 2. Morphological comparison of the studied *Gloeothece* species. Isolates from the current study are compared to literature and to the holotype specimen of the type species of *Gloeothece* (*G. fuscolutea*), and *G. rupestris* as species confused with some of the analyzed strains. BG, blue-green.

Taxon/Strain	Sheath	Colony	Cells (μm)	Color
Gloeothece fuscolutea (holotype)	Colorless to pale yellow, 1–2 concentric lamellations, soft	10–20 μm × 9–14 μm	6–8.5 × 4.7–5	BG
[a]*G. fuscolutea*	Colorless to yellow-brown, 1–2 distinct lamellations, delimited	4–8 (32) cells	6.5–12.5 × 4.4–5.5	Pale BG
G. rupestris (holotype)	Colorless to dark yellow, 1–3 concentric lamellations, firm, minutely warty	16–30 μm × 14–24 μm	6–8 × 3–6.4	Pale BG
[a]*G. rupestris*	Colorless to yellow-brown, distinctly lamellate, delimited	2–8(32) cells	5–9(13–16) × 3.6–6(10)	BG-olive
G. aequatorialis SAG 36.87	Colorless, 1–2 scarcely visible lamellations, soft, delimited	14–36 μm × 12–28 μm	6–14(12) × 5–6	Pale olive
G. aurea CCALA 1111	Colorless, rarely yellow next to cells, 1–2 diffluent lamellations, soft, delimited	13–34 μm × 10–24 μm	5–6(7) × 4–4.5	BG
G. bryophila HA4964-KK2				
G. citriformis PCC 7424	Absent (cells kidney shaped to lemon shaped)	N/A	7–12(11–15) × 6.5–8	Pale BG
G. membranacea SAG 26.84	Colorless, 1(2) lamellations, firm to soft	12–20 μm × 10–18 μm	5.6–9(11) × 4.5–6(8)	Pale gray
[a]*G. membranacea*	Colorless, lamellate, almost diffluent at margin	Small clusters	7–8.8 × 4–6	BG
G. reniformis CCALA 1113	Colorless to tan, 0–1 distinct to diffluent lamellation, outer mucilage delimited	14–28 μm × 9–24 μm	8–10.5(11) × 5–6	Pale gray
G. tepidariorum CCALA 1112	Colorless, 2–4 concentric lamellations, distinctly delimited	18–40 μm × 12–34 μm	5.4–8(9) × 4–5	BG-olive
G. tepidariorum CCALA 1114	Colorless, 2–4 concentric lamellations, distinctly delimited	15–28 μm × 13–22 μm	6–9(11) × 4–4.7	BG-olive
G. tepidariorum CCAP 1430/3	Colorless, 1 lamellation within firm to soft	16–24 μm × 12–18 μm	5.6–9(12) × 4.5–5	Pale olive
G. tepidariorum ACOI604	Colorless (rarely some yellow), 0–2 lamellations, firm to soft	16–40 μm × 14–26 μm	5–8(10) × 4–5.4	BG(olive)
[a]*G. tepidariorum*	Colorless, intensely concentrically lamellate, firm, distinctly delimited	Small clusters	8–15 × 5–6.2	Pale BG
G. verrucosa PCC 7822	Colorless near cells, brown unevenly, 1–2 scarcely visible lamellation, minutely warty	16–36 μm × 12–28 μm	9–18(14–20) × 6–8	BG-pale olive
[a]*G. palea*	Colorless to slightly yellowish, 0–2 indistinct layers, delimited	1–4 cells	3.8–13.5 × 2.5–4.5	Pale BG
[a]*G. dubia*	Pale orange to reddish, colored at margin, sometimes diffluent	2–4 cells	8–11 × 6–8	BG
[a]*G. baileyana*	Bluish, 0–1 lamellations, firm	1(2–4) cells, 20–34 μm × 16–24 μm	12–20 × 8–12	BG
[a]*G. interspersa*	Colorless, wide, 2 lamellations	2–4 cells, rarely 8, 13–18 μm long	7–7.5 × 3.6–4	Pale BG
[a]*G. samoensis*	Yellowish, lamellation scarcely visible	N/A	5–8 × 4–5	Olive-violet BG

[a]Descriptions by Komárek and Anagnostidis (1998).

Basionym: Synechococcus aeruginosus Nägeli (1849) (56, pl. I [1]:E: fig. 1)

Figures 3, a, b, e, f, i, and j; 7a.

Description of the reference strain: Cells solitary or in pairs during division, oval to cylindrical with widely rounded ends, 21–25.2 μm wide and 21.5–39.4 μm long, blue-green to yellowish, granulated. Chromatoplasma more concentrated at cell margin, with reticulate keritomization evident. Mucilaginous sheath absent. Cells dividing by transverse binary fission in a single plane. D1-D1′ helix identical in sequence in both operons, with a basal clamp of 6 bp, a 3′ unilateral bulge of 6 nt, and a large terminal loop with 16 nt. BoxB and V3 helices differing in sequence and structure between both operons and from helices present in *Cyanothece svehlovae.*

Epitype here designated: CBFS A-103-1, herbarium material preserved by drying biomass of the reference strain, SAG 87.79, originally isolated by W. Koch from a bog pool in Ireland in 1977.

Reference strain: Cyanothece aeruginosa SAG 87.79.

Taxonomic notes: Komárek and Cepák (1998) and Komárek et al. (2004) give a more extensive characterization of this strain than given here, including both LM and TEM features. SAG 87.79 was isolated from a cold, oligotrophic bog, and matches exactly

