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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

Table 1
Samples analysed Herbarium voucher, the locality and the GenBank accession numbers.

Voucher number	Taxa	Locality	trnLGenBank accession number	16SGenBank accession number
Spain				
2081	*Flagelliforme*	Yecla, Murcia		KC350467
2287	*Flagelliforme*	Tibi, Alicante		KC350468
2910	*Flagelliforme*	Ronesa, Alicante		KC350471
2910(3)	*Flagelliforme*	Ronesa, Alicante	KC350424	KC350479
2911	*Commune*	Tibi, Alicante	KC350421	KC350462
2912	*Commune*	Tibi, Alicante	KC350422	KC350461
2914	*Commune*	Tibi, Alicante	KC350451	KC350473
2915	*Flagelliforme*	Tibi, Alicante	KC350419	
2916	*Flagelliforme*	Tibi, Alicante	–	KC350474
2918	*Flagelliforme*	Tibi, Alicante	KC350452	KC350475
2919	*Flagelliforme*	Tibi, Alicante	KC350453	KC350476
2920(1)	*Flagelliforme*	Tibi, Alicante	KC350454	KC350477
2920(2)	*Flagelliforme*	Tibi, Alicante	KC350423	KC350478
2921	*Flagelliforme*	Tibi, Alicante	–	KC350480
2922	*Commune*	Tibi, Alicante	KC350425	KC350481
2923	*Flagelliforme*	Tibi, Alicante	KC350456	KC350482
2924	*Commune*	Tibi, Alicante	–	KC350483
2925	*Commune*	Carretera Nac. Murcia-Albacete	KC350427	KC350488
2926	*Commune*	Carretera Nac. Murcia-Albacete	KC350426	KC350487
2927	*Flagelliforme*	Ronesa, Alicante	KC350440	
2928	*Commune*	Tibi, Alicante	KC350434	KC350495
2929	*Commune*	Castalla. Alicante	KC350433	KC350494
2930	*Commune*	Castalla. Alicante	KC350455	KC350493
2931	*Commune*	Castalla. Alicante	–	KC350492
2932	*Commune*	Castalla. Alicante	–	KC350491
2933	*Commune*	Castalla. Alicante	KC350432	KC350490
2935	*Commune*	Castalla. Alicante	KC350431	KC350489
2936	*Commune*	Castalla. Alicante	KC350430	KC350488
2937	*Commune*	Castalla. Alicante	KC350429	KC350487
2938	*Commune*	Castalla. Alicante	KC350435	KC350496
2939	*Commune*	Castalla. Alicante	KC350436	KC350497
2940	*Commune*	Castalla. Alicante	KC350437	KC350498
2941	*Commune*	Castalla. Alicante	KC350438	KC350500
2942	*Commune*	Castalla. Alicante	KC350457	KC350499
2943	*Commune*	Castalla. Alicante	KC350439	KC350501
2944(1)	*Flagelliforme*	Castalla. Alicante	KC350428	KC350486
2944(2)	*Commune*	Castalla. Alicante	–	KC350
3759	*Flagelliforme*	Agost, Alicante	KC350447	KC350506
3760	*Flagelliforme*	Hondón de la Nieves, Alicante,	KC350448	KC350507
3761	*Flagelliforme*	Polígono San Blas, Alicante,	KC350449	KC350508
3762	*Flagelliforme*	Xixona, Alicante	KC350450	KC350509
5177	*Flagelliforme*	Collegats, Lleida	KC350458	KC350502
5179	*Flagelliforme*	Carpwarp, Victoria	KC350442	KC350503
5182	*flagelliforme*	Nowie, Victoria	KC350443	KC350504
Australia				
5180(2)	*Commune*	Manangatang, Victoria	KC350445	KC350466
5180(1)	*Flagelliforme*	Manangatang, Victoria	KC350446	KC350465
5179	*Flagelliforme*	Carpwarp	KC350442	KC350503
5181	*Flagelliforme*	Manangatang, Victoria	KC350460	KC350464
5182	*Flagelliforme*	Nowie, Victoria	KC350443	KC350504
5183	*Flagelliforme*	Speed, Victoria	KC350459	KC350463

an unconstrained MrBayes run were compared to a run with a constraint defined by the samples *N. flagelliforme* specimens, to which a topology prior was applied. We used the stepping stone sampling algorithm with two independent runs setting the number of generations to 5,000,000. Xie, Lewis, Fan, Kuo, & Chen (2011) have shown that stepping stone sampling is more accurate than the harmonic mean of the likelihood values of the MCMC samples.

To study the differentiation between geographical regions we subdivided the samples in seven groups. The grouping was organized in the following manner: *N. commune* from Tibi and *N. flagelliforme* from Tibi; *N. commune* from Castalla; *N. commune* from the remaining localities of Spain; *N. flagelliforme* from the remaining sites of Spain (including one sample from Castalla); *N. commune* from the worldwide sampling (including our sequences from Australia); and, finally *N. flagelliforme* worldwide. The mean values of the p-distances between the sequences of each population and between populations of the aligned sequences were calculated with the help of MEGA 6 (Tamura et al., 2013).

Arlequin 3.5 (Excoffier & Lischer, 2010) was used to estimate the genetic differentiation among the main populations by AMOVA (analysis of molecular variance, Excoffier, Smouse, & Quattro, 1992; Weir, 1996; Weir & Cockerham, 1984). Transition and transversion weight were set to 1 and deletion weight to 0. An analysis of molecular variance was used to estimate the percentage of variance caused by the differences between the two species *N. commune* and *N. flagelliforme*, between populations within these species and individuals within populations. Furthermore we calculated the F_{ST} values of the pairwise comparisons of the populations. The significance of the results was tested by 1000 permutations. Selective neutrality was estimated in Arlequin using Tajimaí's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997) in order to detect possible deviations from neutrality. To adjust for the effects of multiple hypotheses testing in the case of the p-values associated with the neutrality tests, we applied the Hochberg correction (Hochberg, 1988) as implemented in R 3.0.2.

Table 2
Geographical origin, Gene Bank accession numbers and references of taxa from other parts of the world used in molecular analysis.

