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The confirmation of the genus *Glaucospira* (Cyanobacteria) and the occurrence of *Glaucospira laxissima* (G. S. West) comb. nova in Serbia

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Abstract – The work presents the morphology of cells and trichomes of *Glaucospira laxissima* (G.S. West) comb. nova analyzed by light microscopy, confocal laser microscopy (CLM) and transmission electron microscopy (TEM). Cyanobacterial characteristics are confirmed for the first time. It also contains the data on the ecology of this species, found for the first time in Serbia, in a small alkaline lake in a planktic community with the species *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenayya & Subba Raju, *Anabaenopsis elenkinii* V.V. Miller and *Chrysosporum bergii* (Ostenfeld) Zapomělová *et al.*

Cyanobacteria / cytomorphology / confocal laser microscopy / *Glaucospira (not Glaucospira sp.)* / Serbia / taxonomy / TEM

Résumé – La morphologie des cellules et des trichomes de *Glaucospira laxissima* (G.S.West) comb. nov. est étudiée en microscopie optique, en microscopie laser confocale (CLM) et en microscopie électronique à transmission (MET). Ses caractères cyanobactériens sont confirmés pour la première fois. Des données sont apportées sur l'écologie de cette espèce, trouvée pour la première fois en Serbie, dans un petit lac alcalin, au sein d'une communauté planctonique avec les espèces *Cylindrospermopsis raciborskii* (Wołosz.) Seenayya & Subba Raju, *Anabaenopsis elenkinii* V.V. Miller et *Chrysosporum bergii* (Ostenfeld) Zapomělova *et al.*

Cyanobacteria / cytomorphologie / microscopie laser confocale / *Glaucospira (not Glaucospira sp.)* / Serbie / taxonomie / MET

INTRODUCTION

The genus *Glaucospira* (order Pseudanabaenales, family Spirulinaceae) is little-known and considered as problematic. Due to a small number of findings and the lack of material in culture, cyanobacterial characteristics of this taxon have not been proven (Komárek & Anagnostidis, 2005). Majority of the described

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species from nature were found under extreme environmental conditions, mostly outside Europe. Originally, only two species of *Glaucospira* from Ecuador (S. America) and Yellowstone National Park, USA (N. America) were described, but about 15 other species, usually identified as different *Spirulina*-taxa correspond morphologically to this genus. It was also the case of the commonest species "*Spirulina laxissima* G.S.West 1907".

The populations of this cyanobacteria were found in the plankton (rarely in the periphyton) of flat fresh waters, in tropical and subtropical regions of Africa (Lake Tanganyika, Lake Chad, Lake Victoria, Egypt, Zaire, Kenya, Uganda, Tanazania, Zambia), Australia, Asia (India, Japan, Malaysia), America (Brazil, Cuba, Mexico and Florida – Everglades alkaline wetland) (Komárek & Anagnostidis, 2005; Mareš, 2006; Santos & Sant'Anna, 2010; Nogueira *et al.* 2011; Martins *et al*., 2012). In Europe, it was very rarely recorded in Greece, Hungary and in the Caspian Sea (the river Volga estuary) (Komárek & Anagnostidis, 2005). This work describes the presence of this alga in Serbia for the first time, also providing data on the ecology and morphology and demonstrating the presence of cyanobacterial characteristics. Since cytomorphological analyses indicate the taxonomic classification to the genus *Glaucospira* rather than to heterogeneous *Spirulina* (cf. Nübel *et al*., 2000), the new combination of this species, *Glaucospira laxissima* (G.S.West) comb. nova, was proposed.

