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Raphidiopsis mediterranea Skuja represents non-heterocytous life-cycle stages of *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju in Lake Kastoria (Greece), its type locality: Evidence by morphological and phylogenetic analysis

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ABSTRACT

The taxonomical relationship of *Cylindrospermopsis raciborskii* and *Raphidiopsis mediterranea* was studied by morphological and 16S rRNA gene diversity analyses of natural populations from Lake Kastoria, Greece. Samples were obtained during a bloom (23,830 trichomes mL^{-1}) in August 2003. A high diversity of apical cell, trichome, heterocyte and akinete morphology, trichome fragmentation and reproduction was observed. Trichomes were grouped into three dominant morphotypes: the typical and the non-heterocytous morphotype of *C. raciborskii* and the typical morphotype of *R. mediterranea*. A morphometric comparison of the dominant morphotypes showed significant differences in mean values of cell and trichome sizes despite the high overlap in the range of the respective size values. Additionally, two new morphotypes representing developmental stages of the species are described while a new mode of reproduction involving a structurally distinct reproductive cell is described for the first time in planktic Nostocales. A putative life-cycle, common for *C. raciborskii* and *R. mediterranea* is proposed revealing that trichome reproduction of *R. mediterranea* gives rise both to *R. mediterranea* and *C. raciborskii* non-heterocytous morphotypes. The phylogenetic analysis of partial 16S rRNA gene (ca. 920 bp) of the co-existing *Cylindrospermopsis* and *Raphidiopsis* morphotypes revealed only one phylotype which showed 99.54% similarity to *R. mediterranea* HB2 (China) and 99.19% similarity to *C. raciborskii* form 1 (Australia). We propose that all morphotypes comprised stages of the life cycle of *C. raciborkii* whereas *R. mediterranea* from Lake Kastoria (its type locality) represents non-heterocytous stages of *Cylindrospermopsis* complex life cycle.

1. Introduction

The genera *Cylindrospermopsis* and *Raphidiopsis* belong to the order Nostocales, family Nostocaceae in the Botanical classification system (Komárek and Anagnostidis, 1989). *Cylindrospermopsis*, included in the new edition of the Bergey's Manual, belongs to the subsection IV of the cyanobacteria (Rippka et al., 2001) whereas *Raphidiopsis* has not yet been given validly published names under the Bacteriological Code. In the Botanical classification system, the generic delimitation of *Raphidiopsis* and *Cylindrospermopsis* as well as other closely related nostocalean genera is still problematic. The genus *Cylindrospermopsis* is satisfactorily defined on the basis of the heterocytes origin and position while the genus *Raphidiopsis* lacking obligatory heterocytes is classified into the family Nostocaceae by producing akinetes. Both genera are characterized by free-floating, solitary, straight, flexuous or coiled trichomes with subsymmetric structure of trichomes due to the development of akinetes, cylindrical vegetative cells with the additional obligatory feature of narrowed apical cells of trichomes, sharply pointed or needle-like in *Raphidiopsis*. Reproduction mode is trichome fragmentation and akinete germination (Komárek and Anagnostidis, 1989). The genus *Raphidiopsis* includes currently five planktic species (referred by Li et al., 2008) while the genus *Cylindrospermopsis* includes ten planktic species (Komárek and Komárková, 2003; Couté and Bouvy, 2004).

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The validity of the genus *Cylindrospermopsis* has been supported by the long phylogenetic distances that separate it from representatives of other nostocalean genera on the basis of 16S rRNA gene sequences (Wilmotte and Herdman, 2001). On the contrary, strains assigned to *Raphidiopsis curvata* and *Raphidiopsis mediterranea* cluster tightly either with strains of *Cylindrospermopsis raciborskii* (Li et al., 2008) or strains of *Aphanizomenon issatschenkoi* (Wood et al., 2007). Phylogenetic studies of cyanobacteria have demonstrated that genetic relationships sometimes conflict with morphological classification but at the same time a high number of misidentified strains exist (Gkelis et al., 2005; Rajaniemi et al., 2005). This is the case for the strain CAWBG02 which was morphologically identified as *R. mediterranea* but phylogenetically it clustered tightly with *A. issatschenkoi* (Wood et al., 2007).

The juxtaposition of *Raphidiopsis* and *Cylindrospermopsis* based primarily on the botanical species *R. mediterranea* and *C.* (*Anabaenopsis*) *raciborskii* appeared early in the literature by Skuja (1937), who described *R. mediterranea* from Lake Kastoria (Greece). Skuja (1937) observed concomitantly *C. raciborskii* with heterocytes but without akinetes and *R. mediterranea* with akinetes but without heterocytes. The description of the new species was based on this difference but the similarity of their developmental stages gave rise to a question about the close phylogenetic affinity of the two species. As Komárek and Anagnostidis (1989) cite, there exists a possibility that *Raphidiopsis* "represents only the developmental stages from species of other genera with developed akinetes but transitionally without heterocytes". Concerning this taxonomical problem of *Raphidiopsis*, there is an evidence (Komárkova et al., 1999; McGregor and Fabbro, 2000) that, *R. mediterranea*-like trichomes are most likely *C. raciborskii* morphotypes lacking heterocytes.

R. mediterranea is considered a rarely reported cyanobacterium (Li et al., 2008). On the contrary, *C. raciborskii* with tropical origin and first European record from Lake Kastoria is a frequently reported species with expanding geographical distribution (Padisák, 1997). Recently, there has been an exponential increase in the literature on *Cylindrospermopsis* and *Raphidiopsis* due to their ability to produce cyanotoxins (Falconer et al., 1999; Li et al., 2008) and particularly on *C. raciborskii* because of its invasive behaviour at mid-latitudes (Briand et al., 2004). However, the taxonomical problem between *C. raciborskii* and *R. mediterranea* still exists and is crucial in the research of toxic and invasive cyanobacterial species.

