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PHENOTYPIC AND GENETIC DIVERSITIES ARE NOT CORRELATED IN STRAINS OF THE CYANOBACTERIUM *MICROCYSTIS AERUGINOSA* ISOLATED IN SW SPAIN

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ABSTRACT. *Phenotypic and genetic diversities are not correlated in strains of the cyanobacterium Microcystis aeruginosa isolated in SW Spain.* The cyanobacterium *Microcystis aeruginosa* (Kützing) Kützing is notorious for forming extensive and toxic blooms but the genetic structure of natural populations, and in particular during blooms, remains to be explored. In order to add more knowledge about the genetic structure of *M. aeruginosa*, we compared phenotypic and genetic variabilities in seventeen strains of *M. aeruginosa* isolated from three adjacent water bodies in SW Spain (the consecutive reservoirs La Minilla and El Gergal, and small pools in Doñana National Park). For this purpose, a phenetic tree, based on six phenotypic traits (including morphology of cells, photosynthetic and respiratory performance, and toxin production) was compared with a phylogenetic tree built by using the 16S-23S rDNA Internal Transcribed Spacer sequence. Whereas the strains isolated from La Minilla showed a relatively homogeneous phenotype, the phenotypic traits of the remaining strains did not discriminate between the other two water bodies. On the other hand, only a strain isolated in Doñana National Park showed a slightly different ITS sequence, but the sequences from the remaining strains were similar. Possible explanations for this discrepancy between phenotypic and genetic diversities are discussed.

Key words. Genotype, ITS sequencing, *Microcystis*, phenotype.

RESUMEN. *Las diversidades fenotípica y genética de cepas de la cianobacteria Microcystis aeruginosa aisladas en el SW de España no están correlacionadas*. La cianobacteria *Microcystis aeruginosa* (Kützing) Kützing puede dar lugar a extensas floraciones tóxicas pero la estructura genética de las poblaciones naturales, y en particular durante las floraciones, ha sido escasamente estudiada. Con el objeto de profundizar en la estructura genética de *M. aeruginosa*, se han comparado las variabilidades fenotípica y genética de diecisiete cepas aisladas de tres cuerpos de agua adyacentes en el SW de España (los embalses consecutivos de La

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Minilla y El Gergal, y charcas en el Parque Nacional de Doñana). Para este fin, un árbol de similitud fenética basado en seis caracteres fenotípicos (que incluyen la morfología de las células, el rendimiento fotosintético y respiratorio y la producción de toxina) se comparó con un árbol filogenético construido mediante el uso de la secuencia de ITS 16S - 23S del ADNr. Las cepas aisladas en La Minilla mostraron un fenotipo relativamente homogéneo pero no se detectó un patrón de fenotipos que discriminase los otros dos cuerpos de agua. Por otro lado, sólo una cepa aislada en el Parque Nacional de Doñana mostró una secuencia ITS diferente, pero las secuencias de las cepas restantes fueron idénticas. Se discuten las posibles explicaciones para esta discrepancia entre la diversidad fenotípica y genética en *M. aeruginosa*.

Palabras clave. Fenotipo, genotipo, *Microcystis*, secuencia ITS.

