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The polyphasic analysis of two native *Raphidiopsis* isolates supports the unification of the genera *Raphidiopsis* and *Cylindrospermopsis* (Nostocales, Cyanobacteria)

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Abstract: *Raphidiopsis* and *Cylindrospermopsis* are planktic, freshwater bloom-forming cyanobacteria of great concern to human health due to the production of potent cyanotoxins. The presence (in *Cylindrospermopsis*) or absence (in *Raphidiopsis*) of heterocytes is the traditional character used to distinguish them. This has led to misidentifications and to questions about the validity of the genus *Raphidiopsis*. We studied two strains of *R. mediterranea* isolated from Argentinean shallow lakes using a polyphasic approach that included the morphological description of the natural populations and their ultrastructural, physiological and molecular characterisation. Heterocyte differentiation was not observed in the field or in cultures of *R. mediterranea* submitted to nitrogen deprivation. These results support the occurrence of stable native populations of *R. mediterranea* without heterocytes, which would not be a part of the *Cylindrospermopsis* complex life cycle. Based on *16S rRNA*, *16S–23S ITS*, and *cpcBA*-IGS sequences, these two genera are virtually identical. Thus, strains of *Raphidiopsis* and *Cylindrospermopsis* make up a monophyletic lineage in all phylogenetic reconstructions. Furthermore, the 16S–23S ITS secondary structure provided further evidence that these two genera cannot be separated. The intermixed position in the trees points to several losses of heterocytes during the evolution of these cyanobacteria. We conclude that these two genera should not be regarded as separate and distinct generic units and propose their unification under the name *Raphidiopsis*, respecting the principle of priority. Accordingly, we revisited and emended the description of *Raphidiopsis*.

Key Words: *Cylindrospermopsis raciborskii*, Molecular taxonomy, Polyphasic approach, *Raphidiopsis* Argentinean strains, *Raphidiopsis mediterranea*

INTRODUCTION

The taxonomic delimitation of some freshwater planktic nostocalean taxa is still under debate (Komárek & Mareš 2012). This is the case with the genus *Raphidiopsis* Fritsch & Rich, which is identified principally by the obligatory absence of heterocytes throughout the life cycle. *Raphidiopsis* species were included within Nostocales because the differentiation of subterminal akinetes is similar to other nostocaleans, such as *Cylindrospermopsis raciborskii* (Woloszyńska) Seenayya & Subba Raju (Fritsch & Rich 1929; Komárek & Mareš 2012).

The identification of *Raphidiopsis*-like populations based solely on morphology, in particular those of *Raphidiopsis mediterranea* Skuja, is difficult due to the scarcity of diagnostic features and the overlapping characters with *C. raciborskii*. Indeed, the latter species displays wide morphological diversity and phenotypic plasticity (McGregor & Fabbro 2000; Piccini *et al.* 2011) and cannot be distinguished from *R. mediterranea* when heterocytes are absent (Wu *et al.* 2011; Komárek & Mareš 2012).

Raphidiopsis mediterranea and *C. raciborskii* have gained public attention due to their ability to form blooms and produce potent toxins, such as cylindrospermopsin and saxitoxin (Lagos *et al.* 1999; Mohamed 2007; Li *et al.* 2001; McGregor *et al.* 2011). The co-occurrence of both species has been reported in temperate, tropical and subtropical reservoirs (McGregor & Fabbro 2000; Mohamed 2007; Fonseca & Bicudo 2008; Moustaka-Gouni *et al.* 2009).

Because of these morphological similarities, it was proposed that *R. mediterranea*-like trichomes are *C. raciborskii* morphotypes lacking heterocytes (Komárková *et al.* 1999; McGregor & Fabbro 2000; Moustaka-Gouni *et al.* 2009), thus questioning the distinctiveness of *Raphidiopsis* (Komárková *et al.* 1999; McGregor & Fabbro 2000). Furthermore, several phylogenetic studies based on multiple genes (*16S rRNA*, *psbA*, *rbcL*, *rbcS*, *cpcB*) and intergenic sequences [*16S–23S rRNA* internal transcribed spacer (ITS)] demonstrated that species of *Raphidiopsis* and *Cylindrospermopsis* were a polyphyletic group that required revision (Gugger *et al.* 2005; Li *et al.* 2008; Moustaka-Gouni *et al.* 2009; Stucken *et al.* 2009; Wu *et al.* 2011; Komárek 2013; Li *et al.* 2016).

Recently, the joining of *16S rRNA* gene phylogenies with analysis of 16S–23S ITS secondary structure proved to be useful in circumscribing cyanobacterial genera and in recognising intrageneric taxonomic diversity (Johansen *et al.* 2011; Osorio-Santos *et al.* 2014; Sciuto & Moro 2016). The analysis of the 16S–23S ITS secondary structure was recently applied to sequences of *Raphidiopsis* and *Cylindro-*

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spermopsis strains from China (Li *et al*. 2016). Therefore, the potential use of this approach to separate *Raphidiopsis* from *Cylindrospermopsis* strains has not, as yet, been fully assessed.

Here, we describe two natural *R. mediterranea* populations and characterise two strains using a polyphasic approach that includes morphological, ultrastructural and phylogenetic analyses. The information is compared with morphological and molecular data from other *Raphidiopsis* and *Cylindrospermopsis* strains in order to shed light on the controversial taxonomy of these genera. Based on that, we discuss the generic limits of *Raphidiopsis* and *Cylindrospermopsis* and propose the necessary taxonomic changes.

MATERIAL AND METHODS

Sampling was performed seasonally from March 2013 to January 2014 in two shallow lakes located in the province of Buenos Aires: Los Patos (34°50.7′S, 57°57.38′W), an artificial hypertrophic freshwater body (area 0.25 km^2, Z_{max} 1 m), and Los Padres Lake (37°56.28′S, 54°44.18′W), a polimictic water body (area 2 km^2, Z_{mean} 1.8 m). Cyanobacterial blooms have been reported in both shallow lakes, which are used for recreational activities, such as fishing, swimming and sailing (Amé *et al*. 2010; Aguilera *et al*. 2016).

Water samples were obtained using plankton nets (30-μm mesh) and preserved with Transeau solution (1:1) (Bicudo & Menezes 2006). Fixed samples were deposited in the Herbarium of the División Ficología 'Dr Sebastián A. Guarrera', Facultad de Ciencias Naturales y Museo de La Plata (UNLP, Argentina).

The morphological and morphometric characterisation of populations from Los Patos and Los Padres lakes were performed using light microscopy (WILD M20, Heerbrugg, Switzerland; Nikon Eclipse E600; Nikon, Tokyo Japan), including measurements in trichomes ($n = 100$), vegetative cells ($n = 100$) and akinetes ($n = 15$). For phase contrast, photos were obtained using a digital camera (Nikon E995), and Olympus CellSence v1.6 software (Olympus, Center Valley, Pennsylvania USA) was used for image analysis. Differences between isolates were analyzed using Kruskal-Wallis one-way analysis of variance on ranks. Statistical analyses were carried out using the Statistica v7 program (Statsoft Inc., Tulsa, Oklahoma USA).

FCC LP and FCC MDQ strains were isolated from samples collected in October and January 2014 in Los Patos Lake and Los Padres Lake, respectively. The isolates were obtained from single filaments using micropipettes and were grown in MLA medium (Bolch & Blackburn 1996) in a controlled-environment room (22 ± 2°C and 40 μmol m^{-2} s^{-1} light intensity) under a 16:8-h light:dark cycle. The strains are maintained in the FIBA Culture Collections (FCC) in Mar del Plata, Argentina.

Trichomes of FCC LP and FCC MDQ strains were collected by centrifuging at 1100 × *g* for 10 min. Samples were fixed in 2.0% glutaraldehyde in phosphate buffer (pH 7.2–7.4) for 2 h at 4°C and postfixed in 1% osmium tetroxide for 1 h at 4°C. Samples were dehydrated in increasing concentrations of alcohol and embedded in Spurr epoxy resin (Spurr 1969). Ultrathin sections (90 nm) were stained in uranyl acetate and lead citrate and examined with a JEM 1200 EX II transmission electron microscope (JEOL Ltd, Tokyo, Japan). Images were captured using a digital camera (Erlangshen ES 1000 W, model 785, Gatan Inc., Pleasanton, California USA) from the Central Service of Electron Microscopy of the Faculty of Veterinary Sciences, Universidad Nacional de La Plata (Argentina).

Cells were cultured in MLA medium (containing 2 mM Na_2NO_3) and MLA_0 medium (lacking combined nitrogen) for 10 d at 25°C and 40 μmol photons $m^{-2}s^{-1}$ under a 16:8-h light:dark cycle with orbital shaking (120 rpm). Cells in logarithmic growth were collected by centrifugation at 1100 × *g* for 4 min, washed three times with MLA_0 and inoculated in MLA or MLA_0 medium (initial $OD_{750nm} = 0.2$). Additionally, cells were cultivated under 80 μmol photons $m^{-2}s^{-1}$ for 20 d to evaluate changes in trichome morphology. Growth was evaluated every 2 d by optical density (750 nm) and chlorophyll content (International Organization for Standardization 1992). The presence of heterocytes was examined by light microscopy or Alcian blue staining (Huang *et al*. 2005).

Cells were centrifuged at 9300 × *g* for 5 min at room temperature and pulverised in a Retsch MM200 laboratory mill (Retsch GmbH, Haan, Germany) with sterile glass beads (212–300 μm) for 8 min. Total DNA was isolated using the Invisorb Spin Plant Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) according to the manufacturer's instructions. Primers used in this study are listed in supplementary data Table S1.

A fragment of the *rRNA* operon containing the *16S rRNA* gene and the associated *16S–23S* internal transcribed spacer (ITS1) region [1732–1878 base pairs (bp)] was amplified using the primer pair pA/B23S (Edwards *et al*. 1989; Lepère *et al*. 2000) (Table S1) and conditions described by Gkelis *et al*. (2005). The amplification reaction was carried out in a 20-μl final volume containing 10 ng of template DNA, 0.06 μM of each primer and commercial ploymerase chain reaction (PCR) mix with Taq polymerase (Plain PP Master Mix, Top Bio, Prague, Czech Republic). PCR products were purified by electrophoresis in 1% agarose gels and cloned in *Escherichia coli* cells using the standard pGEM T Easy vector system (Promega Corp., Madison, Wisconsin USA). The plasmid containing the insert was purified from one *E. coli* colony per replicate, and the insert was sequenced (Berrendero Gómez *et al*. 2016). The tRNAscan-SE1.21 Web tool was used for search for *tRNA* genes in LP and MDQ sequences obtained with primers pA/B23S (Lowe & Eddy 1997).

The amplification of genes of the phycocyanin operon (*cpcBA*-IGS), nitrogenase operon (*nifH*) and those involved in toxin production (*cyrC* and *sxtA*) was carried out in a 20-μl volume mixture containing 10 ng total DNA, 200 mM dNTPs (Promega), 3 mM $MgCl_2$, 0.20 μM of each primer (GenBiothech SRL, Buenos Aires, Argentina) and 1 U of Pegasus Taq DNA polymerase diluted in buffer. The designed primer pair *cpcBA*-F/*cpcBA*-R (Table S1) was used to amplify the intergenic spacer (IGS) and flanking regions of the *cpcB* and *cpcA* genes of the phycocyanin operon. The nitrogenase reductase gene *nifH* was amplified with the primer pair *nifH*f/*nifH*r (Gugger *et al*. 2005). Thermal

cycling was performed under the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 60°C for 30 s and 72°C for 1 min, and a final extension step at 72°C for 10 min. The sequence of the *cyrC* gene coding for a putative polyketide synthase domain involved in cylindrospermopsin biosynthesis (Mihali *et al*. 2008) was amplified using the primers M4 and K18 (Fergusson & Saint 2003), following the conditions described by McGregor *et al*. (2011). A portion of the *sxtA* gene (C-terminal domain) related to saxitoxin production (Moustafa *et al*. 2009) was amplified using the primer pair sxtA4-F/sxtaA4-R and the cycling conditions described in Hoff-Risseti *et al*. (2013). After separation by agarose gel electrophoresis, the amplification products were sequenced (Macrogen, Seoul, South Korea). Accession numbers for the sequences obtained in this study can be found in Table S2.