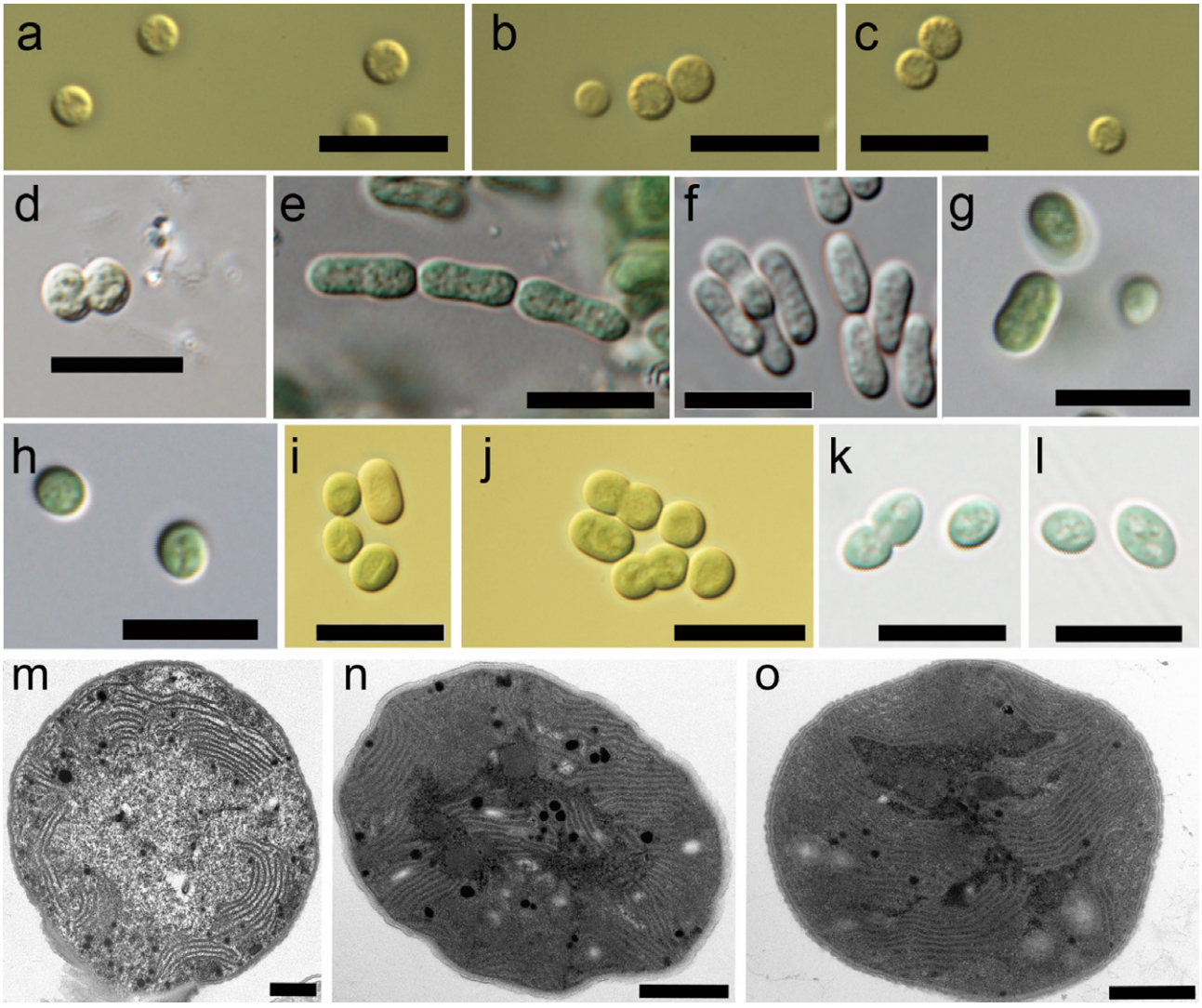

FIG. 13. *Crocosphaera*, *Zehria*, and *Rippkaea* morphology and ultrastructure. Morphology: (a–c) *Crocosphaera watsonii* WH 8501; (d–f) *C. subtropica* ATCC 51142, with cells embedded in diffluent mucilage visible only by staining; (g and h) *C. chwakensis* CCY0110; (i and j) *Zehria floridana* WH 8904; (k and l) *Rippkaea orientalis* PCC 8801. Scales = 10 μm. Ultrastructure: (m) *C. subtropica* ATCC 51142; (n) *C. chwakensis* CCY0110; (o) *R. orientalis* PCC 8801. Scales = 0.5 μm.

the ecology and morphology of the original description given by Nägeli (1849) of the basionym taxon.

Cyanothece svehlovae Mareš, J.R.Johans. et Hauer, sp. nov.

Figures 3, c, d, g, h, k, and l; 7, b–f.

Diagnosis: On average, smaller cells than both *Cyanothece aeruginosa* and *C. major*, which have also been reported from Antarctica. Terrestrial on lichen vegetation, while *C. aeruginosa* and *C. major* are aquatic in the original sense. Secondary structures of conserved domains of the ITS region all differing from those in *C. aeruginosa* SAG 87.79, but especially notable in the shorter length of the V3 helix in both operons. Significantly larger cells than *C. Epiphytica*, which was found in a similar microhabitat (on mosses in Himalaya). Neither halophilic (*C. halobia*, *C. shiloi*) nor planktonic (*C. lineata*).

Description: Cells solitary or in pairs during division, occasionally in small aggregates, oval to shortly cylindrical with widely rounded ends, 10.0–14.5 μm wide and 13.5–23 μm long, blue-green to yellowish, containing granules. Chromatoplasma more concentrated at cell margin, but reticulate keritomization sometimes visible. Mucilaginous sheath absent. Cells dividing by transverse binary fission in a single plane. All secondary structures of conserved domains differing between operons. D1-D1′ helix, with a basal clamp of 5–6 bp, a 3′ unilateral bulge of 5–6 nt, and a large terminal loop with 14–15 nt. BoxB and V3 helices differing in sequence and structure between both operons and from helices present in *C. aeruginosa*. The presence of *nif*D gene confirmed.

Habitat: Polar terrestrial habitat. Lichen vegetation growing on gravel, Antarctica.

Type locality: Queen Maud Land, Antarctica, nearby the Norwegian Meterological station.

Holotype here designated: CBFS A-098-1 herbarium material preserved by drying of biomass of the reference strain, NIVA-CYA 258/1.

Reference strain: Cyanothece svehlovae NIVA-CYA 258/1.

Etymology: Named in honor of Dana Švehlová, the technical assistant of Prof. Jiří Komárek, who has made much of the recent progress in cyanobacterial taxonomy possible.

Taxonomic notes: The material collected by Alex Strømme, University of Oslo was originally designated "A6-89 *Synechococcus*."

Gloeothece aequatorialis Mareš et J.R.Johans., sp. nov.

Figures 4, a and b; 5, a and b; 6, a and b; 8, a–d.

Diagnosis: Similar to *Gloeothece palea* and *G. rupestris*, but differing from those species by greater cell dimensions, absence of yellow pigments in the mucilaginous envelopes (based exclusively on culture observation), and origin in an equatorial tropical habitat in Africa.

Description: Colonies consisting of small cell clusters joined into a gray-green to olive-green smooth macroscopic gelatinous mass covering the substrate, 14–36 μm long by 12–28 μm wide. Mucilaginous envelopes wide, colorless, with 1–2 diffluent, often scarcely visible, concentric lamellations around small clusters of cells. Cells oval to cylindrical, mostly in groups of 4–8, rarely solitary, 5–6 μm wide, 6–14 μm long, with dividing cells 9–12 μm long. Cell contents weakly pigmented, grayish olive to yellowish olive colored, mostly homogenous, sometimes minutely granular. No large storage granules visible in TEM. Secondary structure of the D1-D1′ helix having a terminal loop of 13 nucleotides and two adenine residues on the 5′ strand opposite the unilateral bulge on the 3′ strand, with BoxB helices and V3 helices being unique among all species for which ITS sequence is available at present. The presence of *nif*D gene confirmed.

Habitat: Wet stony wall, gneiss, tropical biome.

Type locality: Yaoundé, Cameroon, Africa.

Holotype here designated: CBFS A-070-1, herbarium material preserved by drying biomass of the reference strain, SAG 36.87.

Reference strain: Gloeothece aequatorialis SAG 36.87.

Etymology: Named for its equatorial origin.

Taxonomic notes: SAG 36.87 was originally misidentified as *Gloeothece rupestris*, and it retains this epithet in the NCBI Nucleotide database. We inspected the type material of *G. rupestris*, which exhibited clearly smaller cells, and an intense yellow-brown pigmentation.

Gloeothece aurea Mareš, Hauer et J.R.Johans., sp. nov.

Figures 4c; 5c; 6c; 8, e–h.

Diagnosis: Cells mostly subspherical to widely oval, typically with a lower length:width ratio than any other *Gloeothece* species. Golden-yellowish pigmentation in the layer of mucilage immediately surrounding the cells.