The aim of this work was to contribute to the taxonomy of *R. mediterranea* and *C. raciborskii* using both morphological and phylogenetic analyses of these cyanobacteria in plankton samples collected from Lake Kastoria, which is the type locality of *R. mediterranea* and the first lake in Europe where the occurrence of *C. raciborskii* was reported. This is the first paper that combines phylogenetic and morphological data of these two closely related species co-existing in a bloom, and proposes a putative life-cycle common for both species showing new developmental stages.

2. Materials and methods

Lake Kastoria is situated at latitude 40°30′N, and longitude 21°18′E in Northern Greece. It covers 24 km^2, has a maximum depth of 8 m and an average depth of 4 m. It is a highly eutrophic system that has a history of toxic cyanobacterial blooms (Cook et al., 2004) and their possible effects on heterotrophic nanoplankton and microbial food web in the lake, have been reported (Moustaka-Gouni et al., 2006). In summer, when high water temperature and poor light conditions prevail and the N:P resource ratio drops below the critical ratio of Redfield, *C. raciborskii* dominates in the phytoplankton (Moustaka-Gouni et al., 2007).

Samples were collected from the shallow area of the lake (2 m depth) during a water bloom in August 2003. Sub-samples were preserved with both Lugol's solution and formaldehyde. Water samples for 16S rRNA analysis were stored in polyethylene bottles and kept under cool and dark conditions until return to the laboratory.

Fresh and preserved samples were examined using an inverted microscope (Nikon ECLIPSE TE2000-S) with phase contrast, and photographs were taken using a digital camera (Nikon DS-L1). Species were identified using the taxonomic paper of Skuja (1937) and the classification system of Komárek and Anagnostidis (1989). Phytoplankton counts (trichomes) were performed using the inverted microscope method (Utermöhl, 1958). Cell and trichome dimensions were measured on freeze-frame micrographs of individuals using the camera's tools (Nikon DS-L1).

The following features were selected to describe the morphology and morphometry of the species studied: trichome shape, length (l) and width (w), the presence or absence of terminal heterocytes, the shape of trichome apical cells, vegetative cells, heterocytes and akinetes, the length and width of the vegetative cells, heterocytes and akinetes and the l:w ratio of vegetative cells. For each of the two investigated species, at least 60 measurements of the trichome length and cell's length and width were made, 30 for heterocytes' and 20 for akinetes' length and width. Differences between mean values were assessed using analysis of variance (ANOVA), followed by a multiple comparison test (LSD). Significant relationships were defined as $p < 0.05$ (Sokal and Rohlf, 1981).

Upon return to the laboratory, 100 mL of lake water was filtered with a Whatman GF/C filter and the filter stored at −20 °C. DNA was extracted using the UltraClean Soil DNA isolation kit (MoBio Laboratories, USA) according to the manufacturer's protocol after slicing the filters with a sterile scalpel. For 16S rRNA PCR amplification, 0.5 μL of the DNA template and the primers BAC8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and BAC907r (5′-CCCGTCAATTCCTTTGAGTTT-3′) were used. Each 50 μL PCR reaction consisted of a 9 min pre-PCR hold at 95 °C, followed by 28 cycles, each of one consisting of a 45 s denaturation step at 95 °C, a 45 s annealing step at 52.5 °C, a 2 min elongation step at 72 °C, and at the end of the 28 cycles, a final 10 min finishing step at 72 °C. All PCR ingredients were prepared with twice-autoclaved ultra pure water, using GoTaq polymerase (Promega, USA) and stringent anti-contamination controls were used during PCR preparation. The PCR products were checked on a 1.2% agarose gel, at 70 V for 45 min under UV light and were purified using the Montage purification kit (Millipore, USA). The purified PCR products were cloned using the TOPO XL PCR cloning kit (Invitrogen, USA) using chemically competent cells according to the manufacturer's specifications. Approximately 100 clones were randomly selected and checked for having the correct insert size (ca. 920 bp). All positive clones were grown in liquid LB medium with kanamycin and their plasmids were purified using the Nucleospin Plasmid QuickPure kit (Macherey-Nagel, Germany). DNA sequencing was done by Macrogen (Korea) using capillary electrophoresis by the BigDye Terminator kit (Applied Biosystems Inc., USA) with the primer M13F (5′-GTAAAACGACGGCCAG-3′). Sequences of about 700 bp of the insert from each clone were compared with those in the DDBJ/EMBL/GenBank databases by FASTA search programs (http://www.ddbj.nig.ac.jp/search/fasta-e.html). Sequences with ≥98% similarity were grouped as identical operational taxonomic units (OTU). For the unique OTUs, additional sequencing was performed with the primer M13R (5′-CAGGAAACAGCTATGAC-3′) and after contig construction of the whole amplified region, detection and omitting chimeric DNAs were performed by the CHECK-CHIMERA program of the Ribosomal Database Project (Maidak et al., 2001).

The sequences were automatically aligned with their closest relatives' data using the Clustal X program (Jeanmougin et al.,

1998) and revised by manual removal of ambiguously aligned regions. Phylogenetic trees were constructed by the neighbour-joining method (Saitou and Nei, 1987) with the Clustal X program. Bootstrap analyses for 1000 replicates were performed to assign confidence levels to the tree topology by using PAUP* version 4.08b (Swofford, 2000).

