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1 **Phylogeography of cylindrospermopsin and paralytic shellfish toxin-producing**
2 **Nostocales cyanobacteria from Mediterranean Europe (Spain)**

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16 **Running title:** Phylogeography of toxic Nostocales cyanobacteria

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26 **Abstract**

27 Planktonic Nostocales cyanobacteria represent a challenge for microbiological research
28 because of the wide range of cyanotoxins they synthesize and their invasive behavior,
29 which is presumably enhanced by global warming. To gain insight into the
30 phylogeography of potentially toxic Nostocales from Mediterranean Europe, 31 strains
31 of *Anabaena* (*A. crassa*, *A. lemmermannii*, *A. mendotae* and *A. planctonica*),
32 *Aphanizomenon* (*A. gracile*, *A. ovalisporum*) and *Cylindrospermopsis raciborskii* were
33 isolated from 14 freshwater bodies in Spain and polyphasically analyzed for their
34 phylogeography, cyanotoxin production and presence of cyanotoxin biosynthesis genes.
35 The potent cytotoxin cylindrospermopsin (CYN) was produced by all 6 *Aphanizomenon*
36 *ovalisporum* strains at high levels (5.7-9.1 µg CYN mg⁻¹ dry weight) with low variation
37 between strains (1.5-3.9-fold) and a marked extracellular release (19-41% dissolved
38 CYN) during exponential growth. Paralytic shellfish poisoning (PSP) neurotoxins
39 (saxitoxin, neo-saxitoxin and decarbamoylsaxitoxin) were detected in 2 *Aphanizomenon*
40 *gracile* strains, both containing the *sxtA* gene. This gene was also amplified in non-PSP-
41 producing *Aphanizomenon gracile* and *Aphanizomenon ovalisporum*. Phylogenetic
42 analyses supported the species identification and confirmed the high similarity of
43 Spanish *Anabaena* and *Aphanizomenon* with other European strains. In contrast,
44 *Cylindrospermopsis raciborskii* from Spain grouped together with American strains and
45 were clearly separate from the rest of the European strains, raising questions about the
46 current assumptions of the phylogeography and spreading routes of *C. raciborskii*. The
47 present study confirms the nostocalean genus *Aphanizomenon* as a major source of
48 CYN and PSP toxins in Europe and demonstrates the presence of the *sxtA* gene in CYN-
49 producing *Aphanizomenon ovalisporum*.

50

51 **Introduction**

52 Planktonic Nostocales cyanobacteria, particularly the genera *Anabaena*,
53 *Aphanizomenon* and *Cylindrospermopsis*, have attracted increasing scientific interest
54 because of their ability to produce most types of cyanotoxins (1) and the apparent
55 spread of some nostocalean from tropical to subtropical and temperate latitudes, a
56 phenomenon that has been associated with global warming (2, 3) . Within these three
57 genera, the alkaloid cylindrospermopsin (CYN), with hepatotoxic, neurotoxic, general
58 cytotoxic and potential carcinogenic effects (4) is produced by strains of
59 *Cylindrospermopsis raciborskii* (5), *Aphanizomenon ovalisporum* (6), *Aphanizomenon*
60 *flos-aquae* (7), *Aphanizomenon gracile* (8), *Anabaena bergii* (although CYN-producing
61 strains have been reclassified as *Aphanizomenon ovalisporum* (9), and *Anabaena*
62 *lapponica* (10). The alkaloid saxitoxin (STX) and its more than 30 derivatives (paralytic
63 shellfish poisoning toxins, PSP toxins) , neurotoxins responsible for the paralytic
64 shellfish poisoning syndrome (4), are synthesized by the freshwater nostocalean,
65 *Anabaena circinalis* (11), by several *Aphanizomenon* spp (12, 13, 14) and by *C.*
66 *raciborskii* (15). The anatoxin-a (ATX) neurotoxin is also produced in strains of
67 *Anabaena* spp. (16, 17) and *Aphanizomenon* spp. (16, 18-20). The hepatotoxins
68 microcystins, although rarely reported in planktonic Nostocales, have been found in
69 *Anabaena* strains mostly from the Baltic region (1, 21).
70 Several gene clusters involved in cyanotoxin biosynthesis have been recently
71 characterized in Nostocales strains: the CYN gene cluster (*cyr* genes) has been
72 identified in 2 strains of *C. raciborskii* (22, 23) and in *Aphanizomenon* sp. 10E6 (24);
73 the saxitoxin gene cluster, which contains more than 26 genes involved in PSP toxin
74 biosynthesis (*sxt* genes), has been described in *C. raciborskii* T3, *A. circinalis*
75 AWQC131C and *Aphanizomenon* sp. NH-5 (25, 26); and the ATX synthetase cluster

has been characterized in *Anabaena* sp. strain 37 (27). In addition, fast and simple PCR methods have been developed to screen for the presence of cyanotoxin genes in Nostocales strains, including those for the simultaneous amplification of the non-ribosomal peptide synthetase (*cyrB/aoaB*) and polyketide synthase (*cyrC/aoaC*) genes of the CYN cluster in *Aphanizomenon* and *Cylindrospermopsis* (28). Methods are also available for the amplification of the *sxtA* gene, a polyketide synthase involved in the first steps of STX biosynthesis in *Anabaena*, *Anabaenopsis* and *Aphanizomenon* strains (19). Overall, the studies have shown the strain-specific nature of toxin production, as illustrated by the non-CYN-producing *Anabaena bergii* and *A. ovalisporum* strains that contain the *cyrA*, *cyrB* and *cyrC* genes (29) but lack the *cyrJ* gene, which is present only in CYN-producing strains (22, 29). Interestingly, the separation between toxin-producing and non-producing strains is linked to biogeographic patterns in certain Nostocales, such as *C. raciborskii*, with CYN-producing strains in Australia and Asia but non-CYN-producing strains in Africa, Europe and North America (30) and PSP-producing strains in Brazil and Uruguay (15, 31). Similarly, *A. circinalis* produces PSPs only in Australia despite the fact that it is a widely distributed species (32). These studies highlight the importance of investigating the phylogeny and toxicity of planktonic Nostocales strains isolated from different geographic areas. In particular, invasive or potentially invasive Nostocales, such as the CYN producers, *C. raciborskii* and *A. ovalisporum*, require attention, as they are apparently expanding from tropical latitudes to subtropical and temperate regions due to global warming (2, 3). In this context, Mediterranean Europe is particularly challenging for Nostocales research, as the region represents a transition area between the tropical/subtropical and temperate Central/North European freshwater ecosystems. However, extensive studies combining phylogeny and cyanotoxin production by planktonic Nostocales strains have mainly

101 been restricted to Central/North European countries (14, 17, 19, 21, 33-36), and very
102 few have focused on southern or Mediterranean Europe, namely, Portugal (37, 38).
103 Spain represents a good location for Nostocales research, as Nostocales were shown to
104 be present in 30 and dominated in 16 out of 47 Spanish water reservoirs studied in
105 1999-2001 (39). Furthermore, nostoclean members have been either confirmed CYN-
106 producers (40) or are good candidates for the production of ATX (41) and PSP (42) in
107 Spanish freshwaters. Therefore, to provide new insight into the cyanotoxin production
108 and phylogeography of planktonic Nostocales from Mediterranean Europe, 31 strains of
109 *Anabaena*, *Aphanizomenon* and *Cylindrospermopsis* were isolated from 14 freshwater
110 bodies in Spain and analyzed using a polyphasic approach, including (1) morphological
111 characterization; (2) analysis of ATX, CYN, MC and PSP toxin production; (3)
112 screening of genes putatively involved in ATX, CYN and STX biosynthesis; and (4)
113 phylogenetic analysis based on the sequence of the intergenic spacer and flanking
114 regions of the *cpcB* and *cpcA* genes of the phycocyanin operon (*cpcBA*-IGS).

