The University of South Bohemia in České Budějovice
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Unsuspected findings about phylogeny and ultrastructure of the enigmatic cyanobacterium *Microcrocis geminata* resulted in its epitypification and novel placement in Geminocystaceae

RNDr. thesis

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Annotation

In present study the epitype originating from a large natural population of the type species *M. geminata* was established. The type material was characterised phylogenetically using cultivation-independent approach, morphologically and ultrastructurally. The phylogenetic placement in Geminocystaceae is supported by the unique parallel thylakoids.

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

In České Budějovice 11. 4. 2024

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Jan Pokorný

Author contributions of the manuscript:

I declare that I have contributed significantly by sample collection and molecular data acquisition. I have also participated in the phylogenetic analyses and writing the original draft. My total contribution to this publication was 40%.

Jan Pokorný

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As co-author of this article, I agree with the above data.

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As co-author of this article, I agree with the above data.

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Abstract

The genus *Microcrocis* belongs to long ago established cyanobacterial taxa for which relevant data required for a modern taxonomic analysis remained unknown for a long time. In present study we proposed an epitype originating from a large natural population of the type species *M. geminata*. Through isolating clean individual colonies from the environmental sample, sufficient material for a single colony PCR and amplification of genomic DNA by MDA method was obtained. Molecular analyses of the 16S rRNA gene determined the unsuspected position of the cyanobacterium within the family Geminocystaceae, forming a distinct branch with two *Merismopedia* sequences. 16S rRNA and ITS analysis clearly separated the two taxa on generic level. The phylogenetic classification is conspicuously consistent with the ultrastructure of parallel thylakoids unique for some members of the family Geminocystaceae. Distinctive morphology of the genus including typical longitudinal division is discussed.

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Unsuspected findings about phylogeny and ultrastructure of the enigmatic cyanobacterium *Microcrocis geminata* resulted in its epitypification and novel placement in Geminocystaceae

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Abstract: The genus *Microcrocis* belongs to long ago established cyanobacterial taxa for which relevant data required for a modern taxonomic analysis remained unknown for a long time. In present study we proposed an epitype originating from a large natural population of the type species *M. geminata*. Through isolating clean individual colonies from the environmental sample, sufficient material for a single colony PCR and amplification of genomic DNA by MDA method was obtained. Molecular analyses of the 16S rRNA gene determined the unsuspected position of the cyanobacterium within the family Geminocystaceae, forming a distinct branch with two *Merismopedia* sequences. 16S rRNA and ITS analysis clearly separated the two taxa on generic level. The phylogenetic classification is conspicuously consistent with the ultrastructure of parallel thylakoids unique for some members of the family Geminocystaceae. Distinctive morphology of the genus including typical longitudinal division is discussed.

Key words: blue–green algae, Geminocystaceae, Merismopediaceae, phylogeny, taxonomy, thylakoids

Introduction

During the first decade of the 21st century, a trend emerged in cyanobacterial taxonomy to characterize new taxa using the full range of possible methods, including the ecology, light and electron microscopy, and primarily phylogenetic analyses of molecular markers. This methodological approach was called the "polyphasic approach" or alternatively "total evidence approach" (Johansen & Casamatta 2005; Komárek 2010; Mareš et al. 2019a). A new taxon may be described – apart from traditional morphological criteria – either via the provision of a sequence or a suitable genetic marker novel to a database and sufficiently distinguishable from others (Dvořák et al. 2018; Li et al. 2019). Additionally, polyphasic, phylogeny–based approach accepting just monophyletic taxa revealed ubiquitous cryptic diversity in the Cyanobacterial systematics (e.g., Johansen et al. 2021). Therefore, the novel approach has led to an explosion of descriptions of new taxa in recent years. In the year 2021 alone, the Web of Science™ database (www.webofscience.com) recorded at least 29 newly described genera and 53 new species. From those numbers follows that describing a new taxon, whether new to science or a cryptic one, is quite common. It is considerably less frequent for authors to apply the polyphasic approach to species that have been described long ago and need "modern verification". The fundamental problems with this type of work are at first, the necessity of examining dated literature that contain frequent ambiguities and is full of incomplete information thus the task requires considerable time commitment (Komárek et al. 2011; Mareš et al. 2013; Aguilera et al. 2018; Mareš et al. 2019a; Mikhailyuk et al. 2019). Secondly, the original or reference material is frequently not available (e.g., Shen et al. 2018). However, avoiding work of this nature may result in describing particular species more than once and therefore undesirable taxonomic inflation (e.g., Ehrenberg 1834; Meyen, 1839; Trevisan 1845; Joosten, 2006). Nevertheless, there are also valuable studies available that confirm the existence and verify the phylogenetic position of traditional taxa by modern molecular methods. Case studies include those of *Aphanothece* Nägeli and *Anathece* (Komárek et Anagnostidis) Komárek, Kaštovský et Jezberová (Komárek et al. 2011), *Hormoscilla* Anagnostidis et Komárek, *Crinalium* Crow, *Starria* Lang (Bohunická et al. 2015) and *Johannesbaptistia* De Toni (Bertold et al. 2020).

The genus *Microcrocis* Richter is often characterized as *Merismopedia* Meyen with oval or more or less elongated cylindrical, never spherical cells. Colonies consisting of numerous cells are flat and microscopic or sometimes even macroscopically visible. Mucilage around the colonies form an envelope with indistinct margins. Cells in young colonies often form regular rows; however, older colonies lose their regularity (Fott 1972). Cells are oval to cylindrical, circular in cross section in subgenus *Microcrocis* and with a clearly polygonal cross section in the subgenus *Beckia* Elenkin (Komárek & Anagnostidis 1999).

Species of *Microcrocis* are mostly freshwater. Exceptions are *M. sabulicola* (Lagerheim) Geitler and *M. marina* (Lagerheim) Komárek et Anagnostidis known to be marine to brackish and *M. gigas* (Ryppowa) Komárek et Anagnostidis, a purely brackish species (Komárek & Anagnostidis 1999). Although *M. pulchella* (Buell) Geitler was described as a freshwater species, it was later found in brackish water as well (Werner & Sant'Anna 2006). Freshwater species preferably inhabit the benthos of oligotrophic to mesotrophic waters but can be secondarily found in plankton, metaphyton or tychoplankton (Fott 1972; Komárek & Anagnostidis 1999; Skácelová & Zapomělová 2010). One undefined species of *Microcrocis* from Lake Vechten, Netherlands, was observed to actively move among detritus including active detachment and reattachment of subcolonies (Frank & Landmann 1988). None of the representatives are known to form aerotopes. A single species, *M. bella* (Beck) Komárek et Anagnostidis, forms granules of unknown composition between the cells (Komárek & Anagnostidis 1999). The type species of the genus is *M. geminata* (Lagerheim) Geitler (Guiry & Guiry 2022).