MATERIAL AND METHODS

Plankton material was collected in September 2011 in Aleksandrovac Lake, which is situated in southernmost part of Serbia (N 42° 29' 22", E 21° 53' 54"), at the elevation of 412 m (Fig. 1). It is an artificial reservoir, constructed in 1963-1964 by creating an earth-fill dam, 8 meters high, on the Aleksandrovac River. The lake area is 12 ha, max. volume 250.000 m^3, length 580 m, width 300 m, maximum depth of 4 m, and average depth of 2 m. The bottom of the lake contains a large quantity of sludge (1.5 m thick by the dam and 5 m near the upstream end of the accumulation). The water mass of the lake covers most of the so-called Aleksandrovac salines. The reservoir covers about 2/3 of the salines, while 1/3 is outside the reservoir. Soda ($NaHCO_3$) appears on the coast in the form of white-powdered lime. This reservoir was originally used for irrigation of agricultural areas, and later for sports and recreational purposes, above all fishing. In the last few years, several fish kills occurred. Complete restoration of the lake was performed from September 2009 to May 2010. The remaining fish was fished out, the water was released from the lake, and a complete rehabilitation (Prokić *et al.*, 2008) was performed. This process included the construction of a pipeline and a canal, 2 027 m long, for the transport of water from water supply, the construction of a drain ditch 2 267 m long to drain away the excess water into the nearest river (Južna Morava), as well as the regulation of lake water itself (removal of 35-40 000 m^3 of sludge, construction of a beach and paths), reconstruction of the buildings and the overflow on the dam. Anti-erosion measures were introduced through planting shrubs on the coast. The salines were not considered within the rehabilitation project.

Phytoplankton samples were gathered by standard methods – phyto-plankton net (net frame 25 cm, mesh net e.g. 22 μm) and Rutner bottle (2 dm^3) in all aspects. All samples were preserved at once in a 4% solution of formaldehyde.

Fig. 1. Study area map. Location of the Aleksandrovac Lake, Southern part of Serbia.

Microscopic identification of Cyanobacteria was based on morphological characteristics using light microscope Amplival-Jena with magnification up to 800-1000x.

For confocal laser microscopy (CLM) analysis, formalin-fixed (neutral buffered 4% formaldehyde in freshwater (lake) water) algae specimens were stained with 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) and observed by Leica TCS SP5 II Basic broadband microscop (Leica Microsystems, Germany).

The same specimens were rinsed in 0.1 M phosphate buffer overnight, additionally fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer and postfixed in 1% OsO_4 in 0.1 M phosphate buffer for transmission electron microscopy (TEM). Dehydration was performed at room temperature in graded ethanol series (30%, 50%, 70%, 95%, 100%) and finished by propylene oxide. The samples were embedded in Araldite (Fluka, USA) and polymerized for 48 h at 60°C. Ultrathin-sections were obtained on Ultracut 6 microtome (Leica Microsystems, Germany), mounted on 300-mesh copper grids, and contrasted with uranyl acetate and lead citrate (Ultrastain I&II). Sections were examined with a Philips CM12 TEM operating at 80 kV and images obtained using iTEM Megaview camera.

Simultaneously with gathering Cyanobacteria samples in the field, the following physical and chemical parameters were measured: water temperature (°C), pH, oxygen concentration ($mg \cdot l^{-1}$), saturation (%), conductivity ($\mu s \cdot cm^{-1}$), nitrate, ammonia and phosphate concentration ($mg \cdot l^{-1}$), and water transparency (Secchi disc) (m) (APHA, 1995).

Identification was confirmed using the literature (Cvijan & Blaženčić, 1996; Hindák, 2001; Komárek & Anagnostidis, 1989, 2005; Whitton, 2008).

Quantitative analysis of phytoplankton was made using Utermöhl metod (Utermöhl, 1958) with inverted microscope Carls Zeiss and it is expressed as number of trichomes per liter.

RESULTS

Taxonomy

The species *Spirulina laxissima* should be nomenclaturally transferred to genus *Glaucospira* Lagerheim 1892 according to cytomorphological characters, as obtained from our analysis (see the following text):

Glaucospira laxissima (G.S.West) Simić *et al.,* **comb. nova**

Basionym: *Spirulina laxissima* G.S.West, *J. Linn. Soc. Bot.* 38: 178, 1907.
Type locality: Tanzania, plankton near Kala; type: slide collection in BM, leg. Cunnington, Nov. 1904 (cf. Drouet, 1968).