Sequences were compared with those available in public databases (National Center for Biotechnology Information and Joint Genome Institute) using BlastN searches. Sequences were chosen based on their high similarities with LP and MDQ strains indicated by BlastN hits and their presence in other phylogenetic studies of *Raphidiopsis* and *Cylindrospermopsis* (Gugger *et al*. 2005; Haande *et al*. 2008; Li *et al*. 2008; Stucken *et al*. 2009; Wu *et al*. 2011; Moreira *et al*. 2015). In the case of the *16S rRNA* gene, only full-length sequences (approximately 1500 bp) were used (Iteman *et al*. 2002). Selected sequences (Table S2) were aligned with FCC LP and FCC MDQ sequences by MAFFT v7.1 (Katoh & Standley 2013) using the G-INS-I algorithm (with default parameters). DECIPHER's Find Chimeras Web tool (Wright *et al*. 2012) was used to identify anomalous *16S rRNA* gene sequences within the multiple sequence alignment. Phylogenetic calculations were run employing Bayesian inference (BI) in MrBayes v3.2.2 (Ronquist *et al*. 2012) and maximum likelihood (ML) and maximum parsimony (MP) analysis in MEGA v6.06 (Tamura *et al*. 2013). For the Bayesian analysis, two runs of eight Markov chains were executed for 40 million generations with default parameters, sampling every 1000 generations (the final average standard deviation of split frequencies was lower than 0.01), and the first 25% of sampled trees were discarded as burn-in. The ML tree was conducted by applying an evolutionary model chosen by jModelTest v2 software (Darriba *et al*. 2012) using the Akaike information criterion: general time reversible + invariant + gamma (GTR + I + G). An MP tree was generated using a heuristic search constrained by random sequence addition (1000), steepest descent and tree bisection and reconnection branch swapping. For ML and MP analysis, a total of 1000 bootstrap replicates were conducted to evaluate the relative support of branches, and only bootstrap values above 50% were indicated at the nodes of the trees. The CIPRES supercomputing facilities (Miller *et al*. 2010) were used for calculation of Bayesian trees. Estimates of evolutionary divergence between sequences were conducted in MEGA v6.06 (Tamura *et al*. 2013) using p-distance and pairwise deletions of gap sites as parameters.

Secondary structures of the semiconserved 16S–23S ITS domains D1–D1′ and Box-B, V2 and V3 helices were predicted using the Mfold Web server (Zuker 2003) set to default settings, choosing secondary structure predictions with minimum free energy. We also estimated ITS secondary structures for available sequences of *Raphidiopsis* and *Cylindrospermopsis* strains.

RESULTS

The search for *Raphidiopsis* strains was the starting point of this study. We sampled Los Patos and Los Padres Lakes (province of Buenos Aires, Argentina) and isolated two strains (FCC LP and FCC MDQ) that were microscopically identified as *R. mediterranea*. Then we carried out a polyphasic study that included the morphological description of the natural populations, ultrastructural and heterocyst differentiation analysis, screening of putative genes involved in toxin biosynthesis and phylogeny inferences based on multisequence analyses.

Morphology of natural populations

Raphidiopsis mediterranea populations present in the two shallow lakes exhibited solitary planktic trichomes. They were subsymmetric, unconstricted, with tapering terminal cells, without mucilaginous sheaths and without heterocytes.

Straight and flexuous trichomes were observed in the populations from Los Patos (Figs 1–5). Terminal cells were conical or gradually attenuated, with rounded or sharply pointed ends. The populations from Los Padres had only straight trichomes, with sharply pointed to needle-like terminal cells (Figs 5–8). Aerotopes were generally present in both populations. Akinetes, found near the ends or slightly off the center in the trichomes, were cylindrical to oval, solitary, in pairs or rows (Figs 3, 4, 6, 7). The morphometric comparison between the two populations revealed significant differences in cell and akinete dimensions (Table 1). However, we found considerable overlap in measured features with other natural populations or isolated strains of *Raphidiopsis* and *Cylindrospermopsis* (Tables 1, S3). Thus, the morphological separation of these taxa was blurred and the identification difficult.

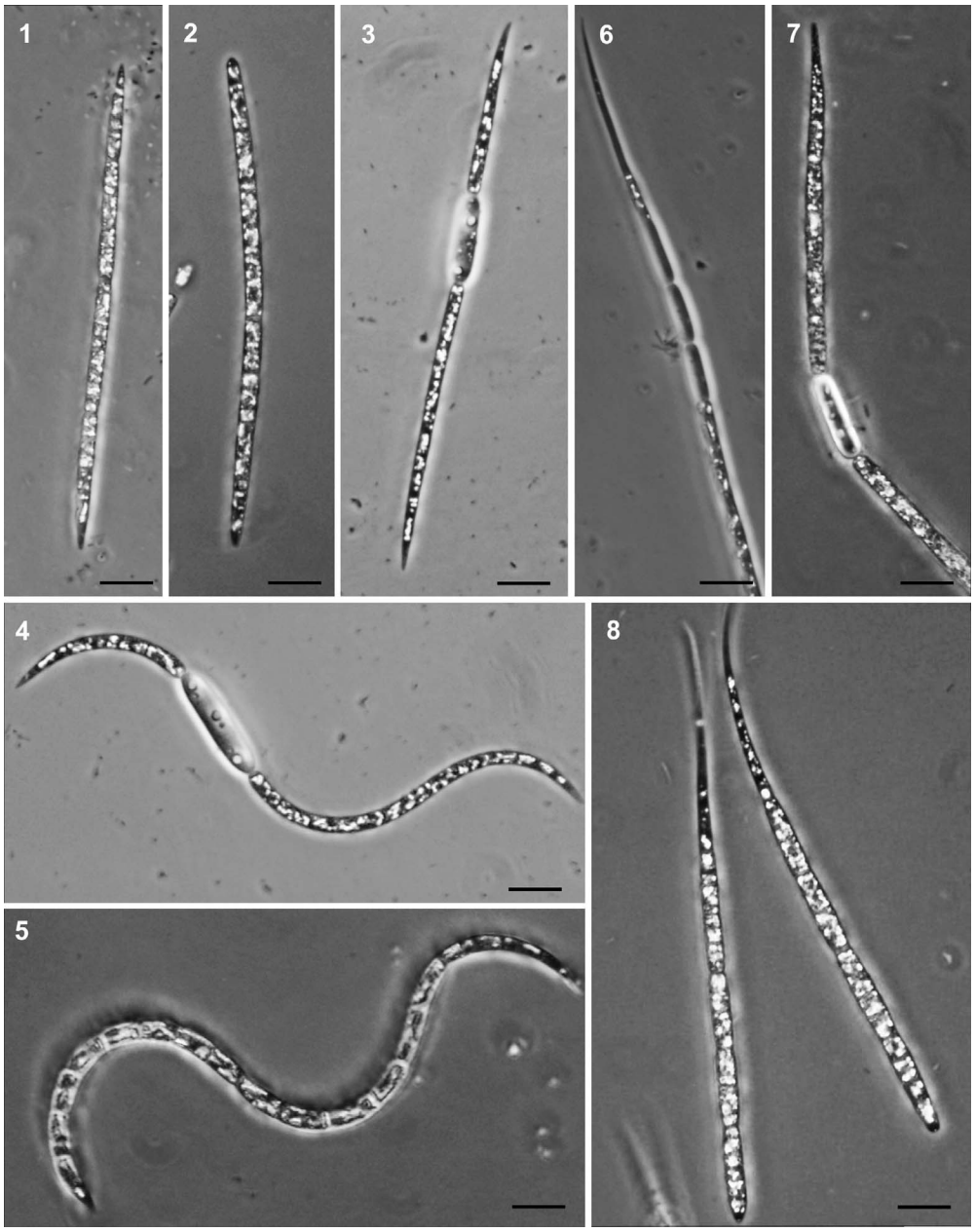
Both *R. mediterranea* native populations were found together with other Nostocales species, such as *Anabaenopsis elenkinii* Miller, *Dolichospermun circinale* (Rabenhorst *ex* Bornet & Flahault) P.Wacklin, L.Hoffmann & J.Komárek, *Sphaerospermopsis aphanizomenoides* (Forti) Zapomelová, Jezberová, Hrouzek, Hisem, Reháková & Komárková and *Aphanizomenon* (*Cuspidothrix*) spp. All of them presented heterocytes when sampled.

Ultrastructure of isolated strains

Comparative transmission electron microscopic analysis revealed that cells of FCC LP and FCC MDQ *Raphidiopsis* strains presented a coiled (wavy) arrangement of thylakoids, dispersed throughout the whole cell but more concentrated in the cell periphery. Both strains showed widened thylakoid membranes (Figs 9–12).

Effect of light and nitrogen source on strain growth and shape

Neither FCC LP nor FCC MDQ isolates grew in media lacking combined nitrogen (MLA_0) (Figs 13, 14). Microscopic



Figs 1–8. Light micrographs (phase contrast) of trichomes of *Raphidiopsis mediterranea* from Los Patos (1–5) (reference strain FCC LP) and Los Padres shallow lakes (6–8) (reference strain FCC MDQ). Scale bars = 10 μm.
Fig. 1. Straight trichome of *R. mediterranea* with pointed apical cells, from Los Patos shallow lake.
Fig. 2. Straight trichome of *R. mediterranea* with conical-rounded apical cells, from Los Patos shallow lake.
Fig. 3. Straight trichome of *R. mediterranea* with akinetes, from Los Patos shallow lake.
Fig. 4. Flexuous trichome of *R. mediterranea* with akinetes, from Los Patos shallow lake.
Fig. 5. Flexuous trichome of *R. mediterranea* from Los Patos shallow lake.
Fig. 6. Straight trichome of *R. mediterranea* with pro-akinetes in rows, from Los Padres shallow lake.
Fig. 7. Straight trichome of *R. mediterranea* with akinete, from Los Padres shallow lake.
Fig. 8. Straight trichomes of *R. mediterranea* with terminal cells with sharply pointed ends, from Los Padres shallow lake.

observations confirmed that they did not differentiate terminal heterocytes when cultured in MLA_0, in agreement with no trichomes staining with Alcian blue. Also, the absence of a complete nitrogenase complex could be inferred since no amplification of the *nifH* gene, the most widely marker used to identify nitrogen-fixing bacteria (Gaby & Buckley 2012), was obtained from genomic DNA (Fig. 15).