Description: Small cell clusters joined into yellow-brownish, macroscopic, irregularly subspherical gelatinous colonies growing on the substrate surface. Envelopes around cells wide, mostly colorless, sometimes infused with golden-yellow pigment in the layer of mucilage immediately surrounding the cells, with 1–2 nearly diffluent, often scarcely visible, concentric lamellations around small clusters of cells, with an outer mucilage that is soft but not diffluent. Colonies 13–34 μm long by 10–24 μm wide. Cells mostly in groups of 2–8, rarely solitary, 4–4.5 μm wide, 5–6 μm long, with dividing cells 7 μm long. Cell contents weakly pigmented, pale olive colored, mostly homogenous. No large granules visible in TEM. Secondary structure of the D1-D1′ helix having a terminal loop of 12 nucleotides and a single cytosine residue on the 5′ strand opposite the unilateral bulge on the 3′ strand, with D1-D1′, BoxB, and V3 helices having closest sequence and structural similarity to these structures in *G. reniformis*, but still being unique among all species for which ITS sequence is available at present. The presence of *nif*D gene confirmed.

Habitat: Calcareous stone surface, tropical rainforest.

Type locality: Palenque ruins, Palenque, Chiapas, Mexico.

Holotype here designated: CBFS A-080-1, herbarium material preserved by drying biomass of the reference strain, CCALA 1111.

Reference strain: Gloeothece aurea CCALA 1111.

Etymology: L. *aureus* = golden, referring to the golden sheath.

Gloeothece bryophila J.R.Johans., sp. nov.

Figures 4, d and e; 5, d and e; 6, d and e; 9, a–c.

Diagnosis: Similar to *Gloeothece palea* and *G. aequatorialis* in cell dimensions, but differing from those species by posessing densely packed colonies with limited mucilage, and rare occurrence of distinct individual sheaths around solitary cells or small clusters of cells.

Description: Colonies when young densely packed with cells not separated by individual sheaths, but possessing soft mucilage not extending more than 3 μm beyond outer margin of cell cluster, with densely granular cells blue-green in color. Colonies when mature with cells more loosely aggregated in colonial mucilage extending up to 5 μm beyond outer margin of individual cells or the cell cluster, sometimes with individual sheaths evident, rarely with a single lamallate layer, with nongranular pale grayish blue-green cytoplasm. Mucilage soft but usually defined, sometimes diffluent, colorless, never yellowish. Cells oval to oval-elliptical, slightly irregular in shape when compressed in colony, 5.2–6.7 μm wide, 6.2–11.2 μm long, with dividing cells 9.6–12.5 μm long. Secondary structure of the D1-D1′

helix having a terminal loop of three nucleotides and four residues (5′–AACA–3′) on the 5′ strand opposite the unilateral bulge on the 3′ strand, with D1-D1′, BoxB, and V3 helices having very close structural similarity to these same structures in *Gloeothece tepidariorum*, but differing in sequence, particularly in the terminal loops.

Habitat: Growing among mosses and liverworts on a wet lava wall, tropical island biome.

Type locality: Thurston Lava Tube, Hawaii Volcanoes National Park, Hawaii, USA.

Holotype here designated: CBFS A101-1, herbarium material preserved by drying biomass of the reference strain, HA4964-KK2.

Reference strain: Gloeothece bryophila HA4964-KK2.

Etymology: Named for the habitat from which it was isolated, among damp mosses.

Gloeothece citriformis Mareš et J.R.Johans., sp. nov.

Figures 4, f and g; 5, f and g; 6, f and g; 9, d–g.

Diagnosis: No mucilaginous structure observed in this species in contrast to all other known *Gloeothece* species. Cells frequently reach lemon-like shape before division. Note: observations are based exclusively on culture material.

Description: Cells solitary, unable to float in liquid medium. Mucilaginous envelopes absent. Cells broadly oval, with some lemon-shaped or slightly arcuated, 7–12 μm long, with longer dividing (constricted and/or with crosswall forming) cells 11–15 μm long, 4.7–8.0 μm wide, pale grayish green, cell content finely granular. No large granules visible in TEM. Secondary structure of the D1-D1′ helix markedly different between operons, with the helix from the operon with $tRNA^{Ile}$ being unique among *Gloeothece* species thus far sequenced by having a basal clamp of 5 bp instead of four, and the smallest number of nucleotides (only 7) in the unilateral bulge on the 3′ strand. The D1-D1′ helix from the operon with no tRNA genes is distinct from all other *Gloeothece* species for which this operon was recovered in the presence of a second unilateral bulge on the 3′ strand (5′–ACAU–3′). BoxB and V3 helices distinct from all other *Gloeothece* species. V3 helix absent in the operon with the $tRNA^{Ile}$ gene.

Habitat: Soil of a rice field, tropical biome.

Type locality: Rice field, Senegal, Africa.

Holotype here designated: CBFS A-69-1, herbarium material preserved by drying biomass of the reference strain, PCC 7424.

Reference strain: Gloeothece citriformis PCC 7424.

Etymology: L. *citriformis* = citrus shaped, named for the cells with constricted ends similar to the shape of the ends of a lemon.

Taxonomic notes: The strain upon which this species is based has been called *Cyanothece* sp. in the Pasteur Culture Collection, NCBI Nucleotide, and published phylogenies and accounts. It is clearly phylogenetically and morphologically separated from the type species of that genus, *C. aeruginosa*, and is clearly a member of *Gloeothece* lineage based on molecular evidence. The absence of mucilaginous envelopes is atypical for the genus, but these may have been secondarily lost in the 46 years since its isolation into culture.

Gloeothece membranacea (Rabenh.) Bornet (1892: 175)

Basionym: Aphanocapsa membranacea Rabenhorst (1865: 49)

This is a valid taxon. We report the following description of strain, SAG 26.84, to facilitate its comparison to other taxa included in this study. This strain matches the ecology and morphology, for this species. We designate a specimen from this strain as the neotype for the species.

Figures 4h; 5h; 6l; 9, h–j.

Description of the reference strain: Colonies 12–20 μm long by 10–18 μm wide, in rapidly growing culture smaller, compact colonies with 8–16 cells. Sheath wide, colorless, with a single lamellation around small clusters of cells contained within one colony, rarely up to two concentric layers, with an outer mucilage that is distinctly delimited to almost diffluent. Cells mostly in groups of 2–4, but up to 16, 4.5–6–(8) μm wide, 5.6–9 μm long, with longer dividing (constricted, and/or with crosswall forming) cells up to 11 μm long. Cell contents finely to coarsely granular. Secondary structure of the D1-D1′ helix having a terminal loop of three nucleotides and four residues (5′–AACA–3′) on the 5′ strand opposite the unilateral bulge on the 3′ strand, with D1-D1′, BoxB, and V3 helices having closest sequence and structural similarity to these structures in *Gloeothece reniformis*, but still being unique among all species for which ITS sequence is available at present. The presence of *nif*D gene confirmed.

Neotype here designated: CBFS A-105-1, herbarium material preserved by drying biomass of the reference strain, SAG 26.84; originally isolated by M.M. Allen from a freshwater locality in California.

Reference strain: Gloeothece membranacea SAG 26.84

Gloeothece reniformis Mareš, Hauer et J.R.Johans., sp. nov.

Figures 4, i and j; 5, i and j; 6, g and h; 10, a–e.

Diagnosis: Cells frequently divide unevenly, reaching a kidney-like shape just before division, and producing daughter cells in a mirrored-diagonal position.