3. Results

The trichomes of the nostocalean cyanobacteria observed in lake water corresponded to the genera *Cylindrospermopsis* and *Raphidiopsis* of the current classification system of Komárek and Anagnostidis (1989). Trichomes were solitary, straight or rarely flexuous, constricted or unconstricted at cross walls, short (range 35.4–256.3 μm; n = 182) and narrow (range 0.8–2.7 μm; n = 273), uniformly or ununiformly wide along the trichomes, mostly with narrowed apical cells, terminal heterocytes or without heterocytes, rarely bearing akinetes (Fig. 1). Gas vacuoles in cells of the trichomes were generally present but trichomes without gas vacuoles were also common.

On the basis of the original description of *R. mediterranea* and the first description of *C. raciborskii* from Lake Kastoria by Skuja (1937) the mixed trichomes of *Raphidiopsis* and *Cylindrospermopsis* were grouped in three morphotypes: (i) 25.9% of the trichomes corresponded to *R. mediterranea* of Skuja (1937) figures (Tafle 1: 5a–f) and identified as typical morphotype of the species (Fig. 1A_{1-11}), (ii) 35.2% of the trichomes corresponded to *C. raciborskii* of Skuja (1937) figures (Tafle 1: 7b–f) and identified as typical morphotype of *C. raciborskii* (Fig. 1B_{1-22}) and (iii) 38.9% of the trichomes corresponded to *C. raciborskii* of Skuja (1937) figure

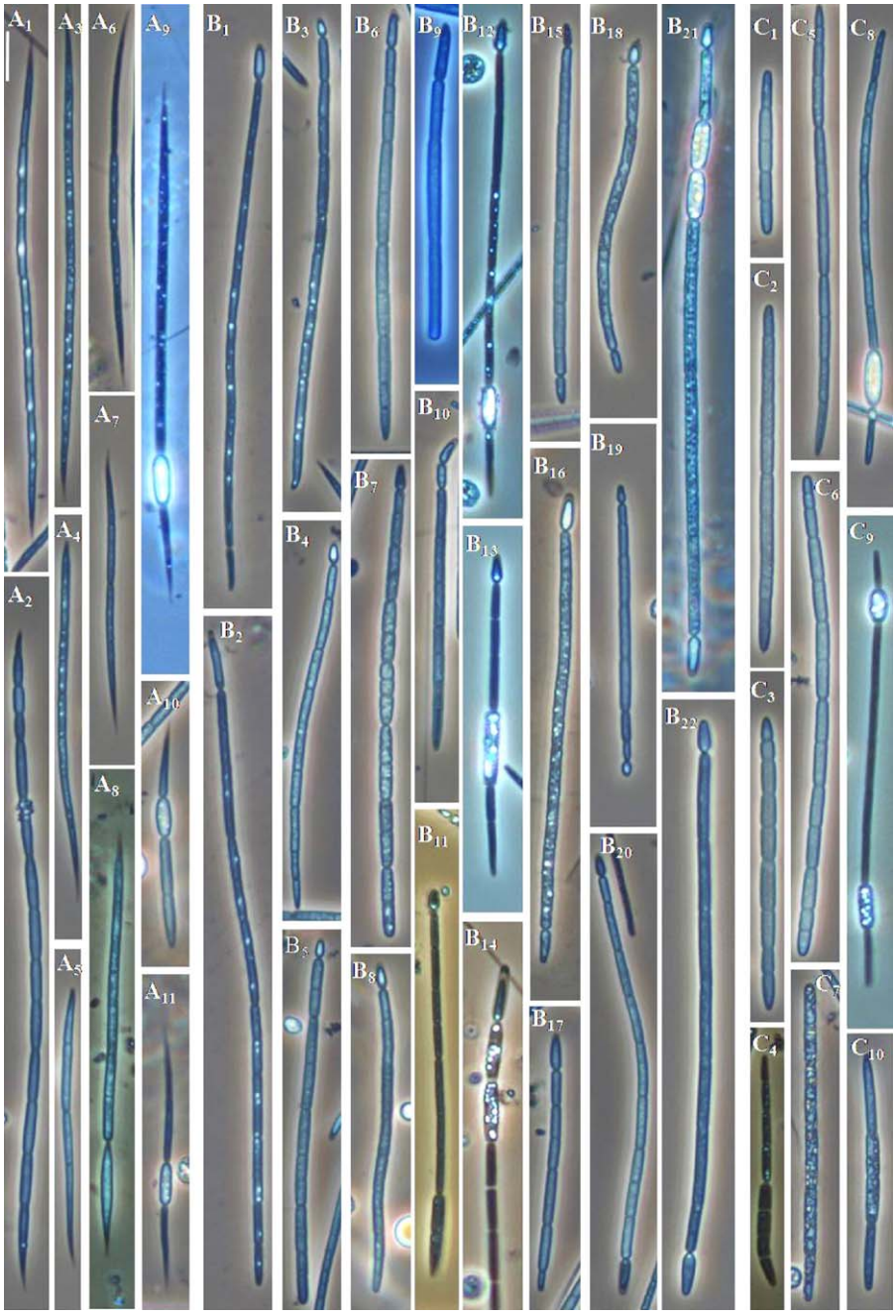

Fig. 1. *Raphidiopsis* and *Cylindrospermopsis* morphotypes. (A–C) Light micrographs (phase contrast) of trichomes. Scale bar 10 μm. (A_1–A_{11}) *R. mediterranea* morphotype variability; (B_1–B_{22}) *C. raciborskii* typical morphotype variability; (C_1–C_{10}) *C. raciborskii* non-heterocytous morphotype variability.

Table 1
C. raciborskii and *R. mediterranea* morphological and morphometric features described in this study in comparison with those described from Lake Kastoria and other localities in the world given in the literature.

