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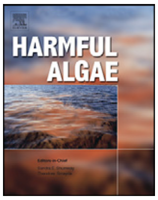

Genetic and eco-physiological differences of South American *Cylindrospermopsis raciborskii* isolates support the hypothesis of multiple ecotypes

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ABSTRACT

The global distribution of the toxic cyanobacterium *Cylindrospermopsis raciborskii* has recently increased, and it has now been identified in tropical, subtropical and temperate freshwater bodies. The mechanisms underlying its success and expansion are still unknown. Several hypotheses have been proposed, including climate change, natural selection and physiological tolerance to different environmental conditions. In this study, we determined the phenotypic and genotypic characteristics of two recently isolated South American strains of *C. raciborskii* obtained from Uruguay. We analyzed the morphology, growth preferences, tolerance to low temperature (14 °C) and toxin production of the strains and performed phylogenetic analyses based on the ITS and *nifH* gene sequences. Both isolates showed significantly different morphology and growth behavior under different light intensities and phosphate supply. When genetic differences were assessed by BOX PCR, cluster analyses revealed that they could also be distinguished genotypically and were clearly distinct from *C. raciborskii* isolated from other continents. Phylogenetic analysis showed that the Uruguayan strains were closely affiliated to other *C. raciborskii* isolated from the Americas, especially to those from Brazil. Similar to previous studies, we found three solid clusters (Africa-Australia, Europe and America) according to the geographical origin of the isolates. Interestingly, based on *nifH* sequences, subclusters were identified in American populations indicating an early spread of the species within the continent. We propose that phenotypic and genetic variability of *C. raciborskii* populations is linked to the existence of different ecotypes whose success is subject to the local environmental conditions.

1. Introduction

Cylindrospermopsis raciborskii is a freshwater cyanobacterium originally described from the tropics (see Padisák, 1997). Interest in this species has increased due to its ability to produce potent toxins and its expansion into subtropical and temperate freshwater bodies (Chapman and Schelske, 1997; Saker and Griffiths, 2000; Hamilton et al., 2005; Hong et al., 2006; Figueredo et al., 2007; Messineo et al., 2010). Whether *C. raciborskii* can be considered an invasive species and which environmental constraints explain its behavior are still under debate. It has been suggested that the invasive capacity of *C. raciborskii* is due to its low light tolerance (subsaturating light intensity, $I_k \leq 26$ μmol photons m^{-2} s^{-1}), and high phosphorus affinity and storage capacity (Padisák and Reynolds, 1998; Istvánovics et al., 2000; Briand et al., 2004; Burford and O'Donohue, 2006). Different hypotheses explaining the dispersal of this species include the central role of ecotype selection (Chonudomkul et al., 2004), the accelerated growth of the species in temperate regions due to increased water temperature driven by climate change (Wiedner et al., 2007) and ecophysiological plasticity that enables *C. raciborskii* to succeed in a wide range of environmental conditions (Briand et al., 2004). However, deeper analyses considering the historical processes that may be responsible for the contemporary geographic distributions of individuals are still required.

Molecular analysis is a powerful tool for studying phylogeography from an evolutionary perspective. Phylogeographic analysis using the 16S–23S internal transcribed spacer (ITS) of the

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ribosomal operon and nitrogenase (*nifH*) gene sequences showed a separation of *C. raciborskii* strains according to their geographic origin into American, European and Australian-African groups (Gugger et al., 2005). A similar separation was found when the phycocyanin intergenic spacer (PC-IGS) and *rpoC1* genes were included in the analyses (Haande et al., 2008). These results suggested an early evolutionary division of *C. raciborskii* populations into American, European and Australia-Africa groups and a recent spread of the species within America and Europe (Dyble et al., 2002; Neilan et al., 2003; Gugger et al., 2005).

According to PCR fingerprinting of short tandem repeated sequences the between-country genetic variability of *C. raciborskii* strains showed to be higher than the within-country variation (Neilan et al., 2003). Using a similar fingerprinting technique, Chonudomkul et al. (2004) found that DNA band patterns distinguished *C. raciborskii* strains from Thailand and Japan, suggesting high variability between isolates. Further information on recently isolated *C. raciborskii* strains is therefore critical for improving our understanding of its phylogeography.

The toxicity of *C. raciborskii* also differs among strains from different continents. Many European strains are hepatotoxic according to mouse bioassays (Bernard et al., 2003; Saker et al., 2003; Fastner et al., 2003) although the toxic compound has not been identified yet. Messineo et al. (2010) detected cylindrospermopsin in an Italian lake with *C. raciborskii* dominance in summer phytoplankton. Cylindrospermopsin-producing strains have been isolated in Australia and Asia (Saker et al., 1999), while no strains producing cylindrospermopsin have yet been found in the Americas or in Africa (Saker et al., 2003). Less information is available about the production of saxitoxin by this species. To date, only Brazilian strains have been found to produce saxitoxins, neosaxitoxins and gonyautoxins (Lagos et al., 1999; Bouvy et al., 2003). Interestingly, it has been shown that toxicity and morphology are not always correlated to phylogeny. For example, Stucken et al. (2009) found toxic and non-toxic strains from the same geographic area, while Chonudomkul et al. (2004) found that morphology (coiled and straight trichomes) was not associated to phylogeny in strains from Thailand and Japan.