Microcrocis geminata was firstly described in the genus *Merismopedia* as *Merismopedium*[1] (subg. *Holopedium*) *geminatum* Lagerheim considering taxonomically relevant the common forming of flat colonies (Lagerheim 1883). The genus *Merismopedium* was divided into subgenera *Merismopedium* and the newly established *Holopedium* (Lagerheim 1883). The subgenus *Holopedium* differed from the subgenus *Merismopedium* by irregularly distributed cells. For this reason, *Merismopedium geminatum* was designated as a member of subgenus *Holopedium* (Lagerheim 1883). Almost a decade later, Richter (1893) described a similar taxon but considered it novel and thus named it *Microcrocis dietelii* Richter and designated it as a type species of a novel genus *Microcrocis*. *Microcrocis dietelii* was considered to be conspecific with *Merismopedium geminatum* by further authors (Lagerheim 1893) and simultaneously the subgenus *Holopedium* was allocated a separate genus with the inclusion of the species *Holopedium geminatum* (Lagerheim 1893). Migula (1905) kept recognizing *Holopedium* as a distinct genus but separated *Holopedium dietelii* and *H. geminatum* as two distinct species, though they just slightly differed in cell length (by 2 μm).

Geitler (1942) re–examined the original sources and focused on the difference between the "isolated" cells in the *Holopedium* colony (original drawings in Lagerheim 1883) and the "adherent" cells of *Microcrocis* (original description of Richter 1893). Considering his own findings as well, Geitler (1942) again separated the genus *Microcrocis* from *Holopedium* and established *M. geminata* as the type species. On the contrary, Fott (1972) suggested synonymizing *Holopedium* and *Microcrocis* given the insufficient difference between the genera as proposed by Lagerheim (1893), while recognising it as *Microcrocis* and not considering the taxonomic priority of *M. geminata*, with the type species *M. dietelii*. Today's accepted type of the genus *Microcrocis* is *M. geminata* (Geitler 1942).

The situation illustrated the limits of the morphological approach, being sometimes arbitrary in the recognizing some morphological traits to be relevant for taxonomy, and thus differing in particular authors. Surprising results uncovering polyphyly and changing taxonomic position have been found from the molecular revisions of many cyanobacterial taxa (e.g., Komárková et al. 2010; Komárek et al. 2016) and therefore the relations between the genus *Microcrocis* and *Merismopedia* (and its subgenera) should be verified as well. Moreover, previous studies, marginally touching the phylogeny of *Merismopedia* suggested its clearly polyphyletic status (Rajaniemi–Wacklin et al. 2006; Shen et al. 2018; Li et al. 2019, 2020). So far, no molecular data concerning *Microcrocis*, not even ultrastructural ones are known (NCBI Resource Coordinators 2018; Mareš et al. 2019b). Therefore, the present study aims to fill the gap of this knowledge.

Material and Methods

Material collection. The original water sample was collected using 20–μm plankton net and 30–ml benthos pipette from a little bay formed by a tributary of a small stream to an unnamed forestall pond near the Stropnice River in the South Bohemian Region, the Czech Republic (pH = 7.6; conductivity = 278 $\mu S.m^{-1}$; transparency = 75 cm; TN = 2,4 $mg.l^{-1}$; TP = 48 $\mu g.l^{-1}$; GPS: 48°52'53.067" N, 14°37'40.563" E) in August 2021 and stored in a refrigerator at 10 °C.

LM and TEM analysis. The natural sample containing *Microcrocis geminata* was observed with an Olympus BX 51 microscope with Nomarski DIC (400× and 1000× magnification) (Olympus Corp., Tokyo, Japan) and its morphology documented using a DP–71digital camera and Olympus cellSens Entry software (Olympus Corp., Tokyo, Japan). The sample was divided in several subsamples intended for different treatment. One subsample made by part of the *Microcrocis* material was prepared for the transmission electron microscopy by the Laboratory of Electron Microscopy, Biology Centre ASCR – Institute of Parasitology,

1 Other orthographic variants of *Merismopedia* are *Merismopoedia*, *Merismopoedium* or *Merismopedium* (Joosten 2006, Guiry and Guiry 2022)

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České Budějovice, Czech Republic. A specimen was preserved in 2.5% glutaraldehyde and 0.1 M cacodylate buffer followed by postfixation with 2% osmium tetroxide. Fixed material was dehydrated in an acetone series (30, 50, 70, 80, 90, 95 and 100%) and embedded in Spurr's resin (Spurr 1969). The 70–nm thick, thin sections were placed on formvar–coated grids, contrasted by uranyl acetate and lead citrate, and analysed by a JEOL JEM 1010 microscope.

Single colony isolation. Another subsample of *Microcrocis* was stored in a TE buffer. Afterwards, templates consisting of clean smaller fragments of colonies were prepared for further molecular analyses. Colonies were mechanically disintegrated and the obtained fragments subsequently isolated using the glass microcapillary method under sterile conditions (Zapomělová et al. 2007; Mareš et al. 2015) under the CX40 microscope (Olympus Corp., Tokyo, Japan) using the 100× magnification. Each colony fragment was washed gradually in at least five droplets of the TE buffer to remove epiphytic bacteria or detritus remnants and each stored in a droplet of the TE buffer in separated PCR eppendorf. In total, 16 fragments were prepared.

