Phylogenetic and morphological evaluation of *Wollea saccata* (Nostocales, Cyanobacteria) isolated from the Yenissei River basin (Eastern Siberia, Russia)

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Abstract: For the first time the genus *Wollea* Bornet et Flahault was recorded in Eastern Siberia (Yenissei River, Karskoe See Basin) and isolated to monoclonal axenic strain ACCS 045. The strain was characterized by a combination of morphological and molecular approaches. It shares the diacritical features of the type–species *W. saccata* (Wolle) Bornet et Flahault and *W. bharadwajae* R.N. Singh such as macroscopic gelatinous colonies, tube–like, trichomes more or less straight or slightly curved, sheaths around trichomes are absent, heterocytes intercalary, and paraheterocytic akinete development at both sides of heteocytes in short series. A morphological comparison shows that it differs from *W. saccata* in the form of colonies being saccule, the form of vegetative cells and the heterocytes being barrel–shaped, the form of terminal cells being conical–rounded, and from *W. bharadwajae* in the form of akinetes being cylindrical or ellipsoid. The first available 16S rRNA gene sequence of *Wollea* appeared poorly related to other cyanobacterial sequences. According to the traditional classification systems based on morphological and ecological observations genus *Wollea* appears closest to *Anabaena* and *Nostoc*. However, the phylogenetic placement of *Wollea* based on 16S rRNA gene sequence was distant from *Nostoc* and the most closely related to taxa in benthic *Anabaena, Sphaerospermopsis*, *Cylindrospermopsis*, and *Raphidiopsis*.

Key words: *Wollea saccata*, cyanobacteria, 16S rRNA gene, taxonomy, morphology

Introduction

The genus *Wollea* includes filamentous and heterocytous colonial cyanobacteria. Originally, the type species was described from stagnant ponds of North America (New Jersey) as *Sphaerozyga saccata* by Wolle (1880, 1887), and later, the new monotypical genus *Wollea* was established by Bornet and Flahault (1888). Later, tree additional species were described from littoral of freshwater ponds and paddy field bottom in India (*W. bharadwayae* R.N. Singh (1942) and *W. udaipurensis* Gupta et Kumar (1968)) and as endophytic into parenchyma of aquatic plant *Lemna trisulca* from freshwater pond in Spain (*W. lemnae* Guerrero (1947)). Furthermore, according to the important generic characters, the following taxons *Anabaena ambigua* Rao, *Anabaena vaginicola* Fritsch et Rich, *Anabaenothrix cylidrica* Randhawa, and *Anabaenothrix epiphytica* Randhawa are included in the genus *Wollea* (Singh 1942).

Currently, the genus *Wollea* belongs to order Nostocales, family Nostocaceae by traditional classification (Komárek & Anagnostidis 1989) and subsection IV.I by bacteriological classification (Castenholtz 2001). *Wollea* spp. are characterized by macroscopic gelatinous colonies which are smooth on the surface, more or less cylindrical or subspherical (sometimes tube–like). Trichomes are more or less straight or slightly curved, uniseriate, not branched, not attenuated or widened at the ends, intensely constricted at cross walls; irregularly or more or less parallel and densely arranged in common, and diffluent mucilage. Terminal cells are rounded. Sheaths around trichomes are absent. Heterocytes are intercalar, solitary. Akinetes arise paraheterocytic in short series at both sides of heteocystes, spherical or oval (Komárek & Hauer 2010). *Wollea* is most morphologically similar to genera *Anabaena* and *Nostoc*.

To date, the phylogenetic relationship between *Wollea* and other genera of family *Nostocales*

have not been determined. In this connection, we investigated morphological characters and molecular organization of 16S rRNA gene of the strain of *W. saccata*.

Material and Methods

The strain *W. saccata* ACCS 045 was isolated from pelagic phytoplankton samples of the Yenissei River (Karskoe See basin) near Igarka city located in Northern of Eastern Siberia. Isolation was carried out by repeated transfers of single trichomes on solid BG_{11} liquid medium without nitrogen (RIPPKA et al. 1979) under continuous light intensity of 30–40 $\mu mol.m^{-2}.s^{-1}$ photons at 22–25 °C. This strain is maintained in culture collection of the Siberian Federal University in Krasnoyarsk, Russia, under the number ACCS 045. Detailed morphological analyses were carried out on cultures during the period of 1–3 months after the isolation from nature using Meiji ML2000 light microscope (Meiji Techno, Japan). Microphotographs were taken with Infiniti I camera (Luminera, Canada).