Taxa	Country	trnLGenBank accession number	16SGenBank accession number	References
Anabaena	¿	AJ228705		n.a.
Nostoc symbiont *Peltigera*	Sweden	AF019919		Paulsrud and Lindblad (1998)
Nostoc commune strain RISA	Ross Ice Shelf	AF204071		Wright et al. (2001)
Nostoc cyanobiont *Nephroma*	Sweden	AF019918		Paulsrud and Lindblad (1998)
Nostoc cyanobiont *Nephroma*	Sweden	AF019917		Paulsrud and Lindblad (1998)
Nostoc cyanobiont *Cycas*	USA	AF095779		Costa et al. (2002)
Nostoc commune strain VER	Switzerland	AF204075		Wright et al. (2001)
Nostoc strain NIVA CYA 308	?	AJ228712		n.a.
Nostoc sp. MALA	UK	AF204077		Wright et al. (2001)
Nostoc sp. ALD776DB	Aldabra Atoll, Indian Ocean	AF204064		
Nostoc commun strain MOA	Mars oasis Alexander Island	AF204073		
Nostoc flagelliforme strain ALD857DB	Aldabra Atoll, Indian Ocean	AF204068		
Nostoc commune strain ALD857DC	Aldabra Atoll, Indian Ocean	AF204069		
Nostoc flagelliforme strain WH015	Mexico	AF204087		
Nostoc symbiont *Peltigera*	Sweden	AF019921		Paulsrud and Lindblad (1998)
Nostoc symbiont *Peltigera*	Sweden	AF019914		Paulsrud and Lindblad (1998)
Nostoc flagelliforme		AJ228710		n.a.
Nostoc Nephroma cyanobiont	Finland	HM448662		Fedrowitz, Kaasalainen, & Rikkinen (2011)
Nostoc sp. MALB	UK	AF204078		Wright et al. (2001)
Nostoc sp. MALC	UK	AF204079		Wright et al. (2001)
Nostoc commune strain WH007A	Italy	AF204099		Wright et al. (2001)
Nostoc commune strain ALD779D	Aldabra atoll Indian Ocean	AF204081		Wright et al. (2001)
Nostoc sp. ALD776DB	Aldabra atoll Indian Ocean	AF204064		Wright et al. (2001)
Nostoc commune strain ALD776DC	Aldabra atoll Indian Ocean	AF204066		Wright et al. (2001)
Nostoc flageliforme strain ALD857DB	Aldabra atoll Indian Ocean	AF204068		Wright et al. (2001)
Nostoc commune strain ALD857DC	Aldabra atoll Indian Ocean	AF204069		Wright et al. (2001)
Nostoc sp LBP	¿	AF204070		Wright et al. (2001)
Nostoc commune strain RISA	Ross ice Shelf	AF204071		Wright et al. (2001)
Nostoc commune strain RISB	Ross ice Shelf	AF204072		Wright et al. (2001)
Nostoc commune strain MOA	Alexander Island	AF204073		Wright et al. (2001)
Nostoc commune strain BBC	USA	AF204074		Wright et al. (2001)
Nostoc commune strain BER	Switzerland	AF204075		Wright et al. (2001)
Nostoc commune strain ENG	USA	AF204076		Wright et al. (2001)
Nostoc sp. MALA	UK	AF204077		Wright et al. (2001)
Nostoc commune strain NZE	New Zealand	AF204080		Wright et al. (2001)
Nostoc sp. ALD8122	Aldabra, Indian Ocean	AF204082		Wright et al. (2001)
Nostoc commune strain WH001	Romania	AF204084		Wright et al. (2001)
Nostoc sp. WH009	Uruguay	AF204085		Wright et al. (2001)
Nostoc commune strain WH013	USA	AF204086		Wright et al. (2001)
Nostoc flagelliforme strain WH015	Mexico	AF204087		Wright et al. (2001)
Nostoc commune strain WH016	Italy	AF204088		Wright et al. (2001)
Nostoc commune strain CHEN	China	AF204089		Wright et al. (2001)
Nostoc commune strain DRH1	China	AF204090		Wright et al. (2001)
Nostoc commune strain HUN	China	AF204091		Wright et al. (2001)
Nostoc commune strain WH002	Romania	AF204092		Wright et al. (2001)
Nostoc commune strain WH004	Germany	AF204093		Wright et al. (2001)
Nostoc commune strain WH012	Indonesia	AF204094		Wright et al. (2001)
Nostoc commune strain WH014	USA	AF204095		Wright et al. (2001)
Nostoc commune strain WH06A	Germany	AF204096		Wright et al. (2001)
Nostoc commune strain WH06B	Germany	AF204097		Wright et al. (2001)
Nostoc sp. UTEX584	UK?	AF204098		Wright et al. (2001)
Nostoc commune strain WH007B	Italy	AF204100		Wright et al. (2001)
Nostoc commune strain SPHAER	??	AF204101		Wright et al. (2001)
Nostoc commune strain TAG	Switzerland	AF204102		Wright et al. (2001)
Nostoc commune strain TEN	USA	AF204103		Wright et al. (2001)
Nostoc sp. TOP	USA	AF204104		Wright et al. (2001)
Nostoc commune strain WH003	Romania	AF204105		Wright et al. (2001)
Nostoc flagelliforme strain WH008	USA	AF204106		Wright et al. (2001)
Nostoc flagelliforme	Aldabra atoll Indian Ocean	AF204067		Wright et al. (2001)
Nostoc commune strain MEL	Australia	AF204083		Wright et al. (2001)
Nostoc sp. ALD776DA	Aldabra atoll, Indian Ocean	AF204065		Wright et al. (2001)
Nostoc commune strain ALD857DA	Aldabra atoll, Indian Ocean	AF204067		Wright et al. (2001)
Nostoc commune var. *flagelliforme* CCAP 1453/33	??		HF678489	n.a.
Nostoc commune UTEX 584	Brazil		AY218833	Fiore et al. (2005)
Nostoc commune CCAP1453/24	South Africa		HE974995	n.a.
Nostoc commune NC1	?		EU784149	n.a.
Nostoc commune	Japan		AB721392	n.a.

Table 2 (*Continued*)

Taxa	Country	trnLGenBank accession number	16SGenBank accession number	References
Nostoc commune	China		AB251863	Arima et al. (2012)
Nostoc commune	France		AB113665	Arima et al. (2012)
Nostoc commune	Antarctica		AB098071	Arima et al. (2012)
Nostoc commune	??		EU586733	n.a.
Nostoc commune OBrien	USA		DQ185223	O'Brien, Miadlikowska, & Lutzoni (2005)
Nostoc commune	India??		KF953518	n.a.
Nostoc flagelliforme	China		GU810186	Gao et al. (2011)
Nostoc flagelliforme	China		EU178143	n.a.
Nostoc flagelliforme CCAP 1453/33	??		KM019940	n.a.
Nostoc indistiguenda	USA		AY577538	Rehaková, Johansen, Casamatta, Li, & Vincent (2007)
Nostoc cf. *indistinguendum*	USA		AY577539	Rehaková, Johansen, Casamatta, Li, & Vincent (2007)
Nostoc cf. *indistinguendum*	USA		AY577540	Rehaková, Johansen, Casamatta, Li, & Vincent (2007)
Nostoc cf. *indistinguendum*	USA		AY577541	Rehaková, Johansen, Casamatta, Li, & Vincent (2007)
Nostoc verrucosum	Japan		AB511947	Arima et al. (2012)
Nostoc verrucosum	Japan		AB494996	Arima et al. (2012)
Nostoc linckia var. *arvense*	¿		AB325907	Arima et al. (2012)
Nostoc carneum	¿		AB325906	Arima et al. (2012)
Mojavia pulchra	USA		AY577534	Rehaková, Johansen, Casamatta, Li, & Vincent (2007)
Nostoc desertorum	USA		AY577537	Rehaková, Johansen, Casamatta, Li, & Vincent (2007)
Anabaena bergei	Israel		FR822623	Ballot et al. (2011)
Nostoc cf. *commune*	Mexico		HQ877827	Ramírez, Hernández-Mariné, Mateo, Berrendero, & Roldán (2011)

Statistical analyses of morphological characters were performed using R program (2.15.3). Pearson correlation analysis was used to identify relationships between vegetative cell and heterocytes size for each species. To compare the means of cell sizes for the two species a Student's *t*-test was applied after checking the normality of data and homogeneity of variances. Finally, a multiple correspondence analysis (MCA) was performed using a matrix of descriptive morphological variables. A hierarchical clustering analysis following the Ward's method for data aggregation was applied to the coordinates of the first five axes of the MCA.