	Trichomes				Vegetative cells		Heterocytes	Akinetes
	Shape	Terminals	*l* (μm)	*w* (μm)	con *l* (μm)	*l*:*w* ratio	*l* × *w* (μm × μm)	*l* × *w* (μm × μm)
C. raciborskii (This paper; Lake Kastoria, Greece)	Straight to flexous	Rounded, bluntly to sharply pointed	35.4–256.3	0.9–2.7	±4.25–16.4	2.2–12.3	3.9–8.9 × 1.2–2.8	5.2–15.5 × 1.7–2.9
C. raciborskii (Skuja, 1937; Lake Kastoria, Greece)	Straight to slightly bent	Rounded, bluntly to sharply pointed	150	2.0–3.0	±2.0–12.0	1.0–4.0	6.0–12.0 × 2.0–2.7	–
C. raciborskii (Singh, 1962; Varanasi ponds, India)	Straight to spirally curved	Rounded, bluntly to sharply pointed	90.0–100.0	2.6–3.0	±12.0–18.0	6.0–8.0	3.4–4.5 × 1.8–3.0	4.5–6.4 × 2.8–3.8
C. raciborskii (Horecká and Komárek (1979); reservoirs and streams, Hungary, Slovakia)	Straight	Conical, pointed-rounded	60.0–250.0 (1570)	(1.8) 2.0–4.0	±2.5–16.0	1.3–8.9[a]	3.4–14.0 × 1.8–4.0	4.5–22.0 × 2.4–5.5
C. raciborskii (Komárkova et al., 1999; Peri laggon, Brazil)	Straight to irregularly bent	Bluntly to sharply pointed	42.4–430.0	1.2–4.4	±3.2–30.0	1.3–14.0	4.8–16.0 × 1.2–4.5	9.6–14.8 × 2.4–5.0
C. raciborskii (McGregor and Fabbro, 2000; reservoirs, Australia)	Straight, coiled, irregularly twisted	Conical, bluntly rounded, rarely sharply pointed	–	1.5–3.2	−4.5–7.0 (8.3)	1.4–4.7[a]	–	–
R. mediterranea (This paper; Lake Kastoria, Greece)	Straight to flexous	Sharply pointed to needle-like	41.3–203	0.8–2.3	±4.1–12.5	2.3–10.2	–	5.8–18.1 × 1.5–2.7
R. mediterranea (Skuja, 1937; Lake Kastoria, Greece)	Straight to slightly bent	Sharply pointed to needle-like	40.0–163.0	1.0–2.5	±2.0–10.0	2.0–4.0	–	6.5–13.0 × 2.5–3.0
R. mediterranea (Li et al., 2008; Wuhan fishpond, China)	Straight	Sharply pointed	–	1.9–2.7	−9.8–10.8	3.6–5.7[a]	–	8.3–12.1 × 2.2–3.1

Note: *l*, legth; *w*, width; con, constrictions; ±, trichomes constricted or unconstricted at cross walls.
[a] Calculated values.

(Tafle 1: 7a) and identified as non-heterocytous morphotype of *C. raciborskii* (Fig. 1C_{1-10}). The three morphotypes reached in total a high number of trichomes in lake water (23,830 trichomes mL^{-1}).

In addition to the forms described by Skuja (1937) from Lake Kastoria, trichomes of both typical and non-heterocytous morphotype of *C. raciborskii* with akinetes (Fig. 1$B_{12-14,21}$ and C_{8-9}, respectively) were rarely observed. Also, two new morphotypes, one of *R. mediterranea* (Fig. 1A_8) and the other of *C. raciborskii* (Fig. 1B_{11} and C_4), were observed. The trichomes of the new *C. raciborskii* morphotype were not uniform in diameter and consisted of two sections with the wider one made of 2–4 cells. The new *R. mediterranea* morphotype was characterized by the development of a morphologically distinct terminal cell. Furthermore, a few trichomes resembling those of the genera *Cylindrospermum* (Fig. 1B_{14}) and *Aphanizomenon* (species *A. issatschenkoi*) (Fig. 1A_6) were observed.

The variability in size and shape of vegetative cells and akinetes of all morphotypes was high (Fig. 1 and Table 1). Most of the vegetative cells were cylindrical, rarely barrel-shaped. Generally, apical cells were narrowed, rounded or bluntly pointed, rarely sharply pointed in *Cylindrospermopsis* (Fig. 1$B_{4,12}$) and sharply pointed to needle-like in *Raphidiopsis* morphotype (Fig. 1A_{1-11}). The needle-like apical cells in *Raphidiopsis* were not found in *Cylindrospermopsis*. Akinetes were from barrel-shaped to oval. The *Cylindrospermopsis* heterocyte variability in shape and size was also high (Fig. 1B_{1-22} and Table 1).

A morphometric comparison of trichome and vegetative cell sizes of the dominant morphotypes showed a high overlapping in the range of the respective values (Fig. 2). However, the trichome and cell mean length and width and the cell *l*:*w* ratio, varied significantly between morphotypes (Table 2). *R. mediterranea* mean trichome length (90.6 μm, 81.2–99.9 μm; mean and 95% confidence interval, respectively) were significantly greater (LSD, $p < 0.05$) than that of *C. raciborskii* typical (73.8 μm, 64.3–83.3 μm) and non-heterocytous (77.7 μm, 73.3–82.1 μm) morphotype (Fig. 2A and Table 2). Also, the *R. mediterranea* mean trichome width (1.4 μm, 1.3–1.4 μm) was significantly smaller (LSD, $p < 0.05$) than that of *C. raciborskii* typical (1.7 μm, 1.6–1.8 μm) and non-heterocytous (1.7 μm, 1.6–1.9 μm), (Fig. 2B and Table 2). The *C. raciborskii* non-heterocytous mean cell length (6.6 μm, 6.2–7.1 μm) was significantly smaller (LSD, $p < 0.05$) than those of typical *C. raciborskii* (7.4 μm, 6.8–7.9 μm) and *R. mediterranea* (7.4 μm, 7.0–7.9 μm) morphotypes (Fig. 2C and Table 2). The mean *l*:*w* ratio value of *R. mediterranea* cells (5.7, 5.3–6.2) was significantly greater (LSD, $p < 0.05$) than those of *C. raciborskii* typical (4.5, 4.1–5.0) and non-heterocytous morphotype (4.0, 3.7–4.4), (Fig. 2D and Table 2).