Extraction of DNA was carried out using a commercial AquaPure Genomic DNA Isolation reagent kit (Bio–Rad, USA), following the manufacturer's protocol. The 16S rRNA gene was amplified using the universal primers 27F (5'–AGAGTTTGATCCTGGCTCAG–3') and 1492R (5'–GGTTACCTTGTTACGACTT–3'), corresponding to *Escherichia coli* positions 8–27 and 1510–1492, respectively. Polymerase chain reaction (PCR) was performed using a Mastercycler Gradient amplifier (Eppendorf, Germany) in the total volume 50 µl, containing 50–100 ng of DNA matrix, 1x reaction buffer, 0.3 µM of each primer, 0.2 µM of each dNTP, 2 µM of $MgSO_4$, and 1 unit of high–fidelity Platinum Taq polymerase (Invitrogen, USA). The PCR was run as follows: primary denaturation at 95 °C for 3 min; 25 cycles: at 94 °C for 30 s, at 55 °C for 40 s, at 68 °C for 1 min 30 s; final extension at 68 °C for 10 min. The sizes, quantity and purity of PCR products were analyzed in a 1.2% agarose gel, using 0.5% TAE buffer.

To generate high–quality sequences, unpolished PCR products were cloned in the vector pCR4–TOPO (Invitrogen, USA), used to transform cells of *E. coli* TOP10. The obtained clones were subjected to restriction analysis to determine whether there was an insert of the proper size in the vector. Isolation of plasmid DNA was performed using a PureLink Quick Plasmid Miniprep kit (Invitrogen, USA), following the manufacturer's protocol. Sequencing was done in two directions, on an ALFexpress II DNA analysis system (Amersham Pharmacia Biotech Ltd, USA), using the universal primers T3 and T7 and a Thermo Sequenase Cy5 Dye Terminator kit. The obtained nucleotide sequence of the 16S rRNA gene were deposited in the database of GenBank NCBI under the accession number GU434226.

A total of 87 sequences from heterocytous taxa including *Wollea saccata* were used. Additional sequences from the databases of GenBank, EMBL, and DDBJ belonging to the genera *Anabaena*, *Anabaenopsis*, *Aphanizomenon*, *Cyanospira*, *Cylindrospermopsis*, *Cylindrospermum*, *Dolichospermum*, *Nodularia*, *Nostoc*, *Raphidiopsis,* *Sphaerospermopsis*, and *Trichormus* were aligned using the ClustalW (1.81) program. *Escherichia coli* ATCC 8739 was used to root all trees. Phylogenetic trees based on the 16S rRNA gene were constructed by neighbour–joining (NJ), maximum–parsimony (MP) and maximum–likelihood (ML) algorithms using PAUP* v4.02b (SWOFFORD 1998). NJ, MP, and ML analysis were bootstrapped with 1000, 1000 and 100 replicates, respectively. The phylogenetic trees had identical topologies. The NJ tree with the indication of bootstrap values obtained with the NJ, MP, and ML approaches is presented in this work.

Results and Discussion

This is the first report on the occurrence of genera *Wollea* in Siberia. *W. saccata* is a freshwater species, which is infrequently reported from the temperate zones of Europe, such as Romania (CĂRĂUȘ 2002), Czech Republic and Slovakia (HINDÁK 2001), the Ukraine (SVIRENKO in ELENKIN 1938), Spain (GUERRERO 1947), and from several localities (mainly standing waters) in North America (KOMÁREK et al. 2003).

Under culture conditions *W. saccata* strain shares the diacritical features of the type–species *W. saccata* and *W. bharadwajae* such as macroscopic gelatinous colonies, tube–like, trichomes more or less straight or slightly curved, sheaths around trichomes are absent, heterocytes intercalary and paraheterocytic akinete development at both sides of heterocytes in short series (Fig. 1–3). A morphological comparison shows that our strain differs from *W. saccata* in the form of colonies being saccule, the form of vegetative cells and heterocytes being barrel–shaped, the form of terminal cells being conical–rounded, and from *W. bharadwajae* in the form of akinetes being cylindrical or ellipsoid (Table 1).