Description: Small cell clusters joined into olive-green to brownish, macroscopic, irregularly subspherical gelatinous colonies growing on the substrate surface. Envelopes around cells wide, colorless to yellow-brown, with a single or no lamellation around small clusters of cells contained within one colony, with inner lamellation distinct to diffluent, with an outer mucilage that is distinctly delimited. Colonies 14–28 μm long by 9–24 μm wide. Cells mostly in groups of 4–8, but up to 16, 5–6 μm wide, 8–10.5 μm long, with longer dividing (constricted and/or with crosswall forming) cells up to 11 μm

long, with division uneven to produce kidney-shaped structures when dividing. Cell contents pale yellowish olive color, finely to coarsely granular. Medium-sized granules (mostly cyanophycin) visible in TEM. Secondary structure of the D1-D1′ helix having a terminal loop of 11 nucleotides and two nucleotides on the 5′ strand opposite the unilateral bulge on the 3′ strand in the operon with no tRNA gene, a terminal loop of 13 nucleotides and one cytosine on the 5′ strand opposite the unilateral bulge on the 3′ strand in the operon with the tRNAIle gene, with D1-D1′, BoxB, and V3 helices having closest sequence and structural similarity to these structures in *Gloeothece aurea*, but still being unique among all species for which ITS sequence is available at present. The presence of *nif*D gene confirmed.

Habitat: Volcanic stone surface, tropical island.

Type locality: Old Pali Highway, Island of Oahu, Hawaii, USA, 21°21′56.5″ N, 157°47′26.6″ W.

Holotype here designated: CBFS A-097-1, herbarium material preserved by drying biomass of the reference strain, CCALA 1113.

Reference strain: Gloeothece reniformis CCALA 1113.

Etymology: L. *reniformis* = kidney-shaped, referring to the shape of the cells.

Gloeothece tepidariorum (A.Braun in Rabenh.) Lagerheim (1883: 44)

Basionym: Gloeocapsa tepidariorum A. Braun in Rabenhorst (1865: 38)

This is a valid taxon. We report the following combined description of the strains examined in this study, to facilitate their comparison to other taxa included in this study.

Figures 4, k and l; 5, m–o; 6, i–l; 10, f–h; 12.

Description of the strains examined in this study: Sheath wide, colorless, rarely with some yellowish coloration in a few colonies, with (0)–1–4 concentric lamellations around small clusters of cells contained within one colony, with an outer mucilage that is distinctly delimited to almost diffluent. Colonies 15–40 μm long by 12–26 μm wide. Cells mostly in groups of 2–8, but up to 32, 4.0–5.4 μm wide, 5–9 μm long, with longer dividing (constricted, with crosswall forming) cells up to 12 μm long. Cell contents blue-green, finely to coarsely granular. Secondary structure of the D1-D1′ helix having a terminal loop of 5 nucleotides and four residues (5′–AACA–3′) on the 5′ strand opposite the unilateral bulge on the 3′ strand, with D1-D1′, BoxB, and V3 helices having very close structural similarity to these same structures in *Gloeothece bryophila*, but differing in sequence, particularly in the terminal loops and central helix. The presence of *nif*D gene confirmed.

Neotype here designated: CBFS A-104-1, herbarium material preserved by drying biomass of the reference strain, CCALA 1112, originally isolated from a wall in a tropical greenhouse in the Botanical Garden in Liberec, Czech Republic.

Reference strain: Gloeothece tepidariorum CCALA 1112.

Additional verified strains: CCALA 1114, CCAP 1430/3 (=PCC 6909), ACOI 604

Taxonomic notes: ACOI 604 is called *Gloeothece rupestris* in the original culture collection. Nevertheless, the material in the holotype of *Gloeothece rupestris* contains far more yellow-brown pigmentation. *G. rupestris* is typically found on calcareous wet rocks in European mountains, while *G. tepidariorum* has been mostly reported from wet walls in tropical greenhouses.

Gloeothece tonkinensis Mareš et Johansen, sp. nov.

Figures 4m; 5k; 6m; 11, a–f.

Diagnosis: Larger cells than any other *Gloeothece* species except *G. baileyana* and *G. verrucosa*, from which it is distinguished by cells being distributed in a delimited but diffluent mucilage lacking visible lamellation, surface structures, or pigmentation (based exclusively on culture observation).

Description: Colonies consisting of irregularly arranged cells in large mucilaginous investment joined into macroscopic dark-brown floating gelatinous mats. Mucilage colorless, without visible lamellation or envelopes around individual cells or small colonies. Cells dividing by transverse binary fission in a single plane, 8–10 μm wide, 8–12 μm long. Cell contents pale to dark brownish-green or brown, mostly homogenous, sometimes granulated. No large granules visible in TEM. Secondary structure of the D1-D1′ helix having a terminal loop of 11 nucleotides and five residues (5′–AAAAA–3′) on the 5′ strand opposite the unilateral bulge on the 3′ strand, with D1-D1′, BoxB helices similar to those in *G. verrucosa*, with a V3 helix unique among all species for which ITS sequence is available at present.

Habitat: Rice field, tropical biome.

Type locality: Rice field, Phu Tho area, Tonkin, Vietnam.

Holotype here designated: Figure 11, a–f.

Reference strain: Gloeothece tonkinensis Viet Nam 01.

Etymology: Named for the Tonkin region of Vietnam.

Taxonomic notes: The culture upon which this species is based, Viet Nam 01, was originally thought to be *Aphanothece stagnina* by those who saw the bloom in the Phu Tho area of Vietnam (Nguyen et al. 2012). It was later described and characterized in detail by Ohki et al. (2014), who identified it as *Cyanothece* Viet Nam 01 and provided the ribosomal sequence including a large fragment of the 16S rRNA gene and the complete 16S-23S ITS region. We tried to obtain the culture of this strain in order to prepare a herbarium mount to use as a holotype satisfying Article 40.1 of the International Code of Nomenclature for Algae, Fungi, and Plants (ICN). The culture was not available even upon request. According to the Nagoya Protocol, to which Vietnam and Japan are both signatories, genetic resources (including algal cultures) cannot be sent out of the country without governmental

permission. Article 40.4 of the ICN states "the type of a name of a new species or infraspecific taxon (fossils excepted: see Art. 8.5) may be an illustration prior to 1 January 2007; on or after that date, the type must be a specimen (except as provided in Art. 40.5)." This would exclude using an illustration as a holotype, except when conditions in Article 40.5 are met. This article states "the type of a name of a new species or infraspecific taxon of microscopic algae or microfungi (fossils excepted: see Art. 8.5) may be an effectively published illustration if there are technical difficulties of specimen preservation or if it is impossible to preserve a specimen that would show the features attributed to the taxon by the author of the name." We consider the unavailability of the culture to us, both due to the refusal of the researchers having possession of the culture to share the culture, and the prohibition by the Nagoya Protocol to share genetic resources with scientists in other countries without extensive application for permission, to satisfy the spirit of *technical difficulties of specimen preservation* in Article 40.5, and so designate the figures published by Ohki et al. 2014 and reprinted with journal permission here as the holotype for this new species.

Gloeothece verrucosa Mareš et Johansen, sp. nov.

Figures 4n; 5l; 6n; 11, g–j.

Diagnosis: Larger cells than any other *Gloeothece* species except *G. baileyana*, which however has blueish envelopes; also distinguished from other taxa by the rusty yellow-brown envelopes with delicate warts on their surface.

Description: Colonies consisting of small cell clusters joined into larger, olive-green to brownish macroscopic irregular gelatinous mats or flocks, 16–36 μm long by 12–28 μm wide. Envelopes wide, colorless near cells, unevenly infused with a rusty brownish-yellow pigment, with 1–2 nearly diffluent, often scarcely visible, concentric lamellations around small clusters of cells, with an outer mucilage that is minutely warty. Cells mostly in groups of 2–8, rarely solitary, 6–8 μm wide, 9–18 μm long, with dividing cells 14–20 μm long. Cell contents weakly pigmented, dirty olive colored, mostly homogenous, sometimes minutely granular. No large granules visible in TEM. Secondary structure of the D1-D1′ helix having a terminal loop of 10 nucleotides and five residues (5′–AACGA–3′) on the 5′ strand opposite the unilateral bulge on the 3′ strand, with a BoxB helix similar to that in *G. tonkinensis*, with a V3 helix uniquely shorter than in all other species for which ITS sequence is available at present.