Regarding morphology, only straight trichomes were observed in *R. mediterranea* FCC MDQ under 40 or 80 μmol photons m^{-2} s^{-1}. *Raphidiopsis mediterranea* FCC LP displayed both straight and flexuous trichomes at the lower light intensity and an increase in the proportion of flexuous trichomes under 80 μmol photons m^{-2} s^{-1}. Interestingly, coiled and curved trichomes resembling *R. curvata* were

Table 1. Morphometric features of *Raphidiopsis mediterranea* populations from this study in comparison with those of *Raphidiopsis* and *Cylindrospermopsis* described in previous studies. Additional information is presented in Table S3.[1]

Genus/species/strain	Trichome form	Vegetative cells (μm)		Heterocytes (μm)	
		Length	Width	Length	Width
C. catemaco CHAB 148	flexuous	4.05–11.56	2.07–3.66	5.03–10.73	2.42–4.39
C. philippinensis CHAB 5180	ND	5.3–13.5	2.75–4.02	5.15–11.89	2.32–4.41
C. raciborskii	straight to flexuous	4.25–16.40	0.9–2.7	3.9–8.9	1.2–2.8
C. raciborskii	straight	(11.5±3.59)	(1.5±0.3)	(5 ± 2)	(1.75 ± 0.5)
C. raciborskii	coiled	(6.0 ± 0.9)	(3.0 ± 0.2)	(5.3 ± 1.2)	(2.7 ± 0.3)
C. raciborskii	straight	(6.5 ± 1.0)	(2.9 ± 0.3)	(5.8 ±1.3)	(2.6 ± 0.4)
R. brookii	straight	[4]–5–10–[13]	2.5–4.5		
R. curvata CHAB 114	spiral	5.95–9.83	2.08–3.2		
R. mediterranea	straight to flexuous	4.1–12.5	0.8–2.3		
R. mediterranea FSS1-150/1	straight	8.7–19.8 (–22.6)	2.3–3.8		
***R. mediterranea* FCC LP**	straight and flexous	**4.3–18 (9.1 ± 2.9) a**	**1.5–3.4 (2.6 ± 0.4) a**		
***R. mediterranea* FCC MDQ**	straight	**5.4–20.5 (11.1 ± 3.4) b**	**1.4–4.9 (3.1 ± 0.8) b**		

[1] Different letters indicate significant differences ($P < 0.05$); numbers in parentheses represent $\bar{X} \pm s$; number between brackets represents outliers; ND, not determined.

observed after 20 d under 80 μmol photons m^{-2} s^{-1} (Figs 16–18).

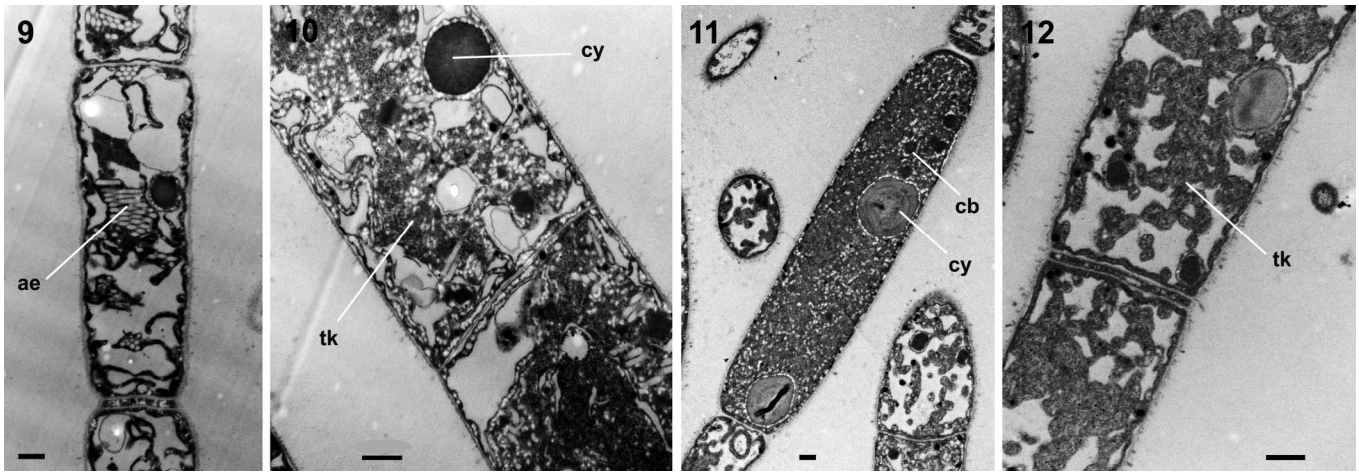
Search for cyanotoxin biosynthesis genes

FCC LP and MDQ strains were assessed for the presence of genes involved in the biosynthesis of saxitoxin and cylindrospermopsin (*sxtA* and *cyrC*, respectively). A *sxtA* gene fragment was amplified only in the FCCMDQ strain, which was highly similar (> 98%) to the sequences from Brazilian *C. raciborskii* (T3, CENA302 and CENA303) and *R. brookii* D9 saxitoxin producing strains. On the other hand, no amplification of the *cyrC* gene was obtained for any of the isolates.

Phylogenetic reconstructions

A total of 21 strains of *Raphidiopsis* and 89 strains of *Cylindrospermopsis* were included in the phylogenetic analysis based on *16S rRNA* gene sequences. The phylogenetic tree failed to provide any resolution between the two genera because *Raphidiopsis* and *Cylindrospermopsis* strains grouped together in a single intermixed cluster with good support values with *Sphaerospermopsis* as the sister taxon (supplementary data Fig. S1). Strains of *Raphidiopsis* were placed in two phylogenetically distinct groups. Group I included FCC LP and FCC MDQ, together with strains of *Raphidiopsis* and *Cylindrospermopsis* from Brazil (CENA 302, CENA 303, ugSPC 820, SPC338, FURGCY1 and ugSPC811), and one strain of *Raphidiopsis* from Senegal (PMC 00.05). Group II contained strains of *R. mediterranea* and *R. curvata* from China (CHABs, HB1 and HB2), the Czech Republic (07-4) and Australia (FSS1-150/1) (Fig. S2).

16S rRNA identity among all the strains [100*(1 – P)] in the *Raphidiopsis* + *Cylindrospermopsis* cluster was between 96.40% and 100%. *Raphidiopsis* and *Cylindrospermopsis* cannot be separated according to the standard 95% cutoff suggested to differentiate genera (Stackebrandt & Goebel 1994). For clarity, only *16S rRNA* percent identities between native strains of *R. mediterranea* (FCC LP and FCC MDQ)



Figs 9–12. Transmission electron microscopic images of *Raphidiopsis mediterranea* FCC LP and FCC MDQ. ae, aerotopes; cb, carboxysomes; cy, cyanophycin granules; tk, thylakoid membranes. Scale bars = 0.5 μm.

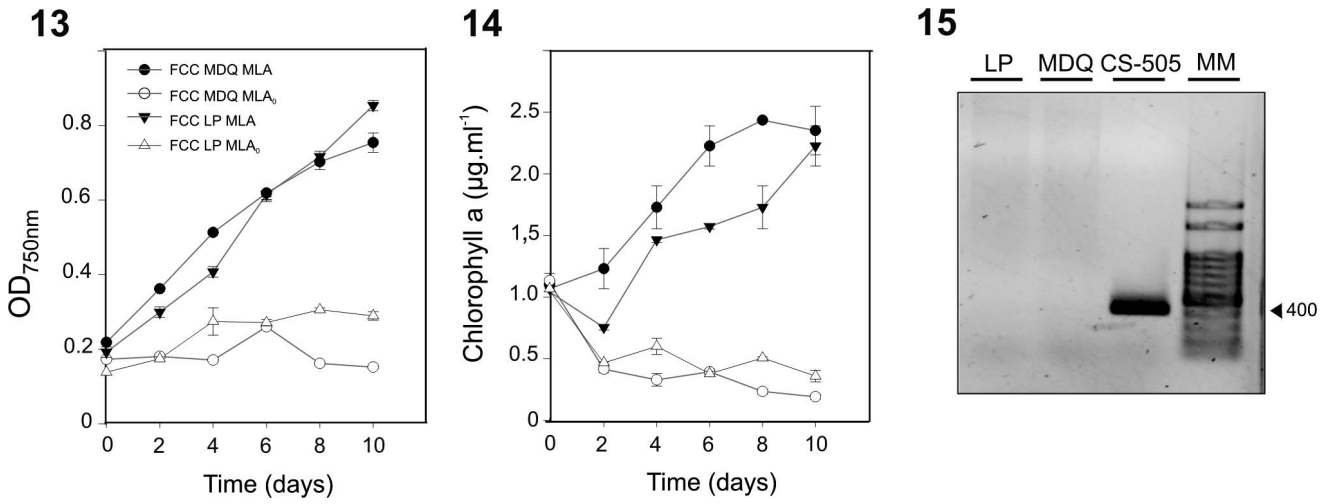
Figs 9, 10. Longitudinal sections of FCC LP trichomes.

Fig. 11. Longitudinal section of FCC MDQ akinete.

Fig. 12. Longitudinal section of FCC MDQ trichome. Longitudinal sections of akinetes (C) and trichomes of FCC MDQ (D, E).

Table 1. Extended

Akinetes (μm)			
Length	Width	Origin	Data source
7.1–17.32	3.28–5.39	China	Li *et al.* (2016)
8.4–24.42	3.1–6.9	China	Li *et al.* (2016)
5.2–15.5	1.7–2.9	Greece	Moustaka-Gouni *et al.* (2009)
(12.5 ± 5.5)	(3.5 ± 0.4)	France	Briand *et al.* (2002)
(9.0 ± 2.1)	(3.9 ± 0.5)	Australia	Saker *et al.* (1999)
(11.4 ± 2.6)	(3.9 ± 0.6)	Australia	Saker *et al.* (1999)
[9]–11–16–[20]	[3]–3.5–4–[5]	United States	Hill (1972)
6.24–16.57	3.12–7.04	China	Li *et al.* (2016)
5.8–18.1	1.5–2.7	Greece	Moustaka-Gouni *et al.* (2009)
7.8–18.8	2.9–5.8	Australia	McGregor *et al.* (2011)
7.2–13.6 (11.7 ± 2.2) a	**2.8–4.2 (3.62 ± 0.6) a**	**Argentina**	**Present study**
4.9–14.5 (9.3 ± 2.8) b	2–**5.3 (3.5 ± 0.7) a**	**Argentina**	**Present study**

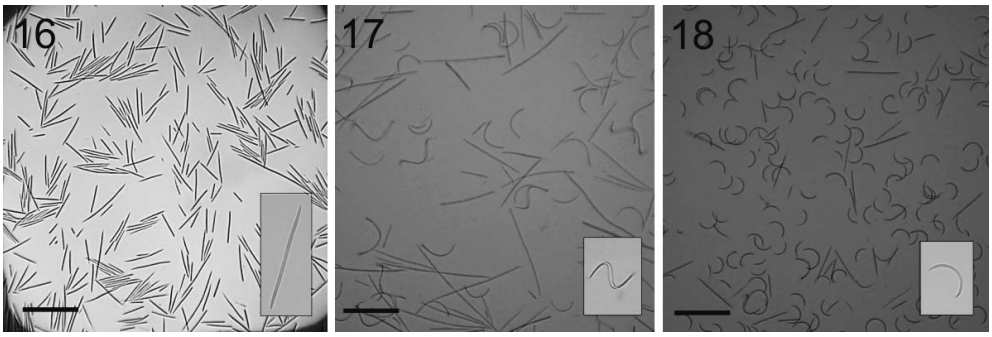


Figs 13–15. Effect of nitrogen source on *Raphidiopsis mediterranea* FCC MDQ and FCC LP growth and detection of the *nifH* gene. Strains were grown in MLA (2 mM Na_2NO_3) or MLA_0 (without combined nitrogen) medium. Data are $\bar{X} \pm s$ of two biological replicates.

Fig. 13. Growth curves of FCC MDQ and FCC LP followed by $OD_{750\ nm}$.

Fig. 14. Growth curves of FCC MDQ and FCC LP followed by chlorophyll *a* content.