According to the traditional classification systems based on morphological and ecological observations, genus *Wollea* appears closest to

Table 1. Comparison of main morphological characteristics of the strain ACCS 045 with species of genera *Wollea*.

Characters	*W. saccata* ACCS 045	*W. saccata* (Wolle) Born. et Flah. according to diagnosis (Bornet & Flahault 1888)	*W. bharadwajae* R.N. Singh according to diagnosis (Singh 1942)
Vegetative cells			
Form	barrel–shaped	from ellipsoid to cylindrical	barrel–shaped
Length (μm)	3.0–5.0	–	2.5–3.5
Width (μm)	5.5–6.6	4.0–5.0	3.5–4.8
Terminal cells			
Form	conical–rounded	–	conical–rounded
Length (μm)	4.0–5.5	–	4.3–5.8
Width (μm)	4.0–4.5	–	2.0–3.0
Heterocytes			
Form	barrel–shaped	ellipsoid or subspherical	barrel–shaped with flat ends
Color	light yellow	yellow or light orange	pale blue–green
Length (μm)	6.0–7.0	–	6.0–7.5
Width (μm)	6.0–7.0	little wider then vegetative cells	5.8–7.0
Akinetes			
Form	cylindrical or ellipsoid	cylindrical	spherical or subspherical
Color	yellowish–brown (brown exospore)	yellowish	yellowish–green (dark–brown exospore and hyaline endospore)
Position	solitary up to 4 in rows aside of both sides of heterocytes	in rows aside heterocytes or distant from them	solitary on either side of heterocytes
Length (μm)	11.0–26.0	15.0–22.0	17.0–20.6
Width (μm)	9.0–11.0	approx. 7.0	14.0–15.7
Colonies			
Form	saccule	sack–like cylindrical	solid cylinder
Length (cm)	0.5–2.0	2.0–10.0	4.0–5.0
Width (mm)	5.0–15.0	2.0–6.0	2.0–2.5
Ecology	littoral of river	stagnant waters	freshwater ponds and pools
Distribution	East Siberia	North America, Europe	North India