Molecular methods. Half of the isolates were used as a template for whole genome amplification via multiple strand displacement amplification (MDA, Spits et al. 2006; Rodrigue et al. 2009), for which Repli–G Mini kit (QIAGEN, Germantown, Maryland, USA) was used. All steps of the protocol were executed in a UV–sterilized laminar box Telstar BIO II A (Telstar, Madrid, Spain). The final mixture was incubated in Biometra T3000 thermocycler (Analytik Jena GmbH, Jena, Germany) for 16 hours at 30 °C and ended with polymerase denaturation at 65 °C. The products of the MDA were checked by gel electrophoresis using 1.5% agarose gel and visualised using the GenoSens 2000 transilluminator (Clinx Science Instruments Co., Ltd, Shanghai, China). The successfully amplified MDA products were used as templates for subsequent PCR amplification of the gene for the 16S rRNA and the adjacent ITS region. Individual 50 µl PCR reactions consisted of 25 µl 2× Plain PP Master Mix (Top–Bio, s.r.o., Vestec, Czech Republic), of 20 pmol of both forward – VRF2 (5'– GGG GAA TTT TCC GCA ATG GG –3') (Johansen et al. 2011 after Nübel et al. 1997) and reverse – VRF1 (5'– CTC TGT GTG CCT AGG TAT CC –3') (Johansen et al. 2011 after Wilmotte et al. 1993) primers, and of 0.5 µl of the template MDA product. Reactions were performed using the Biometra T3000 thermocycler. The program consisted of an initial denaturation at 94 °C followed by 35 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min at 50 °C, and elongation for 2 min at 72 °C, ended by final elongation for 10 min at 72 °C. The successful course of PCR amplification was checked again by gel electrophoresis.

The second half of the isolates was processed using a semi–nested PCR protocol (Janse et al. 2004) modified by Mareš et al. (2015) consisting of two subsequent PCR amplifications, however adjusted in present study in order to obtain the whole 16S rRNA and ITS region. In the first phase, a 50 µl PCR was conducted directly in tubes with isolates and consisted of 25 µl 2× Plain PP Master Mix, 7.5 pmol of both forward (VRF2) and reverse primer (B23S, 5'– CTT CGC CTC TGT GTG CCT AGG T –3') (Lepère et al. 2000) in the Biometra T3000 thermocycler programmed according to the original protocol (Mareš et al. 2015). The second phase was adjusted, and therefore the whole protocol was just two stage PCR and not nested. It consisted of a 50 µl PCR with the same parameters as the first one. 1 µl of the product of the first phase was used as a template. Second phase PCR products were checked by gel electrophoresis, and successfully amplified products were purified using the ExoSAP–IT® method (Bell 2008). Clean PCR products were commercially sequenced using Sanger Sequencing (SEQme s.r.o., Dobříš, Czech Republic), using both forward and reverse primers. To ensure ITS sequences were whole, the PCR products were sequenced using the PCR primers and additional internal primer VRF5F (5'– TGT ACA CAC CGG CCC GTC –3') (Johansen et al. 2011 after Wilmotte et al. 1993).

Phylogenetic analysis. Individual sequences were assembled from separated reads and manually adjusted in the Geneious Prime 2021.2.2 (https://www.geneious.com) software. The matrix including obtained sequences of *Microcrocis geminata* together with the sequences gathered from NCBI (NCBI Resource Coordinators 2018) was aligned by the ClustalW algorithm (Thompson et al. 1994) in the software Mega 10.2.6 (Tamura et al. 2021) using default parameters and afterwards adjusted manually. The final aligned matrix was 1089 sites long and consisted of 121 sequences, including the outgroup. The dataset was analysed in the program MrBayes (Ronquist et al. 2012) using the Bayesian Inference method. The analysis involved two runs of four Markov chains for 10 million generations with the default settings. The Maximum Likelihood analysis was executed by the software PHYML (Guindon et al. 2010). The model GTR+R was chosen by SMS algorithms using both AIC and BIC criteria (Lefort et al. 2017). Support of bootstrap with 1000 repetitions was generated. Additionally, a matrix of pairwise distances was calculated by Mega 10.2.6 (Tamura et al. 2021). It compares the sequences of 16S rRNA gene among the closest relatives of analysed *M. geminata* (here represented by one selected sequence). Standard criteria for generic definition – p–distance above 0,05 (Stackebrandt & Goebel 1994) was used. Additionally, ITS of *M. geminata* and two related strains of *Merismopedia* sp. (AICB1014 and AICB1015), data of which were obtained from NCBI (KJ746509.1 and KJ746510.1) were analysed. Secondary structures of conserved elements of ITS (D1–D1' helix, V2 helix, B–Box and V3 helix) (Iteman et al. 2000) were created using the software mFold WebServer 3.5 with default conditions (Zuker 2003).

Results

Light and transmission electron microscopy

Microcrocis geminata (Lagerheim) Geitler (Fig. 1)

Description: Colonies macroscopic, flat, up to 25 mm^2, consisting of several hundreds to thousands of cells (Fig.1 F, I) irregularly arranged in one plane of the colony. Cells 12–16 µm long and (3) 4–6 (7) µm wide, cylindrical with both ends rounded (Fig. 1 A, B, D, E), circular in the apical cross section and hexagonal in the central part of the cells (Fig. 1 G, H; Fig. 2). Cell division longitudinal, symmetrical on both sides, therefore dividing cells resemble chromosomes (Fig. 1 A, D). No asymmetric division observed.

Epitype (designated here): CBFS A–128–1, dried material obtained from a natural population consisting of macroscopic colonies, kept at Herbarium of the University of South Bohemia, České Budějovice, Czech Republic. (Fig. 1).

Habitat: Epipelic and tychoplanktonic.

Materials analyzed: Natural water sample obtained at 48°52'53.067" N, 14°37'40.563" E.
GenBank accession numbers: 16S–23S rRNA ITS: ON426371–ON426380.

Transmission electron microscopy showed an unusual pattern of thylakoids. The thylakoids, approximately 50–60 in a cell, were transversal, with parallel arrangement and filled the entire cross section of the cell. They were densely stacked so that they resembled parallel lines from a lateral view. This arrangement was so conspicuous that it was indicated also in the light microscope from the side view of the cell. Therefore, the cells appeared striped (Fig. 1 A–D).

Molecular methods

Seven isolated *M. geminata* colony fragments' 16S rRNA + ITS regions were successfully amplified. On the contrary, MDA produced only three successfully amplified genomes and from all of them we got 16S rRNA gene sequences with the ITS region. In total, we obtained 10 sequences, which were deposited at NCBI under accession numbers ON426371–ON426380.

Phylogenetic analysis

All 10 sequences analysed by phylogenetic methods were identical, without a single nucleotide exchange and thus are in the tree represented by a single sequence (Fig. 3). The final alignment consisted of 121 taxa and Oscillatoriales taxa were applied as an outgroup. Both analyses (Bayesian Inference and Maximum Likelihood) placed the species *M. geminata* in the order Chroococcales, in the family Geminocystaceae sensu Tuji et al. (2021) with strong support. The closest relatives of the species are two strains designated as *Merismopedia* sp. (AICB1014 and AICB1015), and the whole clade is sister to the group of the most of Geminocystaceae consisting of *Cyanobacterium* Rippka et Cohen–Bazire, *Geminocystis* Korelusová, Kaštovský et Komárek and *Geminobacterium* Ramos, Brito et Kaštovský (Fig. 3). Therefore, *Microcrocis* is no longer a member of the family Merismopediaceae (Synechococcales).