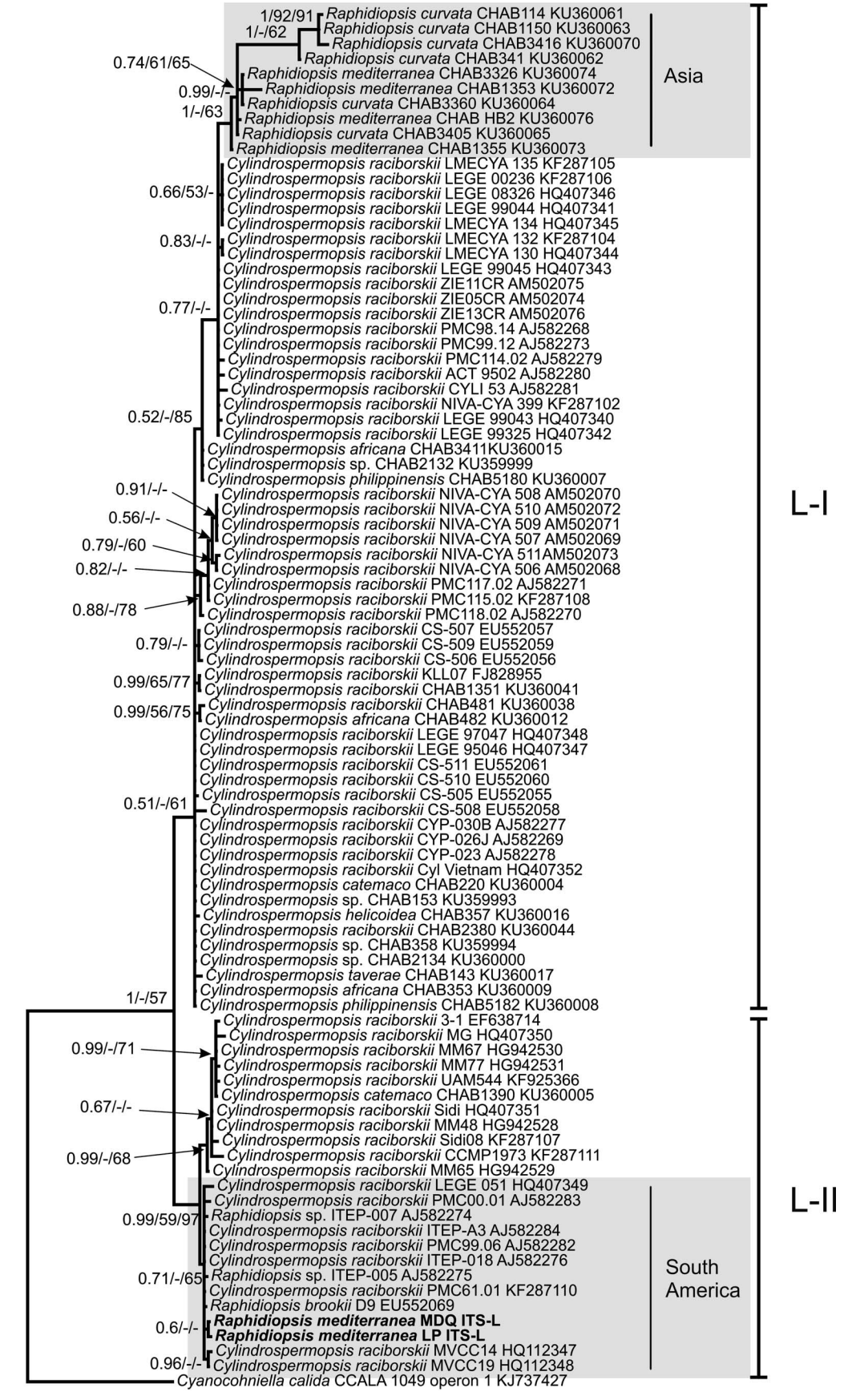
Fig. 15. Electrophoresis in agarose gel of the PCR amplification mixture for *nifH* sequence from genomic DNA corresponding to FCC LP and FCC MDQ (lanes LP and MDQ) and *C. raciborskii* (positive control) (lane CS-505). MM, DNA molecular size marker (100-bp ladder). Arrowhead indicates 400 bp.



Figs 16–18. Light micrographs of trichomes of *Raphidiopsis mediterranea* FCC LP after cultivation in MLA medium under different light conditions. Scale bar = 100 μm.

Fig. 16. Straight trichomes of *R. mediterranea* FCC LP under 40 μmol photons m^{-2} s^{-1}.

Figs 17, 18. Straight and flexuous trichomes of *R. mediterranea* FCC LP under 80 μmol photons m^{-2} s^{-1} at day 8 (19) and at day 14 (20).


1/92/91
1/-/62
Raphidiopsis curvata CHAB114 KU360061
Raphidiopsis curvata CHAB1150 KU360063
Raphidiopsis curvata CHAB3416 KU360070
Raphidiopsis curvata CHAB341 KU360062
0.74/61/65
Raphidiopsis mediterranea CHAB3326 KU360074
0.99/-/-
Raphidiopsis mediterranea CHAB1353 KU360072
Raphidiopsis curvata CHAB3360 KU360064
1/-/63
Raphidiopsis mediterranea CHAB HB2 KU360076
Raphidiopsis curvata CHAB3405 KU360065
Raphidiopsis mediterranea CHAB1355 KU360073
Asia
Cylindrospermopsis raciborskii LMECYA 135 KF287105
Cylindrospermopsis raciborskii LEGE 00236 KF287106
0.66/53/-
Cylindrospermopsis raciborskii LEGE 08326 HQ407346
Cylindrospermopsis raciborskii LEGE 99044 HQ407341
Cylindrospermopsis raciborskii LMECYA 134 HQ407345
0.83/-/-
Cylindrospermopsis raciborskii LMECYA 132 KF287104
Cylindrospermopsis raciborskii LMECYA 130 HQ407344
Cylindrospermopsis raciborskii LEGE 99045 HQ407343
Cylindrospermopsis raciborskii ZIE11CR AM502075
0.77/-/-
Cylindrospermopsis raciborskii ZIE05CR AM502074
Cylindrospermopsis raciborskii ZIE13CR AM502076
Cylindrospermopsis raciborskii PMC98.14 AJ582268
Cylindrospermopsis raciborskii PMC99.12 AJ582273
Cylindrospermopsis raciborskii PMC114.02 AJ582279
Cylindrospermopsis raciborskii ACT 9502 AJ582280
Cylindrospermopsis raciborskii CYLI 53 AJ582281
Cylindrospermopsis raciborskii NIVA-CYA 399 KF287102
0.52/-/85
Cylindrospermopsis raciborskii LEGE 99043 HQ407340
Cylindrospermopsis raciborskii LEGE 99325 HQ407342
Cylindrospermopsis africana CHAB3411KU360015
Cylindrospermopsis sp. CHAB2132 KU359999
Cylindrospermopsis philippinensis CHAB5180 KU360007
0.91/-/-
Cylindrospermopsis raciborskii NIVA-CYA 508 AM502070
Cylindrospermopsis raciborskii NIVA-CYA 510 AM502072
0.56/-/-
Cylindrospermopsis raciborskii NIVA-CYA 509 AM502071
Cylindrospermopsis raciborskii NIVA-CYA 507 AM502069
0.79/-/60
Cylindrospermopsis raciborskii NIVA-CYA 511AM502073
0.82/-/-
Cylindrospermopsis raciborskii NIVA-CYA 506 AM502068
Cylindrospermopsis raciborskii PMC117.02 AJ582271
Cylindrospermopsis raciborskii PMC115.02 KF287108
0.88/-/78
Cylindrospermopsis raciborskii PMC118.02 AJ582270
Cylindrospermopsis raciborskii CS-507 EU552057
0.79/-/-
Cylindrospermopsis raciborskii CS-509 EU552059
Cylindrospermopsis raciborskii CS-506 EU552056
Cylindrospermopsis raciborskii KLL07 FJ828955
0.99/65/77
Cylindrospermopsis raciborskii CHAB1351 KU360041
Cylindrospermopsis raciborskii CHAB481 KU360038
0.99/56/75
Cylindrospermopsis africana CHAB482 KU360012
Cylindrospermopsis raciborskii LEGE 97047 HQ407348
Cylindrospermopsis raciborskii LEGE 95046 HQ407347
Cylindrospermopsis raciborskii CS-511 EU552061
Cylindrospermopsis raciborskii CS-510 EU552060
Cylindrospermopsis raciborskii CS-505 EU552055
0.51/-/61
Cylindrospermopsis raciborskii CS-508 EU552058
Cylindrospermopsis raciborskii CYP-030B AJ582277
Cylindrospermopsis raciborskii CYP-026J AJ582269
Cylindrospermopsis raciborskii CYP-023 AJ582278
Cylindrospermopsis raciborskii Cyl Vietnam HQ407352
Cylindrospermopsis catemaco CHAB220 KU360004
Cylindrospermopsis sp. CHAB153 KU359993
Cylindrospermopsis helicoidea CHAB357 KU360016
Cylindrospermopsis raciborskii CHAB2380 KU360044
Cylindrospermopsis sp. CHAB358 KU359994
Cylindrospermopsis sp. CHAB2134 KU360000
Cylindrospermopsis taverae CHAB143 KU360017
Cylindrospermopsis africana CHAB353 KU360009
1/-/57
Cylindrospermopsis philippinensis CHAB5182 KU360008
L-I
Cylindrospermopsis raciborskii 3-1 EF638714
Cylindrospermopsis raciborskii MG HQ407350
0.99/-/71
Cylindrospermopsis raciborskii MM67 HG942530
Cylindrospermopsis raciborskii MM77 HG942531
Cylindrospermopsis raciborskii UAM544 KF925366
Cylindrospermopsis catemaco CHAB1390 KU360005
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Cylindrospermopsis raciborskii MM48 HG942528
Cylindrospermopsis raciborskii Sidi08 KF287107
0.99/-/68
Cylindrospermopsis raciborskii CCMP1973 KF287111
Cylindrospermopsis raciborskii MM65 HG942529
Cylindrospermopsis raciborskii LEGE 051 HQ407349
Cylindrospermopsis raciborskii PMC00.01 AJ582283
0.99/59/97
Raphidiopsis sp. ITEP-007 AJ582274
Cylindrospermopsis raciborskii ITEP-A3 AJ582284
Cylindrospermopsis raciborskii PMC99.06 AJ582282
Cylindrospermopsis raciborskii ITEP-018 AJ582276
0.71/-/65
Raphidiopsis sp. ITEP-005 AJ582275
Cylindrospermopsis raciborskii PMC61.01 KF287110
Raphidiopsis brookii D9 EU552069
0.6/-/-
Raphidiopsis mediterranea MDQ ITS-L
Raphidiopsis mediterranea LP ITS-L
Cylindrospermopsis raciborskii MVCC14 HQ112347
0.96/-/-
Cylindrospermopsis raciborskii MVCC19 HQ112348
South America
L-II
Cyanocohniella calida CCALA 1049 operon 1 KJ737427
0.02

Table 2. *16S rRNA* percent identities among sequences of *Raphidiopsis mediterranea* strains isolated in this study (in bold) and sequences of representative strains belonging to *Raphidiopsis* and *Cylindrospermopsis raciborskii* genera, selected from the *16S rRNA* phylogenetic reconstruction.