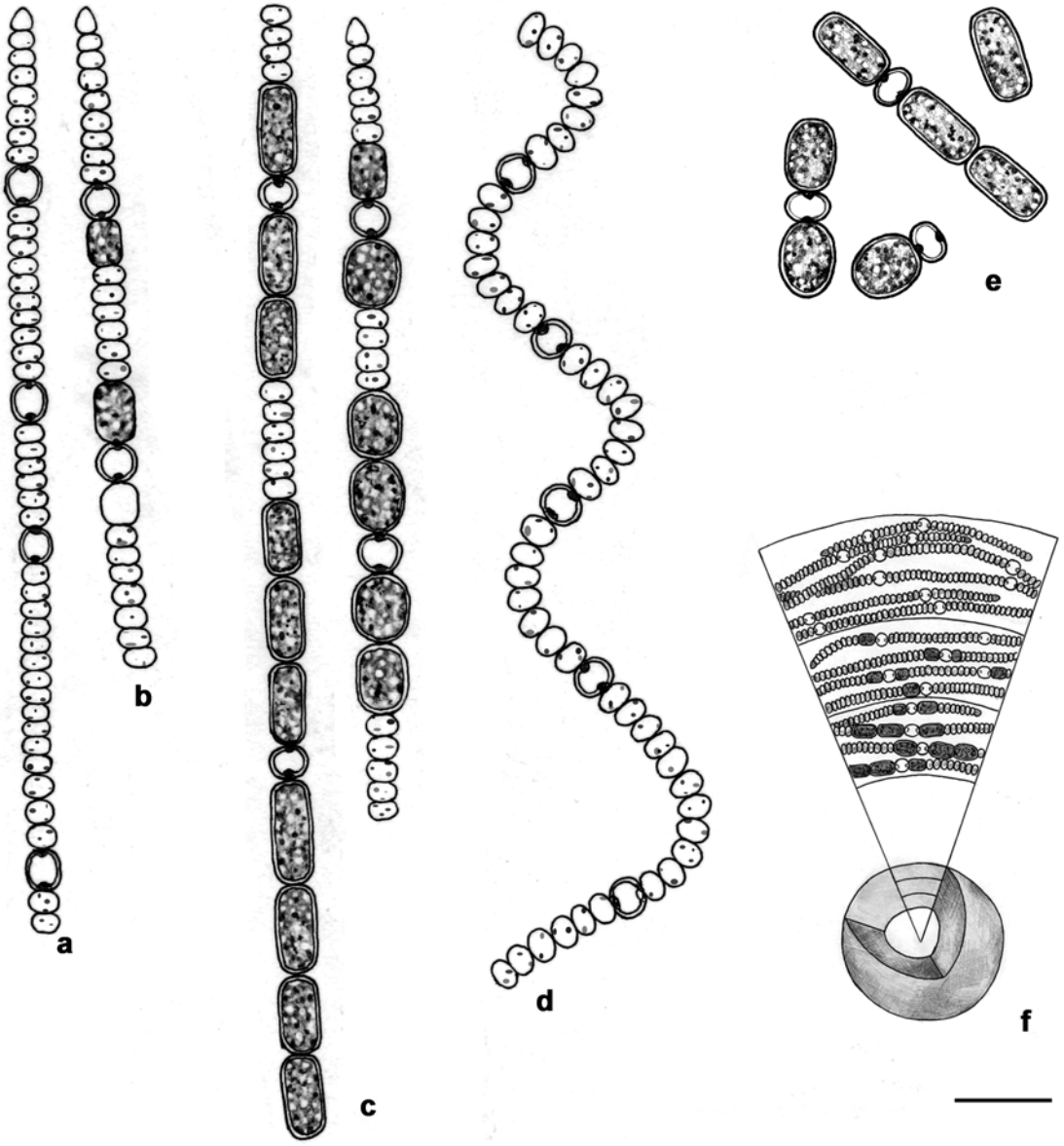


Fig. 1. Iconographs of *Wollea saccata* strain ACCS 045: (a) trichome showing intercalar heterocytes formation; (b) formation of proakinetes; (c) straight trichomes with akinetes at both sides of heterocytes; (d) curved filament of trichome; (e) ellipsoid and cylindrical ripe akinetes with heterocytes; (f) arrangement of trichomes in saccule spherical colony. Scale bar 10 μm (a–e), 30 μm (f.)

Nostoc, *Anabaena* and *Anabaenopsis* (KOMÁREK & ANAGNOSTIDIS 1989). BORNET & FLAHAULT (1888, p. 223) record that trichomes and spores of this genus correspond to genus *Sphaerozyga* (later one of *Anabaena* sections) to which WOLLE (1880, 1887) initially referred species *S. saccata* that was described by him. But the original form of the colonies made them establish this alga as a separate genus *Wollea* placed between *Nostoc* and *Anabaena*. ELENKIN (1938, p. 641) supposes that genus *Wollea* was situated much closer to the typical representatives of genus *Nostoc* than to *Anabaena*. On the basis of the investigation of *W. bharadwajae,* SINGH (1942 , p. 604) was of opinion that genus *Wollea* represents a synthetic genus from which two evolutionary lines, one leading to a Nostocoid habit and another to an Anabaenoid habit, have been evolved.

The *W. saccata* strain ACCS 045 shared less than 95.4% 16S rRNA gene sequence similarity with any other cyanobacterial sequence and formed common subcluster with the strain 133 of *An.* cf. *cylindrical* (similarity 95.4%), closely together with *An. oscillarioides* strains BO HINDÁK 1984/43 (similarity 95.1%) (Fig. 4). Interestingly,