The matrix of pairwise distances, consisted of comparisons of the sequences of 16S rRNA gene among the closest relatives of *M. geminata* supported the distinct position of *Microcrocis* as a separated genus because all numbers of p–distance exceeded the limit 0.05 (Table 1). Also, all sequences of ITS region of *M. geminata* were identical, therefore just one secondary structure was created from our data (Fig. 4). Both *Merismopedia* sp. strains (AICB1014 and AICB1015) ITS were almost identical, differing just in two sites (A–G exchange in non–conservative region and A–G exchange in B–Box) and the conserved structures were alike (Fig. 4). Therefore, the strains probably represented single species. Its comparison with *M. geminata* showed clear differences in D–stem, V2 region, and B–Box concerning the length and the structure (Fig. 4). Such differences supported separation of the two taxa on generic level. Both taxa *Merismopedia* sp. and *M. geminata* contained two tRNA, Ile and Ala. On the other hand, they both completely missed V3 structure (Fig 4).

In addition to the changed placement of *M. geminata*, our results allowed us to propose further taxonomic clarification. Enough material was collected from the natural population; therefore, an herbarium entry was created, which we here propose as an epitype.

Discussion

Taxon distribution

Information about the occurrence of the species *M. geminata* in the territory of the Czech Republic is scarce, especially in comparison with much more common representatives of the genus *Merismopedia*. The species was first found in 1972 in the Smyslov Pond near Blatná and in a ditch in the

Table 1. The matrix of pairwise distances, consisting of comparisons of the sequences of 16S rRNA gene among the closest relatives of *Microcrocis geminata* (represented by one selected sequence) was calculated using p–distance. Numbers above 0.05 support the distinct position of *Microcrocis geminata* as a separated genus.

	JX504430	**JX504454**	**KF856396**	**KF856398**	**KJ746509**	**KJ746510**
JX504430 Uncultured bacterium clone bac326c						
JX504454 Uncultured bacterium clone bac118c	0.0009					
KF856396 Uncultured cyanobacterium clone BF311C	0.0449	0.0440				
KF856398 Uncultured cyanobacterium clone BF27C34	0.0449	0.0439	0.0065			
KJ746509 *Merismopedia* sp. AICB1014	0.0704	0.0694	0.0805	0.0804		
KJ746510 *Merismopedia* sp. AICB1015	0.0704	0.0694	0.0804	0.0804	0.0019	
Microcrocis geminata	**0.0565**	**0.0555**	**0.0636**	**0.0636**	**0.0648**	**0.0648**

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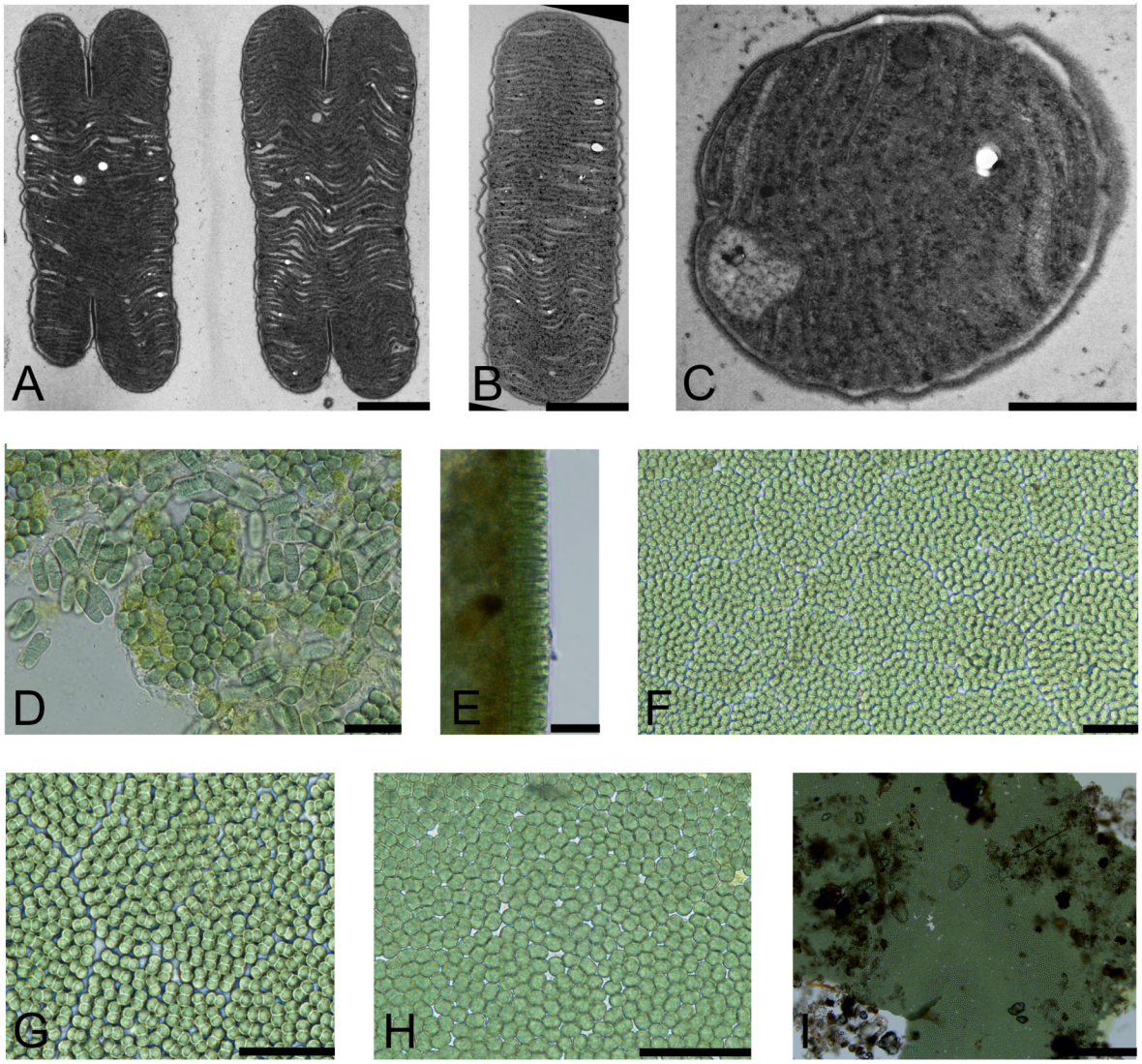