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 *C. raciborskii* CHAB358 (China)															
2 *C. raciborskii* T3 (Brazil)	99.53														
3 *C. raciborskii* Vietnam (Vietnam)	99.84	99.70													
4 *C. raciborskii* LMECYA 130 (Portugal)	99.84	99.26	99.77												
5 *C. raciborskii* CS-505 (Australia)	99.68	99.71	99.85	99.41											
6 *C. raciborskii* NIVACYA 508 (Uganda)	99.68	99.56	99.85	99.41	99.85										
7 *C. raciborskii* PMC 115.02 (Senegal)	99.68	99.56	99.85	99.55	99.85	100.00									
8 *C. raciborskii* Sidi (Tunisia)	99.53	99.72	99.70	99.26	99.57	99.56	99.56								
9 *C. raciborskii* DMKU 51003 (Thailand)	99.76	99.56	99.85	99.39	99.85	99.85	99.85	99.56							
10 *C. raciborskii* MM 69 (New Zealand)	96.43	96.83	96.60	96.60	96.75	96.76	96.75	96.75	96.67						
11 *R. mediterranea* FCC LP	**99.45**	**99.65**	**99.62**	**99.18**	**99.65**	**99.49**	**99.48**	**99.50**	**99.49**	**96.60**					
12 *R. mediterranea* FCC MDQ	**99.53**	**99.65**	**99.70**	**99.18**	**99.65**	**99.49**	**99.48**	**99.50**	**99.56**	**96.60**	**99.86**				
13 *R. brookii* D9 (Brazil)	99.45	99.78	99.62	99.18	99.65	99.49	99.48	99.78	99.49	96.75	99.72	99.58			
14 *R. mediterranea* CHAB 1353 (China)	99.37	99.21	99.53	99.53	99.53	99.68	99.68	99.37	99.61	96.44	99.13	99.21	99.29		
15 *R. curvata* CHA B114 (China)	99.21	99.37	99.37	99.37	99.53	99.37	99.37	99.29	99.45	96.52	99.29	99.37	99.45	99.45	
16 Cyanocohniella calida CCALA 1049[1]	94.58	93.88	93.86	93.61	94.05	93.69	93.69	93.78	93.99	92.45	94.05	94.05	94.05	94.40	94.40

[1] Out-group taxon in the *16S rRNA* gene phylogenies.

and some representatives of the focus taxa from different regions are reported in Table 2. The two strains of *R. mediterranea* (FCC MDQ with straight trichomes and FCC LP with straight, flexuous and curved trichomes) were 99.86% identical. Taking into consideration the *16S rRNA* identity cutoff of 98.7% recommended for recognition of bacterial species (Stackebrandt & Ebers 2006), p-distances give some evidence of speciation in the *Raphidiopsis* + *Cylindrospermopsis* clade.

Analysis of the *16S-23S* ITS regions for native strains of *R. mediterranea* revealed that they had at least two operons, one with two *tRNA* genes ($tRNA^{Ala}$ and $tRNA^{Ile}$) and the other with no *tRNA* genes. The phylogenetic trees based on ITS-L and ITS-S sequences showed that *Raphidiopsis* strains form two distinct subclades (Figs 19, 20). *Raphidiopsis mediterranea* FCC LP and FCC MDQ clustered with other *Raphidiopsis* and *C. raciborskii* strains; whereas, Asian *Raphidiopsis* isolates (*R. mediterranea* and *R. curvata*) were placed in another subclade. Similar clusters were obtained from phylogenetic analyses when *16S rRNA* sequences were coupled with the associated ITS regions (Figs S3, S4). This indicated that trichome shape (straight, flexuous or curved) is not correlated with phylogeny in strains of *Raphidiopsis* (Figs 16–18).

Additionally, we looked for the 7-bp AGAAACT fragment in the ITS-L sequences, which is supposed to be conserved and specific for *Raphidiopsis* strains according to Li *et al.* (2016). Contrary to predictions, we found that the AGAAACT fragment was absent in *Raphidiopsis* strains from South America; whereas, it was present in all European strains of *Cylindrospermopsis* (Fig. 21).

All *Raphidiopsis* and *Cylindrospermopsis* were placed together in the phylogenies based on *cpcBA*-IGS sequences (Fig. S5). Isolates FCC MDQ and FCC LP grouped with Brazilian *R. brookii* D9 (group P-II), separated from cluster P-I, which included *R. mediterranea* and *R. curvata* from China (CHAB) and South Africa (AB2011/37 and AB2011/25) (Fig. 22).

16S-23S ITS secondary structure analyses

Hypothetical ITS secondary structures were estimated for *R. mediterranea* FCC and MDQ and all *Raphidiopsis* and *Cylindrospermopsis* sequences available at GenBank (n = 126; Table S4). Comparisons of D1–D1′ helices indicated that there are at least 18 types in *Raphidiopsis* and *Cylindrospermopsis*, which were classified in nine ribosomal operons, five containing both *tRNA* genes (B1, B2, B3, B4 and B5; Figs 23–33) and four containing no *tRNA* genes (N1, N2, N3 and N4) (Figs S6–S12). While the basal portion of the helix was conserved in all analysed strains, the middle region had two repeating configurations, one found in operons B1, B2 and B3 and the other in B4 and B5 (Figs 23–28, 29–33, see arrows). The D1–D1′ helix was not useful for separating genera. For example, identical D1–D1′ helices were found in *R. mediterranea* FCC, MDQ and *C. raciborskii* LEGE 051, PMC 99.96, PMC61.01 from Brazil and *C. raciborskii* MVCC 14 and MVCC 19 from Uruguay (operon B1) (Fig. 23). *Raphidiopsis mediterranea* CHAB 1353, *R. curvata* CHAB 1150, *C. africana* CHAB485 and *C. raciborskii* CHAB 480 showed the same helix (operon B5) (Table S4; Figs 31–33). The differences in the D1–D1′ helices between *Raphidiopsis* sequences suggest that strains from South America (operon B1) and Asia (operons B4 and B5) conform to different species. Outgroup taxa were more

←

Fig. 19. Phylogenetic relationships of *Raphidiopsis* and *Cylindrospermopsis* strains derived from ITS-L sequences using topology given by Bayesian analysis. *Cyanocohniella calida* CCALA 1049 operon 1 (KJ737427) was selected as the out-group taxon. Grey boxes indicate branches that contain *Raphidiopsis* strains. Support values (BI/ML/MP) for the branches are indicated at the nodes of the tree when bootstrap values are above 50% and BI is above 0.5. Strains isolated in this study are indicated in bold.

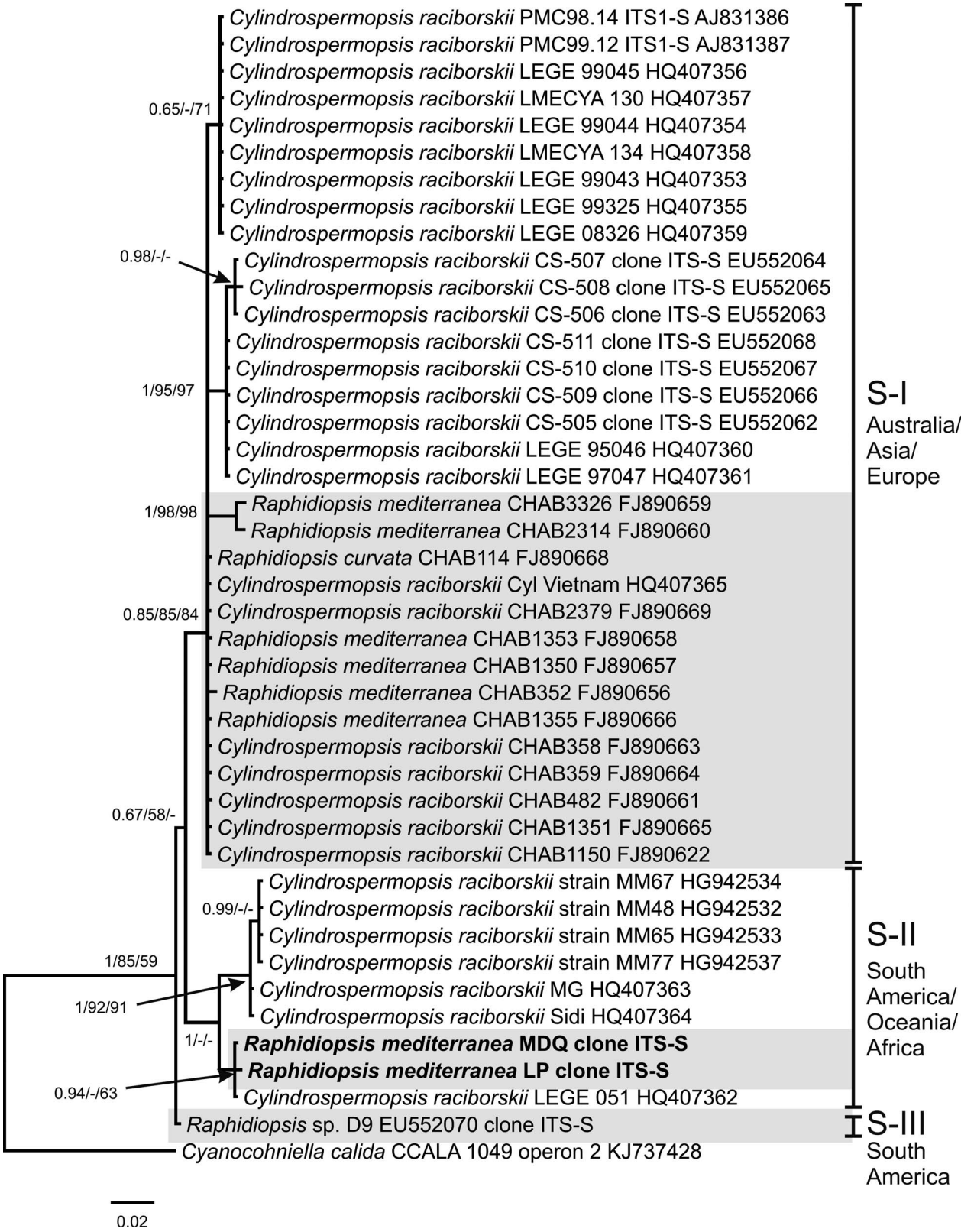


Fig. 20. Phylogenetic relationships of *Raphidiopsis* and *Cylindrospermopsis* strains derived from ITS-S sequences using topology given by Bayesian analysis. *Cyanocohniella calida* CCALA 1049 operon 2 (KJ737428) was selected as the out-group taxon. Support values (BI/ML/MP) for the branches are indicated at the nodes of the tree when bootstrap values are above 50% and BI is above 0.5. Strains isolated in this study are indicated in bold.