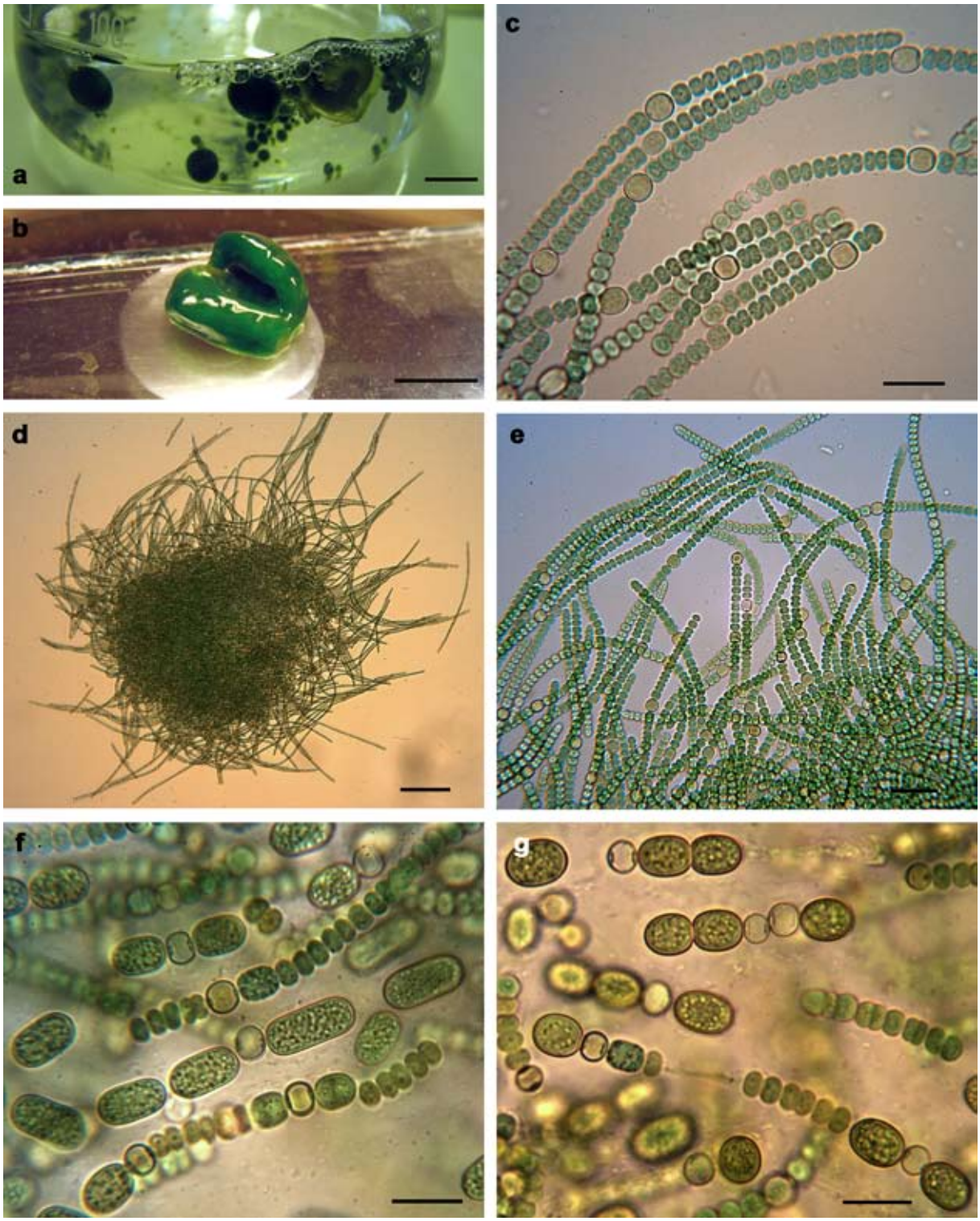


Fig. 2. Microphotographs of *Wollea saccata* strain ACC 045: (a, b) saccule spherical form of colony; (c) young trichomes with intercalary heterocytes and conical–rounded terminal cells; (d) young colony without apparent mucilage; (e) arrangement of trichomes in the young colonies; (f, g) akinetes formation (cylindrical and ellipsoid). Scale bar 1 sm (a–b), 50 μm (d), 20 μm (e), 10 μm (c, f, g).