Fig. 1. The ultrastructure and overall morphology of the cells and colony of *Microcrocis geminata*. Transmission electron micrographs: the longitudinal section through a pair of dividing cells (A) and longitudinal (B) and perpendicular (C) cross sections of vegetative cells. Light micrographs: both the lateral and the transversal views of the cells (D), lateral (E) and front views to the whole colony (F, G, H and I). All lateral views show clear striation – the pattern caused by the parallel arrangement of thylakoids. The transversal views focused on rounded ends of the cells (F, G,) and transversal focus on "hexagonal" cross section in the middle part of the cells (H) illustrate the cell morphology. Formation of subcolonies was detected (F). Scale bars 2 µm (A – C); 20 µm (D, E, F, H, I); 200 µm (G).

Emil Mine near Řevničov (Fott 1972) and later in 2010 in a forest pond near the village Otěvěk (Kaštovský et al. 2010). It is considered a rare taxon also according to the comprehensive surveys of cyanobacteria in other European countries. The most frequent occurrence was documented in Ukraine – in about twenty localities (Tsarenko et al. 2006), further two localities are known from Serbia (Cvijan & Blazencic 1996). *M. geminata* was reported in Poland (Sieminska & Wolowski 2003), Great Britain (John et al., 2011), Germany (Hellish et al., 2018) and Scandinavia (Karlson et al., 2018) without the frequency of occurrence specified. A single colony was found in the Netherlands (Frank & Landmann 1988), and one locality is known from Slovakia (Hindák 2005). On the contrary, Slovenia (Vrhovšek et al. 2006) and Greece (Gkelis et al. 2016), for example, do not report any occurrence at all. Nevertheless, *M. geminata* was also repeatedly reported from seawater in Romania (Caraus 2003), the discovery of which can indicate confusion with another species. Overall, the absence of more modern data for such a rare species is not surprising.

We collected a sufficient amount of material; therefore, an herbarium entry was proposed as a novel epitype. The epitype material does not come from the original type locality (meso–eutrophic pools with macrophytic algae vegetation near Leipzig, Germany), but corresponds to it in habitat character and geographical region. Similarly, the morphology of the specimen corresponds to the original description of *M. geminata* concerning the colony style, cell shapes and dimensions (Richter 1893). We believe that in this specific case it is useful to admit such material as an epitype, as well as in similar cases (Mühlsteinová et al. 2019).

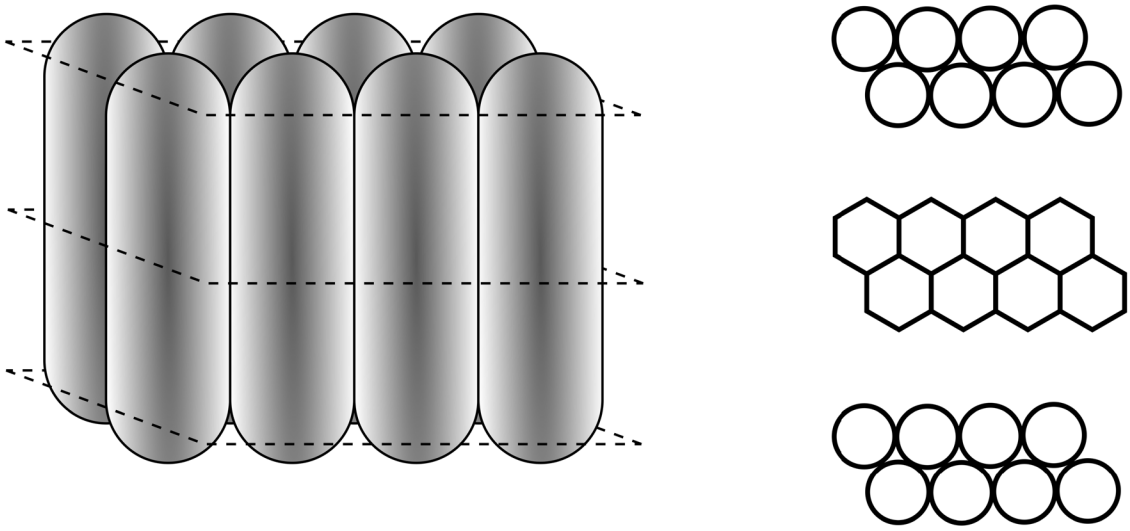

Fig. 2. Diagram of morphology of *Microcrocis geminata*, illustrating the overall cell shape and arrangement of the cells in the colony. The cross–sectional views in three different microscope focus planes are plotted. The shape of the cell appears round on the apexes, and transitions into hexagonal in the middle of the cell.

Morphology

According to Algaebase (Guiry & Guiry 2022) and Index Nominum Algarum (Silva 2022), the genus *Microcrocis* includes 10 accepted species (Tab. 2). Many of them were originally described as *Holopedium* or *Merismopedia* spp. or as members of the genus *Beckia*, today considered a subgenus of *Microcrocis* (Lagerheim 1883, 1893; Richter 1893; Migula 1905; Beck 1929; Tiffany 1934; Buell 1938; Geitler 1942; Skuja 1948; Komárek & Anagnostidis 1995). The genus compiles relatively uniform morphotypes of multicellular flat colonies with more or less oval cells differing mostly by cell size (Komárek & Anagnostidis 1999).