Cytomorphology (Table 1, Figs 2-11)

Trichomes are solitary, free floating in the plankton, of pale blue-green color, with more or less homogeneous content (Figs 2-3). Trichomes are relatively short, their length is from 36.8 to 60 μm. Trichomes are bent, regularly freely coiled, coil number is from 1 to 3(5). Coil length is from 17.5 to 27.6 μm, the distance between coils is variable from 4.7 to 9.7 μm (Figs 2-4). Trichomes are multicellular, cell number in trichome is 9-14 (Fig. 4). Cells are long and cylindrical, their length is from 2.7 to 6.1 μm, cell width is from 0.5 to 0.6 μm. Cell wall thickness ranges from 26.5-38 nm (Figs 5-6). Thylakoids are parietal, concentric, two parallel to the cell wall (Figs 8-9, arows). Around trichomes are very fine mucilaginous, diffluent envelopes. Thicknesses of envelopes are variable, from 0.3 to 1.5 μm (Figs 10-11).

Distribution

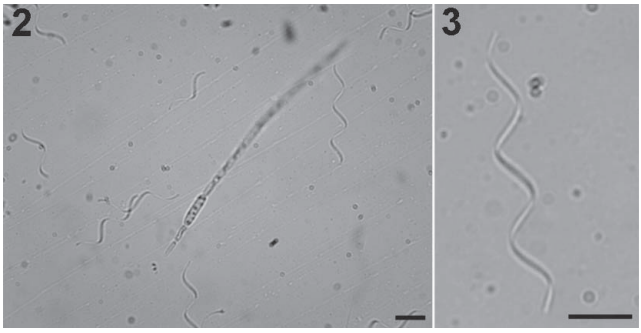
Trichomes were found in Aleksandrovac Lake (Fig. 1) in September 2011. Physical and chemical parameters measured (average values) there are presented in Table 2. The total abundance of Cyanobacteria was 1 033 427 trichomes per liter, dominated by *Glaucospira laxissima* (764 735 trichomes per liter; 74%), sub-dominant was *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenayya & Subba Raju (237 688 trichomes per liter; 23%), *Anabaenopsis elenkinii* V.V.Miller and *Chrysosporum bergii* (Ostenfeld) Zapomělová *et al.* were found in traces (less than 5%).

DISCUSSION

Trichomes of *Glaucospira laxissima* (syn. *Spirulina laxissima*) found in Aleksandrovac Lake, morphologically exactly correspond to the best available description of *Spirulina laxissima* (cf. Komárek & Anagnostidis, 2005) (Table 1). The morphology of the whole species *Glaucospira laxissima* including both literary data and our material is as follows (Table 1): solitary filaments (trichomes), short or long, thin (up to 3 μm wide), regularly and very loosely spirally coiled and screw-like with wide and long screws, sometimes slightly flexible, regularly cylindrical along the whole length, not attenuated at the ends, not constricted at the invisible (in LM) cross-walls. Cells are pale blue-green or yellowish with homogeneous content, sometimes with a few fine granules, always

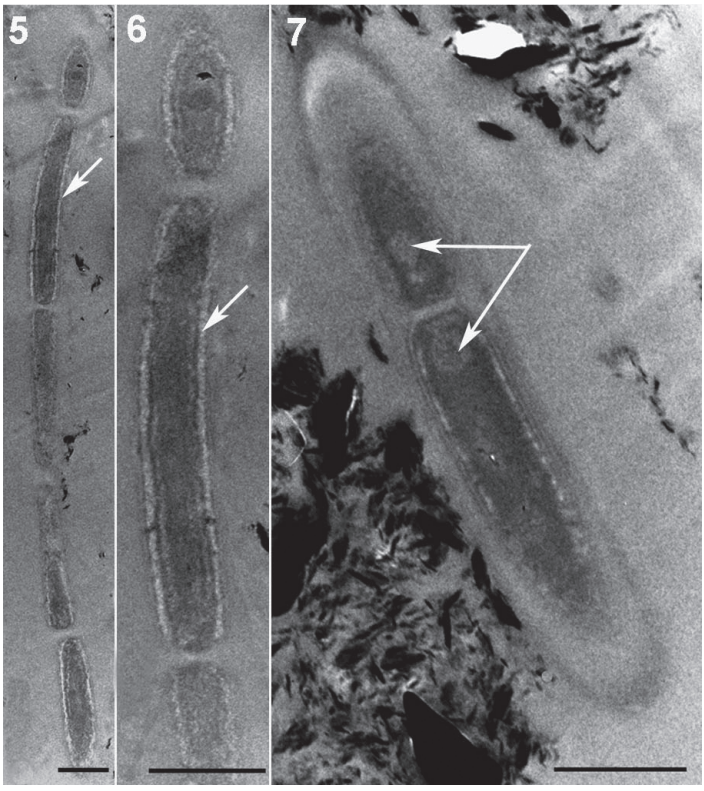
Table 1. Morphological features of *Glaucospira laxissima*