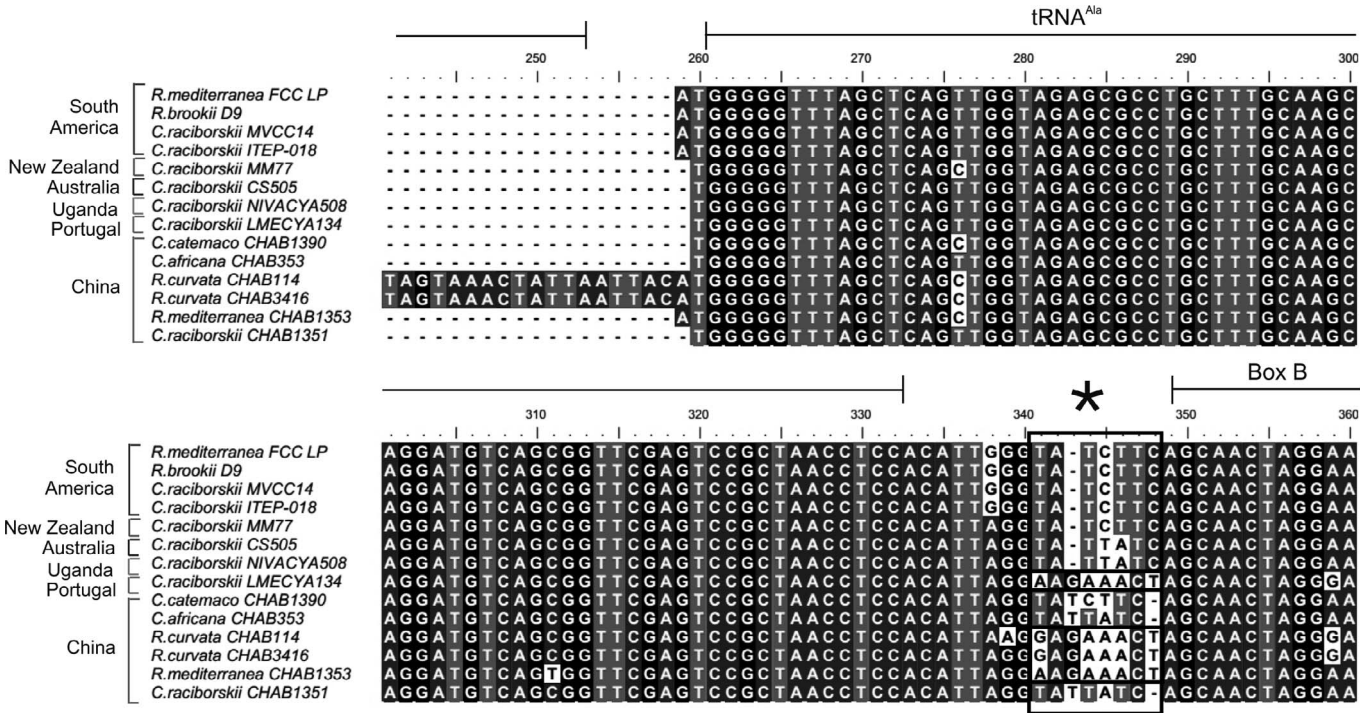


Fig. 21. Partial alignment of ITS-L sequences obtained for *Cylindrospermopsis* and *Raphidiopsis* strains from different regions. South America: *R. mediterranea* FCC LP (Argentina); *R. brookii* D9 (Brazil); *C. raciborskii* ITEP-018 (Brazil). The box with * indicates the ITS-L fragment conserved in *Raphidiopsis* strains (RAGAAACT), according to Li *et al.* (2016). Note that the fragment is present in *C. raciborskii* NIVA-CYA 508 (Portugal) and is absent in *Raphidiopsis* from South America.

divergent in sequence but retained structural similarity (Figs S13–S15).

Two conserved types of Box-B helices were found in *Raphidiopsis* and *Cylindrospermopsis* strains (Figs 34–37). The V2 helix was absent in all *Cylindrospermopsis* strains; whereas, two types were found in *R. curvata* strains from China: (1) in *R. curvata* CHAB 1150 and CHAB 114 (Fig. 38) and (2) in *R. curvata* CHAB 3416 and HB1 (Fig. 39). Eight highly similar V3 helices were present in *Raphidiopsis* and *Cylindrospermopsis* strains but absent in others, such as *C. raciborskii* Sidi, MG, MM 65, *R. mediterranea* LP, MDQ and *Raphidiopsis* ITEP-005 (Table S4; Figs 40–49). Box-B, V2 and V3 helices of outgroup taxa are depicted in Figs S16–S26.

DISCUSSION

In this study, we characterised two native strains of *R. mediterranea* (FCC LP and FCC MDQ) and used the morphology of natural populations together with ultrastructural, physiological and molecular data. The comparison with previous reports indicates that the morphological and genetic diversity within the *Raphidiopsis* + *Cylindrospermopsis* group is not enough to distinguish the two genera as distinct units. But based on *16S rRNA*, *16S–23S ITS* and *cpcBA*-IGS sequences, the two genera are virtually identical. Strains of *Raphidiopsis* and *Cylindrospermopsis* conform to a monophyletic lineage in the phylogenetic reconstructions with strong statistical support in all of the analyses (BI, MP and ML). Additionally, the secondary structure analyses of the ITS regions do not support the separation of *Raphidiopsis* from *Cylindrospermopsis*. Considering the evidence presented here, we conclude that these two genera should not be regarded as separate and distinct generic units. Therefore, we propose the unification of both genera under the name *Raphidiopsis*, respecting the principle of priority (McNeill *et al.* 2012). Accordingly, we emend the description of the genus *Raphidiopsis* and transfer *Cylindrospermopsis* species to *Raphidiopsis*.

The identification of species of *Raphidiopsis* such as *R. mediterranea* and *R. curvata* from nature or culture relies principally on trichome morphology (straight versus curved) and heterocyte absence (Li *et al.* 2001, 2008; Wu *et al.* 2011; Komárek 2013). However, those morphological differences are not sufficiently supported either by physiological properties or by phylogenetic analysis (Li *et al.* 2008; Wu *et al.* 2011). Based on trichome morphology in field and in culture, *Raphidiopsis* populations from Los Padres (only straight trichomes) and from Los Patos (straight and flexuous trichomes) were identified as *R. mediterranea*. However, in culture, trichome morphology of strain FCC LP (Los Patos) changed qualitatively from straight (under 40 photons μmol m^{-2} s^{-1}) to curved (under 80 photons μmol m^{-2} s^{-1}) (Figs 16–18). The effect of light intensity on trichome coiling suggests that its morphology is a variable trait that could lead to misidentification between *R. mediterranea* and *R. curvata*. This is in line with our phylogenetic reconstructions that show that strains with

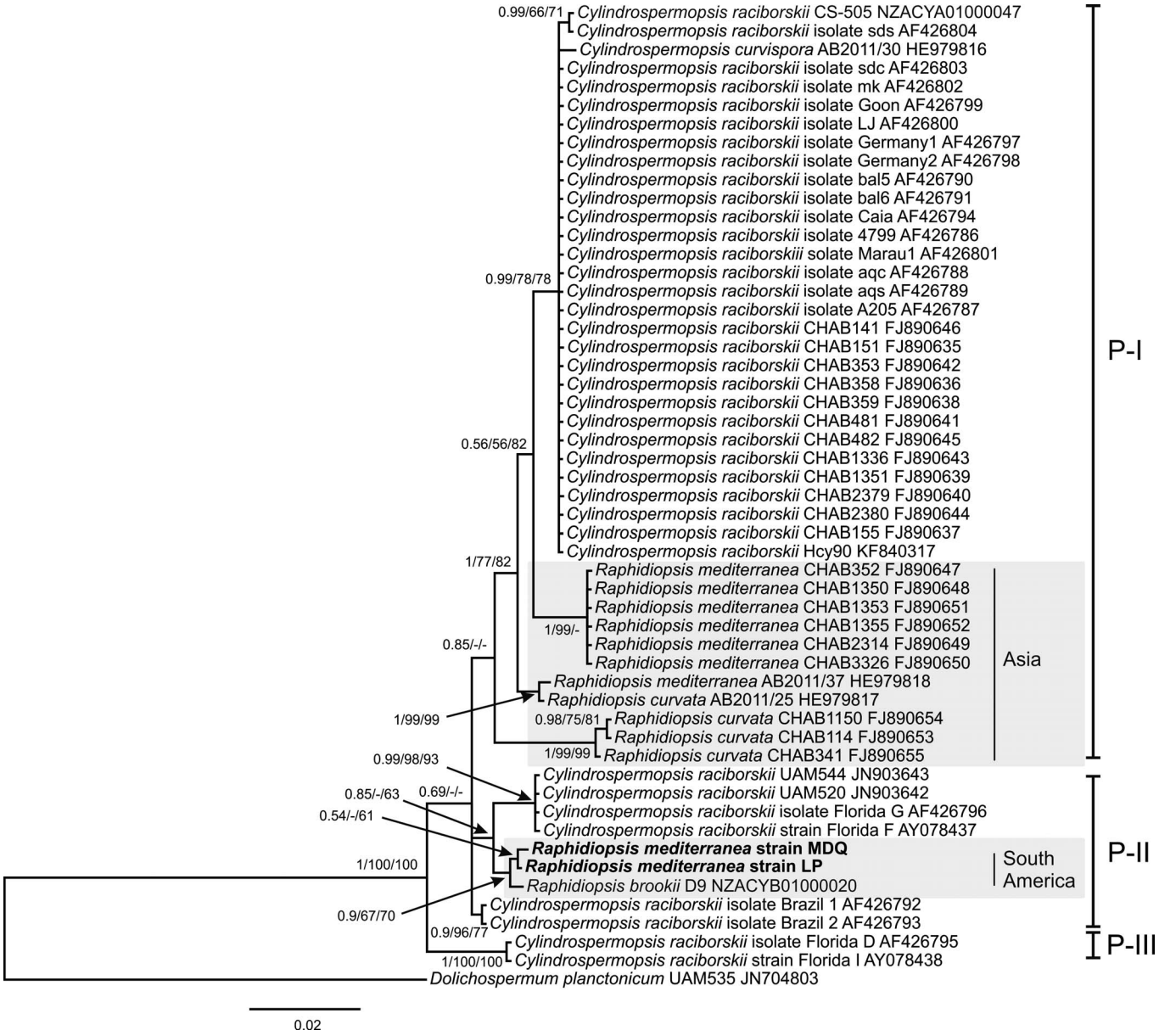
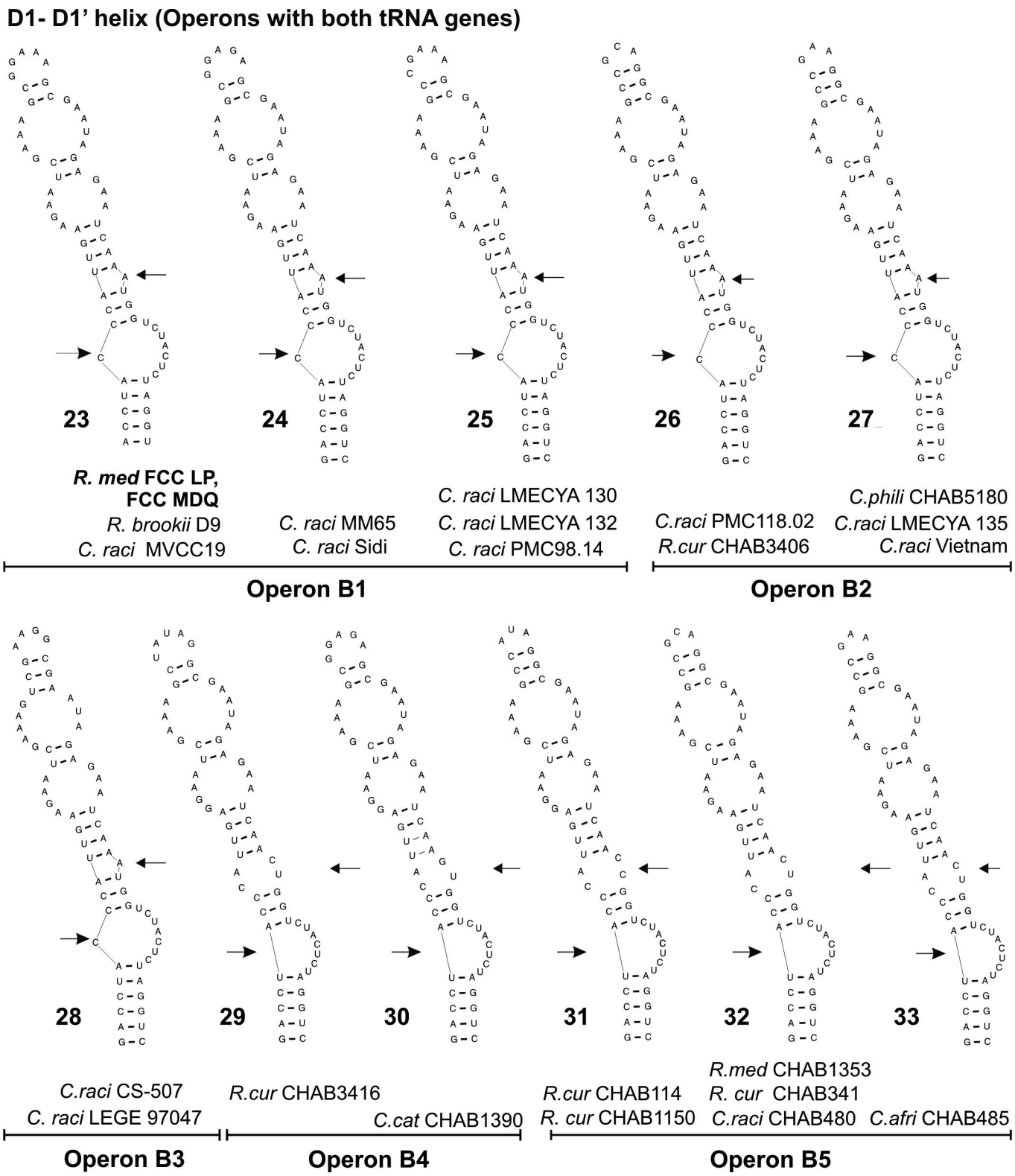
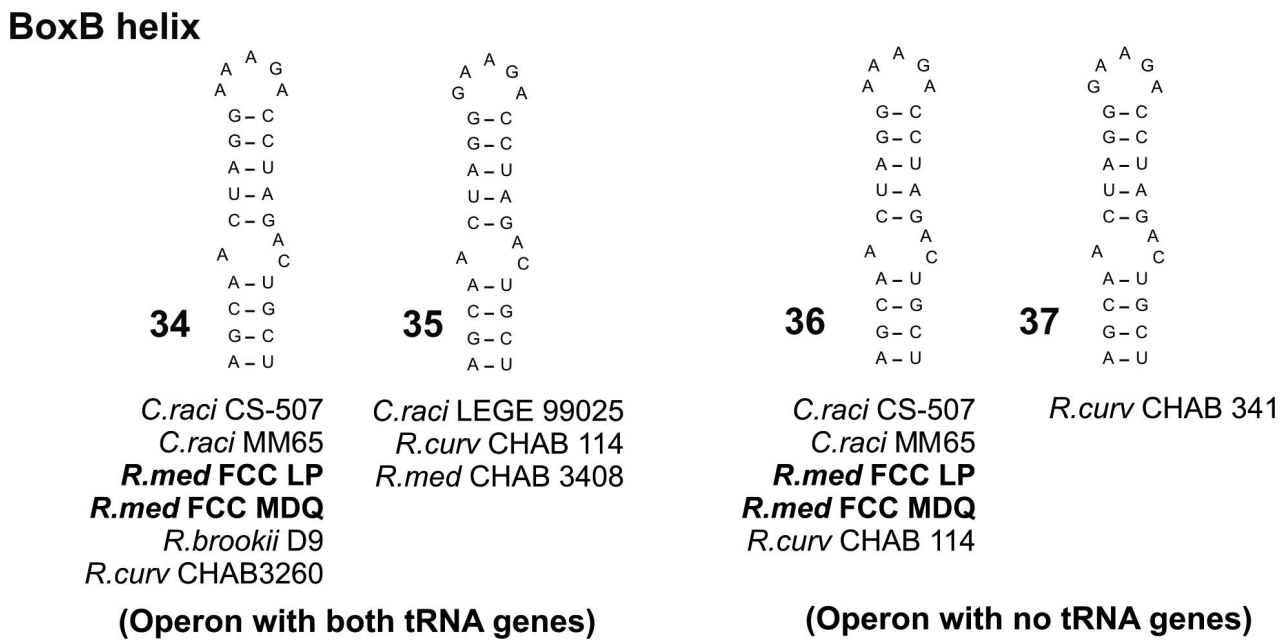