3. Results

3.1. Variability of the habitat

On red clay soils, slightly sloped (<15%) under bush vegetation from south-east Spain the cylindrical thalli grew alone but on poorly developed soils on calcareous rocks, with slight or no slope and under *Pinus* vegetation (sometimes more or less disturbed) in several areas from Spain and south Australia both sheet and cylindrical thalli cohabited and several intermediary forms were observed (Fig. 1). The vesiculous morph was observed only once in a small hollow on calcareous rock where rainfall water accumulated during a short period of time as were the cylindrical thalli growing epiphytically on *Collema* lichen on fully exposed vertical rock walls

3.2. Morphological and ultrastructural variability

Sheets are usually lobated or perforated with smooth, verrucose or warty surface. Cylindrical thalli were generally branched and blackish in the dry state becoming cylindrical or ribbon-like when hydrated, from 1 to 5 mm in diameter (width) and brownish-green in color; small sheets with revolute margins were observed occasionally in apical parts (Fig. 2). The surface was smooth or slightly striated. Vesiculous thalli were spheric or subspheric, from 2 to 30 mm in diameter, grayish in color, as a result of the calcium carbonate incrustation.

No ordination of the filaments was observed in sheets or vesicules but were uniformly parallel disposed in cylindrical thalli (Fig. 2). The density of the filaments was homogeneous in cylindrical thalli and homogeneous or slightly denser in the outer part in sheets and vesicules. Sheaths were always narrow in the vesiculous thalli but wide and lamellated in the others, hyaline in the internal part and yellow in the external part in all cases. The trichomes were short or fairly long and the heterocytes were numerous in all cases. Akinetes (young) were observed in only three of the sheet samples. Fungal hyphae and bacteria were fairly abundant inside thalli.

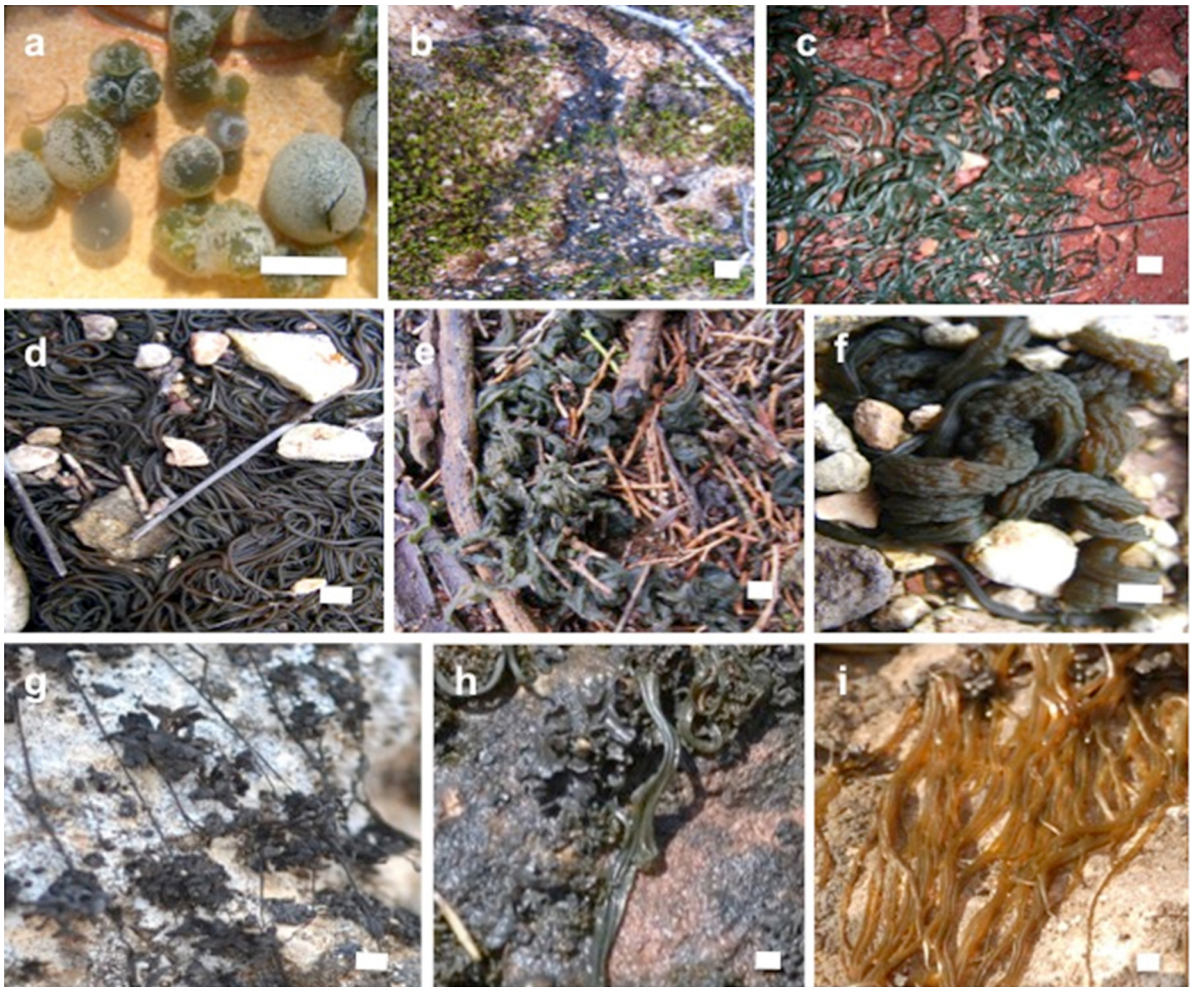

Fig. 1. Morphology and habitat variability of *Nostoc commune* and *N. flagelliforme*. (a) Vesiculous aquatic colonies. (b–d) Edaphic colonies from SE Spain and SE Australia. (e) Sheets between *Pinus* leaves (SE Australia). (f) Striates sheets ending in cylindrical filaments from dry soils of SE Australia. (g–i) Colonies associated with lichens on vertical rock walls (NE Spain). Scales bars: 1 cm.