115 **Materials and methods**

116 **Study sites and water sampling**

117 The Nostocales strains were isolated during the years 2005–2010 from 13 freshwater
118 reservoirs and 1 urban pond located in the following 5 Spanish watershed areas (Table
119 1): Cantábrico and Miño-Sil (Northwestern Spain); Tajo (Central Spain); Guadiana
120 (Central-Southwestern Spain); and Atlántica Andaluza (Southern Spain). These
121 watershed areas cover a maximum distance of approximately 700 km from the Trasona
122 reservoir (Northwestern Spain) to the Arcos reservoir (Southern Spain). Most of the
123 water bodies are eutrophic or hypertrophic. Their average depths range from 1.5 m
124 (Juan Carlos I pond) to 16.6 m (Alange reservoir) with a maximum of 1.5–62 m (Table
125 1).

126 The water samples were collected from May to mid-November using a 5-L water
127 sampler (Uwitec, Austria) and were stored at 4°C. Samples were transported to the
128 laboratory within 24 hours for subsequent analyses.

129 **Isolation, morphological characterization and culturing of Nostocales strains**

130 The *A. ovalisporum* strains, UAM 287, UAM 289 and UAM 290, were isolated as
131 described (40). The remaining 28 Nostocales strains were isolated from field water
132 samples that were left undisturbed at room temperature until a layer of floating
133 cyanobacteria formed on the surface. One milliliter of floating material was then
134 harvested with a Pasteur pipette, and single Nostocales filaments were picked under a
135 dissecting microscope (Leica MZ75). The filaments were subsequently transferred to
136 multi-well plates containing 2 mL of BG11₀ medium (43) and kept at 28°C under a
137 continuous white light of 10–20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Successfully grown Nostocales
138 were checked for species identification under an optical Olympus BH-2 microscope
139 equipped with a Leica DFC300 FX camera (Leica Microsystems, Germany) following
140 (44, 45). Cell dimensions (width, length) were measured at 500X magnification with the
141 aid of Leica QWin software (Leica Microsystems, Germany) in a minimum of 100 cells
142 from 10 filaments. After unequivocal species identification, cultures were transferred to
143 Erlenmeyer flasks containing BG11₀ medium and maintained at 28°C under continuous
144 white light of 10–20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ within the Autonoma de Madrid University
145 culture collection.

146 **Genomic DNA extraction, PCR amplification and sequencing**

147 Culture material from each strain (2 mL) was harvested during exponential growth and
148 centrifuged (10,000 x g, 5 min). Genomic DNA was extracted from the cell pellet using
149 the UltraClean TM kit (MO BIO laboratories, Inc.). The PCR reactions were performed
150 using the *Taq* PCR core kit (Qiagen GmbH, Germany). The reaction mixture (20 μL)

151 contained 0.1 μL of Taq DNA polymerase ($5 \text{ U } \mu\text{L}^{-1}$), 0.5 μL of deoxynucleoside
 152 triphosphate mix (10 mM), 2 μL of 10X Qiagen PCR buffer, 1 μL of each forward and
 153 reverse primer (10 μM) and 1 μL of genomic DNA ($1\text{-}10 \text{ ng } \mu\text{L}^{-1}$).
 154 The amplification of the intergenic spacer and flanking regions of the *cpcB* and *cpcA*
 155 genes of the phycocyanin operon (*cpcBA*-IGS) was performed in all 31 strains with the
 156 PC β f and PC α r (46) primers as previously described (19). The *nifH* gene was amplified
 157 in *C. raciborskii* strains with the CNF and CNR primers (47) and PCR conditions as
 158 described by Diez et al. (48). The presence of the non-ribosomal peptide synthetase
 159 (*cyrB/aoaB*) and polyketide synthase (*cyrC/aoaC*) genes of the CYN cluster was
 160 screened in all 31 strains by the multiplex PCR protocol designed by Fergusson and
 161 Saint (28) with primer pairs, M13/M14 and M4/K18, respectively. Since the multiplex
 162 method by Fergusson and Saint (28) might fail to amplify one of the individual
 163 fragments in particular strains (7), *cyrB/aoaB* and *cyrC/aoaC* genes were also checked
 164 individually following (49) for *cyrB/aoaB*, and (50) for the *cyrC* gene. The *sxtA* gene of
 165 the STX cluster was screened using the sxtaf and sxtar primers as previously described
 166 (19). The presence of the gene encoding the polyketide synthase (PKS) fragment of the
 167 putative ATX biosynthesis gene cluster was determined using the atxoaf and atxar
 168 primers as described (18). Genomic DNA obtained from several cyanotoxin-producing
 169 Nostocales was used as positive controls and included the *A. ovalisporum* UAM 290
 170 (40) strain for the *cyrB* and *cyrC* genes; *A. gracile* AB2008/19 (19) for *sxtA*; and *A.*
 171 *issatschenkoi* SP33 (18) for the PKS-encoding gene of the ATX cluster.
 172 The amplified products of *cpcBA*-IGS, *nifH* and *sxtA* were purified using the QIAquick
 173 PCR purification kit (Qiagen, Germany), and the DNA was eluted in buffer according to
 174 the manufacturer's instructions. The 3 genes were sequenced separately using the Big
 175 Dye Terminator v3.1 Cycle Sequencing kit in an ABI Prism 3730 Genetic Analyzer

176 (Applied Biosystems, Germany) according to the manufacturer's instructions. The
177 sequencing reactions were performed with the same forward and reverse primers used in
178 the PCR.

179 **Phylogenetic analysis**

180 The phylogeny of all 31 Nostocales strains was investigated by aligning their *cpcBA*-
181 IGS sequences with 77 Nostocales sequences from GenBank of the National Center for
182 Biotechnology Information (NCBI) using the Clustal W version 1.4 software.
183 Additionally, the phylogeography of *C. raciborskii* was investigated by aligning the
184 *cpcBA*-IGS and *nifH* sequences of *C. raciborskii* UAM 520 and UAM 544 from this
185 study with 27 and 44 *C. raciborskii* sequences from GenBank, respectively.