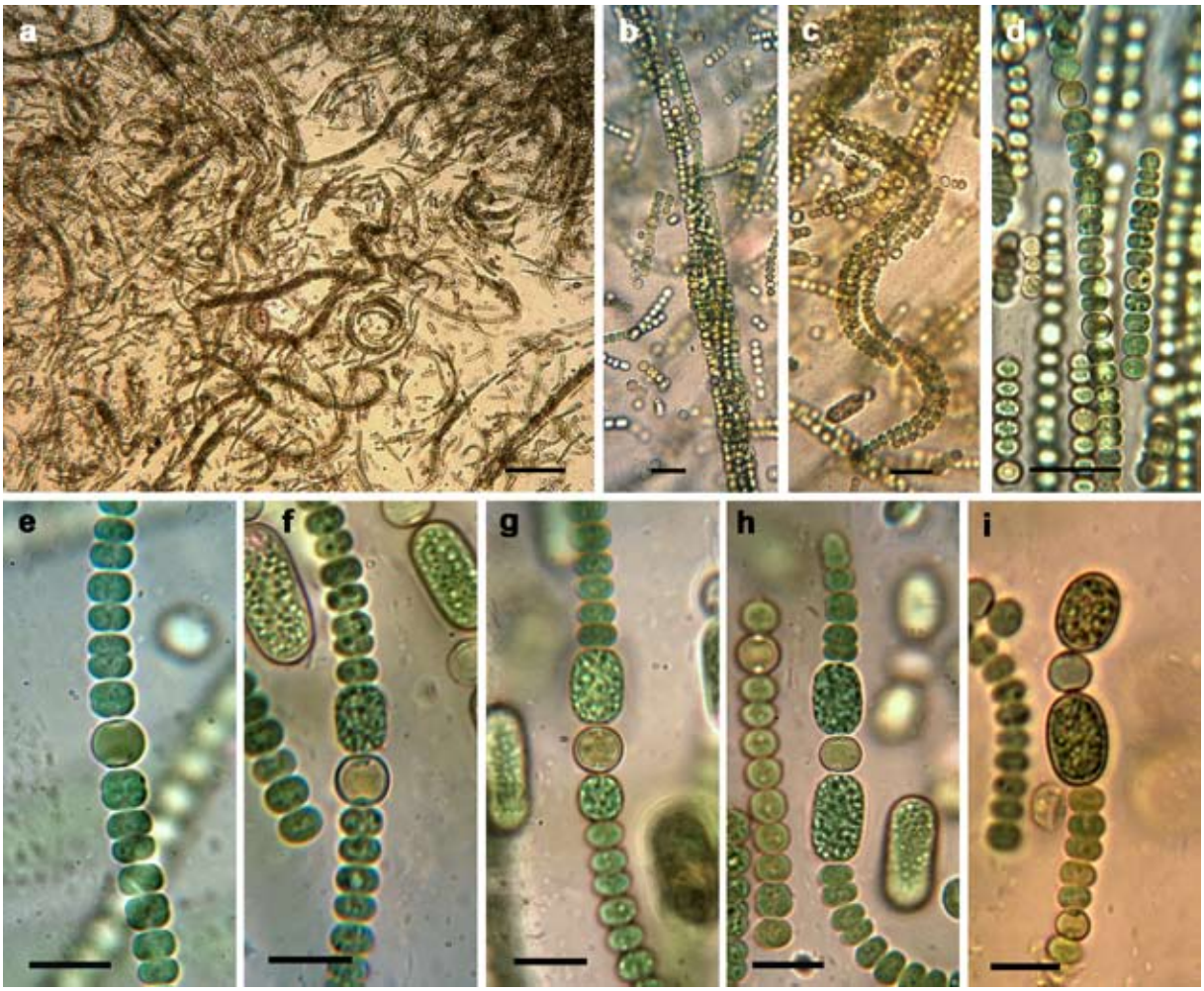