INTRODUCTION

The cyanobacterium *Microcystis aeruginosa* (Kützing) Kützing is notorious for forming extensive and toxic blooms in nutrient-rich freshwater bodies worldwide, constituting a serious threat for both humans (Codd *et al*. 1999; Carmichael *et al*. 2001; Azevedo *et al*. 2002; Chen *et al*. 2009a) and wildlife (Carmichael & Hui 2006; López-Rodas *et al*. 2008; Chen *et al*. 2009b). The population structure in *M. aeruginosa* is mainly clonal and, when blooms occur, the structure is comparable to epidemics of pathogens (Tanabe & Watanabe, 2011). Although the genetic structure of *M. aeruginosa* populations at large geographical scales has been addressed (Yoshida *et al*. 2008; van Gremberghe *et al*. 2011), knowledge of the genetic structure at local scales (in particular, during single blooms) has scarcely been studied (Carrillo *et al*. 2003; Martín *et al*. 2004; Briand *et al*. 2009). For instance, a comparison of the ITS sequence in a large array of strains of *M. aeruginosa* available in the GenBank database showed that some highly conserved genotypes are found worldwide (Humbert *et al*. 2005). Moreover, at a global scale, it was demonstrated that *M. aeruginosa* lacks phylogeographic structure, which suggests that this species might have a truly cosmopolitan distribution (van Gremberghe *et al*. 2011). At a local scale, the studies on genetic structure of *M. aeruginosa* populations have been mainly focused on genes involved in microcystins (Wilson *et al*. 2005; Briand *et al*. 2009; Sabart *et al*., 2009). However, an approach based on several phenotypic traits could help us better understand the genetic structure of natural populations of *M. aeruginosa*. Consistent with this idea, genetics has been found to explain a very high percentage of the phenotypic variability in several strains isolated from SW Spain (Carrillo *et al*. 2003; Martín *et al*. 2004; Bañares-España *et al*. 2006, 2007; López-Rodas *et al*. 2006; Rico *et al*. 2006).

The aim of this work was to study the possible correlation between phenotypic and genetic diversities, in strains of *M. aeruginosa* isolated from three different but connected water bodies in SW Spain. It can be hypothesized that the high phenotypic variability in several morphological and physiological traits could be due to the coexistence of different genetic variants. For this reason, we compared a phenotypic similarity tree with a similarity tree based on rDNA sequences. Moreover, since the three water bodies where the strains of *M. aeruginosa* originated are connected within the same river basin, we hypothesized that the phenotypic and genetic similarities would be greater the nearer the water bodies are to each other.

MATERIALS AND METHODS

Collection sites and culture strains

Seventeen different strains of *M. aeruginosa* were isolated from three different water bodies in SW Spain (Doñana National Park, and

Water body	Date of isolation	Code of the strains
La Minilla	XII 1999	MaM1-MaM9
El Gergal	IV-VI 1999	MaG4-MaG6
Doñana	IV 2001	MaD3-MaD5, MaD7 and MaD8

Table 1. Isolation data of *Microcystis aeruginosa* strains from three water bodies in SW Spain, and codes of the strains in the Algal Culture Collection of the Veterinary School (UCM, Spain). *Fecha de los aislamientos de las cepas de Microcystis aeruginosa en tres masas de agua del SW de España, y códigos de las cepas en la Colección de Cultivos de Algas de la Facultad de Veterinaria (UCM, España).*

La Minilla and El Gergal reservoirs; tab. 1). La Minilla and El Gergal are separated by around 35 km but are connected by the river Rivera de Huelva, which is a tributary of the Gualdalquivir River. Doñana National Park is located along the final stretch of the Guadalquivir River (around 125 km from El Gergal reservoir).

All the strains are deposited in the Culture Collection of the Veterinary School (UCM, Spain). Only colonies with clear *M. aeruginosa* morphotypes, fitting in the middle of the range of cell and colony size, were isolated. Isolation procedures and culture methods were as described in Carrillo *et al*. (2003), López-Rodas *et al*. (2006) and Rico *et al*. (2006).

Phenetic analysis

A neighbour-joining clustering of the seventeen strains of *M. aeruginosa*, based on Euclidean distances, was performed by using dark respiration rate, gross photosynthetic capacity, maximum quantum yield, acclimated maximal growth rate, per-cell microcystin production, and area of cell cross-section, as phenotypic variables; these data were compiled from López-Rodas *et al*. (2006) and Rico *et al*. (2006). The traits selected were those that showed the greatest contribution of genetics to the phenotypic diversity. Previous to the clustering analysis, the phenotypic variables were typified (by subtracting the mean, and dividing by the standard deviation) in order to avoid an unbalanced contribution of any of them.