Fig. 22. Phylogenetic relationships of *Raphidiopsis* and *Cylindrospermopsis* strains derived from *cpcBA*-GIS (580–601 bp), using topology from Bayesian analysis. *Dolichospermum planctonicum* UAM535 (JN704803) was selected as the outgroup. Support values (BI/ML/MP) for the branches are indicated at the nodes of trees when bootstrap values are above 50% and BI is above 0.5. Strains isolated in this study are indicated in bold.

coiled trichomes (*R. curvata*) intermix with straight ones (*R. mediterranea*) (Figs 19–20, 22). Additionally, phylogenetic trees point to the existence of at least two *Raphidiopsis* subgroups, one from Asia (*R. curvata* + *R. mediterranea*) and the other from South America (*R. mediterranea* + *R. brookii*), suggesting a possible divergence into species or subspecies. This hypothesis is also supported by ITS secondary structure analyses, which indicate differences between *Raphidiopsis* strains from South America and Asia at the level of D1–D1′ domain. Considering the phylogenetic results together with the lack of clear distinction between coiled and straight forms, we raise the question of whether trichome form is a sufficiently stable character to differentiate *Raphidiopsis* species. Further studies should be conducted to evaluate this at the species level. Coiled and straight forms also occur in populations of *C. raciborskii* (Saker *et al*. 1999; Dyble *et al*. 2002). Indeed, *R. mediterranea* has been considered a morphotype corresponding to the straight form of *C. raciborskii*; whereas, *R. curvata* has been considered the coiled morphotype of *C. raciborskii* (McGregor & Fabbro 2000). As seen in *Raphidiopsis* (Li *et al*. 2008; Wu *et al*. 2011; present study), genetic comparisons of coiled and straight *C. raciborskii* indicate that they are genetically



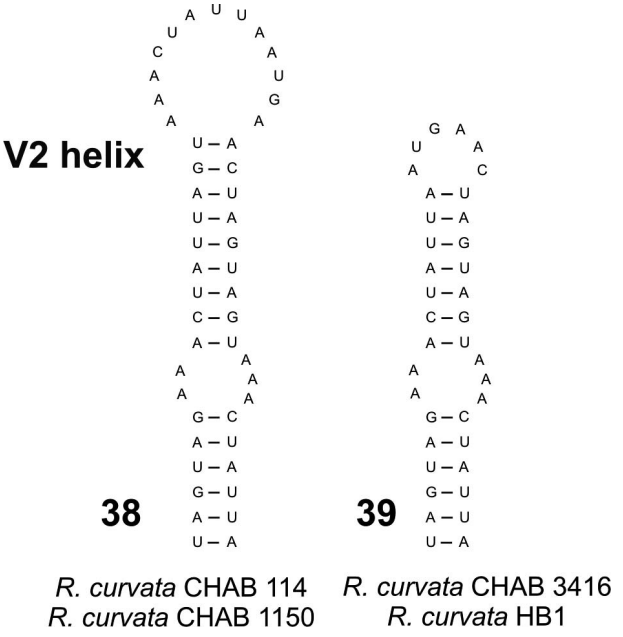
Figs 23–33. D1–D1′ helices of the 16S–23S ITS for *Raphidiopsis* and *Cylindrospermopsis* strains. Nine distinct operons can be discerned based upon differences in this helix in the 216 strains analysed (Table S4). Five operons with both *tRNA* genes are shown (B1, B2, B3, B4, B5). Operons with no *tRNA* genes are shown in Figs S6–S12. *C. afri*, *C. africana*; *C. cat*, *C. catemaco*; *C. phili*, *C. philippinensis*; *C. raci*, *C. raciborskii*; *R. cur*, *R. curvata*; *R. med*, *R. mediterranea*.



Figs 34–37. Box-B helices of the 16S–23S ITS for 216 *Raphidiopsis* and *Cylindrospermopsis* strains (Table S4). *C. raci*, *C. raciborskii*; *R. cur*, *R. curvata*; *R. med*, *R. mediterranea*.

identical based on *16S rRNA*, *nifH* and *cpcBA*-IGS sequences (Saker *et al*. 1999; Dyble *et al*. 2002).

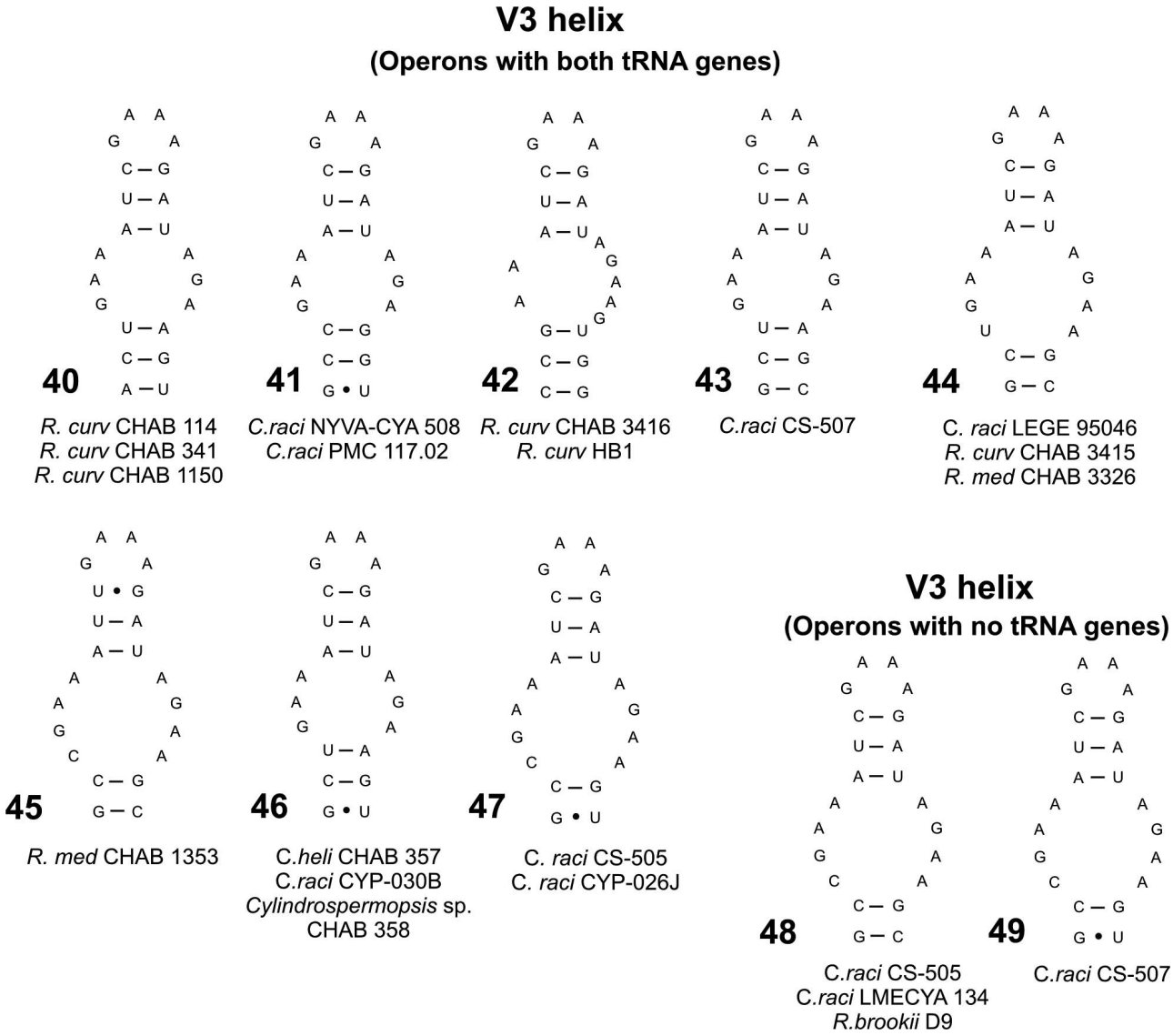
The lack of heterocytes in the two populations of *R. mediterranea* (during 1 yr of monitoring) and in the two isolates (FCC LP and FCC MDQ) cultured in a medium without combined nitrogen, together with the absence of *nifH* sequences, supports the occurrence of stable native *R. mediterranea* populations. Consequently, these populations do not represent a part of the *Cylindrospermopsis* complex life cycle, as proposed for *R. mediterranea* from Lake Kastoria (Moustaka-Gouni *et al*. 2009).