186 The phylogenetic analyses were conducted with the Mega 5.0 software. All missing
187 data and gaps were excluded from the analysis by choosing the "complete deletion"
188 option; final datasets were obtained containing 432 positions for *cpcBA*-IGS from all
189 Nostocales, and 466 positions and 297 positions for *C. raciborskii cpcBA*-IGS and *nifH*,
190 respectively. Phylogenetic trees were constructed using the Neighbor-Joining (NJ),
191 Maximum Parsimony (MP) and Maximum Likelihood (ML) methods. In the NJ
192 analysis, the Jukes-Cantor substitution model was chosen. The best-fitting evolutionary
193 models for the ML analyses, selected using the BIC criterion, were the Tamura 3-
194 parameter + G model for *cpcBA* in all 31 Nostocales and the Kimura 2-parameter for
195 *cpcBA*-IGS and *nifH* in *C. raciborskii*. Bootstrap replicates (1,000) were performed for
196 all methods. Because the trees generated by the three methods showed a similar
197 topology, only the NJ trees are represented. The GenBank sequences from *Microcystis*
198 *aeruginosa* NIVA-CYA 66 (AM421579) and *Anabaena sp.* PCC 9109 (AY7688419)
199 were used as outgroups for the *cpcBA*-IGS and *nifH* trees, respectively. Sequence
200 similarities were calculated with the EzTaxon Server version 2.1.

201 **Cyanotoxin analysis**

202 **(i) LC-MS/MS and HPLC-PDA**

203 Cyanotoxins (ATX, CYN, MC and PSP toxins) were measured in the sestonic fraction
204 of all 31 cultures from GF/F filters (Whatman, UK) saturated with 5–25 mL of
205 exponentially grown cultures after low-vacuum filtration. The filtrate was also stored
206 for the analysis of dissolved CYN. Both types of samples were kept at –20 °C until
207 analysis.

208 ATX was extracted into 100% methanol and analyzed according to (41) on a Waters
209 Alliance 2695 HPLC system equipped with a 996 PDA (Waters, USA). CYN, MC and
210 PSP toxins were quantified by ESI LC-MS/MS on a Varian 500 MS Ion Trap Mass
211 Spectrometer (Agilent Technologies, USA) supported by two Varian 212 LC
212 chromatographic pumps and a 410 autosampler. MCs (MC-LR, MC-RR, MC-YR) were
213 extracted into 90% methanol (v/v) and analyzed by ESI LC-MS/MS (51). CYN from
214 the filters was extracted with Milli-Q water (52), and dissolved CYN was solid-phase
215 extracted (53). Both fractions were quantified by ESI LC-MS/MS as described (52).
216 PSP toxins from filters were extracted into acetonitrile/water/formic acid (80:19.9:0.1),
217 and the variants, gonyautoxin 5 (GTX5), neosaxitoxin (NEO), saxitoxin (STX) and
218 decarbamoylsaxitoxin (dcSTX), were determined by ESI LC-MS/MS as previously
219 described (42).

220 **(ii) ELISA of STX producers**

221 The total saxitoxin content of cultures that were PSP-positive in the LC-MS/MS
222 analysis was quantified using the Abraxis saxitoxin enzyme-linked immunosorbent
223 assay (ELISA) (Abraxis LLC, USA), which shows a lower detection limit (0.02 µg eq.
224 STX L⁻¹) than the LC-MS/MS technique, although it does not provide information on
225 the PSP-toxins variants. Culture samples were harvested and GF/F-filtered as described

above. Saxitoxin from the filters was extracted into 80% methanol (v/v) and analyzed by ELISA following the manufacturer's instructions. The dissolved fraction was directly analyzed by ELISA without prior extraction. ELISA absorbance readings were performed at 450 nm on a Biotek Synergy HT multi-mode microplate reader (Biotek Instruments, USA). Total STX ($\mu\text{g eq. STX L}^{-1}$; sum of sestonic and dissolved) was standardized to biomass parameters (dry weight and cell concentration) determined as described (52) in culture samples taken simultaneously with those for the STX analysis.

(iii) CYN production/release in *Aphanizomenon ovalisporum* strains

Batch cultures of 6 *A. ovalisporum* strains from 3 Spanish water bodies (Table 2) were grown in duplicate in BG11₀ medium (43) at 28°C under continuous white light (60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and supplemented with sterile air bubbling. This condition simulates the culture conditions promoting the maximum CYN production in *A. ovalisporum* UAM 289 according to (52). Culture material (15–25 mL) was harvested during exponential growth, GF/F-filtered and analyzed for sestonic and dissolved CYN by ESI LC-MS/MS as described above. The CYN concentrations ($\mu\text{g CYN L}^{-1}$) were standardized to biomass parameters (dry weight, biovolume, cell concentration and chlorophyll *a* concentration) determined as described (52) in culture samples taken simultaneously with those obtained for the CYN analysis.

Nucleotide sequence accession numbers. The sequence data from *cpcBA*-IGS, *sxtA* and *nifH* were submitted to the NCBI GenBank under the accession numbers listed in Table S1 (supplemental material).

Results

Morphological identification

The 31 Nostocales strains were distributed into 7 species within the nostocacean genera, *Anabaena*, *Aphanizomenon* and *Cylindrospermopsis* (Fig. 1; Table 2). The *Anabaena*

strains comprised the coiled-types, *A. crassa* (Lemmermann) Komárková-Legnerová et Cronberg (1 strain), *A. lemmermannii* Richter in Lemmermann (1 strain), and *A. mendotae* Trelease (1 strain), and the straight-type, *A. planctonica* Brunnthaler (2 strains). The *Aphanizomenon* strains comprised 2 species of solitary straight filaments, *A. gracile* (Lemmermann) Lemmermann (18 strains) and *A. ovalisporum* Forti (6 strains). Two strains were identified as *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba-Raju.

Cyanotoxins and cyanotoxin biosynthesis genes

Cyanotoxins were detected in 8 of the 31 Nostocales strains analyzed (25.8%); CYN was found in 6 strains, and PSP was found in 2 strains. Neither MC nor ATX were detected in any of the strains (Table 2).

(i) CYN production by *A. ovalisporum*

CYN was found in all 6 *A. ovalisporum* strains from the Juan Carlos I pond and the Alange and Arcos reservoirs. Both the *cyrB/aoaB* and *cyrC/aoaC* genes of the CYN cluster were amplified in all *A. ovalisporum* strains (Table 2).