Habitat: Soil of rice fields, tropical biome.

Type locality: Rice fields of Central Rice Research Institute, Cuttack, Orissa, India.

Holotype here designated: CBFS A071-1, herbarium material of the reference strain, PCC 7822, preserved by drying.

Reference strain: Gloeothece verrucosa PCC 7822.

Etymology: L. *verrucosus* = warty, referring to the wartiness of the mucilage.

Taxonomic notes: Despite the presence of distinct mucilaginous envelopes with yellowish pigmentation and lamellations, the strain upon which this species is based has been called *Cyanothece* sp. in the Pasteur Culture Collection, NCBI Nucleotide, and published phylogenies and accounts. Like *Gloeothece citriformis*, it is clearly phylogenetically and morphologically separated from the type species of that genus, *C. aeruginosa*, and is clearly a member of *Gloeothece* based on both morphological and molecular evidence.

Crocosphaera Zehr, Rachel A.Foster, Waterbury et E.Webb, gen. nov.

Cells solitary, in pairs, or in amorphous aggregates, capable of producing large amounts of diffluent mucilage, spherical to elongated, with dispersed thylakoids in fasciculated packets, with high concentrations of urobilin-containing phycoerythrin light-harvesting pigments. Diazotrophic. Marine in tropical oceans.

Type species: Crocosphaera watsonii

Etymology: Crocos Gr. Noun = crocus, orange-colored; *sphaera* Gr. Noun = ball or sphere; an orange sphere due to autofluorescence of high urobilin phycoerythrin.

Crocosphaera watsonii Zehr, Rachel A.Foster, Waterbury et E.Webb, sp. nov.

Figures 4t; 5p; 6q; 13, a–c; Zehr et al. (2001) (fig. 3c).

Diagnosis: Differing from all other species by the complete absence of oval or elongated cells. Percent dissimilarity based on aligned ITS sequences among strains in the genus <0.6%, percent dissimilarity to other species in the genus 12.5%–19.0%.

Description: Cells mostly solitary in nature, but also producing cell aggregates through production of mucilage, spherical, 2.5–6 μm in diameter. Confirmed diazotroph. Secondary structure of the D1-D1′ helix with a single adenosine residue on the 5′ strand opposite the basal unilateral bulge on the 3′ strand, and with a subterminal 3′ unilateral bulge characteristic of both *Crocosphaera* species and *Zehria floridana*. BoxB and V3 helices distinct from all other *Crocosphaera* species.

Habitat: Open warm oligotrophic tropical ocean.

Type locality: Tropical Atlantic Ocean, off Brazil (28° S, 48° W).

Holotype here designated: CBFS A-098-1, herbarium material preserved by drying biomass of the reference strain, WH8501.

Reference strain: Crocosphaera watsonii WH8501

Additional strains: Crocosphaera watsonii WH0002, WH0003, WH0401, WH0402, WH8502.

Etymology: In honor of Stanley W. Watson, American marine microbiologist.

Taxonomic notes: This taxon has been mentioned in over 200 publications (Guiry and Guiry 2018), even though it was never formally proposed,

described, or had authorities cited; thus, it was invalid. We validate it here and describe it minimally, recognizing that there is an extensive amount that is known about this interesting set of strains isolated from the tropical waters off the coast of Brazil. The cultivated *Crocosphaera* strains are currently divided into two phenotypic groups, with large (5–6 μm) and small (2–4 μm) cell diameters, based on growth properties at 25°C (Webb et al. 2009). The strains separate into two groups on the basis of phylogenomic reconstructions (25 genes totaling 25 kb; Bench et al. 2016). The holotype was prepared from WH8501, which belongs to the smaller-sized clade.

Crocosphaera subtropica Mareš et Johansen sp. nov.

Figures 4u; 5q; 6r; 13, d–f, m.

Diagnosis: Differing from all other species by the production of markedly elongate cells in stationary phase which can exceed a 3:1 length-to-width ratio, and also through the production of short temporary chains of cells. Also with a D1-D1′ helix shorter in nucleotides than in all other species. Percent dissimilarity based on aligned ITS sequences among strains in the genus = 0.0%, percent dissimilarity to other species in the genus 12.5%–18.8%.

Description: Cells solitary and spherical in exponential phase, without mucilage production or clumping, with dispersed thylakoids in fasciculated packets, 3.3–4.4 μm in diameter, becoming thin and elongated in stationary growth phase, often medianly constricted, clumping in small aggregates without visible mucilage, sometimes forming short chains, 1.2–1.8 μm wide, 2.8–7.1 μm long. Secondary structure of the D1-D1′ helix shorter than in all other *Crocosphaera* species, lacking the subterminal 3′ unilateral bulge and with nucleotides on the 5′ strand opposite the basal unilateral bulge on the 3′ strand. BoxB helix most similar in structure to that in *C. watsonii*, but with four nucleotide substitutions and two fewer nucleotides in the terminal helix.

Habitat: Subtropical ocean, intertidal area.

Type locality: Intertidal area, sea water, Texas, USA.

Holotype here designated: CBFS A-078-1, herbarium material preserved by drying biomass of the reference strain, ATCC51142.

Reference strain: Crocosphaera subtropica ATCC51142.

Etymology: Named for the subtropical waters from which it was collected in the Gulf of Mexico off the coast of Texas.

Taxonomic notes: The strain upon which this species is based has been known in the literature and public databases as *Cyanothece* sp. It clearly does not belong to that genus based on phylogenetic and ultrastructural evidence.

Crocosphaera chwakensis Mareš et Johansen sp. nov.

Figures 4v; 5r; 6s; 13, g, h and n.

Diagnosis: Most similar to *Crocosphaera watsonii*, particularly in exponential growth phase, but differing from that taxon through the production of cylindically elongated cells in stationary phase. Also with a uniquely long and branched D1-D1′ helix unique among all cyanobacteria for which ITS structure analysis have been completed. Percent dissimilarity based on aligned ITS sequences to other species in the genus 14.3%–19.0%.

Description: Cells spherical to cylindrically elongated, with clumping not observed, with dispersed thylakoids in fasciculated packets, 3.0–4.4 μm wide, 3.7–6.7 μm long. Secondary structure of the D1-D1′ helix highly unusual, with terminal branching in the helix and a large unpaired loop in central helix. BoxB helix identical in length to that of *Crocosphaera* species, but differing at the terminus.

Habitat: Marine sediment, tropical ocean.

Type locality: Chwaka, Zanzibar.

Holotype here designated: CBFS A-079-1, herbarium material preserved by drying biomass of the reference strain, CCY0110.

Reference strain: Crocosphaera chwakensis CCY0110.

Etymology: Named for the collection locality: Chwaka, Zanzibar.

Taxonomic notes: The strain upon which this species is based has been known in the literature and public databases as *Cyanothece* sp. It clearly does not belong to that genus based on phylogenetic and ultrastructural evidence.

Crocosphaera sp. 1

Figures 4w; 5s; 6t.

Diagnosis: Most similar to *C. subtropica* in morphology, particularly in cell shape and degree of clumping, but without markedly elongated cells. Most similar to *C. watsonii* in secondary structures of the 16S-23S ITS region, but having a BoxB helix different in structure and sequence from that taxon. Percent dissimilarity based on aligned ITS sequences among strains in this species <1.2%, percent -dissimilarity to other species in the genus 12.5%–16.7%.