Reproduction by trichome fragmentation was not rare in the studied cyanobacteria (Fig. 3). In *Raphidiopsis*, the process of fragmentation resulted in short trichomes of either similar or different morphologies (needle-like, sharply and bluntly pointed or rounded terminal cells). Some of the segments (hormocytes) were produced by central division of the trichome (Fig. 3A) whereas most of the trichomes disintegrated into several hormocytes (Fig. 3B). Rapid size reduction before trichome fragmentation was observed both in *Raphidiopsis* and *Cylindros-*

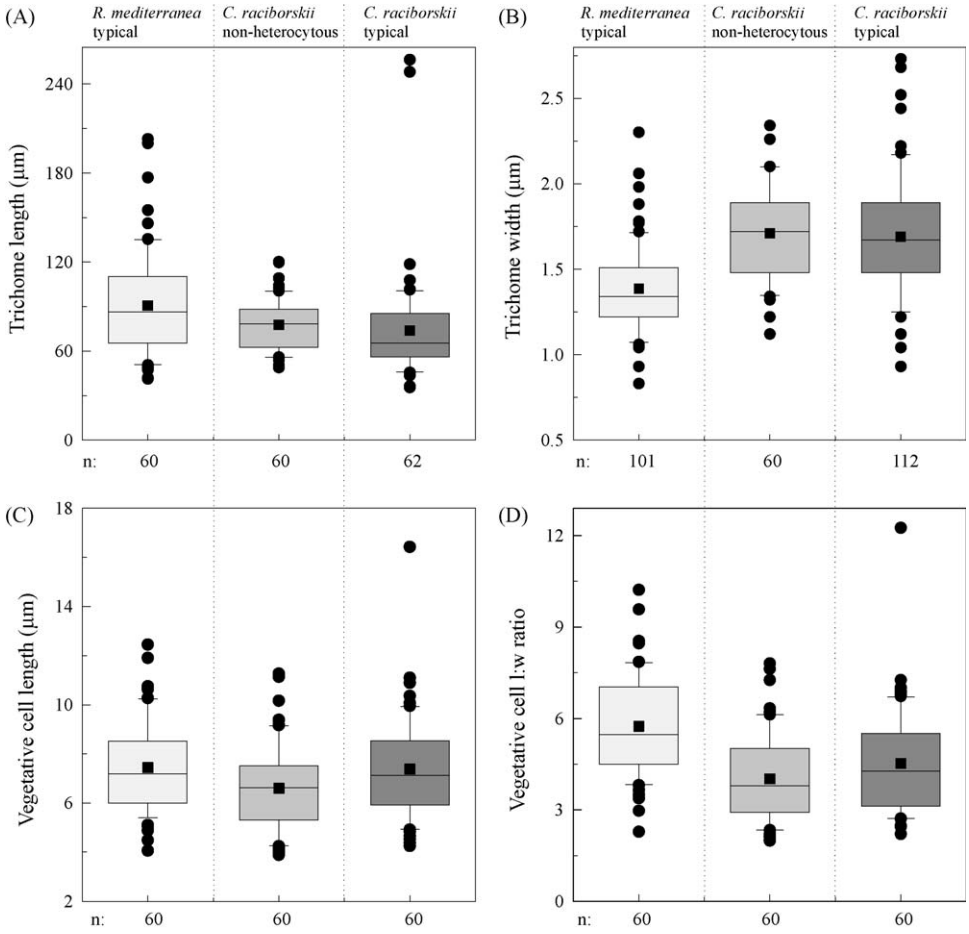

Fig. 2. Box and whisker plots of trichome length (A), trichome width (B), vegetative cell length (C) and vegetative cell *l*:*w* ratio (D), of *R. mediterranea* morphotype and *C. raciborskii* typical and non-heterocytous morphotype. The box represents the 25th–75th percentiles and the median value, the bars the 10th and 90th percentiles. Outlying values (●) and the mean value (■) for each morphotype are given, where *n* is the total number of trichomes or cells of each morphotype measured.

Table 2
ANOVA components (df, *F* value and *p* value) and pairwise multiple comparison LSD test of trichome length, trichome width, vegetative cell length and cell *l*:*w* ratio means in the three identified morphotypes (*R. mediterranea*, *Rm*; *C. raciborskii* typical, Cr_t; *C. raciborskii* non-heterocytous,Cr_{nh}).

Source of variation	ANOVA components			LSD test pairwise comparison[b]			
	df[a]	*F*	*p*	*Rm*	Cr_t	Cr_t	Cr_{nh}
Trichome length	179	4.651	0.011			○——○	
Trichome width	270	34.724	0.000			○——○	
Vegetative cell length	177	3.647	0.028	○——○			
Vegetative cell *l*:*w* ratio	177	18.418	0.000			○——○	

[a] Degrees of freedom (df) identifies the variation within morphotype groups (df for variation between groups equals in all cases to 2).
[b] ○——○ indicates pair of means whose 95% confidence intervals overlap (LSD test, $p > 0.05$).

permopsis (Fig. 3C and D). Central division by narrow constriction was observed in typical *Cylindrospermopsis* morphotype (Fig. 3E). In addition to trichome fragmentation, a new mode of trichome reproduction was observed in a few *Raphidiopsis* trichomes. A structurally distinct cell developed from one of the apical cells of a typical *Raphidiopsis* trichome (Fig. 3F and G). During its development there was a gradual disappearance of gas vacuoles (Fig. 3F). This mode of trichome reproduction resulted in short trichomes of both *Raphidiopsis* and *Cylindrospermopsis* morphology in regard to apical cells.