Figs 38, 39. V2 helices of the 16S–23S ITS for 216 *Raphidiopsis* and *Cylindrospermopsis* strains (Table S4). V2 helices appear only in operons with both *tRNA* genes, so operons N1 and N2 are not represented.

Many attempts have been made to distinguish *Raphidiopsis* from *Cylindrospermopsis* with genetic markers (Li *et al*. 2008; Moustaka-Gouni *et al*. 2009; McGregor *et al*. 2011; Wu *et al*. 2011; Li *et al*. 2016). We used *16S rRNA*, *16S-23S* ITS and *cpcBA*-IGS sequences to provide stronger molecular resolution. However, similar tree topologies were obtained in the three cases, with *Raphidiopsis* strains grouping with those of *Cylindrospermopsis* in a highly defined cluster. Furthermore, 16S–23S ITS secondary structure analysis, recently proposed to be very useful to circumscribe cyanobacterial genera (Johansen *et al*. 2011; Osorio-Santos *et al*. 2014; Sciuto & Moro 2016), failed to distinguish *Raphidiopsis* from *Cylindrospermopsis* (Figs 23–49).

In a detailed genome comparison of *C. raciborskii* CS-505 and *R. brookii* D9, Stucken *et al*. (2010) found that the genomes shared a set of 2539 genes with more than 90% average sequence identity. Based on those results, the authors proposed that the sequenced strains were congeneric. Wu *et al*. (2011), Moustaka-Gouni *et al*. (2009) and Li *et al* (2016) arrived at the same conclusion after performing molecular phylogenies based on multiple gene loci (*16S rRNA*, *psbA*, *rbcL*, *rbcS* and *cpcB*). *Raphidiopsis brookii* D9 is unable to fix nitrogen and lacks the *nifH* genes. Furthermore, strain D9 does not produce heterocytes despite having 39 of the 55 genes related to heterocyte differentiation present in *C. raciborskii* CS-505 (Stucken *et al*. 2010). Accordingly, the authors concluded that the ability to produce heterocytes and the metabolic ability to fix atmospheric nitrogen (diazotrophy) might have been lost in



Figs 40–49. V3 helices of the 16S–23S ITS for 216 *Raphidiopsis* and *Cylindrospermopsis* strains (Table S4). *C. raci*, *C. raciborskii*; *C. heli*, *C. helicoidea*; *R. cur*, *R. curvata*; *R. med*, *R. mediterranea*.

R. brookii D9. Other authors (McGregor *et al*. 2011; Li *et al*. 2016) considered that differences in the genomes (i.e. the presence of *nifH* and heterocyte differentiation genes in *C. raciborskii* CS-505 and the absence in *R. brookii* D9) and the resulting physiology and autapomorphic characters (presence or absence of heterocytes presence/heterocyte absence) were sufficient to recognise *Raphidiopsis* and *Cylindrospermopsis* as distinct genera. However, if they were distinct, this should be reflected in a phylogeny analysis, that is, with their sequences grouping in two separate and unique clusters. In the present study, phylogenetic analyses clearly showed a geographical distinction between South American and Chinese *Raphidiopsis*. Thus, contrary to predictions, strains of *R. mediterranea* isolated in this study were more closely related to South American *Raphidiopsis* and *C. raciborskii* than to *R. mediterranea* and *R. curvata* isolated from other regions (such as China, the Czech Republic and Australia), in agreement with previous studies (Gugger *et al*. 2005).

Recently, the molecular separation of *Raphidiopsis* and *Cylindrospermopsis* was proposed based on the presence of a 7-bp ITS-L fragment sequence supposed to be conserved only in *Raphidiopsis* (Li *et al*. 2016). Accordingly, a primer pair (Raphi-R/Raphi-F) was designed for targeting only *Raphidiopsis* strains and was successful in identifying Chinese strains. However, a comparative analysis of *Raphidiopsis* and *Cylindrospermopsis* ITS-L sequences deposited to date in databases revealed that the proposed AGAAACT-conserved fragment is not useful for targeting strains of *Raphidiopsis* specifically because it is absent in *Raphidiopsis* strains from South America. Moreover, it is present in all European *Cylindrospermopsis* strains (Fig. 21).

The existence of at least two *Raphidiopsis* lines, one from Asia (*R. curvata* + *R. mediterranea*) and the other from

South America (*R. mediterranea* + *R. brookii*) (Figs 19, 20), together with the presence of specific ITS-L sequences in *Raphidiopsis* found in China (Li *et al*. 2016) but absent in South American strains, points to the existence of different *Raphidiopsis* genotypes. In light of our results, we conclude that the intermixed position of *Raphidiopsis* and *Cylindrospermopsis* in the phylogenetic trees could be ascribed to a recent species/subspecies/ecotype divergence. Moreover, our data suggest that the main diagnostic feature of the genus (i.e. the absence of heterocytes; Komárek & Mareš 2012) may be a consequence of independent events of secondary loss occurring in different geographic populations. Populations without heterocytes (*Raphidiopsis*) might have arisen from heterocytous ones (*Cylindrospermopsis*) multiple times by natural selection, for example, in nitrogen-rich environments. This hypothesis is further supported by ecophysiological inferences. Although *C. raciborskii* can fix nitrogen, recent studies have questioned whether this capacity provides an ecological advantage enabling the species to form blooms while nitrogen deficient (Moisander *et al*. 2012). On the one hand, *C. raciborskii* develops high biomass under high nitrogen availability but reaches very low biomass when relying on diazotrophic conditions (Wood *et al*. 2014; Yema *et al*. 2016). On the other hand, nitrogen fixation in *C. raciborskii* only supports cell maintenance and allows little growth (Burford *et al*. 2006; Willis *et al*. 2016); whereas, *C. raciborskii* CS-506 neither produces heterocytes nor grows when lacking combined nitrogen (Willis *et al*. 2015).

In conclusion, *Raphidiopsis* and *Cylindrospermopsis* strains group intermixed in a single cluster and cannot be distinguished as distinct generic units based on different genetic markers and ITS secondary structure analyses. Therefore, we propose the unification of both genera under the name of *Raphidiopsis*, respecting the principle of priority.

Emended description and nomenclatural changes proposed in this study

Raphidiopsis Fritsch & Rich emend. Aguilera, Berrendero Gómez, Kaštovský, Echenique & Salerno

SYNONYM: *Cylindrospermopsis* Seenayya & Subba Raju, 1972.

EMENDED DESCRIPTION: Filaments solitary, free floating, without sheaths and gelatinous envelopes, straight, flexuous, or coiled. Trichomes uniserial, thin (< 4 μm in diameter) with or without constrictions at cross-walls, isopolar or subsymmetric, with or without heterocytes. If heterocytes present, only terminal, and oval, ovoid or conical, sometimes slightly curved and drop-like, unipored, and usually developed asynchronously at both ends of the trichome. Trichomes without heterocytes usually slightly attenuated toward both ends (or one end after trichome disintegration), apical cells conical-rounded or pointed but not elongated in cellular hair-like ends. Vegetative cells cylindrical or barrel-shaped, usually distinctly longer than wide, pale blue-green, yellowish or olive-green, facultative gas vesicles (aggregated in aerotopes). Akinetes solitary or in pairs, ellipsoidal or cylindrical, in coiled specimens usually slightly curved, usually developing distant from heterocytes, rarely adjacent to apical heterocytes. Reproduction by disintegration of trichomes and by akinetes.

ECOLOGY: Only planktic, freshwater species, sometimes forming water blooms.

TYPE SPECIES: *Raphidiopsis curvata* Fritsch & Rich 1930: 91, fig. 32.

New combinations

Raphidiopsis acuminato-crispa (Couvy & Bouvy) Aguilera, Berrendero Gómez, Kaštovský, Echenique & Salerno *comb. nov*.

BASIONYM: *Cylindrospermopsis acuminato-crispa* Coute & Bouvy 2004. Archives für Hydrobiology/Algological Studies (Cyanobacterial Research 5) 113: 69, figs 1–14.

Raphidiopsis africana (Komárek & Kling) Aguilera, Berrendero Gómez, Kaštovský, Echenique & Salerno *comb. nov*.

BASIONYM: *Cylindrospermopsis africana* Komárek & Kling 2001. Algological Studies 61: 38.

Raphidiopsis catemaco (Komárková-Legnerová & Tavera) Aguilera, Berrendero Gómez, Kaštovský, Echenique & Salerno *comb. nov*.

BASIONYM: *Cylindrospermopsis catemaco* Komárková-Legnerová & Tavera 1996. Archive für Hydrobiology/Algological Studies 83: 413–414.

Raphidiopsis curvispora (Watanabe) Aguilera, Berrendero Gómez, Kaštovský, Echenique & Salerno *comb. nov*.

BASIONYM: *Cylindrospermopsis curvispora* Watanabe 1995. Bulletin of the National Museum of Nature and Science, Series B (Botany) 21 (2): 48.

Raphidiopsis cuspis (Komárek & Kling) Aguilera, Berrendero Gómez, Kaštovský, Echenique & Salerno *comb. nov*.

BASIONYM: *Cylindrospermopsis cuspis* Komárek & Kling 1991. Archive für Hydrobiology/Algological Studies 61: 38.

Raphidiopsis gangetica (G.U.Nair) Aguilera, Berrendero Gómez, Kaštovský, Echenique & Salerno *comb. nov*.

BASIONYM: *Anabaenopsis gangetica* G.U. Nair 1967. Hydrobiologia 30 (1): 147, fig. 3.

Raphidiopsis helicoidea (Cronberg & Komárek) Aguilera, Berrendero Gómez, Kaštovský, Echenique & Salerno *comb. nov*.

BASIONYM: *Cylindrospermopsis helicoidea* Cronberg & Komárek 2004. Nova Hedwigia 78 (1–2): 71–106.

Raphidiopsis philippinensis (W.R.Taylor) Aguilera, Berrendero Gómez, Kaštovský, Echenique & Salerno *comb. nov*.

BASIONYM: *Anabaenopsis philippinensis* W.R. Taylor 1932. American Journal of Botany 19: 462, pl. XL [40], figs 1–7.

Raphidiopsis raciborskii (Woloszynska) Aguilera, Berrendero Gómez, Kaštovský, Echenique & Salerno *comb. nov*.

BASIONYM: *Anabaena raciborskii* Woloszynska 1912. Bulletin International de L'Académie des Sciences de Cracovie. Classe des

Sciences Mathématiques et Naturelles. Série B: Sciences Naturelles 684, fig. 10.

Raphidiopsis sinuosa (Coute, Leitao & Sarmento) Aguilera, Berrendero Gómez, Kaštovský, Echenique & Salerno *comb. nov.*

BASIONYM: *Cylindrospermopsis sinuosa* Coute, Leitao & Sarmento 2003. Archive für Hydrobiology/Algological Studies (Cyanobacterial Research 3) 111: 1–15.

Raphidiopsis taverae (Komárek & Komárková-Legnerová) Aguilera, Berrendero Gómez, Kaštovský, Echenique & Salerno *comb. nov.*

BASIONYM: *Cylindrospermopsis taverae* Komárek & Komárková-Legnerová 2002. Preslia 74: 229.

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SUPPLEMENTARY DATA

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