DNA sequencing and phylogenetic analysis

The nuclear marker rDNA Internal Transcribed Spacer sequence (ITS region thereafter) was selected to analyse the within-populations structure of *M. aeruginosa* from the different sites. This marker was chosen because it has been widely used for intraspecific studies due to its high sequence variation (Hillis & Dixon 1991). DNA extractions were carried out on the 16S-23S ribosomal region using an ExtraGen® kit. DNA obtained this way was amplified using PCR. The primer used for the DNA extraction was AF387609, yielding 580 bp, which is the entire ITS region. After the analyses, all sequences were double-checked with the AF387609 primer. The sequence legibility was high for all the PCR products, and no equivocal sites were observed. Sequences were edited and aligned by using CLUSTAL W (Thompson *et al*. 1994; available from http://www.ebi.ac.uk). A phylogenetic tree was carried out using a neighbour-joining approach based on Euclidean distances.

Statistical analysis

Comparisons of phenotypic traits among the strains from different geographical origins were performed using the non-parametric Kruskall-Wallis test; when significant differences were found, the Mann-Whitney test, with

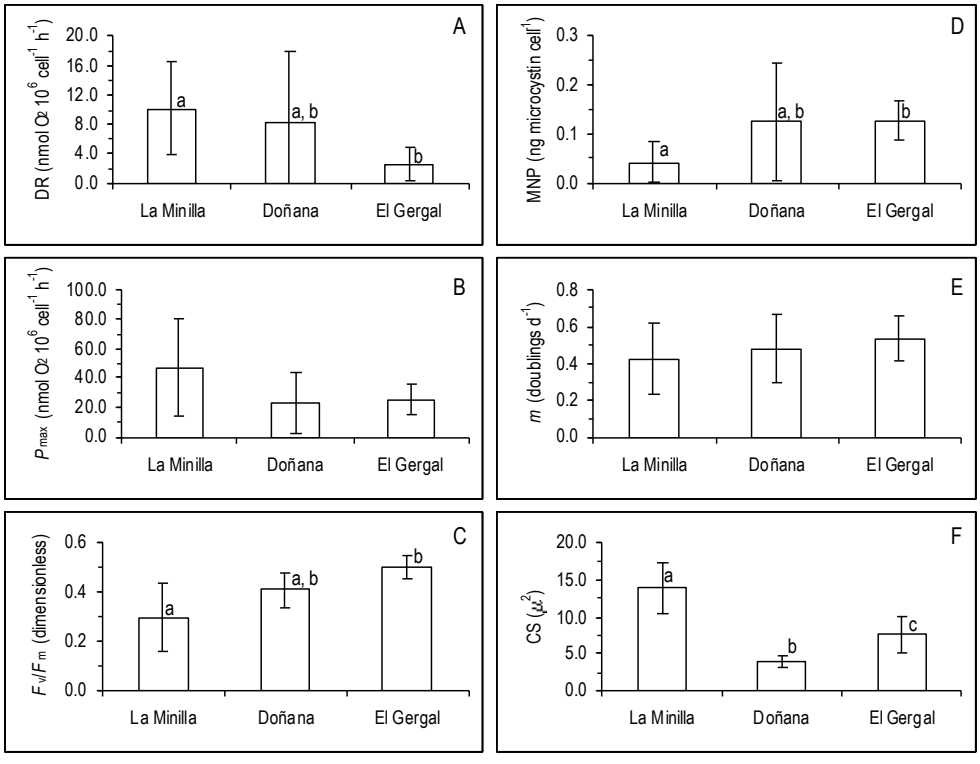

Figura 1. Differences in quantitative phenotypic traits among strains of *Microcystis aeruginosa* isolated from three different water bodies in SW Spain (mean ± SD; $n = 7$, 5 and 3 strains from La Minilla, Doñana and El Gergal, respectively). Significant differences among locations, detected by Kruskall-Wallis analysis and post-hoc Mann-Whitney test, are indicated by different letters next to bars. 1A: DR, dark respiration rate ($H = 7.528$, $p = 0.02319$); 1B: P_{max}, gross photosynthetic capacity ($H = 2.358$, $p = 0.3076$); 1C: F_v/F_m, maximum quantum yield ($H = 7.027$, $p = 0.02979$); 1D: MNP, microcystin net production ($H = 5.435$, $p = 0.06603$); 1E: m, acclimated maximal growth rate ($H = 1.009$, $p = 0.6039$); 1F: CS, area of cell cross-section ($H = 12.75$, $p = 0.0017$). *Diferencias en caracteres fenotípicos en cepas de Microcystis aeruginosa aisladas de tres masas de agua del SW de España (media ± DE; n = 7, 5 y 3 cepas de La Minilla, Doñana y El Gergal, respectivamente). Las diferencias significativas entre las localidads, detectadas mediante el test de Kruskall-Wallis y el test a posteriori de Mann-Whitney, se indican mediante diferentes letras junto a las barras. 1A: DR, tasa de respiración en oscuridad (H = 7.528, p = 0.02319); 1B: P_{max}, capacidad fotosintética bruta (H = 2.358, p = 0.3076); 1C: F_v/F_m, rendimiento cuántico máximo (H = 7.027, p = 0.02979); 1D: MNP, producción neta de microcistina (H = 5.435, p = 0.06603); 1E: m, tasa de crecimiento máxima (H = 1.009, p = 0.6039); 1F: CS, sección transversal de la célula (H = 12.75, p = 0.0017).*