There is growing evidence that natural microbial populations are composed of several ecotypes, that is, a clade of microorganisms that shares ecological characteristics. Genetic diversity within the ecotype is limited by a cohesive force, such as periodic selection or genetic drift, or both (Cohan, 2005). For instance, ecotypes have been discovered for several taxa such as *Prochlorococcus* (Moore et al., 1998), SAR11 clade (Carlson et al., 2009) and *Alteromonas macleodii* (Ivars-Martinez et al., 2008). In these studies, the authors found that the different ecotypes were observed to have divergent environmental preferences, including depth, light intensity, high or low nutrients concentrations and organic matter sources. While the mechanisms underlying the prevalence of one ecotype over others are not well understood, they should involve physiological adaptations (Carlson et al., 2009). The worldwide spread of *C. raciborskii* suggests the existence of multiple ecotypes with differences in their physiology, which could explain the success of this species in different environments. In this sense, Saker and Neilan (2001) distinguished different ecotypes by their growth rates and cylindrospermopsin production under nitrogen stress. Moreover, Chonudomkul et al. (2004) found differences in temperature tolerance between ecotypes.

Studies addressing morphological parameters also improve the understanding of *C. raciborskii* success at invading new environments, since morphology reflects organisms' ecological aptitude and responses to environmental change (Kruk et al., 2010; Whitfield, 2001). For example, it has been demonstrated that *C. raciborskii* filament morphology changes in response to the presence of different nitrogen sources (Saker and Neilan, 2001). Thus, different *C. raciborskii* morphotypes might have different fitness and result in ecotypes with differences in their morphology. Moreover, morphological variation related to ecophysiology can have serious implications in classic taxonomic identification especially in less studied areas (Komárek and Komárková, 2003; Komárek, 2010).

The relationship between toxicity, genetic diversity, morphology and ecophysiology in *C. raciborskii* has therefore not yet been elucidated and there is no consensus about the mechanism underlying the worldwide expansion of this species. In order to understand the distribution patterns and ecological preferences of *C. raciborskii*, integrative studies linking phylogeny, physiology (including toxin production) and morphology are needed. We propose that the success of *C. raciborskii* and its increasing ability to form blooms in higher latitudes is related to the existence of ecotypes which allow this species to adapt to different environmental conditions within and between continents. Therefore, our objective was to analyze *C. raciborskii* isolates from shallow water bodies in Uruguay and to determine the existence of ecotypes by studying morphological, physiological and genetic characteristics. With this purpose, we first compared their size, shape and growth under different experimental conditions and then we described their toxicity and genetic profile (rep-PCR). Finally, we carried out phylogenetic analyses on the sequences obtained by the ITS, 16S rRNA and *nifH* genes.

2. Materials and methods

2.1. Isolates and culture conditions of *C. raciborskii*

Isolate MVCC14 was collected in Laguna Blanca (34°53′S, 54°20′W), an eutrophic shallow lagoon (area: 0.41 km^2, Z_{max}: 2.6 m, total phosphorus: 2.76 μM) used as a drinking water supply (Vidal and Kruk, 2008). Isolate MVCC19 was obtained from Lago Javier (34°51′S, 56°02′W), a eutrophic artificial lake (area: 0.10 km^2, Z_{max}: 9.8 m, total phosphorus: 1.97 μM; Vidal and Kruk, 2008). In addition, two *C. raciborskii* strains from other countries were used for rep-PCR analyses, CCMP1973 from USA (purchased at the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, USA) and CYP011K from Australia (kindly provided by Andrew Humpage, SA Water and University of Adelaide Medical School, Adelaide, Australia). In order to minimize laboratory-induced differences between cultures (Lakeman et al., 2009), both isolates were obtained from eutrophic lakes in Uruguay during a single sampling campaign (in January 2007 which corresponds to summer in Southern Hemisphere) and maintained in the laboratory under identical culture conditions. All isolates were incubated in BG11 culture medium, modified to have 25% of the $CuSO_4$ of the original recipe after Stanier et al. (1971). Isolates were maintained at 26 °C, normal summer temperature at the Uruguayan lakes where this species inhabits (Vidal and Kruk, 2008). Light intensity was 80 μmol photons m^{-2} s^{-1} provided by daylight fluorescent tubes with a 16:8 h light:dark photoperiod. Static cultures were maintained in 500 ml borosilicate culture bottles that were continuously bubbled with saturated humid air, pre-bubbled in ultrapure sterilized water and pre-filtered. Cultures kept under these conditions were used to determine morphology and to detect toxins.

2.2. Isolate morphology

We estimated the morphological characteristics of the trichomes of MVCC14 and MVCC19 including length, width, volume (V) and surface-volume ratios (S/V) based on the measurement of 60 trichomes under a light microscope (Olympus