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Should ecomorphs be conserved? The case of *Nostoc flagelliforme*, an endangered extremophile cyanobacteria

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ABSTRACT

Nostoc flagelliforme has been reported from deserts of all continents and more recently from semiarid environments in south-east Spain and Australia. Its cylindrical thalli are very conspicuous on soils after rains and can be easily differentiated from other taxa and it is considered endangered in some countries, e. g. China. It was firstly described as variety *flagelliforme* of *Nostoc commune* but later was considered a separate species. The morphology, fine structure and ecology of populations of both taxa from Australia and different regions of Spain were studied and 16S rRNA and *trn*L^{Leu} (UAA) genes were sequenced to identify their intraspecific genetic variation. The morphological study revealed the presence of several intermediate morphs, from spheres and sheets to ribbons followed by the cylindrical thalli and the overlapping of cell dimensions, permitting the differentiation of cylindrical morphs from the others. The molecular data did not allow for a distinction between *N. commune* and *N. flagelliforme* which is clearly polyphyletic and both show a high genetic diversity. The presence of cylindrical thalli seems to be related to extreme conditions of aridity, usually on soils but occasionally on rock walls. The ecotype that *N. flagelliforme* represents deserves to be protected for itself and for the evolutionary process it represents opening up the possibility of implementation of recovery measures and even opportunities to explore its biotechnological production from a different point of view.

1. Introduction

Nostoc flagelliforme has been reported in most deserts and arid zones of the world: Europe (France and Spain); America (Mexico, USA); Africa (Morocco, Somalia, South Africa, Seychelles islands, Aldabra Atoll); Asia (China, Mongolia), and Australia (South Australia, Western Australia and Northern Territory) (Aboal, Cristobal, & Marín-Murcia, 2010; Caraus, 2002; Frémy, 1929; Gao, 1998; Geitler, 1930–1932; Scherer & Zhong, 1991; Skinner & Entwisle, 2001; Wright, Prickett, Helm, & Potts, 2001).

In the semiarid regions of south-east Spain *N. flagelliforme* is fairly abundant on clay and silt soils with a slight slope. During the dry periods this species remains inconspicuous but after the rains the masses of cylindrical thalli are clearly visible (Aboal et al., 2010). Earlier Australian records were herbarium specimens from deserts (Skinner & Entwisle, 2001).

The separation of the variety *flagelliforme* from *Nostoc commune* was proposed by Bornet and Flahault (1885–1887) based on the cylindrical thallus and the parallel arrangement of the trichomes, indicating that it was also more frequent on sandy soils. Mollenhauer, Bengtsson, & Lindstrom (1999) attempted to clarify *Nostoc* taxonomy and more recently the phylogeny of the genus has been studied, particularly the macroscopic species and those from deserts (García-Pichel et al., 2001), however the genomic information was inconclusive.

Several authors point out the importance of a polyphasic approach to unravel the phylogenetic relationships between species and to generate a more natural taxonomy on cyanobacteria (Komárek & Mares, 2012; Sciuto et al., 2011). However there is still scarce information on the genetic variation of natural populations. Yet species like *N. flagelliforme* are considered endangered in China where it has been intensely collected for its use as a delicacy in

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Chinese cuisine (Takenaka et al., 1998). The increasing rarity of the species has promoted a trade of substitutes and the parallel development of methods of production to avoid its exploitation (Li et al., 2011; Gao & Changpeng, 2003).

Nostoc seems to be a genetically complex genus (Wright et al., 2001) with a high level of morphological diversity and a broad ecological range.

In an effort to resolve the present endangered status of *N. flagelliforme* we undertook a study of the morphological, ultra-structural, ecological and genomic variability (*trnL*^{Leu}(UAA), 16 S rRNA). This was carried out on the basis of samples collected across semiarid areas from south-east Spain and Australia and data from all continents obtained from GeneBank.

2. Materials and methods

2.1. Study area

Most of the samples were collected from Keuper (Triassic) clay and silt soils, sandy soils or calcareous rocks, flat or slightly sloped (<15%), at 200–900 m altitude in localities of south-east Spain. The climate was semi-arid, Mediterranean with mean temperatures of 13–19 °C and 200–350 mm precipitation. Even when south-east Spain was more intensely prospected some samples were also collected from other Spanish regions with higher latitude or rainfall. Most samples were subaerial, with only one that was collected in a small hole in a calcareous rock and another that was epiphytic on a calcareous saxicolous lichen (*Collema*). Samples from southern Australia were also collected in semiarid regions with mean average temperatures ranging between 9–25 °C and precipitation between 224 and 324 mm. All of the Australian samples were collected from carbonate sands and calcarosols, low relief (<10% slope).

2.2. Sampling and collection

Samples were collected in paper envelopes or steril plastic vials and desiccated at ambient temperatures or preserved with 3.7% formalin. Australian samples were collected and preserved in the same manner and sent by airmail to Murcia. A representative fraction of all samples was deposited in MUB-ALGAS Herbarium from Murcia University. The field material studied and sequenced with indication of the GenBank accession numbers are compiled in Table 1.