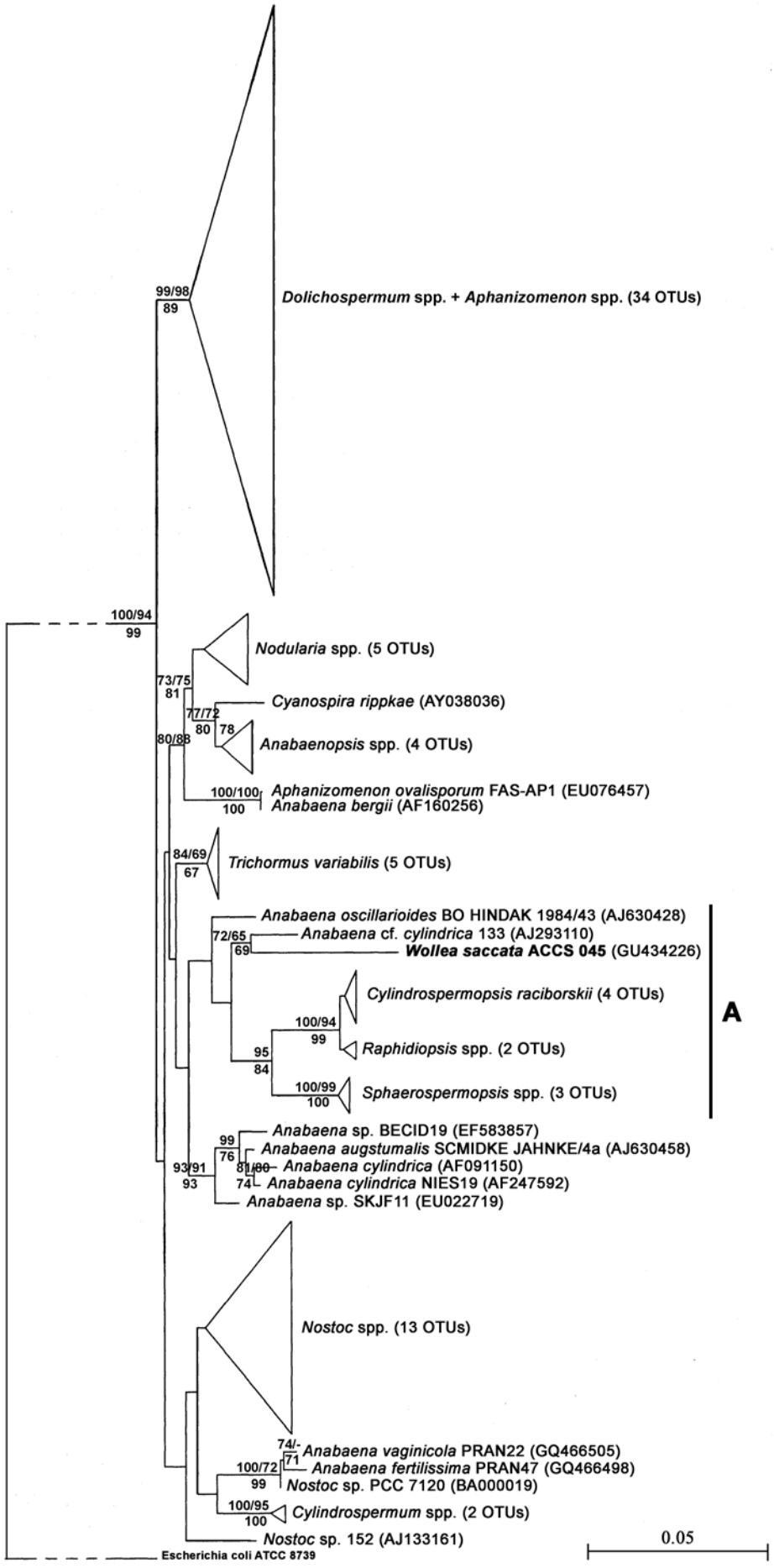