Characteristics of species Glaucospira laxissima	*Serbia (this paper)*	*Komárek & Anagnostidis (2005)*	*Santos & Sant'Anna (2010)*	*Martins et al. (2012) (as* Spirulina laxissima*)*
Trichomes colour	pale blue-green	pale blue-green		
Trichomes	solitary	solitary	solitary	solitary
Trichomes length (μm)	36.8-60 (43.3)	up to 100		17-40
Number of coils in trichomes (μm)	1-5			
Wide distance between coils (μm)	14.7-20.5	highly variable (5.2-8.8) 10-22 (25)	8.0-12.5	6-14
Coils length (μm)	17. 5-27.6 (23.6)			
Coils height (μm)	4.7-9.7	coils left-handed, variably wide and long,4-6.6 (8.2)		2.5-4
Cell length (μm)	2.7-6.1 (3.7)		5.0-6.2	
Number of cells in trichomes	9-14			
Cell width (μm)	0.5-0.6	0.5-0.7-1.3	1.4-2.0	1.2-2.0
Mucus around trichomes	yes (visible only in TEM)	no		no
Cell wall thickness (nm)	26.5-38			
End of trichomes	apical cells obtuse-rounded	apical cells obtuse-rounded		
Reproduction	fragmentation			
Motility	not detected sample were fixed	usually intensely motile (rotation)		motile
Water/community	lake/plankton	freshwater, saline/ plankton	wetland/ plankton	coastal lagoons/ plankton

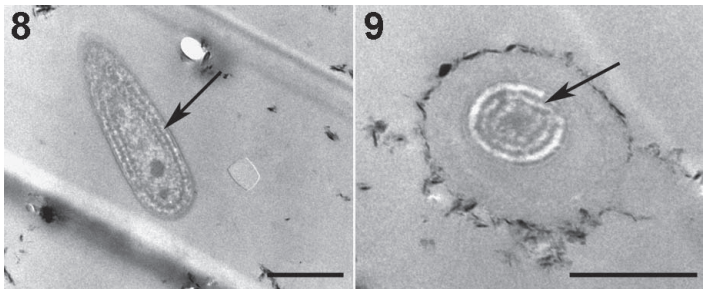


Figs 2-3. **2.** Trichomes of *Glaucospira laxissima* and *Cylindrospermopsis raciborskii* under the light microscope (LM); **3.** Trichome of *G. laxissima* under the light microscope (LM). Scale bar 10 μm.

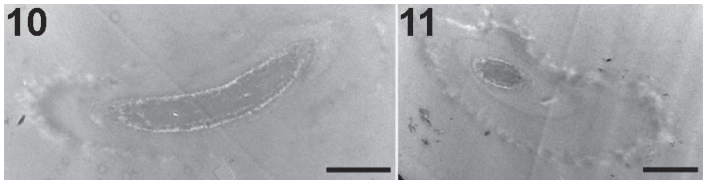
Fig. 4. Multicellular trichomes of *Glaucospira laxissima* under the confocal laser microscopy (CLM). Autofluroescence of chlorophyll was used to indicate the septation of *G. laxissima*. Scale bar 5 μm.



Figs 5-7. Transmission electron microscopy (TEM) photomicrographs of *Glaucospira laxissima*: **5-6.** Multicellular trichomes; cell wall (arrows); **7.** Molecules of DNA (arrows). Scale bar 1 μm.



Figs 8-9. Transmission electron microscopy (TEM) photomicrographs of parietal thylakoids in cells (arrow). **8.** The thylakoids are concentric and parallel to the cell wall (arrow). **9.** Cross section. Scale bar 1 μm.



Figs 10-11. **10.** Mucilaginous envelope around cells. **11.** Cross section. Scale bar 1 μm.