For the molecular study we added samples available at GenBank (Table 2). In the case of the *trnL*^{Leu} (UAA) region we essentially choose the sequences published by Wright et al. (2001), because they are a good representation of *N. flagelliforme*, *N. commune* and some related taxa, and include samples from all continents. We further added all the samples identified in Genbank as *N. flagelliforme* or *N. commune* var. *flagelliforme*. In the case of the 16S rRNA region we added a representation of the available sequences that had a similar or greater length as the fragment sequenced by us. *Anabaena* sequences were used in both cases to root the trees.

2.3. Morphological studies

The material was rehydrated and then studied with a stereomicroscope. The fine sections were observed with a light microscope OLYMPUS ZH equipped with inter-differential contrast and a digital camera. The bionomic study was undertaken with the OLYMPUS Cell P® digital imaging software. Firstly the material was identified and separated according to the taxonomic characters indicated in the monography of Komárek (2013) and then it was studied based on the following morphological characters: thallus form (sheets, ribbon-like, cylindrical or vesiculous); external surface; inner and outer ordination of filaments in cross sections; density of filaments; structure and color of the sheaths in the inner and outer regions; dimensions of vegetative cells; frequency and dimensions of heterocytes; and, presence of fungal hyphae and bacteria inside thalli. At least twenty different filaments were measured of each morphotype at 1200×, and the mean and extreme values were calculated.

2.4. Ultrastructural studies

A part of the material was preserved in the field with 2% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M cacodylate buffer, postfixed with 8% osmium tetroxide for 2 h at 4 °C, dehydrated in an acetone series and embedded in Spurr's resin. The ultrathin sections were stained with uranyl acetate and lead citrate (Kaneko, Danev, Nagayama, & Nakamoto, 2006) and observed with the transmission electron microscope PHILIPS TECNAI equipped with a digital camera in the Microscopy Service of Murcia University.

2.5. Extraction of DNA and sequencing

Total DNA was extracted from the material using the NaOH extraction method as explained in Werner, Ros, & Guerra (2002). The 16S rRNA gene was amplified using the primers 5′ GGG GAA TTT TCC GCA ATG GG 3′ (after Nübel, García-Pichel, & Muyzer, 1997) and primer 5′ GAC GGG CCG GTG TGT ACA 3′ (after Wilmotte, Van der Auwera, & De Wachter, 1993). For the amplification of the trnL^{Leu} (UAA) intron we used the primers LEUI1 (5′-TGT GGC GGA ATG GTA GAC GCT AC-3′) and LEUI2 (5′-GAC TTG AAC CCA CAC GAC-3′) of Wright et al. (2001). Both genetic regions were selected because prior studies made available sequence information at a broad geographic scale of *Nostoc* specimens and covering related taxa and have shown within species variability necessary to calculate population genetic parameters.

The final concentration of the primers was 400 μM. 4 μL of stock DNA were added as template. 200 μM of each dNTP, 2 mM $MgCl_2$, 2 units DreamTaq Green DNA polymerase (Fermentas), 1 μL BLOTTO (10% skimmed milk powder and 0.2% NaN_3 in water) and the buffer provided by the enzyme supplier were added. BLOTTO attenuates PCR inhibition caused by plant compounds (De Boer et al., 1995). The amplification conditions were as follows: 3 min at 94 °C, 35 cycles with 30 s at 94 °C; 60 s at 50 °C and 2 min at 72 °C; and, a final 7 min extension step at 72 °C. Amplification products were controlled on 1% agarose gels and successful reactions were cleaned with the help of the GenElute PCR Clean-Up Kit (Sigma–Aldrich). Cycle sequencing was performed using a standard protocol at the installations of Secugen (Madrid). Successful amplifications were sequenced with the help of the amplification primers.

2.6. Data analysis

The sequences were edited using Bioedit 5.0.9 (Hall, 1999) and aligned manually. The alignment is available from the authors on request. P-distances between the sequences and the number of pairwise distances of the aligned sequences were calculated with the help of MEGA 6 (Tamura et al., 2013). The data were analyzed by Bayesian inference as implemented with MrBayes 3.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Instead of selecting an appropriate substitution model we used a sampling across the substitution model space in the Bayesian MCMC analysis itself (Huelsenbeck, Larget, & Alfaro, 2004) removing the need for *a priori* model testing. Three runs were conducted with 10,000,000 generations. Trees were sampled every 10,000th generation and the first 200 trees were discarded (burn-in) in order to exclude the trees before the chain reached the stationary phase. Trees were edited with the help of TreeGraph2 (Stöver & Müller, 2010).

The monophyly of the *N. flagelliforme* specimens was tested with MrBayes 3.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). In order to do so, the marginal likelihoods of