the Bonferroni correction, was applied. All the statistical analyses were carried out in accordance with Zar (1999). The statistical tests and the neighbour-joining trees were performed using the free software PAST ver. 2.17 (Hammer *et al*. 2001) accessible at http://nhm2.uio.no/norlex/past/download.html.

RESULTS

The area of the cell cross-section (an estimator of the size of the cells since they are spheres) was the only phenotypic trait that showed significant differences among the three different geographical origins of the strains

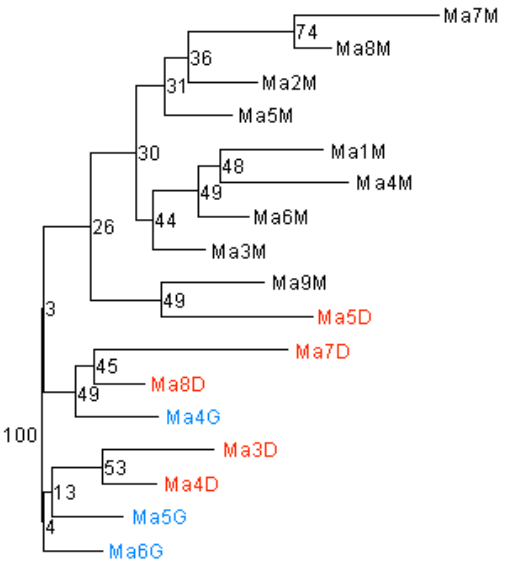

Figura 2. Neighbour-joining clustering for seventeen strains of *Microcystis aeruginosa* isolated from three different water bodies in SW Spain (La Minilla, M code label; Doñana,D code label; and El Gergal, G code label), based on six quantitative phenotypic traits (dark respiration rate, gross photosynthetic capacity, maximum quantum yield, microcystin net production, acclimated maximal growth rate and area of cell cross-section). The bootstrap value (*n* = 10,000) is indicated at the base of each branch. *Agrupación por máxima similitud de diecisiete cepas de Microcystis aeruginosa aisladas de tres diferentes masas de agua en el SW de España (La Minilla, etiqueta de código de M; Doñana, etiqueta de código D, y El Gergal, etiqueta de código G), a partir de seis caracteres fenotípicos cuantitativos (tasa de respiración en oscuridad, capacidad fotosintética bruta, rendimiento cuántico máximo, producción neta de microcistina, tasa de crecimiento máxima y sección transversal de la célula. El valor de bootstrap (n = 10 000) se indica en la base de cada rama.*

(fig. 1F). Although other phenotypic traits also showed significant differences in accordance with their origins (i.e. dark respiration rate, maximum quantum yield and microcystin net production), the post-hoc analysis demonstrated that at least at one of the locations, no significant differences were detected in comparison to the other sites (figs 1A, 1C and 1D). On the other hand, all the strains (independently of their origins) showed a similar gross photosynthetic capacity (fig. 1B) and acclimated maximal growth rate (fig. 1E).