Description: Colonies in the form of small clumps or sheet-like aggregates, with evident mucilage, not aggregating or producing mucilage in exponential phase of growth. Cells spherical to oval, with dispersed thylakoids in fasciculated packets, 2.4–4.4 μm wide, 2.7–5.6 μm long. Confirmed diazotrophic. Secondary structure of the D1-D1′ helix as in *C. watsonii*. Sequence of the BoxB helix also most similar to *C. watsonii*.

Reference strains: KO38U6 and KO25B (Ohki et al. 2008).

Taxonomic notes: This taxon was reported from coastal waters near Singapore, and was collected and characterized more extensively in Ohki et al. (2008). The strain was not available upon request, and consequently an immobilized holotype specimen could not be prepared in accordance with requirements of the International Code of Nomenclature of for Algae, Fungi, and Plants. Consequently, it cannot be named in the present work,

although it is clearly a species separate from the other described species.

Crocosphaera sp. 2

Diagnosis: Similar to *Crocosphaera chwakensis* in morphology, particularly in cell shape (markedly elongated cells are present). No rRNA ITS sequences are available, the putative species is separated solely based on the formation of a separate clade in the 16S rRNA gene tree (Fig. 1).

Description: Cells spherical, oval to cylindrical, with dispersed thylakoids in fasciculated packets, ~2–4 μm wide, 3–6 μm long. Colonies in the form of small clumps or short rows, not aggregating or producing mucilage in exponential phase of growth. Confirmed diazotrophic.

Reference strains: KNU CB MAL-031 and KNU CB MAL-058 (Park et al. 2014).

Taxonomic notes: This taxon was reported from coastal waters near Korea, and was collected and characterized more extensively in Park et al. (2014). The strains were not available upon request, and consequently an immobilized holotype specimen could not be prepared in accordance with requirements of the International Code of Nomenclature for Algae, Fungi, and Plants. Consequently, it cannot be named in the present work, although it is probably a species separate from the other described species.

Zehria Johansen et Mareš, gen. nov.

Diagnosis: Morphologically identical to *Crocosphaera*, but differing phylogenetically and having shorter BoxB helix in the 16S-23S ITS (26 nt vs. 32–33 nt). Differs from other morphologically similar coccoid genera through the absence of mucilage surrounding cells as well as by the ability to fix atmospheric N_2. Note: observations are based exclusively on culture material.

Marine in tropical oceans.

Description: Cells solitary or briefly in pairs following binary fission, without mucilage, short bacilloid to spherical, 1.0–2.0 times as long as wide. Diazotrophic.

Type species: Zehria floridana

Etymology: Zehria = Named in honor of Jonathan Zehr, a prominant researcher in the field of diazotrophic cyanobacteria.

Zehria floridana Johansen et Mareš, gen. et sp. nov.

Figure 13, i and j.

Diagnosis: Morphologically identical to *Crocosphaera*, but differing phylogenetically. Differs from other morphologically similar coccoid genera through the absence of mucilage surrounding cells as well as by the ability to fix atmospheric N_2. Differs from the *Zehria* strains from Singapore in its smaller cell size. Note: observations are based exclusively on culture material.

Description: Cells solitary or briefly in pairs following binary fission, without mucilage, short bacilloid to spherical, 1.0–1.7 times as long as wide, 4–5.2 μm wide, 4–8 μm long, dividing cells showing constriction of the wall, 7–11 μm long.

Habitat: In tropical marine surface waters.

Type locality: Coastal Mangroves, Florida

Holotype here designated: CBFS A-100-1 herbarium material preserved by drying biomass of the reference strain, WH8904.

Reference strain: Zehria floridana WH8904

Etymology: floridana = for the site of isolation, Florida mangroves.

Taxonomic notes: The strain upon which this species is based has been known in the literature and public databases as *Cyanothece* sp. It cannot remain in that genus based on phylogenetic and ultrastructural evidence. Unfortunately, we do not have 16S-23S ITS sequence for this strain, so a definitive molecular separation from the Singapore strain is not possible at this time.

Zehria sp.

Figures 4, q–s; 5, v and w; 6, v–x. Morphology illustrated in Ohki et al. (2008) (Figs. 2 and 3).

Diagnosis: Morphologically identical to *Crocosphaera*, but differing phylogenetically and having a shorter BoxB helix in the 16S-23S ITS (26 nt vs. 32–33 nt). Differs from other morpholgically similar coccoid genera through the absence of mucilage surrounding cells as well as by the ability to fix atmospheric N_2. Note: observations are based exclusively on culture material.

Description: Cells solitary or briefly in pairs following binary fission, without mucilage, short bacilloid to spherical, 1.0–2.0 times as long as wide, 2.0–4.0 μm wide, 2.0–5.6 μm long, dividing cells showing constriction of the wall up to 7.0 μm long. The secondary structures of conserved domains are similar to those in some *Crocosphaera* species. The D1-D1′ helix possesses the subterminal 3′ unilateral bulge characteristic of *C. watsonii* and *Crocosphaera* sp., and the V3 helices are exceptionally short in both genera as well. Only the Box B helix is clearly separated by its shorter length and considerably different sequence and secondary structure.

Habitat: under mangrove forest, surface of sand and sediment, on the thallus of seaweed, on rock surfaces, all in intertidal zone.

Source locality: Diverse localities around Singapore, Malaysia, and Japan (Ohki et al. 2008, table 1).

Strains in the species: SK40, KO11DG, KO68DG (Ohki et al. 2008)

These strains of *Zehria* were isolated from coastal waters near Singapore (Ohki et al. 2008), a different ocean, different continent, and different climate than WH8904. We suspect that the Singapore strains belong to a separate species based on phylogenetic evidence, but the strains are not yet publicly available. We hope that at some future point their taxonomic status will be resolved more definitively. For now, we place them provisionally in *Zehria* sp.

Rippkaea orientalis Johansen et Mareš, gen. et sp. nov.

Figures 4p; 5u; 6p; 13, k, l and o.

Diagnosis: No spatially delimited mucilaginous structures observed in this species in contrast to all other known *Aphanothece* and *Gloethece* species. Note: observations are based exclusively on culture material.

Description: Cells solitary, unable to float in liquid medium. Mucilaginous envelopes absent. Cells broadly oval to cylindrical with rounded ends, 4–9 μm long, 3–5 μm wide, bright to pale blue-green, cell content homogenous or finely granular. No large granules visible in TEM. Secondary structure of the D1-D1′ helix distinct from all other taxa in this study, with a short helix on the 3′ side of the central helix, and a single adenosine residue on the 5′ strand opposite the unilateral bulge on the 3′ strand, with D1-D1′, BoxB, and V3 helices all unique among the ITS regions sudied in this work.

Habitat: Soil of a rice field, tropical biome.

Type locality: Rice field, Ping-Tong district, Southern Taiwan.

Holotype here designated: CBFS A-017-1, herbarium material preserved by drying biomass of the reference strain, PCC 8801.

Reference strain: Rippkaea orientalis PCC 8801.

Etymology: Rippkaea = in honor of Rosmarie Rippka, a prominent cyanobacterial researcher who ushered in the period of using strains to study cyanobacteria; *orientalis* = eastern, from Asia (China).

Taxonomic notes: The strain upon which this species is based has been known in the literature and public databases as *Cyanothece* sp. It cannot remain in that genus based on phylogenetic and ultrastructural evidence.