Of the 100 clones analysed, 19 were either false positives or chimeras and were excluded from further analysis. Of the 81 clones analysed for 16S rDNA diversity, only phylotype NK2-532 (GenBank accession number **EU376201**) was clustered in the *Raphidiopsis*/*Cylindrospermopsis* clade. It was closely related to *R. mediterranea* HB2 (99.54% similarity) and to *C. raciborskii* form 1 (99.19% similarity) (Fig. 4).

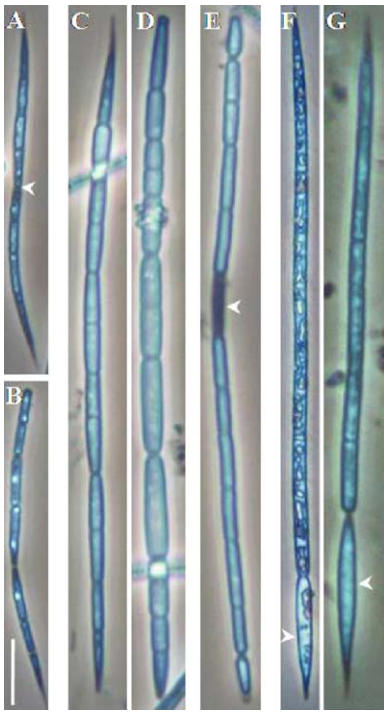

Fig. 3. Trichome reproduction in *Raphidiopsis* and *Cylindrospermopsis*. (A–G) Light micrographs (phase contrast) of reproduction stages. Scale bar 10 μm. (A) *R. mediterranea* trichome fragmentation by central division (arrow head); (B) *R. mediterranea* trichome disintegration; (C–D) *Raphidiopsis* and *Cylindrospermopsis* cell size reduction before trichome fragmentation; (E) *C. raciborskii* trichome central division by narrow constriction (arrow head); (F–G) *R. mediterranea* trichome reproduction by development of a structually distinct terminal cell (arrow head).

4. Discussion

In Lake Kastoria, *Cylindrospermopsis* and *Raphidiopsis* trichomes exhibited a high morphological diversity displaying not only the typical morphological features of the genera (Komárek and Anagnostidis, 1989) and particularly of the species *C. raciborskii* and *R. mediterranea* (Skuja, 1937) but also the morphological features of two new morphotypes. The first morphotype, an irregular developmental stage of *C. raciborskii,* had trichomes consisting of two sections of different diameters formed probably by asynchronous cell division. The second morphotype represented a *R. mediterranea* reproductive stage and was characterized by the development of a morphologically distinct cell in the terminal of the trichome. To the best of our knowledge, this is the first report of a structurally distinct reproductive stage in the developmental cycle in planktic Nostocales (Komárek and Anagnostidis, 1989; Rippka et al., 2001).

In addition to the above-mentioned morphotypes, few trichomes similar to *Cylindrospermum* sp. and *A. issatschenkoi* were observed. The *Cylindrospermum*-like morphotype forming aerotopes in vegetative cells were assigned to *Cylindrospermopsis* (Komárek and Anagnostidis, 1989) while the *A. issatschenkoi*-like morphotype was assigned to *R. mediterranea* according to Skuja (1937) figure (Tafle 1: 5g). Phylogenetic analysis of the mixed trichomes of the nostocalean cyanobacteria in Lake Kastoria did not reveal any phylotype related either to *Cylindrospermum* or *A. issatschenkoi.*

In Table 1, the lake's *C. raciborskii* and *R. mediterranea* morphological features are presented with those of populations from other world localities given in the literature. Comparing with the features of temperate, subtropical and tropical populations, the species from Lake Kastoria from our study showed a high morphological variability consistent with other observations (Horecká and Komárek, 1979; Komárkova et al., 1999) having the thinner and shorter trichomes.

Cylindrospermopsis and *Raphidiopsis* morphotypes differed significantly by mean length and width of the trichomes and the *l*:*w* ratio of the cells. However, there was a high overlap in the range of size values indicating that all trichomes belong to one population. The mean *l*:*w* ratio of the cells was significantly greater in *Raphidiopsis* than in *Cylindrospermopsis* morphotypes. Padisák (2003) observed that young trichomes of *Cylindrospermopsis* having acuminate ends, that resemble *R. mediterranea*, were narrower than older trichomes, typical of *C. raciborskii*.

Most of our *Raphidiopsis* trichomes were similar to the young trichomes of *C. raciborskii* reported by Singh (1962), Hindák (1988) and Padisák (2003) and the *Raphidiopsis*-like environmental morphotypes of *C. raciborskii* reported by Komárkova et al. (1999) and McGregor and Fabbro (2000). Moore et al. (2004) in their study on akinete germination in *C. raciborskii* provide documentation on morphology of early stages of its life-cycle, important to explain the morphology of developmental and environmental stages reported in the literature. The consideration that *Raphidiopsis*-like trichomes are most likely *Cylindrospermopsis* non-heterocytous trichomes is supported by the above discussion whereas the validity of the existence of the genus *Raphidiopsis* has already been questioned (Komárkova et al., 1999; McGregor and Fabbro, 2000).