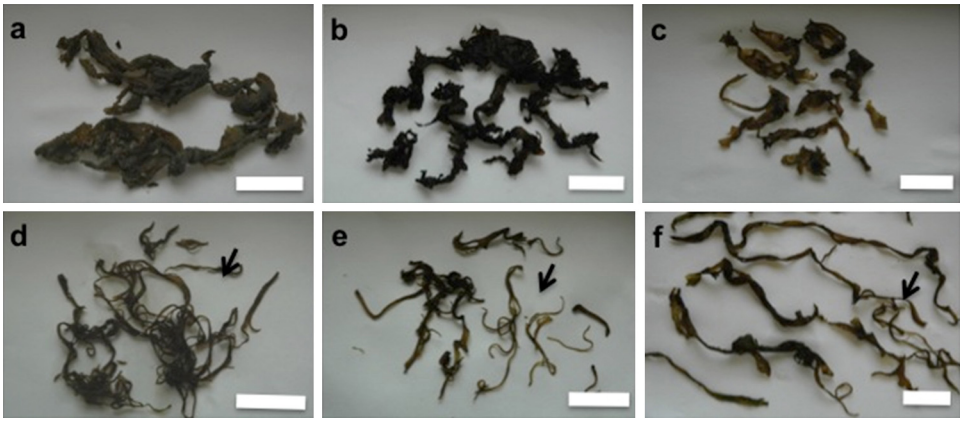

Fig. 2. Morphological variability of *Nostoc* thalli. (a–c) Sheets and intermediate forms. (d–f) Ribbon-like to cylindrical thalli and intermediate forms (arrows). All material from SE Spain. Scale bars: 5 cm.

Vegetative cells were 4.89 ± 0.28 to 6.08 ± 0.29 μm in diameter in sheets and 5.30 ± 0.25 to 6.18 ± 0.44 μm in diameter in cylindrical thalli. The heterocytes were 5.41 ± 0.36 to 7.50 ± 0.43 μm in diameter in sheets and 5.56 ± 0.52 to 6.87 ± 0.77 μm in diameter in cylindrical thalli. No visible differences were observed in the structure of the mucilage with TEM in sheets but two different types of mucilage were visible in cylindrical thalli: one less electrodense in inner parts and denser in the external areas (Fig. 3).

The bionomic characteristics of both species showed fairly high variation (Fig. 4) and no significant differences were detected by the *t*-student test ($p = 0.99$) between the mean size of vegetative cells or heterocytes of sheets and cylindrical thalli. Significant correlations

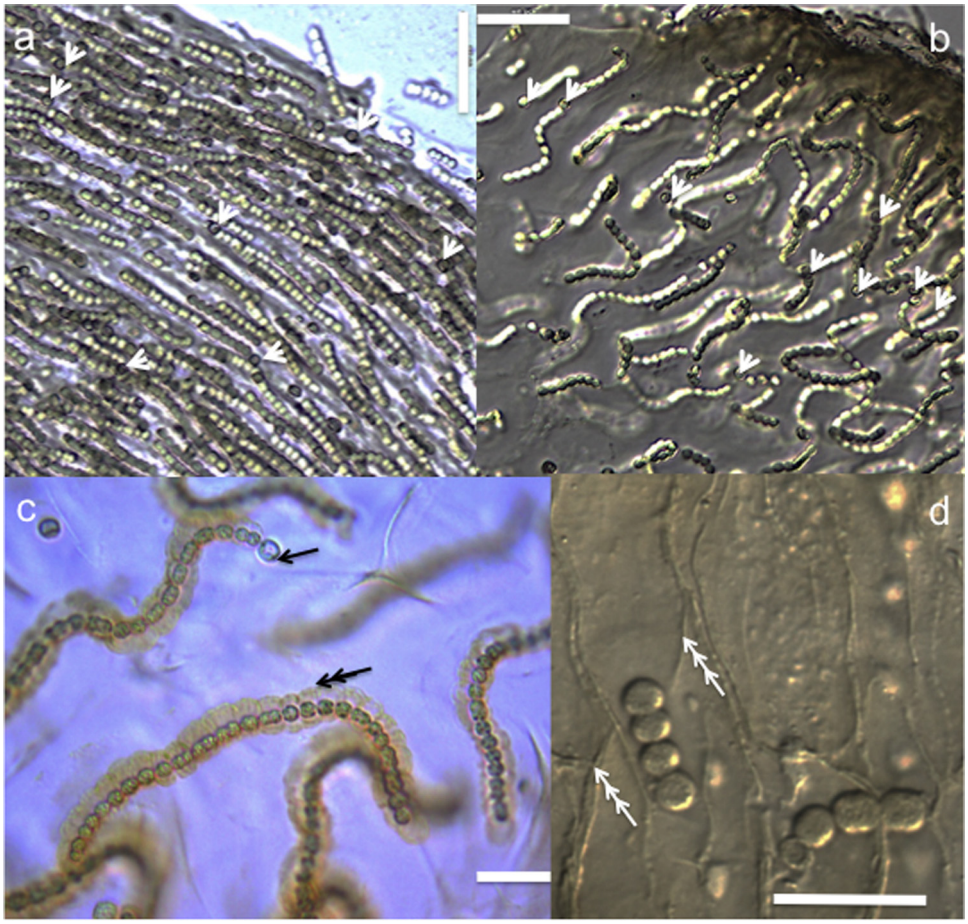

Fig. 3. Variability of colonies with light microscopy. (a) Disposition of filaments in outer part of morph *flagelliforme* with low magnificatiom. (b) Disposition of filaments in outer part of morph *commune* with low magnification. (c) Detail of coiled filaments with sheaths. (d) Trichomes and fungal hyphae in the inner part of the thalli. Heterocysts are marked with simple arrows, sheaths with double arrows and fungal hyphae with triple arrows. Scale bars: (a–c) 50 μm and (d) 20 μm.