DISCUSSION

Taxonomy of cyanobacteria assigned to the Chroococcales in its narrowed, most recent taxonomic sense (Komárek et al. 2014, Mareš 2018) has been largely misunderstood, and the circumscription of genera widely misinterpreted. This unfavorable situation has most likely arisen due to the scarcity of distinct morphological traits and a relatively high level of evolutionary convergence (homoplasy) in unicellular cyanobacterial taxa (Schirrmeister et al. 2011, Dvořák et al. 2014). In the current study, we endeavor to provide one of the first steps toward a conservative but accurate view of the evolutionary relationships within the unicellular oval- to rod-shaped cyanobacteria with or without mucilaginous envelopes and non-parietal organization of thylakoids (Table 3). We focus on the existing botanical genera *Gloeothece* and

TABLE 3. Main diagnostic features distinguishing the cyanobacterial genera studied in this work.

Genus	Cell shape	Colony formation	Thylakoid arrangement	Habitat	rRNA ITS features
Cyanothece	Oval to cylindrical	No	Special (radial with a central network)	Freshwater, terrestrial	0 or 2 tRNA genes, base of D1-D1′ helix with 5–6 bp and no residues on 5′ strand opposite the 3′ basal bulge
Gloeothece	Oval to cylindrical	Yes (rarely absent); cells usually with individual envelopes	Irregular fascicles	Freshwater, terrestrial	0 or 1 tRNA genes, base of D1-D1′ helix with 1–5 residues on 5′ strand opposite the 3′ basal bulge
Crocosphaera	Spherical to cylindrical	No	Irregular fascicles	Marine	0 or 1 tRNA genes, D1-D1′ helix highly variable with 1–2 residues on 5′ strand opposite the 3′ basal bulge, V3 helix very short
Zehria[a]	Spherical to cylindrical	No	Irregular fascicles	Marine	0 or 1 tRNA genes, D1-D1′ helix with large subapical bilateral bulge, V3 helix very short
Rippkaea[a]	Oval to cylindrical	No	Irregular fascicles	Semi-terrestrial (soil of a rice field)	1 tRNA gene, D1-D1′ helix with short side helix on 3′ strand, V3 helix long
Aphanothece	Oval to cylindrical	Yes; individual cell envelopes scarce (only some species at margins of large colonies)	Irregular fascicles	Freshwater, terrestrial (some species marine, but identity not confirmed by molecular methods)	1 tRNA gene, D1-D1′ helix long and unbranched, V3 helix very short
"Cyanothece" PCC 7425[a]	Cylindrical	No	Parietal	Semi-terrestrial (soil of a rice field)	2 tRNA genes, base of D1-D1′ helix with 0 residues on 5′ strand opposite the 3′ basal bulge

[a]Morphology known only from cultures.

Cyanothece, further discussing their evolutionary relatives, particularly *Aphanothece*, *Crocosphaera*, *Rippkaea*, and *Zehria*, based on both rRNA operon sequences and protein-coding gene data. We omit from our discussions several morphologically close but phylogenetically distant genera such as *Halothece* and *Cyanobacterium*, which have been given attention elswhere (Margheri et al. 2008, Korelusová et al. 2009).

The Cyanothece *lineage*. Our results on the representatives of *Cyanothece* sensu stricto are in full accordance with the modern definition of the genus provided by Komárek and Cepák (1998). The two sequenced strains of *Cyanothece* (SAG 87.79 and NIVA-CYA 258/1) show a relatively high sequence divergence. Since NIVA-CYA 258/1 was isolated from lichen vegetation in Antarctica, its ecology and morphology does not fully match that of *C. aeruginosa*, and it is here recognized as a separate species. The clustering of *Cyanothece* in a special and derived phylogenetic lineage close to filamentous taxa is congruent with previous reports (Bohunická et al. 2015b). This vast evolutionary divergence further highlights the need for designation of suitable taxonomic names for unrelated isolates attributed incorrectly to *Cyanothece* in culture collections and sequence databases.

The Gloeothece *lineage*. The evolutionary reconstruction based on both 16S rRNA data alone and the 16S rRNA gene accompanied by two protein-coding loci unequivocally revealed a monophyletic clade, which was identified as the traditional genus *Gloeothece*, as defined using the recently conserved generitype *G. fusco-lutea* (Mareš et al. 2013c). The phylogenetic cluster contains relatively divergent 16S rRNA sequences (as low as 92.6% identity) that could easily be recognized as two or three genera (Perkerson et al. 2011, Mühlsteinová et al. 2014a,b, Řeháková et al. 2014, Bohunická et al. 2015a, Miscoe et al. 2016). It is uncertain however, to which of the subclades the not yet sequenced type species (*G. fuscolutea*) belongs. Given the morphological, ultrastructural, and ecological uniformity within the clade at present, we chose to use the conservative single-genus approach. As the clade becomes better represented in molecular phylogenies and as the type species is eventually sequenced, it seems very possible that more genus-level clusters will become separated. Conveniently, the cluster contains the reference strain of the bacteriological "form-genus" *Gloeothece*, *G. membranacea* PCC 6501 (Rippka et al. 1979). The most prominent morphological trait separating *Gloeothece* from other genera is the presence of concentrically lamellated envelopes around individual cells that give rise to several-celled gelatinous colonies as a result of cell proliferation. Some strains within the genus, especially those with terrestrial or aerophytic life strategies (such as the members of *G. tepidariorum*, *G. aurea*, *G. reniformis*, but also the possibly aquatic *G. verrucosa*), facultatively exhibit production of yellow-brown pigments in the sheath material. This can be explained by the increased need for UV-screen substances that would protect their photosynthetic apparatus in (semi-)terrestrial habitats (Hauer et al. 2015).

Contributing to the ecological definition of *Gloeothece*, all sequenced strains within the genus were isolated from freshwater or terrestrial ecosystems, with no marine representatives. Furthermore, all *Gloeothece* strains, in which the ability or at least genetic potential for dinitrogen fixation was assessed, were positive in this trait (Mullineaux et al. 1980, Latysheva et al. 2012, Ohki et al. 2014, *nif*D analysis in this study). The nitrogenase activity in *Gloeothece* has been reported for a long time and studied in detail in some laboratory strains (Meeks et al. 1978, Mullineaux et al. 1980, 1981, 1983, Thomas et al. 1982, Maryan et al. 1986, Gallon et al. 1988, Ortega-Calvo and Stal 1991, Ohki et al. 2014). However, the specific ecological roles and adaptations provided by this ability in the source ecosystems of *Gloeothece* have rarely been discussed (Compaoré and Stal 2010). Another component of a typical life strategy in *Gloeothece* is extensive production of extracellular polysaccharide substances (EPS) that play important roles in substrate utilization (Ortega-Calvo and Stal 1994, Saiz-Jimenez 1997, Pereira et al. 2011a), resilience to toxic substances (Pereira et al. 2011a), tolerance to desiccation, and biofilm formation (Gorbushina 2007, Hauer et al. 2015). On the other hand, the production of mucilaginous structures is facultative and environmentally dependent in many cyanobacteria (Strunecký et al. 2013). Specifically in *Gloeothece*, this ability may demonstrably be lost as a result of mutation in culture as proved in *G. tepidariorum* PCC 6909 (Micheletti et al. 2008). In the light of this evidence, absence of lamellated envelopes in *Gloeothece tonkinensis* Viet Nam 01 (Ohki et al. 2014) and complete absence of visible EPS in *Gloeothece citriformis* PCC 7424 (Rippka et al. 1979, this study) could be an artifact of cultivation. More material phylogenetically close to these strains needs to be studied before we can reach a conclusion concerning the possible natural occurrence and survival of sheathless species or populations of *Gloeothece*.