The high CYN content in all 6 *A. ovalisporum* strains allowed for a detailed analysis of the intracellular and extracellular fractions in batch cultures during exponential growth (Table 3). Strain UAM 289 showed the highest content of total CYN (sum of sestonic and dissolved) at 9.1 $\mu\text{g CYN mg}^{-1}$ dry weight (DW) 2.4 $\mu\text{g CYN mm}^{-3}$, 0.5 $\mu\text{g CYN } \mu\text{g}^{-1}$ Chl *a* and 190.6 fg CYN cell⁻¹. Differences among the strains ranged from 1.5-fold (when expressed as $\mu\text{g CYN } \mu\text{g}^{-1}$ Chl *a*) to 3.9-fold (when expressed as fg CYN cell⁻¹), although these differences were not statistically significant ($p > 0.05$; one-way ANOVA). No geographical pattern could be observed, e.g., the total CYN ($\mu\text{g mg}^{-1}$ DW) in UAM 287, UAM 289 and UAM 290 from Juan Carlos I pond showed a 1.54-

fold variation and was almost identical to the 1.55-fold variation observed in all 6 strains from 3 water bodies. The average dissolved CYN content ranged from 19.5%-41.5% in the different strains, representing a 2-fold variation; however, the variation was not statistically significant ($p > 0.05$; one-way ANOVA). Intra-site variations were also as broad as inter-site variations.

(ii) PSP production by *A. gracile* and detection of the *sxtA* gene in *Aphanizomenon* spp.

PSP toxins were detected by ESI-LC MS/MS in 2 *A. gracile* strains from the Rosarito reservoir (UAM 529 and UAM 531) of the total 18 *A. gracile* strains analyzed (Table 2). Specifically, STX and dcSTX were detected in UAM 529, and STX, NEO and dcSTX were detected in UAM 531. GTX5 was not detected in either of the 2 strains. Analysis by ELISA showed total STX contents (sum of sestonic and dissolved; $n = 2$) of $0.17 \pm 0.02 \mu\text{g eq. STX mg}^{-1} \text{ DW}$ (or $1.7 \pm 0.1 \text{ fg eq. STX cell}^{-1}$) in UAM 529; and $0.41 \pm 0.14 \mu\text{g eq. STX mg}^{-1} \text{ DW}$ (or $2.4 \pm 0.8 \text{ fg eq. STX cell}^{-1}$) in UAM 531. The differences between the strains were not statistically significant ($p > 0.05$; Student's *t* test).

A fragment of the *sxtA* gene was amplified in PSP-producing *A. gracile* (UAM 529 and UAM 531) and non-PSP-producing *Aphanizomenon* spp. (*A. gracile* UAM 508 and all 6 *A. ovalisporum* strains) (Table 2). *SxtA* amplification was not observed in any of the remaining 22 Nostocales strains. Partial *sxtA* sequences (612 bp) from *A. gracile* and *A. ovalisporum* in our study (Table S1 in supplementary material) shared a 99.8% similarity and differed in just 1 bp among the 612 aligned bp. They also shared a 99.5%-100% sequence similarity with 27 *sxtA* sequences from GenBank, including PSP-producing and non-producing *Anabaena*, *Anabaenopsis* and *Aphanizomenon* spp. (554-bp alignment; data not shown).

300 **Phylogenetic analyses**

301 **(i) Nostocales phylogeny**

302 The *cpcBA*-IGS tree (Fig. 2) showed a distribution of the strains into 4 main clusters
303 supported by high bootstrap values as well as by *cpcBA* intergenic spacer (IGS) lengths
304 specific to each cluster (Fig. 2). The 18 *A. gracile* strains from this study grouped
305 together in cluster I that included mostly GenBank sequences of *A. gracile* but also of
306 unclearly identified *Aphanizomenon* sp strains. *A. lemmermanni* UAM 506 and *A.*
307 *mendotae* UAM 518 were also located in cluster I (Fig. 2). *A. crassa* UAM 502 and the
308 2 *A. planctonica* from our study were grouped in cluster II together with GenBank
309 sequences of several coiled and straight *Anabaena* spp. The 2 *Cylindrospermopsis*
310 *raciborskii* strains from Spain clustered within the single-species cluster III. The 5 *A.*
311 *ovalisporum* strains were grouped within the highly homogeneous cluster IV (100%
312 similarity; 485-bp alignment), which also contained 2CYN-producing *Anabaena bergii*
313 sequences (ANA283A and ANA366B) from GenBank.

314 The CYN-producing *A. ovalisporum* strains from Spain clustered with many other
315 CYN-producing *A. ovalisporum* strains but also with 2 non-CYN-producing *A.*
316 *ovalisporum* strains from Israel (AB2010/05 and AB2010/06). All of these strains
317 showed identical *cpcBA*-IGS sequences (485-bp alignment).

318 The 2 PSP-producing *A. gracile* strains from Spain (UAM 529 and UAM 531) were
319 grouped within the Ia subcluster together with the remaining 15 non-producing strains
320 from Spain and a number of PSP-producing and non-producing *A. gracile* strains from
321 GenBank (Fig. 2). The non-PSP-producing *A. gracile* UAM521 strain was placed in the
322 Ib subcluster (Fig. 2), which showed 95.8–96.8% similarity with subcluster Ia (506-bp
323 alignment).

324 **(ii) *C. raciborskii* phylogeography**

325 *cpcBA*-IGS phylogeny of all Nostocales (Fig. 2) revealed variability in sequences and
 326 IGS lengths within the *C. raciborskii* cluster (III). The *C. raciborskii* phylogeny was
 327 therefore examined in more detail by a separate *cpcBA*-IGS tree (Fig. 3) including 27
 328 *cpcBA*-IGS sequences of *C. raciborskii* from different continents derived from
 329 GenBank. Fig. 3 shows that *C. raciborskii* strains UAM 520 and UAM 544 from Spain
 330 grouped together with 2 American (Floridian) strains from GenBank within the very
 331 homogeneous cluster III (100% similarity in a 504-bp alignment; Table 4). This cluster
 332 was clearly separated from cluster I, which included the rest of European (Hungarian,
 333 German and Portuguese) *C. raciborskii* strains from GenBank together with Australian
 334 and African strains (Fig. 3). As shown in Table 4, clusters I and III shared a *cpcBA*-IGS
 335 sequence similarity below 97.5% and differed in IGS lengths (113-bp in cluster III
 336 including Spanish strains vs. 97-bp in cluster I with European GenBank strains). The
 337 sequence similarities of cluster III (containing Spanish strains) ranged from 98.4% with
 338 cluster II (including the Brazilian PSP-producing strains) to 96.4% with cluster IV
 339 (comprising strains Florida D and Florida I), and the IGS lengths were again different
 340 (Table 4).

341 The *C. raciborskii* phylogeography shown by the *cpcBA*-IGS tree was verified by
 342 analyzing *nifH* sequences. The *nifH* tree (Fig. 4) confirmed the *C. raciborskii* UAM 520
 343 and UAM 544 grouping with American strains (USA, Mexico) from Genbank within a
 344 bigger cluster including GenBank sequences from other strains from the USA, Brazil
 345 and Uruguay. Similar to what was shown in the *cpcBA*-IGS tree (Fig. 4), the *nifH*
 346 sequences of UAM 520 and UAM 544 from Spain were clearly separate from other
 347 *nifH* sequences from European *C. raciborskii* strains obtained from GenBank (Hungary,
 348 Portugal, Germany, France), the latter grouping close to the Australian/African cluster
 349 (Fig. 4).