However, both the long persistence (10 years) of *Raphidiopsis* strains in the laboratory without forming heterocytes and their inability to grow without a combined nitrogen compound have been considered the crucial differences from *C. raciborskii* (Li et al., 2008). In Lake Kastoria, when the N:P resource ratio dropped below

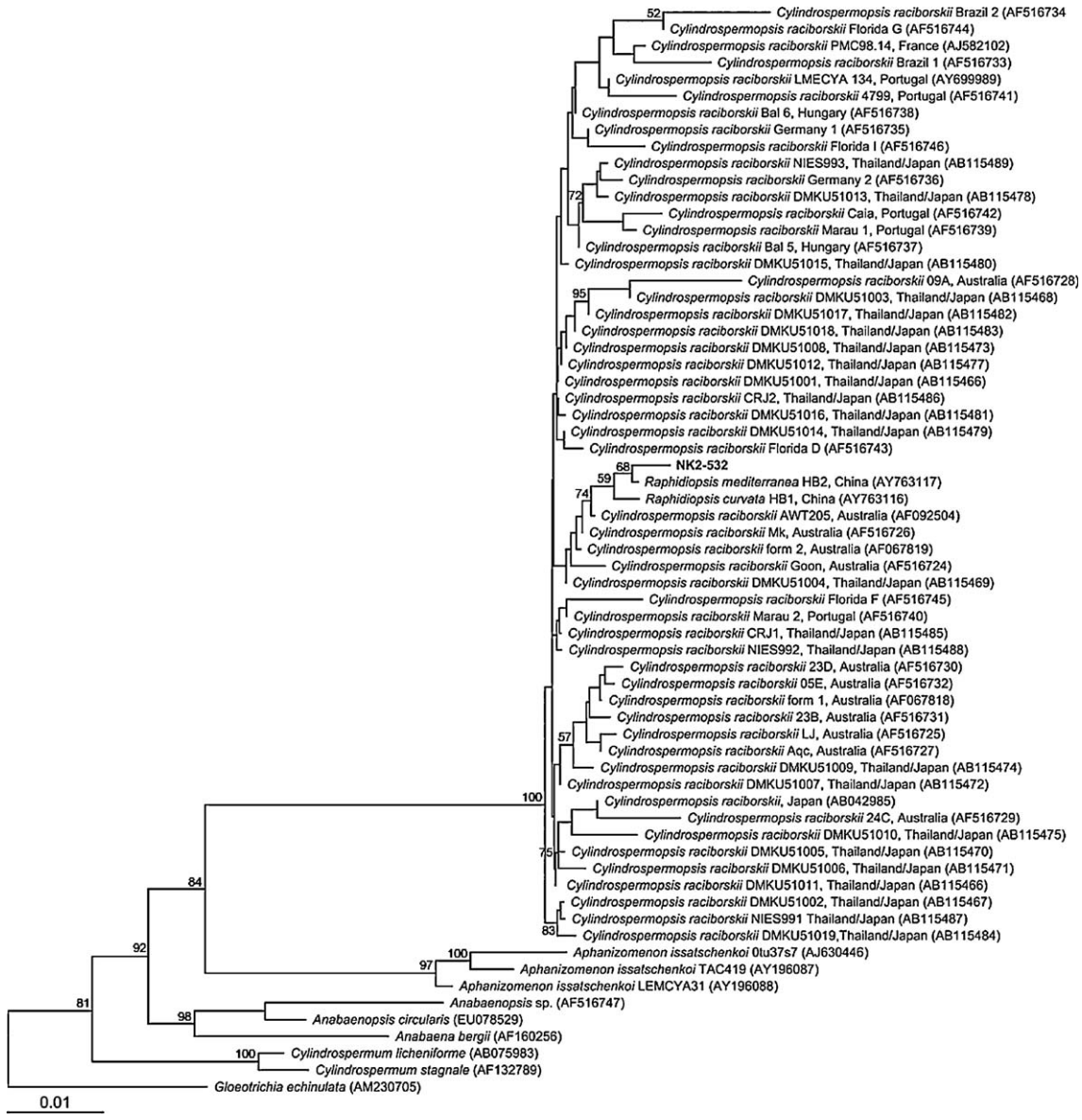

Fig. 4. Neighbour-joining phylogenetic tree showing the relationship of the of Lake Kastoria cyanobacterial phylotype NK2-532 with other Nostocales strains. Numbers at nodes represent the bootstrap percentages from 1000 replicates. Values below 50% are not shown. Bar indicates the number of substitutions per site. *Gloeotrichia echinulata* (Rivulariaceae) was used as an outgroup.

the critical ratio of Redfield in summer (Moustaka-Gouni et al., 2007) *Raphidiopsis* trichomes were almost undetectable (less than 5% of *C. raciborskii* density). The lack of heterocytes in *Raphidiopsis* morphotype in Lake Kastoria could be explained by the inability of the needle-like apical cells of the trichomes to divide and transform into terminal heterocytes. This might support the lack of diagnostic value of the absence of heterocytes in nostocalean cyanobacteria although the heterocyte is supposed to be one of the more reliable morphological characters (Rajaniemi et al., 2005).

In Lake Kastoria, the *Raphidiopsis* needle-like apical cells were not found in *Cylindrospermopsis*, whereas sharply pointed apical cells were observed both in *Raphidiopsis* and *Cylindrospermopsis* trichomes. Furthermore, the reproduction of *R. mediterranea* trichomes either by fragmentation or by the development of a distinct reproductive cell gave rise to short trichomes of both *R. mediterranea* and *C. raciborskii* non-heterocytous morphotypes linking their life-cycles. The phenomenon known as "rapid size reduction" (McGregor and Fabbro, 2000) was observed both in *C. raciborskii* and *R. mediterranea* in trichome positions where fragmentation occurs. Based on these results it could be postulated that *R. mediterranea* and *C. raciborskii* represent different stages in the life-cycle of one species. In parallel studies in other Greek freshwaters, where *R. mediterranea* and *C. raciborskii* co-exist, the same reproduction modes and similar morphotypes as life-cycle stages of one species were observed as well (Moustaka-Gouni et al., unpublished data).