At the infrageneric level, we were able to identify all European *Gloeothece* isolates as members of two existing species – *G. membranacea* and *G. tepidariorum*. On the other hand, strains originating from tropical countries distant from Europe were recognized as taxa new to science. This is not surprising, since the non-European regions are still very understudied. In accordance with the current hypotheses on the distribution of cyanobacterial species (Nabout et al. 2013, Ribeiro et al. 2018), an enormous unexplored taxonomic diversity can be expected in specific tropical microhabitats. Although describing new species based on a single isolate is

not a favorable practice in cyanobacterial taxonomy, the strains (SAG 36.87, PCC 7424, PCC 7822, HA4964, Viet Nam 01, CCALA 1111, CCALA 1113) were so unique in both genotypic and phenotypic characters that leaving them unnamed would probably create more confusion than establishing new species. We realize that the definition of such taxa may need future amendment as more data are collected.

The Crocosphaera *lineage.* It has been known for some time that several groups of nitrogen-fixing unicellular cyanobacteria occur in coastal oceanic waters (Zehr et al. 2001, Ohki et al. 2008, Park et al. 2014). Unfortunately, the taxonomy applied to the isolates of these organisms by their collectors has been inconsistent with phylogeny and insufficient in terms of both the International Code of Botanical Nomenclature and the International Code of Nomenclature of Prokaryotes. The single taxon with a species epithet, *C. watsonii*, was introduced without a description (Komárek and Hauer 2013), while other putative species were only vaguely characterized based on the "form-genus" concept (Castenholz 2001) and designated as genera *Cyanothece*, *Gloeothece*, or *Gloeocapsa* without a species name. In the current study, we correct this confusion by validating the genus and type species utilizing the material of its reference strain *C. watsonii* WH8501 and describe two new species of *Crocosphaera* for those strains that were placed in incorrect genera in the past. All species of *Crocosphaera* are marine diazotrophic coccoid cyanobacteria with a highly uniform morphology, ultrastructure, and habitat of origin and cluster tightly with the generitype, *C. watsonii*. Some of the newly recognized *Crocosphaera* strains are important laboratory cultures that have been subjected to genome sequencing and numerous experimental studies, and despite their cryptic nature we have described them as new species because of their significance to scientific progress. The precedent for describing cryptic species taxonomically rather than leaving them undesignated in paraphyletic or polyphyletic morphospecies was set in the past few years (Osorio-Santos et al. 2014, Bohunická et al. 2015a).

The species in *Crocosphaera* are fairly cryptic, and we rely on percent dissimilarity based on aligned ITS sequences to other species in the genus as one of the major criteria for species recognition. This follows precedents set in a number of cyanobacterial taxonomy papers (Erwin and Thacker 2008, Osorio-Santos et al. 2014, Pietrasiak et al. 2014, Bohunická et al. 2015a, González-Resendiz et al. 2018a,b, Mai et al. 2018, Vázquez-Martínez et al. 2018). These papers have shown that there is generally a marked discontinuity between percent dissimilarity within species (<3.0%) and percent dissimilarity between species (>7.0%). So far, this criterion has been consistent with phylogenetic and morphological separation of close sister species.

Intriguingly, we found a number of endosymbiotic coccoid cyanobacterial descendants to be closely related to *Crocosphaera*. Endosymbionts (spheroid bodies) of rhopalodiacean diatom genera, such as *Epithemia* and *Rhopalodia*, were demonstrated to be capable of nitrogen fixation that presumably provides an advantage to the host (Nakayama et al. 2011, Nakayama and Inagaki 2014). Besides, these endosymbionts exhibit a morphology and even thylakoid arrangement similar to those of *Crocosphaera* (Bergman et al. 1997). Another related lineage is represented by *Candidatus Atelocyanobacterium thalassa* (so-called cyanobacterium UCYN-A), a Prymnesiophyte endosymbiont group (Thompson et al. 2012, 2014, Hagino et al. 2013). These widely distributed cyanobacteria (Krupke et al. 2014, Messer et al. 2015) have already lost the ability of independent photosynthesis (Bothe et al. 2010, Tripp et al. 2010). Altogether, the data strongly support the hypothesis that *Crocosphaera* and these plastid-like diazotrophic endosymbionts had a close common ancestor (Bombar et al. 2014). In fact, the endosymbiotic cyanobacteria of a marine diatom *Climacodium frauenfeldianum* analyzed by Carpenter and Janson (2000) clustered inside the *C. watsonii* cluster in our 16S rRNA tree, suggesting a recent origin of this symbiotic association. Consequently, our results suggest that the endosymbiosis between *Crocosphaera*-like cyanobacteria and various eukaryote hosts may have occurred repeatedly. We hope that the taxonomic treatment of *Crocosphaera* given here will help to provide a clear backgroud for future research in this fascinating field that has implications for the global nitrogen cycle. Calculation of the probable number and chronology of endosymbiotic events as well as assessment of the level of obligation in the symbiotic relationship are desirable for understanding the evolution of cyanobacteria in this clade.

The Zehria *lineage.* A new genus is erected here to harbor five strains of coccoid marine diazotrophic cyanobacteria (Fig. 1). Four of them were isolated from the coast of Singapore together with *Crocosphaera* sp. 1 (Ohki et al. 2008). The remaining one, reference strain of *Z. Floridana*, was isolated from coastal mangroves in Florida (Ehrenreich et al. 2005), suggesting possible wide distribution of the genus. Morphologically and ultrastructurally, the genus is rather cryptic and cannot be easily distinguished from *Crocosphaera* (Fig. 13), yet it holds a clearly separate phylogenetic position and exhibits unique traits in the secondary structure of the rRNA ITS. By establishing this new taxon, we hope to facilitate further studies to disentangle the intriguing evolution of the single-celled marine nitrogen-fixing cyanobacteria.

The Rippkaea *lineage.* Two strains (PCC 8801 and PCC 8802) designated here as a new genus and species, *Rippkaea orientalis*, were originally isolated from rice fields in Taiwan (as *Synechococcus* RF-1 and RF-2; Huang and Chow 1988). They are phylogenetically

close although not monophyletic with *Aphanothece* (Fig. 1) and exhibit similar properties, except the formation of distinct EPS structures (Castenholz 2001, this study). Recently, the genus *Aphanothece* was subjected to polyphasic study and taxonomic revision (Komárek et al. 2011). Members of the *Aphanothece* sensu stricto cluster typically form amorphous gelatinous colonies in benthic or terrestrial habitats, and their cells are oval to cylindrical, with fascicles of thyalkoids crossing the whole cell. The absence of colony formation in *Rippkaea* again may be constitutive or it may have been induced by culture conditions or completely lost as a result of mutation during the decades in an artificial environment. *Aphanothece* as the basal taxon in the broad lineage containing *Rippkaea*, *Zehria*, and *Crocopshaera* has long been known to possess the ability to fix atmospheric nitrogen (Singh 1973, 1977, Kostyaev 1990). This metabolic ability is present in *Rippkaea* as well (Huang and Chow 1988) and seems to be rather ancestral in Chroococcales.

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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:

Table S1. List of all strains examined in this study, with NCBI GenBank accession numbers.

Table S2. List of primers used during the course of this study.

Table S3. Pairwise percent identity (1 − p-distance) × 100)) in the 16S rRNA gene sequence among coccoid cyanobacteria analyzed in this study. Individual genera are highlighted by colours, intra- and intergeneric sequence identity ranges are given above the table.