350 Discussion

351 The present study confirmed that 8 out of 31 (25.8%) Nostocales strains from Spain
352 produced cyanotoxins, with 6 of the strains producing CYN and 2 PSP toxins. Neither
353 ATX-producing nor MC-producing Nostocales were found. This result is not surprising
354 considering that ATX is rare in Spanish water reservoirs (41) and that MC is associated
355 with the chroococcalean genus *Microcystis* in the Mediterranean region (54, 55).

356 Here, we demonstrated CYN production by six *A. ovalisporum* strains from 3 water
357 bodies in Spain. *A. ovalisporum* is a major source of CYN not only in subtropical and
358 tropical areas, of Australia, USA (Florida) and Israel (9), but also in Mediterranean
359 Europe where *A. ovalisporum* can be considered the main CYN producer to date (56-
360 58). Studies have shown levels of 9.4-18 $\mu\text{g CYN L}^{-1}$ during *A. ovalisporum* blooms in
361 Spain and Italy (57, 58). *A. ovalisporum* is characterized by its genetic homogeneity
362 worldwide (9; and this study) and an unusually high proportion of CYN-producing
363 strains, although 2 non-CYN producing strains have been recently reported from Lake
364 Kinneret, Israel (29). CYN contents seem to vary slightly among strains (e.g., 1.5–3.9-
365 fold in our study). *A. ovalisporum* has also shown substantial CYN release during
366 exponential growth (19-41% dissolved CYN) as has been observed in other CYN
367 producers (59-61). Considering the ecological plasticity of *A. ovalisporum* revealed in
368 this study in which this species was present from the shallow 1.5-m deep Juan Carlos I
369 pond to the thermally stratified 62-m deep Alange water reservoir and the forecasted
370 spread of *A. ovalisporum* to colder regions under increasing temperature scenarios (2,
371 3), the reports of CYN-producing *A. ovalisporum* are likely to grow in number and
372 geographic distribution in the coming years.

373 The finding of PSP toxins in 2 *A. gracile* strains from the Rosarito reservoir supports
374 the involvement of *Aphanizomenon* spp. in PSP production in Spanish freshwaters (42).

375 Together with findings from Portugal (14), France (34) and Germany (19), these data
 376 confirm this widespread cyanobacterium as a major PSP-producer in European
 377 freshwaters. The profile of PSP variants appears to be species-specific and related to the
 378 content (including deletions) and arrangement of *sxt* genes in each PSP-producing strain
 379 (62; and references therein). Thus, *Aphanizomenon* spp. synthesize STX, NEO, GTX5
 380 and dcSTX, whereas *Lyngbya wollei* and *A. circinalis* produce more than 9 variants
 381 (62). The STX, NEO and dcSTX production in *A. gracile* strains from Spain resembles
 382 the toxin profile in French and Portuguese strains (14, 34). German strains also produce
 383 GTX5, a less toxic PSP variant (4) detected in an *Aphanizomenon gracile*-like
 384 dominated population of cyanobacteria in Casas de Millán pond (Spain) (42). *A. gracile*
 385 has also been confirmed to be a CYN producer in Poland (8) and is linked to CYN
 386 production in other countries of Central and Northern Europe (63-66). The *cpcBA*-IGS
 387 phylogeny (Fig. 2) indicated a separation of PSP- and CYN-producing *A. gracile*-like
 388 strains into 2 different groups (Ia and Ib). The PSP-producing *A. gracile* strains from
 389 Spain are included in a well-supported monospecific group (subcluster Ia) clearly
 390 distinguishable (below 96.8% *cpcBA*-IGS sequence similarity) from a more
 391 heterogeneous group (Ib) that includes only some *A. gracile* strains (e.g., UAM 521
 392 from Spain) together with many unclearly identified *Aphanizomenon* sp., such as CYN-
 393 producing *Aphanizomenon flos-aquae*/*gracile* from Germany (9).
 394 The *sxtA* gene encodes a PKS-like molecule that catalyzes the first steps of STX
 395 synthesis (25). The gene was detected in 2 PSP-producing *A. gracile* strains and in non-
 396 PSP-producing *A. gracile* and *A. ovalisporum* strains. These data support previous
 397 results of non-PSP-producing but *sxtA*-positive *Anabaena*, *Anabaenopsis* and
 398 *Aphanizomenon* strains (19, 34). To our knowledge, the presence of *sxtA* genes in *A.*
 399 *ovalisporum* and in a CYN-producing strain in general has not been reported prior to the

400 present study. Our results are in accord with those demonstrating the presence of *cyr*
 401 genes in several non-CYN-producing *Anabaena bergii* and *Aphanizomenon*
 402 *ovalisporum* strains (29) and in ATX-producing *A. issatschenkoi* CAWBG02 (24), and
 403 of the small *cyr*-like gene fragment (orf2) identified in PSP-producing *Lyngbya wollei*
 404 (62). These findings together with the lack of phylogenetic separation between toxic and
 405 non-toxic strains (9, 19, 29; and the present study) suggest a complex evolutionary
 406 history of horizontal gene transfer and events of insertions, deletions and recombination
 407 that have shaped toxin biosynthesis genes in cyanobacteria (24, 67); such a complex
 408 evolutionary history represents one of the emerging challenges for microbiological
 409 research.

410 *C. raciborskii* has been intensively studied in the last two decades due to its potential
 411 for CYN (5) and PSP production (15) and its invasive behavior (3; and references
 412 therein). Studies based on several phylogenetic markers (*cpcBA*-IGS, *nifH*, 16S-23S
 413 ITS) have suggested the existence of genetically delimited European, African-
 414 Australian, and American *C. raciborskii* groups (30, 31, 68, 69). The *cpcBA*-IGS and
 415 *nifH*-based phylogeny in the current study (Fig. 3 and 4) demonstrated that *C.*
 416 *raciborskii* UAM 520 and 544 from 2 Spanish water bodies were almost identical to
 417 American strains and grouped separately from the rest of the European (French,
 418 German, Hungarian) strains from GenBank, and they even differed in IGS length. This
 419 grouping is in contrast to current assumptions on the genetic homogeneity of European
 420 *C. raciborskii* and its clear separation from American populations (30, 31, 68). To
 421 further consolidate this interesting finding, we sequenced the ITS1-L region in *C.*
 422 *raciborskii* UAM 544 following (30). The ITS1-L tree (Fig. S1, in supplemental
 423 material) confirmed that Spanish *C. raciborskii* UAM 544 was clearly separated from
 424 the rest of European strains (France, Germany, Hungary, Portugal) and grouped very

close to American strains together with 2 other Mediterranean *C. raciborskii* strains from Tunisia. The phylogeography depicted by our study may indicate that either different *C. raciborskii* genotypes/ecotypes occur inside Europe as already observed inside America (30, 31, 68) and Africa (70) or that American and Spanish (or Mediterranean?) populations have undergone relatively recent trans-oceanic exchanges by transport of trichomes or akinetes by migratory birds or human activities (3, 31). Therefore, our findings indicate the need for a thorough revision of the current assumptions on the phylogeography and spreading routes of *C. raciborski* by including a greater number of strains from Mediterranean regions in future phylogenetic studies. In summary, the present study shows the toxicological importance of planktonic Nostocales in general and the genus *Aphanizomenon* in particular with *A. gracile* proving to be a widespread PSP-producer and *A. ovalisporum* comprising the main CYN-producer in Mediterranean Europe with great spreading potential. Our findings indicate that even in an extensively studied microbial group, such as planktonic Nostocales cyanobacteria, there is still a place for the discovery of intriguing phylogeographic patterns, such as that of *C. raciborskii*, which provides new avenues for future microbiological research.