The neighbour-joining phenotypic tree for the seventeen strains showed that the strains isolated from La Minilla are closely similar, but a clear pattern among the strains isolated from Doñana and El Gergal was not found (fig. 2).

The neighbour-joining phylogenetic tree for the seventeen strains, based on the 16S-23S ITS region sequencing, showed that all the strains were similar, with the exception of one strain, Ma7D, isolated from Doñana National Park (Euclidean distance = 0.024; data not shown); this strain is characterized by the highest production of microcystin among all the strains (López-Rodas *et al*. 2006).

DISCUSSION

Only the area of the cross section of the cells of *M. aeruginosa* allows us to characterize the three water bodies where the strains were isolated. The remaining phenotypic traits were not correlated with the strain origins (fig. 1). Although the strains isolated from La Minilla showed a relatively homogeneous phenotype (fig. 2), the strains from the other two water bodies did not show any clear pattern, suggesting that the phenotypic variability within a given water body could be similar or greater than among different water bodies. It must be emphasized that La Minilla and El Gergal are connected by the same river, although separated by a distance of around 35 km, and that the sampling of the strains of *M. aeruginosa* was carried out during the same year (tab. 1). On the other hand, water pools in Doñana National Park ,where strains were isolated two years later than those from La Minilla and El Gergal, are located about 125 km from El Gergal. For this reason, the hypothesis of a higher similarity of the strains isolated from La Minilla and El

Gergal, in comparison to those isolated from Doñana, must be rejected.

No genetic diversity was detected based on the 16S-23S rDNA, with the exception of a single strain from Doñana; this strain is characterized by very high levels of toxin production per cell (Carrillo *et al*. 2003; López-Rodas *et al*. 2006). Since we did not sequence any gene involved in the synthesis of microcystin, we cannot explore the genetic diversity for toxins. However, it is worth noting that the high phenotypic diversity found (with a homogeneous pattern for the strains from La Minilla and no clear pattern for the strains isolated from El Gergal and Doñana) is not reflected by the 16S-23S rDNA sequences. The lack of correlation of genetic and phenotypic variabilities suggests that a low-diversity genetic pool can produce different phenotypes. However, another possible explanation is that the 16S-23S rDNA sequences are not an adequate proxy to detect genetic diversity. In fact, a very high genetic diversity has been proposed for the strains of *M. aeruginosa* studied in this work by using different Quantitative Genetics approaches (Carrillo *et al*. 2003; Martín *et al*. 2004; Bañares-España *et al*. 2006, 2007; López-Rodas *et al*. 2006; Rico *et al*. 2006; Rouco *et al*. 2011). Further studies using specific gene sequences, such as for toxin production, could help to achieve a better understanding of the genetic structure of *M. aeruginosa* populations. In this sense, it has been found that marked changes occurred in the ITS genotype composition of a *M. aeruginosa* population during the development of the bloom in a reservoir in France (Briand *et al*. 2009). These changes led progressively to the selection of one dominant ITS genotype throughout the entire reservoir. At the same time, a decrease occurred in the proportion of the mcyB+ genotype, with a significant negative correlation between this proportion and that of the dominant ITS genotype during the bloom. Thus, it appeared that favourable conditions for *M. aeruginosa* cell growth led to the selection of a non-microcystin-producing genotype, whereas potentially microcystin-producing genotypes were dominant in this population before and after the bloom, when environmental conditions were less favourable for growth (Briand *et al*. 2009).

The genetic structure of natural populations of *M. aeruginosa*, at a local scale, and a short time period, remains as a relevant scientific problem. The results of our study contribute by adding a paradoxical result showing that phenotypic and genetic diversities are not correlated.

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