Based just on the morphological data, it had been assumed that *Microcrocis* was related to the genus *Merismopedia* (Fott 1972; Komárek & Anagnostidis 1999; Guiry & Guiry 2022). Early growth stages of some other taxa (apart from *Merismopedia* also *Synechocystis*) (Komárek & Anagnostidis 1999) are often morphologically very similar to the early growth stages of *Microcrocis*. This fact and the similar pattern of regular colony formation has led to joint classification (Lagerheim 1883, 1893). However, unlike other cyanobacteria with tabular colonies such as *Coccopedia* Troitzkaja, *Cyanotetras* Hindák, *Pannus* Hickel or *Merismopedia*, cells of *Microcrocis* are oval, cylindrical, elongate or almost rod–shaped with rounded ends (Geitler 1942; Fott 1972; Hindák 1992; Komárek & Anagnostidis 1999). Moreover, except for one species – *Microcrocis gigas*, which was suspiciously originally described as *Merismopedia gigas* (Komárek & Anagnostidis 1995) – all *Microcrocis* species have irregularly arranged cells (Komárek & Anagnostidis 1999). Similar irregularity is present among the genera *Pannus* and *Mantellum* Dangeard. However, colonies of *Pannus* are lobate or clathrate and not regularly tabular (da Silva Malone et al. 2014), and the genus *Mantellum* is known to include strictly epiphytic species (Hindák 2002). Even though some *Merismopedia* species (e.g., *M. convoluta* Brébisson ex Kützing, *M. revoluta* Askenazy) with macroscopic colonies are also known, none of them possess oval cells or irregular cell arrangement (Kützing 1849; Askenazy 1894; Komárek & Anagnostidis 1999). On the other hand, the genus includes some uncharacteristically short–celled species as *Microcrocis irregularis* (Lagerheim) Geitler and *M. obvoluta* (Tiffany) Frank et Landman (Lagerheim 1883; Tiffany 1934; Geitler 1942). But none of the species possess spherical cells as known in *Merismopedia* (Komárek & Anagnostidis 1999).

Cell division of *Microcrocis* is rather specific. Cells of *M. geminata* divide along the longer axis (Fig. 1. A, D), which is quite rare. This phenomenon has been described in the earlier literature – see, for example, *M. geminata* (Richter 1893), *M. marina* (Niell & Manadon 1978) as well as *M. pulchella* (Buell 1938) and *Microcrocis* sp. (Frank & Landman 1988). Other cyanobacteria with cylindrical cells (*Aphanothece, Cyanothece* Komárek, *Cyanobium* Rippka et Cohen–Bazire, *Gloeothece* Nägeli, *Myxobaktron* Schmidle, *Rhabdogloea* Schröder, *Rhabdoderma* Schmidle et Lauterborn, *Synechococcus* Nägeli) all divide transversely (Komárek & Anagnostidis 1999) similarly as short filamentous cyanobacteria, such as *Romeria* M. Koczwara, *Borzia* Cohn ex Gomont or *Hormoscilla* Anagnostidis et Komárek (Komárek & Anagnostidis 2005). The only other colonial genera with longitudinal division are *Snowella* Elenkin, *Woronichinia* Elenkin and *Gomphosphaeria* Kützing. However, they possess a rather elliptical cell shape and three–dimensional colonies (Komárek & Anagnostidis 1999). None of these taxa were shown to be closely related to *Microcrocis geminata* in the present study.

It is occasionally mentioned in literature that *Microcrocis* may still divide asymmetrically or symmetrically

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Table 2. The morphological and ecological delimitation of existing species of the genus *Microcrocis*.

	Cells	Cells width (µm)	Cells lenght (µm)	Habitat; occurence	Colony
Microcrocis bella (Beck) Komárek et Anagnostidis 1995	cylindrical	3.4–4	cca 7	freshwater benthos; Austria, Sweden	microscopic, irregular
Microcrocis geminata (Lagerheim) Geitler 1942	rod–shaped	3.4–6 (7)	12–16	freshwater benthos, tychoplankon; temperate north hemisphere and Tierra del Fuego	macroscopic, irregular
Microcrocis gigas (Ryppowa) Komárek et Anagnostidis 1995	cylindrical to rod–shaped	4–6	7.6–12	freshwater benthos, metaphyton; Romania	macroscopic, regular
Microcrocis granulata (Skuja) Skuja 1966	cylindrical, granulated	3.5–4.7	6.5–8	freshwater tychoplankton; Sweden	macroscopic, irregular
Microcrocis irregularis (Lagerheim) Geitler 1942	ellipsoidal or slightly cylindrical	(1) 1.5–3	2–4.5	freshwater tychoplankton; temperate north hemisphere	microscopic, irregular
Microcrocis marina (Lagerheim) Komárek et Anagnostidis 1995	oval to rod–shaped	4–7	6–19	brackish; France, Spain, Sweden	macroscopic, irregular to regular
Microcrocis obvoluta (Tiffany) T.H.Frank et A.G.Landman, nom. inval. 1988	oval	3–5 (6.8)	(4.6) 5.4–7	freshwater benthos; temperate northern hemisphere	macroscopic, irregular
Microcrocis pulchella (Buell) Geitler 1942	oval to cylindrical	(2) 2.3–3.6	(3.2) 3.8–6	freshwater and brackish, tychoplankton; N. and S. America	microscopic, irregular
Microcrocis sabulicola (Lagerheim) Geitler 1942	oval	3–4 (5)	(3) 5–6	marine and brackish epipsammon; Western Baltic, England	macroscopic, approx. regular
Microcrocis solea (Komarenko) Komárek et Anagnostidis 1995	cylindrical	5–6	9–10	plankton of river; Yakutia, Russia	microscopic, irregular

transversally (Komárek & Anagnostidis 1999; Whitton 2011). Instead, our observations suggest that this can be an inaccurate interpretation of the original figure of *M. geminata* (Richter 1942). Figures of the lateral and proximal views, plotted above, may resemble the division by exocytes as occurs e.g., in the genus *Chamaesiphon* Braun. After all, according to our research, transversal division was not observed.

On the other hand, observation of our specimen revealed clearly hexagonal cross sections of the cells in their middle part, especially in the middle of the colony (Fig. 2). We assumed that the hexagonal cross section may be caused by cells being in the colony arranged close to each other for spatial reasons. Therefore, it is not a unique morphological and taxonomically relevant trait. The apical, slightly narrowed parts of the cells do not fit so closely to each other as in the middle part. Therefore, the apical cross sections appear rounded when focused (Fig. 1G), as well as the cross sections of cells on the edge of the colony. This misinterpretation might mistakenly lead to the separation of the genus or subgenus *Beckia* from other *Microcrocis* species (Elenkin 1933; Komárek & Anagnostidis 1999).

Ultrastructure

The novel position of *Microcrocis geminata* in Geminocystaceae is also supported by ultrastructural data. Its observation using transmission electron microscopy revealed the unique parallel position of thylakoids (Fig. 1A–C). Only two other representatives of Geminocystaceae possessed parallel thylakoids so far, namely members of *Cyanobacterium* and *Geminocystis* (Korelusová et al. 2009; Mareš et al. 2019b). Thus, the theory positing that a parallel arrangement originating (unlike, e.g., parietal thylakoids) from a single evolutionary event from (more likely) fascicular or parietal thylakoids can still be valid (Mareš et al. 2019b). However, the parallel thylakoid architecture is not a universal morphological marker of the family Geminocystaceae, considering radial thylakoids of *Annamia* spp. (Tuji et al. 2021) and irregular thylakoids of *Geminobacterium atlanticum* Ramos, Brito et Kaštovský (Brito et al. 2017). Moreover, for two other members of the family Geminocystaceae and the closest relatives of *M. geminata,* strains *Merismopedia* sp. AICB1014 and AICB1015 (as well as for many other *Merismopedia* taxa) the character of thylakoids is not known.