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697 **Figure legends**

698 **FIG. 1. Photomicrographs of the Nostocales species studied.** (a) *Anabaena crassa*;
699 (b) *Anabaena lemmermannii*; (c) *Anabaena mendotae*; (d) *Anabaena planctonica*; (e)
700 *Aphanizomenon gracile*; (f) *Aphanizomenon ovalisporum*; (g)-(i) Akinetes of: (g)
701 *Aphanizomenon ovalisporum*, (h) *Aphanizomenon gracile* and (i) *Cylindrospermopsis*
702 *raciborskii*; (j) *Cylindrospermopsis raciborskii*. Scale bars indicate 20 μ m.

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704 **FIG. 2. Neighbor-joining tree based on partial *cpcBA*-IGS sequences from 108**
705 **Nostocales cyanobacterial strains.** Strains from this study are marked in bold.
706 Accession numbers are given in parenthesis (strains from GenBank) or listed in Table
707 S1 (strains from this study). Node values are based on 1,000 bootstrap replicates
708 (NJ/MP/ML). Only values above 65 are shown. Scale bar indicates 5% sequence
709 divergence. The lengths (base pairs) of the IGS sequences are indicated next to the
710 respective clusters. CYN: cylindrospermopsin; PSP: paralytic shellfish toxins; +,
711 detected; -, not detected. Information on cyanotoxin production of the GenBank strains
712 was retrieved from (9), (19), (29), (30).

713 **FIG. 3. Neighbor-joining tree based on partial *cpcBA*-IGS sequences from 29**
714 ***Cylindrospermopsis raciborskii* strains.** Strains from this study are marked in bold.
715 Accession numbers are given in parenthesis (strains from GenBank) or listed in Table
716 S1 (strains from this study). Node values are based on 1,000 bootstrap replicates
717 (NJ/MP/ML). Only values above 65 are shown. Scale bar indicates 5% sequence
718 divergence. The lengths (base pairs) of the IGS sequences are indicated next to the
719 respective clusters. CYN: cylindrospermopsin; PSP: paralytic shellfish toxins; +,

720 detected; —, not detected. Information on cyanotoxin production of GenBank strains was
721 retrieved from (30).

722 **FIG. 4. Neighbor-joining tree based on partial *nifH* sequences from 48**
723 ***Cylindrospermopsis raciborskii* strains.** Strains from this study are marked in bold.
724 Accession numbers are given in parenthesis (strains from GenBank) or listed in Table
725 S1 (strains from this study). Node values are based on 1,000 bootstrap replicates
726 (NJ/MP/ML). Scale bar indicates 2% sequence divergence.
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728 **Tables**

729 **TABLE 1. Characteristics of the water bodies from which Nostocales strains were**

730 **isolated.** All the water bodies studied are reservoirs except the urban pond, Juan Carlos

731 I. C: central; N: north; S: south; W: west.

Watershed	Water body	Vmax (hm ³)	Depth (m)		Coordinates (latitude/longitude)	Trophic status
			Mean	Max		
Atlántica andaluza (S)	Arcos	14.0	4.8	12	36°45' N/5°47' W	Meso-eutrophic
Cantábrico (NW)	La Barca	31.1	17.5	62	43°18' N/6°18' W	Eutrophic-hypertrophic
	Trasona	4.1	6.7	13	43°27' N/5°52' W	Hypertrophic
Guadiana (C/SW)	Alange	851.6	16.6	53	38°47' N/6°15' W	Eutrophic
	Brovales	6.98	4.4	17	38°21' N/6°41' W	Hypertrophic
	Nogales	14.9	9.8	29	38°33' N /6°44' W	Hypertrophic
	Valuengo	19.3	13.3	18	38°18' N /6°40' W	Hypertrophic
	Vega del Jabalón	33.5	5.3	18	38°45' N/3°46' W	Hypertrophic
	Vicario	32.9	3.4	18	39°03' N/3°59' W	Hypertrophic
Miño-Sil (NW)	Cachamuiña	2.0	7.4	16	42°20' N/7°48' W	Eutrophic
Tajo (C)	Juan Carlos I	< 1	1.5	1.5	40°27' N/3°36' W	Hypertrophic
	Navalcán	33.9	4.5	20	40°02' N/5°06' W	Hypertrophic
	Rosarito	85	5.8	33	40°06' N /5°18' W	Hypertrophic
	Santillana	91.2	8.7	40	40°42' N/3°49' W	Hypertrophic

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743 **TABLE 2. Cyanotoxins and cyanotoxin biosynthesis genes in the Nostocales**
744 **strains.** ^a, +, detected; –, not detected.

Species and strain	Water body	Detection of ^a :							
		ATX		CYN		MC	PSP toxins		
		HPLC-PDA	PKS	LC-MS/MS	PS/PKS	LC-MS/MS	LC-MS/MS	<i>sxtA</i>	
<i>Anabaena</i>									
<i>A. crassa</i>									
UAM 502	Trasona	—	—	—	—	—	—	—	
<i>A. lemmermannii</i>									
UAM 506	Trasona	—	—	—	—	—	—	—	
<i>A. mendotae</i>									
UAM 518	Santillana	—	—	—	—	—	—	—	
<i>A. planctonica</i>									
UAM 516	La Barca	—	—	—	—	—	—	—	
UAM 517		—	—	—	—	—	—	—	
<i>Aphanizomenon</i>									
<i>A. gracile</i>									
UAM 508	Brovales	—	—	—	—	—	—	+	
UAM 521	Cachamuiña	—	—	—	—	—	—	—	
UAM 538	Navalcán	—	—	—	—	—	—	—	
UAM 539		—	—	—	—	—	—	—	
UAM 540		—	—	—	—	—	—	—	
UAM 541		—	—	—	—	—	—	—	
UAM 542		—	—	—	—	—	—	—	
UAM 509		Nogales	—	—	—	—	—	—	—
UAM 519		Rosarito	—	—	—	—	—	—	—
UAM 528			—	—	—	—	—	—	—
UAM 529			—	—	—	—	—	+	+
UAM 530			—	—	—	—	—	—	—
UAM 531	—		—	—	—	—	+	+	
UAM 510	Valuengo	—	—	—	—	—	—	—	
UAM 511	V. del Jabalón	—	—	—	—	—	—	—	
UAM 543		—	—	—	—	—	—	—	
UAM 526	Vicario	—	—	—	—	—	—	—	
UAM 527		—	—	—	—	—	—	—	
<i>A. ovalisporum</i>									
UAM 536	Alange	—	—	+	+	—	—	+	
UAM 537		—	—	+	+	—	—	+	
UAM 507	Arcos	—	—	+	+	—	—	+	
UAM 287	Juan Carlos I	—	—	+	+	—	—	+	
UAM 289		—	—	+	+	—	—	+	
UAM 290		—	—	+	+	—	—	+	
<i>Cylindrospermopsis</i>									
<i>C. raciborskii</i>									
UAM 520	V. del Jabalón	—	—	—	—	—	—	—	
UAM 544	Vicario	—	—	—	—	—	—	—	