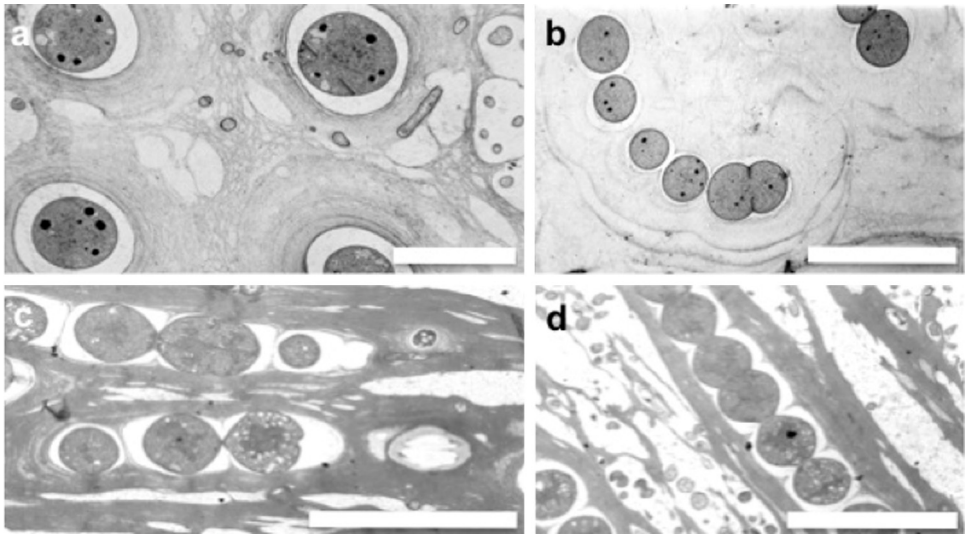

Fig. 4. Variability in ultrastructure. (a, b) Sheets: homogeneous and light mucilage between cells and sheaths (double arrows) of filaments. (c, d) Cylindrical thalli: dense mucilage and higher presence of fungal hyphae (arrows). Scale bars: 10 μm.

were neither found between the cell size of vegetative cells nor heterocytes of both species (Fig. 5).

The cluster analysis performed with the five first axes of the MRA analysis separate clearly cylindrical thalli from sheets-ribbon-like and vesiculous thalli (Fig.6).

3.3. Genetic variation

We obtained 48 new sequences for the *trnL* intron and 42 for the 16S rRNA gene (partial sequence). Within the *trnL* intron, we observed both classes of heptamers which were described in differ-

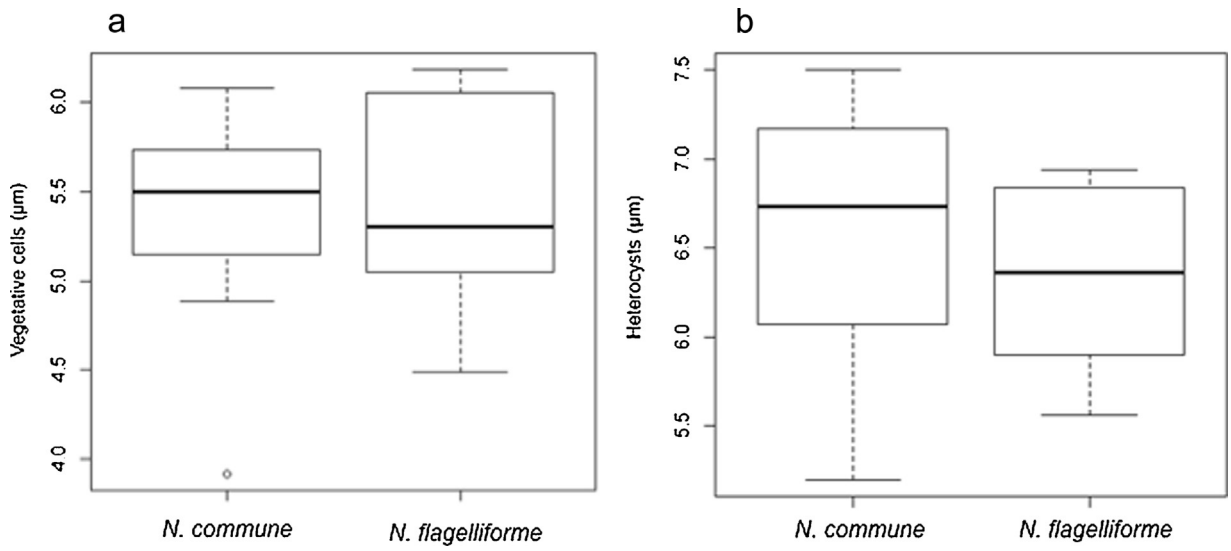

Fig. 5. Variability of vegetative cells and heterocyte from specimens assigned to *N. flagelliforme* and *N. commune*. (a) Vegetative cells. (b) Heterocyte. Mean, median and standard deviation are indicated.

Table 3
Estimates of evolutionary divergence over 16S rRNA sequence pairs between groups (below diagonal) and within groups (diagonal). The number of base differences per sequence from averaging over all sequence pairs between groups and within groups are shown. All ambiguous positions were removed for each sequence pair.

N. commune Castalla	9.731						
N. commune Tibi	11.795	13.600					
N. commune Spain	9.923	12.250	14.000				
N. commune World	17.951	18.848	17.045	23.655			
N. flagelliforme Tibi	11.325	12.500	10.222	18.677	10.194		
N. flagelliforme Spain	12.231	13.389	11.083	18.727	11.056	12.667	
N. flagelliforme World	12.419	14.407	11.944	19.273	12.037	12.926	14.000

Table 4
Estimates of evolutionary divergence over *trn*L sequence pairs between groups (below diagonal) and within groups (diagonal). The number of base differences per sequence from averaging over all sequence pairs between groups and within groups are shown. All ambiguous positions were removed for each sequence pair.

N. commune Castalla	8.876						
N. commune Tibi	11.881	12.694					
N. commune Spain	10.507	13.200	13.500				
N. commune World	11.390	11.560	12.311	12.623			
N. flagelliforme Tibi	12.139	13.828	12.545	13.727	12.255		
N. flagelliforme Spain	7.613	9.911	9.400	7.720	9.073	0.800	
N. flagelliforme World	14.154	14.641	14.815	15.187	15.112	12.138	16.192

ent copy-numbers from the P6b stem-loop (Costa et al., 2002). Both classes were found in all three major populations mentioned above and in samples classified as *N. commune* and *N. flagelliforme*. This region was excluded from further analyses, because previous studies have shown that this region does not correspond with species phylogeny (Oksanen et al., 2004).

Both regions showed similar numbers of pairwise differences between the sequences belonging to the joint *N. commune* and *N. flagelliforme* sequences, which ranged from 0 to 44 (mean 12.402) in the case of the *trnL* intron and 0–47 (mean 14.488) for the 16S rRNA gene. Within each of the supposed species *N. commune* and *N. flagelliforme* these numbers did not change much. In *trn*L within *N. commune* we found between 0 and 41 (mean 11.628) differences, within *N. flagelliforme* 0–25 (mean 13.286) differences, and in the case of the 16SrRNA gene within *N. commune* 0–47 (mean 15.704) differences and within *N. flagelliforme* 0–44 (mean 12.069) differences. The distance values within and between geographical groups for both genetic regions are given in Tables 3 and 4. Remarkably, the distances between populations of the two species were in the range of the values within populations of the two species. The F_{ST} values, which describe the differentiation among the groups, were low (Tables 5 and 6) and the major part of the variability was observed within the populations. Pairwise F_{ST} values in the comparison between the seven groups were also low, although in part statistically significant (Table 7). The neutrality tests gave no significant deviation from neutrality when the Hochberg correction for multiple hypothesis testing was applied (Tables 6 and 7). As in prior studies on *N. commune* and related taxa, the molecular analyses resulted in trees with poor resolution of many clades (Figs. 7 and 8). There were also some clear inconsistencies between the trees obtained using the *trnL* region and the 16S rRNA gene. Furthermore, the morphological characteristics are only partly reflected by the gene trees. For example, samples with *flagelliforme* morphology are scattered all over the trees and some clades unite samples with very distinctive morphology.