In a recent phylogenetic analysis, Gugger et al. (2005) showed that the partial 16S rRNA gene and the 16S-23S internally transcribed spacer (ITS1) sequences of two *Raphidiopsis* strains were identical to 15 corresponding sequences of *Cylindrospermopsis* strains, indicating the delimitation problem of these genera. In our study, the phylogenetic analysis of the mixture of trichomes from Lake Kastoria corresponding morphologically to *C. raciborskii* and *R. mediterranea* revealed only one phylotype, closely related to the Chinese strains *R. mediterranea* HB2 and *R. curvata* HB1 (Li et al., 2008). However, these phylotypes are phylogenetically indistinguishable from closely related *Cylindrospermopsis* strains enhancing, thus, the evidence that *Raphidiopsis* and *Cylindrospermopsis*

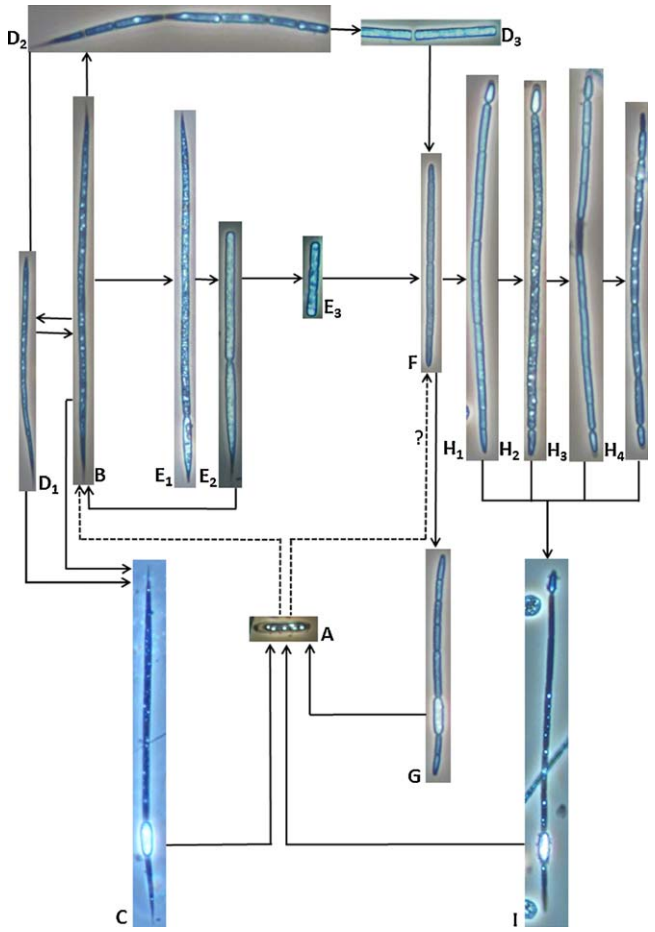

Fig. 5. A putative life-cycle for *C. raciborskii*. (A) Akinete germination phase 2; (B) *Raphidiopsis*-like trichome; (C) *Raphidiopsis*-like trichome with akinete; (D_1–D_3) *Raphidiopsis*-like trichome segments; (E_1–E_3) *Raphidiopsis*-like trichome reproduction stage and segments; (F) *C. raciborskii* non-heterocytous trichome; (G) *C. raciborskii* non-heterocytous trichome with akinete; (H_1–H_4) *C. raciborskii* heterocytous trichomes and their reproduction stages; (I) *C. raciborskii* heterocytous trichome with akinete. Lines and arrows indicate life-cycle routes: solid lines indicate the observed routes, dashed lines and question mark indicate the hypothetical routes.

are practically the same genus. A taxonomic revision might reduce the taxonomic problem existing in these genera for decades.

Considering the above discussion, the *Raphidiopsis* and *Cylindrospermopsis* morphotypes in Lake Kastoria were assigned to *C. raciborskii*. A putative life-cycle of *C. raciborskii*, based on the results of this study, is proposed in Fig. 5. Starting point of the proposed cycle is the morphological phase 2 of akinete germination (Moore et al., 2004) observed in the lake water. However, the role of germlings morphology in the formation of young trichomes (only *Raphidiopsis*-like trichomes?) has not been clarified in the proposed life-cycle. The new reproduction mode of *Raphidiopsis*-like trichomes, the disintegration in hormocytes and their development in new trichomes constitute the key processes linking *Raphidiopsis* and *Cylindrospermopsis* morphotypes in one life-cycle.

In conclusion, this is the first report of phylogenetic and morphological data of co-existing natural populations of *C. raciborskii* and *R. mediterranea* from Lake Kastoria, the first site of their record in Europe. A putative life-cycle has been constructed based on the results of the study revealing new developmental stages. The linkage of the morphotypes of both species in one life-cycle and their correspondence to only one phylotype suggest that *Raphidiopsis* and *Cylindrospermopsis* constitute one genus, and consequently a taxonomic revision is needed. *R. mediterranea* might in fact represent non-heterocytous life-cycle stages of *C. raciborskii*. Further research is needed, using both natural populations and ecotypes from different freshwaters supplemented by laboratory strains and single-cell approaches in order to enlighten the underinvestigated complete life-cycle stages and diversity of *Cylindrospermopsis* genus.

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