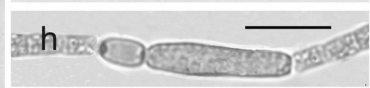
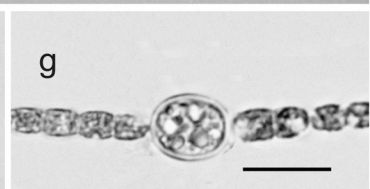
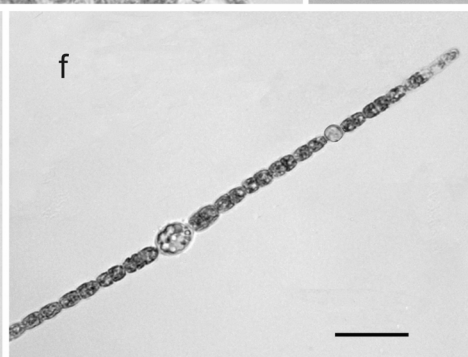
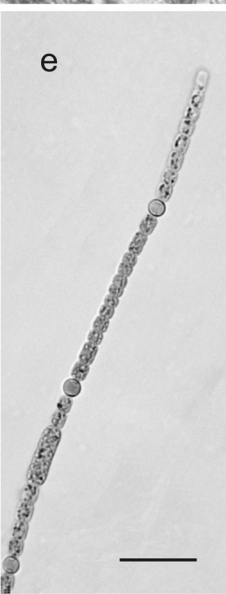
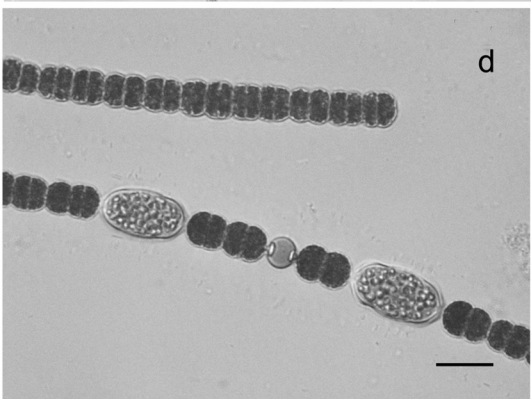
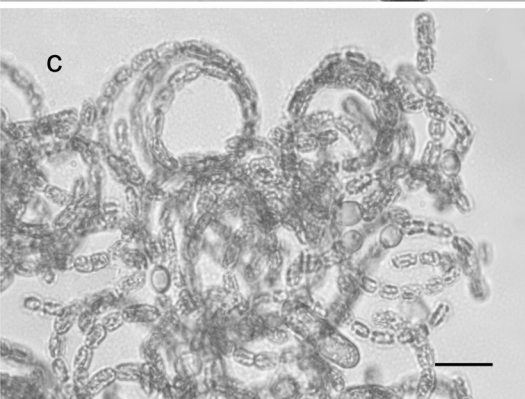
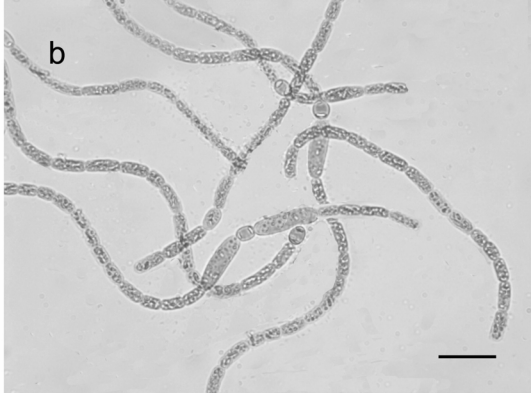
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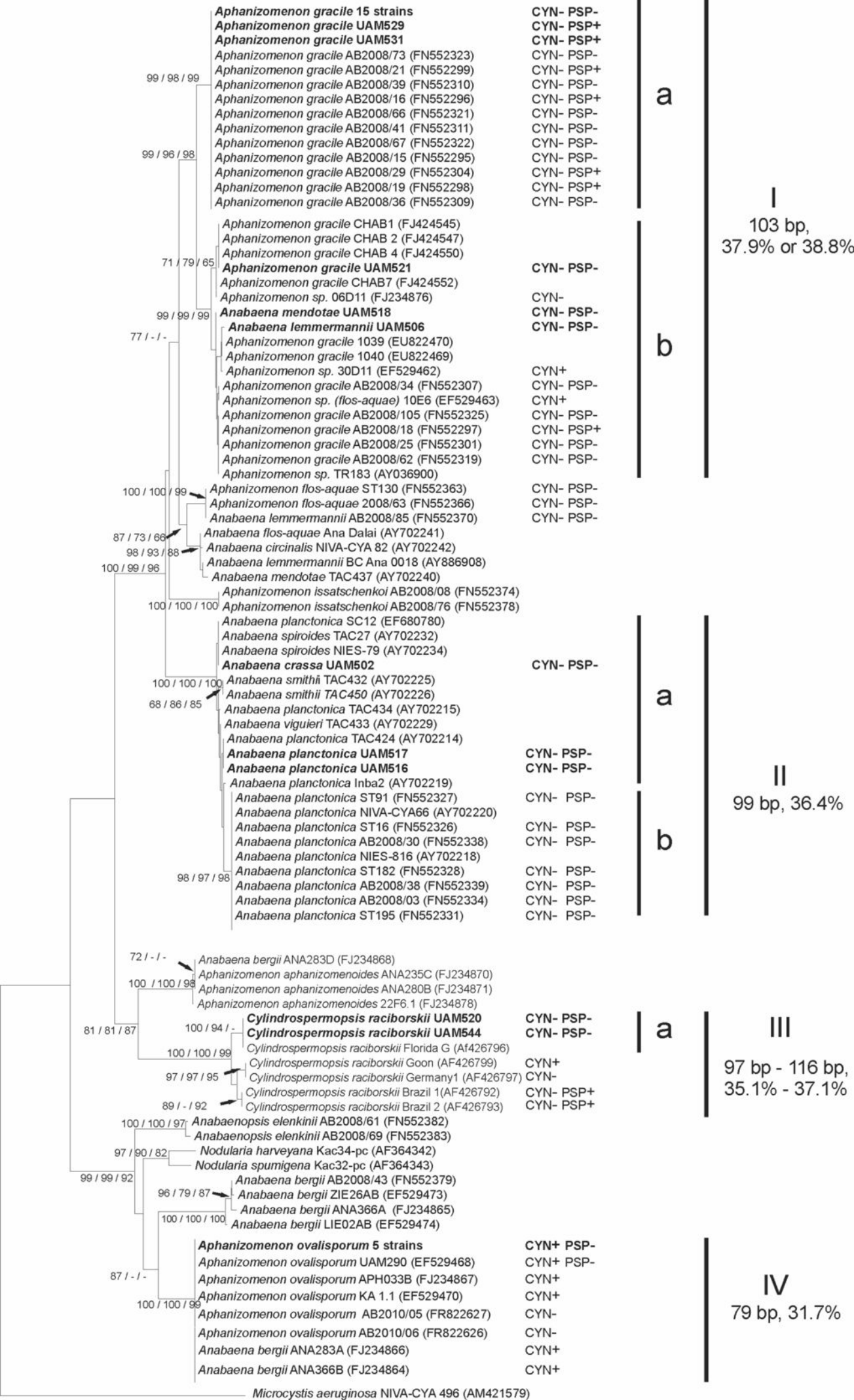
746 **TABLE 3. Comparison of cylindrospermopsin content in exponentially growing**
747 **batch cultures of *Aphanizomenon ovalisporum*. Values are expressed as the mean \pm**
748 **SD (n = 2).**