Fig. 3. *Wollea saccata* strain ACC 045, arrangement of the trichomes in the mature colony and paraheterocytic development of akinetes: (a) filaments of trichomes in the mucilaginous colony; (b) twisted strand of trichomes; (c) curved filament of trichomes; (d) parallel arrangement of the single trichomes; (e) segment of trichome with intercalary heterocyte; (f) trichome with proakinete at one side of heterocyte; (g, h) trichome with proakinetes at both sides of heterocyte; (i) trichome with ripe ellipsoid akinetes. Scale bar 60 μm (a), 30 μm (b, c), 20 μm (d) and 10 μm (e–i).

Fig. 4. Neighbour–joining tree based on 16S rRNA gene sequences (1193 bp) showing phylogenetic position of studied strain ACCS 045 of *Wollea saccata* (in bold). Values above nodes indicate bootstrap support over 65 % for the NJ/MP analyses, while values below nodes indicate bootstrap support over 65 % for the ML analysis. OTUs – operational taxonomic units.

W. saccata, *An. cylindrical* and *An. oscillarioides* are characterized by similar formation of akinetes at both sides of a heterocyte (paraheterocytic) and conical (conical–rounded) form of terminal cells. Moreover, disregarding the peculiar macroscopic colonial structure of *Wollea*, and considering only trichomes and cells (including heterocytes and akinetes) morphology, strain ACCS 045 is very similar to *An. oscillarioides* and *An. turkestanica* (KISSELEV) KOMÁREK (former *An. oscillarioides* f. *turkestanica* (KISSELEV) ELENKIN).

The strain of *Wollea* was placed close to *Raphidiopsis* FRITSCH et RICH (16S rRNA sequence similarity with *W. saccata* less than 92.8%), *Cylindrospermopsis raciborskii* (WOŁOSZ.) SEENAYA et SUBBA Raju (similarity less than 92.8%), *Sphaerospermopsis* ZAPOMĚLOVÁ, JEZBEROVÁ, HROUZEK, HISEM, ŘEHÁKOVÁ et KOMÁRKOVÁ (similarity less than 93.6%) and benthic *Anabaena* (including *An. augstumalis* SCHMIDKE JAHNKE/4a (similarity 95.2%)) clusters. The benthic *Anabaena*–types (former subg. *Anabaena*) forming mats and never producing gas vesicles in cells were found clearly genetically separated from the planktic cluster (former subg. *Dolichospermum*) containing free–floating solitary trichomes or their small groups (GUGGER


99/98
89
Dolichospermum spp. + *Aphanizomenon* spp. (34 OTUs)
100/94
99
Nodularia spp. (5 OTUs)
73/75
81
Cyanospira rippkae (AY038036)
77/72
80
78
Anabaenopsis spp. (4 OTUs)
80/88
100/100
100
Aphanizomenon ovalisporum FAS-AP1 (EU076457)
Anabaena bergii (AF160256)
84/69
67
Trichormus variabilis (5 OTUs)
Anabaena oscillarioides BO HINDAK 1984/43 (AJ630428)
72/65
69
Anabaena cf. *cylindrica* 133 (AJ293110)
***Wollea saccata* ACCS 045** (GU434226)
Cylindrospermopsis raciborskii (4 OTUs)
100/94
99
Raphidiopsis spp. (2 OTUs)
95
84
100/99
100
Sphaerospermopsis spp. (3 OTUs)
A
Anabaena sp. BECID19 (EF583857)
99
76
Anabaena augstumalis SCMIDKE JAHNKE/4a (AJ630458)
93/91
93
81/80
Anabaena cylindrica (AF091150)
74
Anabaena cylindrica NIES19 (AF247592)
Anabaena sp. SKJF11 (EU022719)
Nostoc spp. (13 OTUs)
74/-
71
Anabaena vaginicola PRAN22 (GQ466505)
100/72
Anabaena fertilissima PRAN47 (GQ466498)
99
Nostoc sp. PCC 7120 (BA000019)
100/95
100
Cylindrospermum spp. (2 OTUs)
Nostoc sp. 152 (AJ133161)
Escherichia coli ATCC 8739
0.05

et al. 2002; RAJANIEMI et al. 2005). Recently, on the basis of polyphasic data the new genera *Sphaerospermopsis* (ZAPOMĚLOVÁ et al. 2010) and *Dolichospermum* (WACKLIN et al. 2009) have been separated from *Anabaena*.

All members of cluster A (Fig. 4) are characterized paraheterocytic type formation of akinetes except genera *Raphidiopsis*. Certainly, heterocysts clearly influence the position of akinetes in the filament, yet it has been impossible to provide a unifying model to explain the different types of the akinete development in, e.g., *Nostoc* and *Wollea*, in which akinetes form midway between and adjacent to heterocysts, respectively. We suppose that as with the varied triggers for akinete development, it may be that akinete positioning is controlled by different mechanism in different cyanobacteria. But not numerous data available till now including molecular genetics of akinetes and their formation does not allow to estimate evolutionary relevance of different akinete development types.

Thus, our results show that the phylogenetic placement of genera *Wollea* based on 16S rRNA gene sequence was distant from *Nostoc* and the most closely related to taxa in benthic *Anabaena, Sphaerospermopsis*, *Cylindrospermopsis*, and *Raphidiopsis*.

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