Table 2. Mean value of physical and chemical parameters of Aleksandrovac Lake in September, 2011

Parameters	*Mean value*
Water temperature (°C)	21.6
pH	10.21
Oxygen ($mg \cdot l^{-1}$)	7.36
Saturation (%)	85.80
Nitrate ($mg \cdot l^{-1}$)	1.85
Phosphate ($mg \cdot l^{-1}$)	0.33
Ammonia ($mg \cdot l^{-1}$)	0.14
Conductivity ($\mu s \cdot cm^{-1}$)	423
Water transparency (m)	0.6

distinctly longer than wide. Cell division crosswise to the trichome axis. Reproduction is probably only by trichome disintegration. In all descriptions of *Glaucospira laxissima* (and *Spirulina laxissima*) this species is without sheath (Komárek & Anagnostidis, 2005; Santos & Sant'Anna, 2010; Martins *et al*., 2012). Sheaths or envelopes also are not clearly visible in light microscope in our material (Table 1, Figs 2-3). A very fine mucilaginous and diffluent envelope is visible around trichomes only in the EM sections (Figs 10-11).

Santos & Sant'Anna (2010) describe a similar *Glaucospira* sp. from one salt lake in Brazil (Table 1). They describe the single, short trichomes with regular

or irregular coils. Trichome length is 17-40 μm, and the width is 1.2-2.0 μm. Coil length is from 2.5 to 4 μm, the distance between coils is from 6 to 14 μm. The cells have homogeneous content without granules and green colour. Trichomes are moving, rotating in both directions. Komárek & Anagnostidis (2005) also mentioned that the trichomes usually move vigorously (rotation). The similar trichomes (as *Spirulina laxissima)* from Brazilian wetlands were also described by Martins *et al.*, (2012) (Table 1). Trichomes not constricted at cross-walls are solitary and loosely curved. Cell contents are blue-green and homogenous. The population showed trichomes wider than those cited by Komárek & Anagnostidis (2005) (Table 1).

The *Glaucospira* genus is an insufficiently known one, rarely found in the plankton of stagnant waters, usually in tropical countries (Komárek & Anagnostidis, 2005). Similar cyanobacteria are very rare in North America (from thermal springs in Yellowstone National Park, USA), and only one species is known from Europe (Greece, Hungary, Caspian Sea – estuary of river Volga). Few species, known from plankton of lakes and reservoirs and from metaphyton among water plants in swamps in tropical regions are recorded from African lakes and also from Australia, Asia (India, Japan, Malaysia), America (Brazil, Cuba, Mexico, Ecuador and Venezuela) (Komárek & Anagnostidis, 2005; Nogueira *et al.*, 2011). Mareš (2006) found trichomes this cyanobacteria (as *Spirulina laxissima*) in periphyton of Everglades alkaline wetland in Florida.

Santos & Sant'Anna (2010) provide data on the appearance of *Glaucospira* sp. with *Anabaenopsis elenkinii* in a Brazilian salt lake Salina de Meio. This locality has extreme conditions during periods of water level fluctuation and succession of droughts and floods (pH > 10, conductivity > 3 800 μS·cm^{-1}, temperature 28.2°C). Nogueira *et al.* (2011) found *Glaucospira* sp. in a fishery pond (Lake Jaó – a shallow artificial lake) in a planktic community with 31 taxa of Cyanobacteria. *Cylindrospermopsis raciborskii* was also present in the community of Cyanobacteria. The Jaó Lake is a shallow alkaline lake (pH was from 7.5 to 9.1, conductivity was from 110.8 to 137.4 μS·cm^{-1}, temperature > 25°C). Similar situation was recorded in Serbia, when mass occurrence of this alga was discovered in a lake, formed on the saline bottom. This is the first record of the genus *Glaucospira* in the waters of Serbia (Cvijan & Blaženčić, 1996; Sedmak & Svirčev, 2011).

Modern taxonomy of cyanobacteria is based on the polyphasic approach, including molecular analyses. However, there are currently many species, the culturing of which is difficult and their phylogenetic position remains to be investigated in future. *Glaucospira*, which occurs very rarely, belongs to this taxonomic category. Nevertheless, considering the characteristic morphological and ultrastructural traits reported in this study, we find it useful to classify *Glaucospira* in a corresponding separate genus.

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