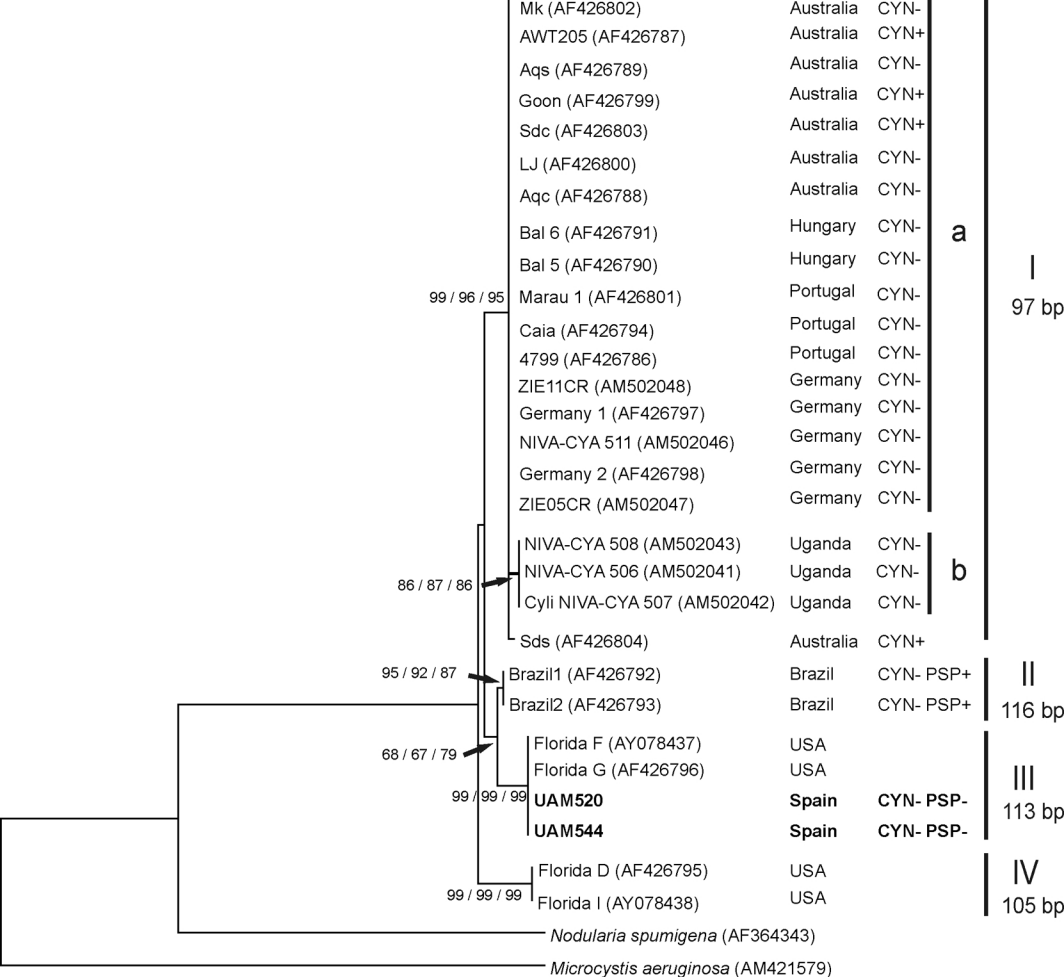
Strain	Water body	Total CYN				Dissolved CYN (% of total CYN)
		($\mu\text{g mg}^{-1}$ DW)	($\mu\text{g mm}^{-3}$)	(fg cell $^{-1}$)	($\mu\text{g } \mu\text{g}^{-1}$ Chl <i>a</i>)	
UAM 287	Juan Carlos I	9.1 \pm 2.9	2.1 \pm 1.5	115.2 \pm 81.1	0.5 \pm 0.3	35.3 \pm 0.8
UAM 289	Juan Carlos I	6.4 \pm 0.6	2.4 \pm 0.3	190.6 \pm 26.6	0.5 \pm 0.1	25.2 \pm 3.7
UAM 290	Juan Carlos I	5.9 \pm 1.4	2.1 \pm 0.5	110.4 \pm 27.1	0.5 \pm 0.1	19.4 \pm 5.3
UAM 507	Arcos	7.3 \pm 2.2	1.4 \pm 0.4	75.7 \pm 21.6	0.4 \pm 0.2	31.1 \pm 15.3
UAM 536	Alange	7.6 \pm 0.2	1.1 \pm 0.1	59.5 \pm 5.4	0.3 \pm 0.1	31.5 \pm 9.2
UAM 537	Alange	5.7 \pm 0.9	0.9 \pm 0.1	48.7 \pm 5.4	0.3 \pm 0.1	41.5 \pm 4.9

TABLE 4. Features of *Cylindrospermopsis raciborskii* strains from different continents included in the *cpcBA*-IGS tree (see FIG. 3). ^a, DNA similarities (%), calculated as substitutions per site (504-bp alignment).

Cluster	Geographical origin	<i>cpcBA</i> -IGS similarity ^a		IGS length (bp)
		Intra-cluster	vs. cluster III	
I		99.8–100	97.1–97.5	97
Subcluster Ia	Africa (Uganda), Australia, Europe (Germany, Hungary and Portugal)	99.8–100	97.3–97.5	97
Subcluster Ib	Africa (Uganda)	99.8	97.1	97
II	America (Brazil)	100	98.4	116
III	America (USA), Europe (Spain)	100	–	113
IV	America (USA)	100	96.4	105







a

b

I

97 bp

II

116 bp

III

113 bp

IV

105 bp