Phylogenetic and taxonomic analysis

In comparison to the MDA, the semi–nested PCR protocol was more reliable in terms of successfully amplified DNA products. Thus, the modified protocol of gradual PCR (Mareš et al. 2015) adjusted in present study seems to be a more reliable method for basic 16S rRNA and ITS sequencing than the MDA followed by the PCR (Lara et

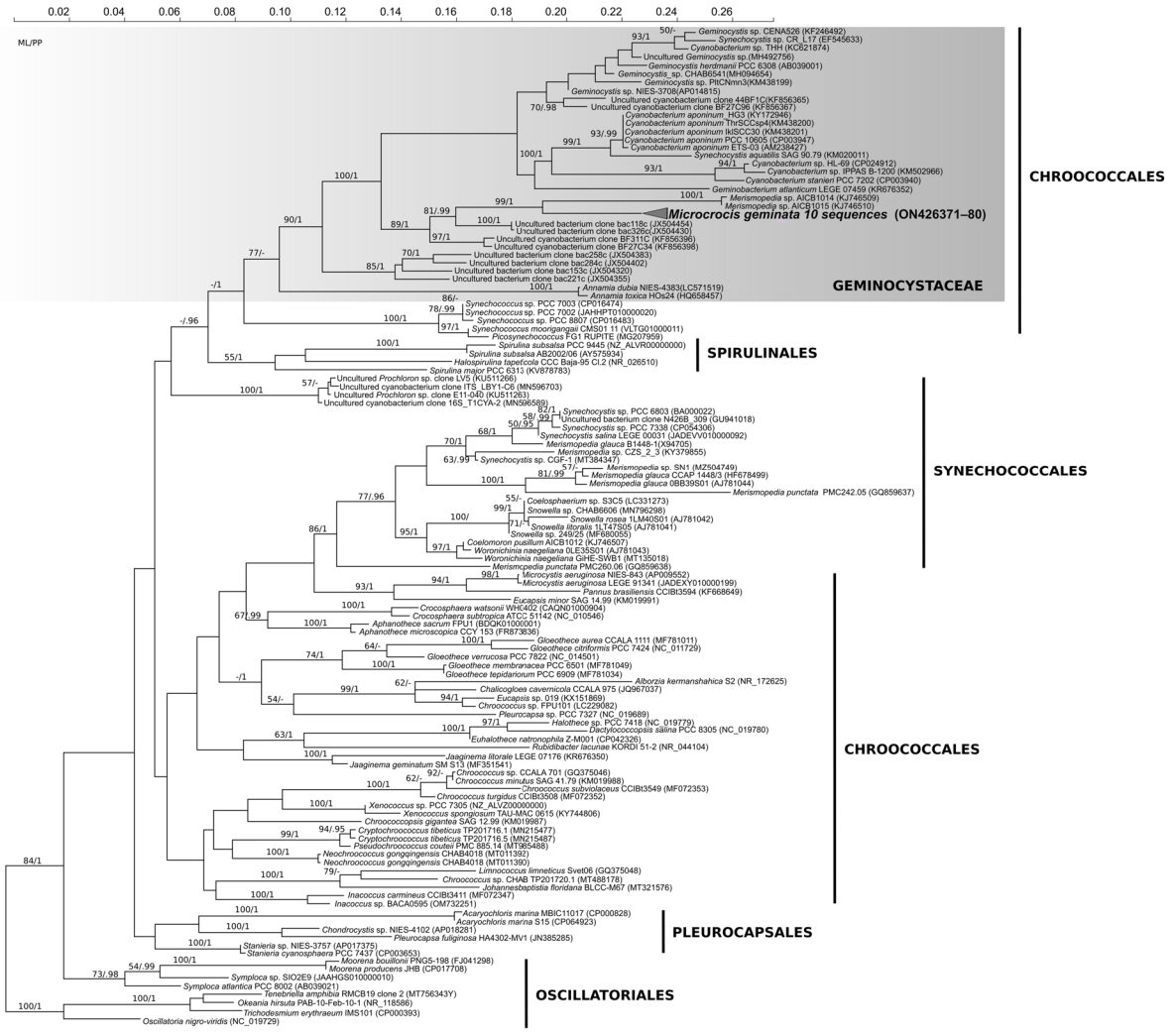

Fig. 3 The phylogenetic tree constructed from the 16S rRNA gene sequences of Chroococcales with a part of Oscillatoriales applied as an outgroup. With the paraphyletic Chroococcales partly interfere the orders Pleurocapsales, Spirulinales and part of Synechococcales, also included in the analyses. Topology represents the best ML tree. Support of bootstrap of Maximum Likelihood analysis above 50 (ML) and posterior probabilities of Bayesian Inference analysis above 0.95 (PP) are recorded. Our 10 sequences of *M. geminata* are highlighted in bold font.

al. 2013). MDA amplification provide however, the possibility to obtain data from additional markers, as well as whole genome data (Rodrigue et al. 2009).

On the basis of the morphological data, traditional systematics assumed that genera *Microcrocis* and *Merismopedia* were close relatives and as members of the family Merismopediaceae were classified in the order Synechococcales (Fott 1972; Komárek & Anagnostidis 1999; Komárek et al. 2014). Our analyses confirmed two strains *Merismopedia* sp. (AICB1014 and AICB1015) as the closest relatives of *M. geminata*. Though the strains were determined without a specific epithet, they definitely possessed the typical *Merismopedia*–like morphology (Drugă, pers. com.; Tuji et al. 2021). The shape of the cells of both strains corresponded to *Merismopedia*, and not *Microcrocis* being clearly spherical and the cells in colonies were arranged regularly in tetrads and its multiples (Drugă, pers. com.). The group clustered surprisingly inside Chroococcales, in the family Geminocystaceae sister to the group of genera *Cyanobacterium, Geminocystis* and *Geminobacterium* (Kaštovský et al. 2006; Brito et al. 2017; Tuji et al. 2021).