The tests for monophyly of *N. flagelliforme* resulted in values of ln = −3094.63 for unconstrained topology and ln = −3289.82 for the constrained topology (*N. flagelliforme* monophyletic) in the case of the *trnL* intron and ln = −3736.21 (unconstrained) and ln = −3866.88 (constrained) for the 16SrRNA gene. A difference of 5 log units can be considered as very strong evidence in favor of the better model (Kass & Raftery, 1995). With differences of the ln values of the marginal likelihood of more than 100, the monophyly of *N. flagelliforme* can be rejected using this criterion.

4. Discussion

4.1. Morphological variability

Even when morphology, ultrastructure and habitat characteristics seem to differentiate between morphs, several transition forms were also observed both in Spain and Australia. In south-east Spain the spherical thalli developed in a place where the water accumulated for short periods of time. The sheets were found on sandy soils and the cylindrical forms were only observed on slightly sloped silt or clay soils, but sometimes sheets has cylindrical proliferations. In Australia there were no particular patterns for the distribution of either morphs. However, in spite of extensive surveys, the cylindrical thalli had not yet been located in the semi-arid savannah regions of northern Australia (Williams & Büdel, unpublished data).

Table 5
Results of the AMOVA test for the *trn*L region. The observed differences are mostly due to the variation within the defined populations and in a minor degree to the variation found among populations within species. The variation among species is very low and statistically not significant.

Source of variation	*d.f.*	Sum of squares	Variance components	Percentage of variation	Fixation index	*p*-value
Among species	1	19.026	0.12695 Va	2.143	F_{CT}: 0.02141	0.19550
Among populations within species	5	58.731	0.51941 Vb	8.12	F_{SC}: 0.08299	0.00587
Within populations	86	493.566	5.73914 Vc	89.74	F_{ST}: 0.10263	0.00098
Total	92	571.323	6.39550	100		

Table 6
Results of the AMOVA test for the 16SrRNA region. The observed differences are mostly due to the variation within the defined populations. The percentage of variation found among populations within species and among species is low and not significant ($p < 0.05$).

Source of variation	*d.f.*	Sum of squares	Variance components	Percentage of variation	Fixation index	*p*-value
Among species	1	19.447	0.37570 Va	5.03	F_{CT}: 0.05025	0.05376
Among populations within species	5	44.236	0.26923 Vb	3.60	F_{SC}: 0.03792	0.13783
Within populations	49	334.746	6.83155 Vc	91.37	F_{ST}: 0.08626	0.01857
Total	55	398.429	7.47647	100		

Nevertheless, *N. commune* is widespread throughout all Australian climatic zones; coastal, desert and savannah (Skinner & Entwisle, 2001; Williams & Büdel, 2012). Yet, only *N. commune* has been found on acidic soils across the semi-arid Mulga Lands of Australia (Williams & Büdel, 2012). The cylindrical thalli found among *Eucalyptus* leaves and on the edges of bush tracks in winter, apparently in microsites where there was evidence of rain-wash, were substituted the following autumn by sheets. In the same sampling period (autumn) at a nearby site there had been a recent rain event, and masses of cylindrical thalli were found on a degraded clay dam slope. In the arid desert sites cylindrical thalli appeared scarce and mainly visible after rain.

Bornet and Flahault (1888) based mainly on morphology and habitat considered the cylindrical morph an extreme morphological variation of *N. commune* and described the evolution from balls to sheets with venations and finally to filaments (the veins). Whitton (2002) mentions that in U.K. intermediate forms between commune and flagelliforme are frequent but they never reach the filiform state.

4.2. Genetic variation

Both trees based on two independent markers show that the morphospecies *N. flagelliforme* is clearly polyphyletic. Consequently, there are clear contradictions between morphology and genetic data on the one hand and the two investigated DNA regions on the other hand. Wright et al. (2001) found genetically different strains that shared morphologies and dissimilar morphologies in strains that shared identical *trnL* markers. We tested to which degree constraining the topology of the trees forcing *N. flagelliforme* to a monophyletic clade affects the marginal likelihood and the results confirm convincingly that *N. flagelliforme* is not a monophyletic entity. The marginal likelihood changed by more than 100 ln units while 5 units are already considered to deliver clear evidence (Kass & Raftery, 1995).

High sequence diversity was found within the populations and a relatively low separation between populations. The *N. flagelliforme* samples from the Tibi locality were highly diverse and at the same time some of the Australian samples were closely aligned with Spanish specimens. This indicates that the areas were colonized independently at different times and that *Nostoc* has a very high dis-

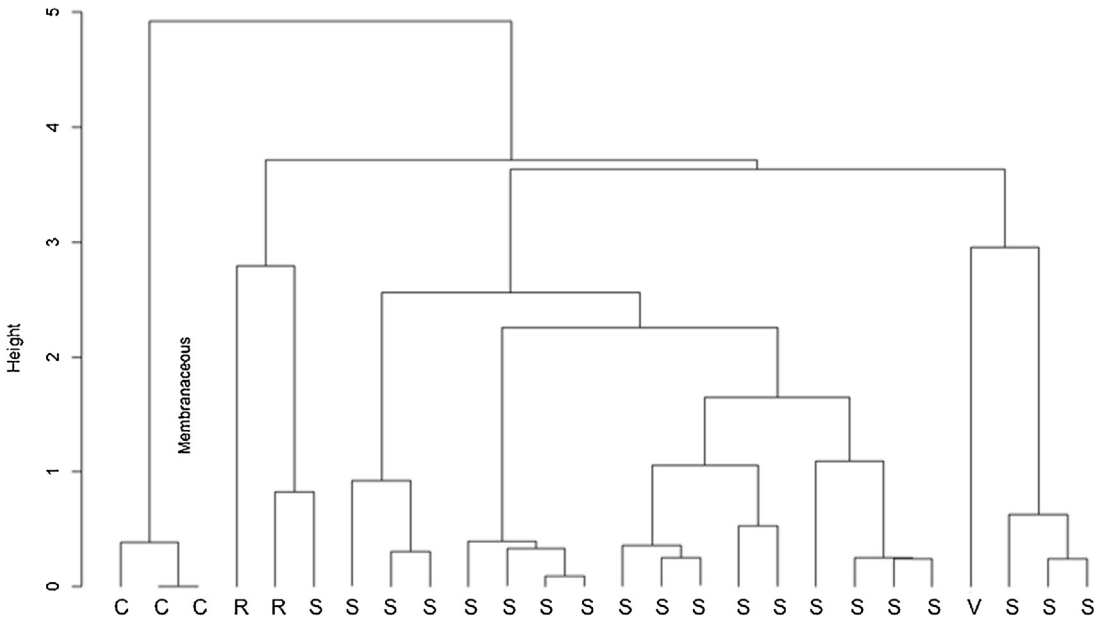

Fig. 6. Hierarchical cluster analysis performed with the coordinates of the first five axes of the multiple correspondence analysis (MCA) with the matrix of morphological variables (C = cylindrical, R = ribbon-like, S = sheets, V = vesicules).