Previous molecular studies that had analysed sequences of *Merismopedia* taxa suggested its clearly polyphyletic status (Rajaniemi–Wacklin et al. 2006; Furtado et al. 2009; Shen et al. 2018; Li et al. 2019, 2020). Individual *Merismopedia* lineages occurred in the order Synechococcales in the vicinity of the genera *Synechocystis* s.str., *Snowella* and *Woronichinia* (Palinska et al. 1996; Rajaniemi–Wacklin et al. 2006; Thomazeau et al. 2010; Albrecht et al. 2017), core Synechococcales (Furtado et al. 2009; Shen et al. 2018) as well as Chroococcales sensu auct. (Shen et al. 2018; Tuji et al. 2021). Even though the lineage related to *Synechocystis*, *Woronichinia* and *Snowella* included strains designated as the type species *M. punctata*, and it is according to Thomazeau et al. (2010) highly probable that the core of this genus will be found in the vicinity of the species *Merismopedia*

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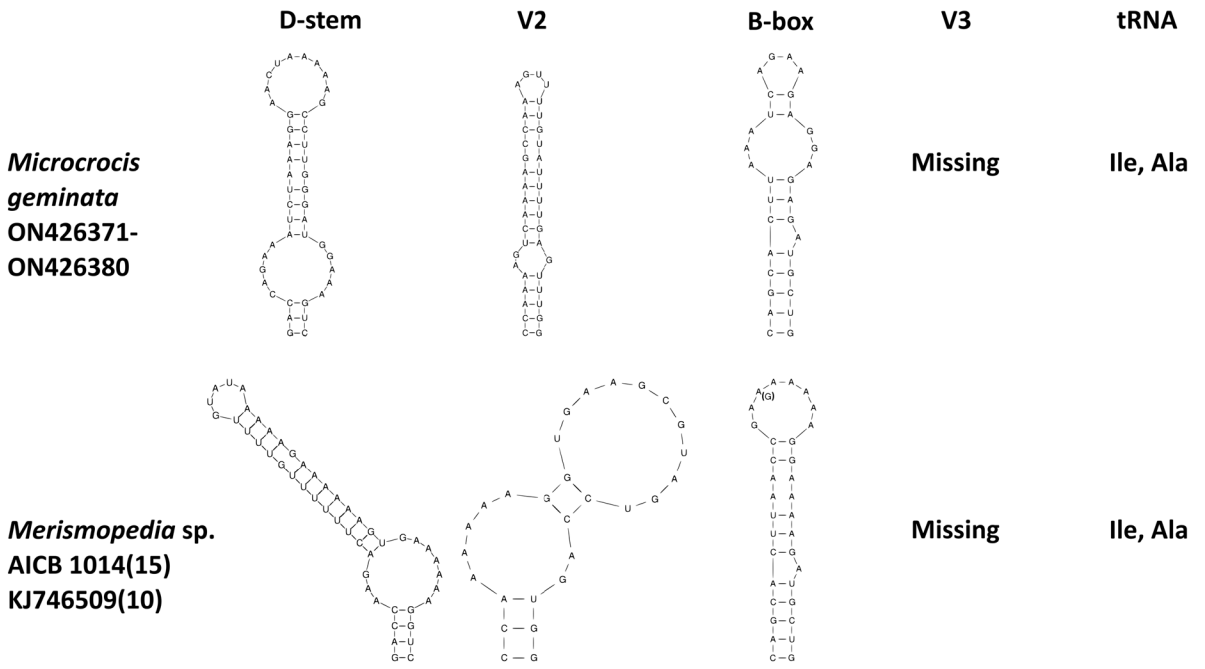

Fig. 4. Conserved elements of secondary structure of ITS of *Microcrocis geminata* and its comparison with the ITS structures of two related strains *Merismopedia* sp. (AICB1014 and AICB1015). A to G exchange in B–Box sequence of *Merismopedia* sp. AICB1015 against AICB1014 is written with a slash and highlighted by brackets.

punctata/glauca in the Synechococcales (Komárek et al. 2014). The reference material and therefore its sequence is not available, so the position of true *Merismopedia* and thus all Merismopediaceae have not been determined so far (Shen et al. 2018).

Consequently, it is still possible that historical assumption of close relationship of *Microcrocis* and *Merismopedia* is valid. *M. geminata* is certainly related to the *Merismopedia* morphotype represented by strains AICB1014 and AICB1015. However, despite this close relationship supported by molecular phylogeny (Fig. 3), the matrix of pairwise distances containing the closest relative taxa of *M. geminata* clearly separated *M. geminata* from both *Merismopedia* sp. strains. The analysis confirmed, that *Microcrocis* is a distinct genus (Table 1), which was also proved by comparison of ITS structures of the two taxa, because of significant differences in D–stem, V2 region, and B–Box, concerning both the length and the structure (Fig. 4). According to our analyses, both taxa missed terminal element V3 (Fig. 4). The element V3 is also missing in ITS sequence of *Geminocystis papuanica* (EF555569) and *Geminobacterium atlanticum* (KR676352). Also, V3 stem of other *Geminocystaceae* taxa is remarkably short (Korelusová et al. 2009; Brito et al. 2017). The reduction is specific for most of the examined Geminocystaceae except for *Annamia dubia* (Tuji et al. 2021) (Fig. 4), the basal taxon, which also differs from the rest of Geminocystaceae by its filamentous morphology and thylakoid ultrastructure (Mareš et al. 2019b; Tuji et al. 2021). All examined Geminocystaceae ITS contained Ile and Ala tRNA, including *A. dubia* and our examined taxa. On the other hand, we have observed V2 element in between those two tRNA sequences of both examined taxa *M. geminata* and *Merismopedia* sp. (however unusual in case of *Merismopedia* sp.), which was unique in Geminocystaceae, in comparison with *Geminocystis, Geminobacterium* and *Cyanobacterium* taxa (Korelusová et al. 2009; Brito et al. 2017), and also *Annamia* (Tuji et al. 2021).

All the data presented in this study together confirmed recognition of *Microcrocis* as a separate genus with its novel and seemingly surprising placement in the family Geminocystaceae (Chroococcales). The position in Geminocystaceae is supported by ultrastructure (parallel thylakoid architecture) and 16S rRNA sequence analysis. On the other hand, its distinction is supported by ITS secondary structure and unique combination of morphological characteristics (unusual cell shape, division and formation of a typical tabular colony with irregular cells arrangement).

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