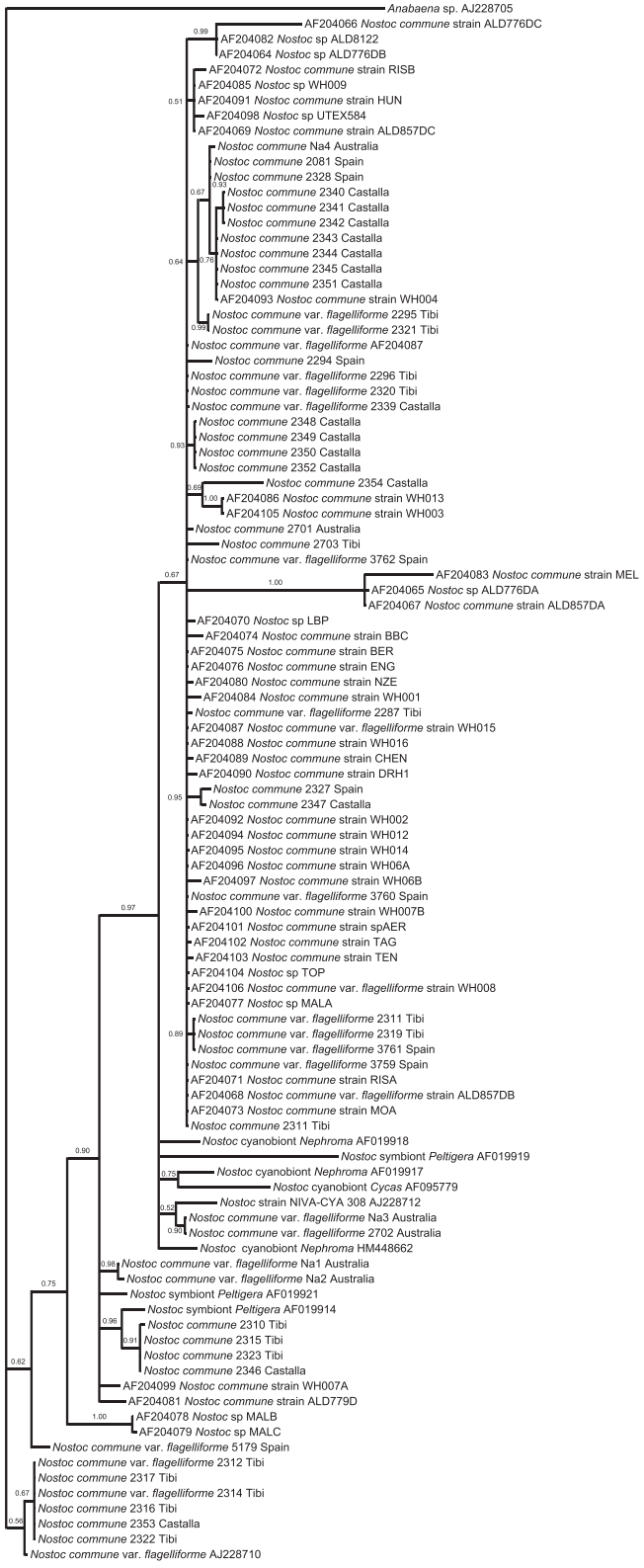

Fig. 7. Bayesian tree based on the *trnL* sequence data after the exclusion of the hypervariable P6b stem-loop. The incongruencies between morphology and molecular data are apparent. The geographic origin of the newly sequenced specimens is given. Sequences from other sources are indicated by their GenBank accession number and strain information when available. Posterior probability values are given at the clades.

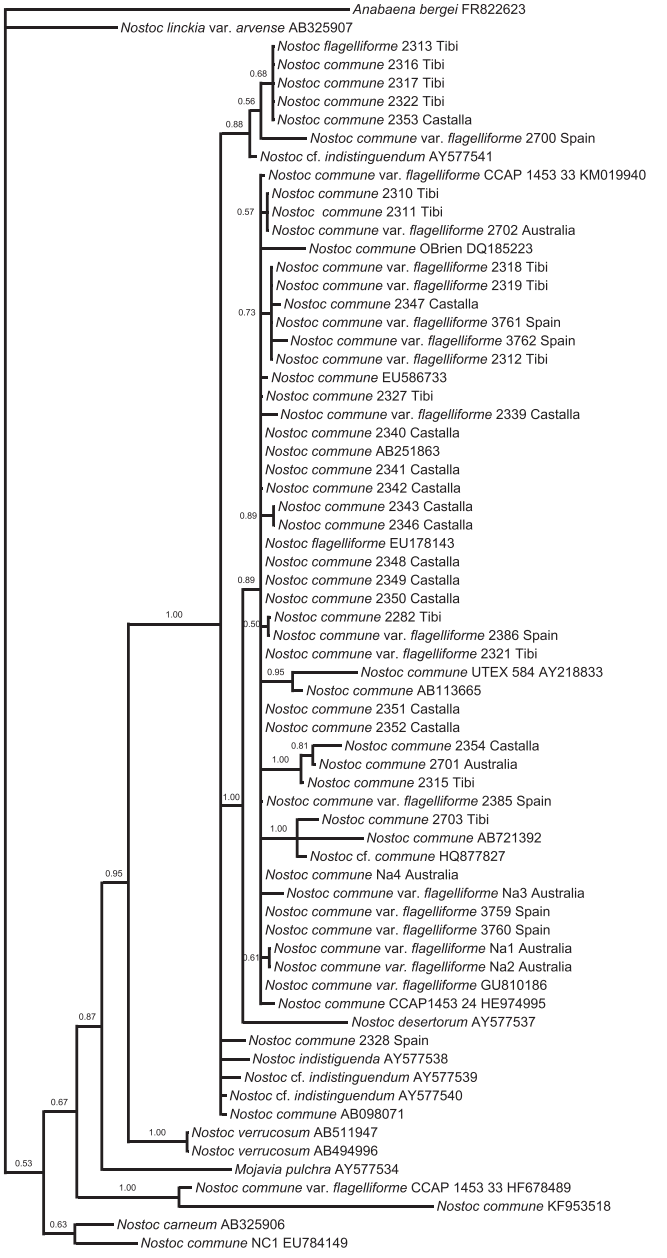

Fig. 8. Bayesian tree based on the 16S rRNA sequence. Samples from the same locality are widely distributed over the tree, while occasionally samples from distant localities cluster together. The geographic origin of the newly sequenced specimens is given. Sequences from other sources are indicated by their GenBank accession number and strain information when available. Posterior probability values are given at the clades.

persal capacity. Similar low genetic variability between continents was also shown recently for some genera of red algae (Necchi et al., 2013) and the existence of cosmopolitan species like *Microcoleus chthonoplastes* has been proved by several authors (García-Pichel et al., 1996). However in other cases the dispersal barriers and allopatric speciation seem to play important roles in the evolution of species like *Microcoleus vaginatus* (Dvorak et al., 2012).

It seems that most, if not all, cyanobacteria possess multiple copies of the genome. This explains why sometimes clonal isolates of a filament give rise to phenotypically diverse filaments within a single culture flask (Pott, 2000). It is unknown the role that gene exchange (lateral transfer) and recombination play on cyanobacteria evolution (Castenholz, 1992; Rudi, Skulberg, & Jakobsen, 1998) but some authors think that the low rate of evolutionary change of

Table 7
Population pairwise F_{ST} values for the *trn*L region (below diagonal and 16S rRNA (above diagonal) calculated by Arlequin. Significant values ($p < 0.05$) are indicated by bold letters. Although some comparisons show significant values, the data show that the differentiation between the populations are in the low range of what is observed between natural populations.

N. commune Castalla		0.028	−0.121	0.116	**0.121**	0.099	0.076
N. commune Tibi	**0.103**		−0.120	0.019	0.057	0.019	**0.142**
N. commune Spain	−0.037	0.011		−0.178	0.058	−0.183	0.047
N. commune World	**0.115**	0.027	0.030		**0.102**	0.032	**0.136**
N. flagelliforme Tibi	**0.136**	0.098	-0.021	**0.143**		−0.028	0.057
N. flagelliforme Spain	**0.238**	0.229	**0.239**	0.063	0.173		0.034
N. flagelliforme World	**0.119**	0.009	−0.013	**0.107**	0.056	0.176	

the group could be the price or the consequence for being able to colonize extreme environments where DNA damage may be accelerated (Potts, 2000).

In a rare species we expect a low genetic diversity because the genetic diversity is closely linked to the population number by the equation: $\theta = 2\ Nu$ where θ is a measure of the genetic diversity, N indicates the effective population size and u stands for the mutations rate. If we assume that there is no difference in the mutation rate between *N. commune* and *N. flagelliforme*, and given that θ has very similar values for *N. commune* and *N. flagelliforme*, there is a clear contradiction, if we think that *N. flagelliforme* is a clearly distinct and genetically isolated taxonomic unit from *N. commune.* Furthermore, the F_{ST} values show a very weak separation between the two supposed species and the major part of the observed variation is found within populations. The part of the genetic variability that can be attributed to differences between the two supposed species is very low. All these data together further support the view already sustained by the phylogenetic analysis that from a taxonomic point of view, *N. flagelliforme* is not an isolated entity and that there is a constant gene flow between *N. commune* and *N. flagelliforme*. These findings do not exclude the possibility that the distinctive morphology of *N. flagelliforme* has a genetic basis. But if this is the case, there must be convergent evolution or exchange of genetic material between *N. commune* and *N. flagelliforme*.

4.3. Conservation

Based on the study of a long series of historical data and herbaria Mollenhauer et al. (1999) considered that *N. commune* has become a vulnerable, rare or extinct species in some areas of Central Europe while it is abundant elsewhere. It is claimed that the widespread use of phosphate and nitrate-based fertilizers have eradicated *Nostoc* from many soils where it would otherwise proliferate (Scherer & Zong, 1991).

N. flagelliforme is considered endangered in China and is at present protected by law, and its collection, acquisition and commercialization is forbidden (Gao, 1998). In Europe it has been very rarely reported and its addition to the red lists of some countries would be justified. It seems that the cylindrical thalli of the variety only develop in extreme arid conditions which force a very low growth rate and it probably represents a morphological adaptation (ecotype) to aridity conditions that merits conservation. Even when all measures of conservation are based on species some authors claim that the conservation goal should be to conserve ecological and evolutionary processes rather than specific phenotypic variants (Moritz, 1999; Forsman et al., 2010). The environment triggers the expression of those regions in the DNA that provide the most appropriate characters required for survival at any particular time (Morales, Trainor, & Schlichting, 2002; Lemay, Donnelly, & Russello, 2013). This gene regulation and the resulting phenotypic variation are often marked and drastic (Mazel & Marliere, 1989) and several studies have provided evidence for rapid restoration of adaptative phenotypes following experimental translocations (Reznick, Shaw, Rodd, & Shaw, 1997). All this provides new insights into the implementation of conservation measures like translocation, which in this case will not have the risks associated with sexual reproduction (Hufford & Mazer, 2003). "*Ex situ* conservation is potentially critically important, but habitat protection of the wild populations should be the first recourse, and *ex situ* conservation should be used only as a last resort" (Brodie, Andersen, Kawachi, & Millar, 2009). As protection of habitats is not always possible *ex situ* cultivation may be a powerful means. Threatened and endangered species may be preserved in culture when their natural habitat is degraded and unable to support the survival of the species but it is difficult to determine how much genetic diversity is required for the recovery of the endangered species (Brodie et al., 2009). Cryopreservation offers an alternative to reduce costs of maintaining active growing cultures and is being used in most culture collection at present. Even when this technique is not adequate for some algae it is frequently used for cyanophytes (Day, 2007).

Herbaria may also contribute to conservation especially of prokaryotic organisms. The high tolerance of *Nostoc* to desiccation in known and some strains recovered growth after 87 years preserved in a herbarium (Lipman, 1941).

4.4. Biotechnology and conservation

N. flagelliforme is extremely threatened in China due to its extensive use as delicacy or in traditional medicine, but this is not an isolated case. Several plants are endangered as a consequence of intensive collection or the deterioration of their habitats (Tirkey et al., 2014). A potential biotechnological application or economic interest may represent an added value for environment managers to include species in red lists and at the same time biotechnology may help in the implementation of conservation measures (*ex situ* conservation, multiplication and translocation, for instance). As far as is known, no *ex situ* conserved species has been reintroduced (Brodie et al., 2009), but the success of these methods will depend between other factors of genetic diversity and there is no legislation covering the number of isolations or cultures to be conserved.

As a conclusion: the best conservation measure is always the habitat conservation but habitat conservation should not exclude "*ex situ*" preservation. Several strains are deposited (life or cryopreserved) in culture collections all around the world but taking into account the great resistance to dessication of *Nostoc* in nature the deposit of specimens in herbaria should be encouraged as a very useful method for long-term preservation, avoiding at the same time the problems of mutations in cultured cyanobacteria (Kanesaki et al., 2011).

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