Differential patterns of evolution and distribution of the symbiotic behaviour in nostocacean cyanobacteria

Dimitra Papaefthimiou,¹ Pavel Hrouzek,^{2,3,4} Maria Angela Mugnai,^{5,6} Alena Lukesova,⁷ Silvia Turicchia,^{5,8} Ulla Rasmussen¹ and Stefano Ventura⁵

Correspondence

Stefano Ventura
stefano.ventura@ise.cnr.it

¹Department of Botany, Stockholm University, S-10691 Stockholm, Sweden

²Department of Botany, Faculty of Biological Sciences, University of South Bohemia, České Budějovice, Czech Republic

³Institute of Physical Biology, University of South Bohemia, Nové Hrad, Czech Republic

⁴Institute of Microbiology, Department of Autotrophic Microorganisms, Academy of Sciences of the Czech Republic, Opatovický Mlýn, Třeboň, Czech Republic

⁵Institute of Ecosystem Study, National Research Council of Italy, via Madonna del Piano 10, 50019 Sesto Fiorentino, Italy

⁶PhD School in Science for Conservation of the Cultural Heritage, University of Florence, Italy

⁷Institute of Soil Biology, Biology Centre AS CR, v. v. i., Na Sádkách 7, 37005 České Budějovice, Czech Republic

⁸PhD School in Polar Science, University of Siena, Italy

Many cyanobacteria commonly identified as belonging to the genus *Nostoc* are well-known cyanobionts (symbionts) of a wide variety of plants and fungi. They form symbioses with bryophytes, pteridophytes, gymnosperms and angiosperms that are considerably different in the type of reciprocal interaction between the host and the cyanobiont. The phylogenetic and taxonomic relationships among cyanobionts isolated from different hosts and *Nostoc* strains isolated from free-living conditions are still not well understood. We compared phylogeny and morphology of symbiotic cyanobacteria originating from different host plants (genera *Gunnera*, *Azolla*, *Cycas*, *Dioon*, *Encephalartos*, *Macrozamia* and *Anthoceros*) with free-living *Nostoc* isolates originating from different habitats. After preliminary clustering with ARDRA (amplified rDNA restriction analysis), phylogeny was reconstructed on the basis of 16S rRNA gene sequences and compared with morphological characterization, obtaining several supported clusters. Two main *Nostoc* clusters harboured almost all cyanobionts of *Gunnera*, *Anthoceros* and of several cycads, together with free-living strains of the species *Nostoc muscorum*, *Nostoc calcicola*, *Nostoc edaphicum*, *Nostoc ellipsosporum* and strains related to *Nostoc commune*. We suggest that the frequent occurrence of symbiotic strains within these clusters is explained by the intensive hormogonia production that was observed in many of the strains studied. However, no evidence for discrimination between symbiotic and free-living strains, either by molecular or morphological approaches, could be found. Sequences of *Azolla* cyanobiont filaments, taken directly from leaf cavities, clustered tightly with sequences from the planktic cyanobacterium *Cylindrospermopsis raciborskii*, from the benthic *Anabaena cylindrica* 133 and from *Anabaena oscillarioides* HINDAK 1984/43, with high bootstrap values. The phylogenetic analysis showed that two distinct patterns of evolution of symbiotic behaviour might exist for the nostocacean cyanobacteria, one leading to symbioses of *Nostoc* species with a wide variety of plants, the other leading to the association of a unique cyanobacterial type with the water fern *Azolla*.

Abbreviations: AKLC, akinete-like cells; ARDRA, amplified rDNA restriction analysis; ITS, internal transcribed spacer; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences determined in this study are AM711522–AM711554.

A supplementary figure showing the ARDRA of symbiotic and free-living cyanobacteria performed on the 16S rRNA gene + ITS region is available with the online version of this paper.

INTRODUCTION

Filamentous cyanobacteria of the genus *Nostoc* are known to be the main symbiotic partners of a wide range of eukaryotic organisms, where the hosts are found among all kingdoms: protists, animals, fungi and plants (Rai *et al.*, 2002). According to the International Code of Botanical Nomenclature, the genus *Nostoc* belongs to the order Nostocales and, in *Bergey's Manual of Systematic Bacteriology*, it is affiliated to subsection IV of *Cyanobacteria* (Rippka *et al.*, 2001). In recent phylogenetic studies, using several molecular marker genes (small subunit rRNA, *rpoB*, *rbcLX* and *nifD* or *nifH*), the closest relatives to the genus *Nostoc* were shown to be the genera *Anabaena*, *Aphanizomenon* and *Trichormus*, also members of the family Nostocaceae (Rajaniemi *et al.*, 2005; Svenning *et al.*, 2005; Henson *et al.*, 2002, 2004; Lohtander *et al.*, 2003). All members of the genus *Nostoc* are characterized by a complex life cycle that includes differentiation into heterocytes (nitrogen-fixing cells), akinetes (resting spores) and hormogonia (motile filaments), serving for dispersion and as infective units in symbiosis (Lazaroff, 1966; Lazaroff & Vishniac, 1961; Mollenhauer, 1970; Rippka *et al.*, 1979). Four *Nostoc* groups, three of which corresponded to the type species *Nostoc muscorum*, *Nostoc edaphicum* and *Nostoc calcicola*, have been resolved on the basis of their life cycle (Hrouzek *et al.*, 2003; Rajaniemi *et al.*, 2005) but, following the analysis of 81 additional 16S rRNA gene sequences of cyanobacterial strains identified as belonging to the genus *Nostoc*, many more clusters were obtained. These clusters were separated to an extent, indicating that they might represent different taxa (Hrouzek *et al.*, 2005).

The symbiotic relationships of nostocacean cyanobacteria with the plant kingdom involve plants from the divisions Bryophyta (mosses, liverworts and hornworts), Pteridophyta (the genus *Azolla*), gymnosperms (family Cycadaceae) and angiosperms (family Gunneraceae). Identification and diversity studies of the cyanobionts from the individual plant hosts, with the exception of *Azolla*, have revealed high strain diversity both within and among different host species (Rasmussen & Svenning, 1998; Nilsson *et al.*, 2000; Guevara *et al.*, 2002; Zheng *et al.*, 2002; West & Adams, 1997; Costa *et al.*, 2001; Rasmussen & Nilsson, 2002). Among the cyanobacteria associated with cycads, the species *N. muscorum* and *Nostoc punctiforme* have been identified (Costa *et al.*, 1999).

The symbiosis with the water fern *Azolla* is well studied, mainly due to the potential use of *Azolla* as a fertilizer in rice fields (Van Hove & Lejeune, 2002), and also for its unique perpetual nature (Lechno-Yossef & Nierzwicki-Bauer, 2002). The *Azolla* cyanobionts, together with symbionts belonging to other bacterial phyla, are hosted within specialized cavities in the fern leaves (Lechno-Yossef & Nierzwicki-Bauer, 2002). Originally, the cyanobionts of *Azolla* were described as *Anabaena azollae* by Strasburger (1884). However, morphological studies of the cyanobacterial populations inhabiting the cavity, performed on

presumed cultivated cyanobionts, yielded misleading or incomplete results on the taxonomic position of the cyanobionts (Gebhardt & Nierzwicki-Bauer, 1991). Comparative molecular studies have revealed that cultured cyanobacteria isolated from the fern were indeed different from the cyanobiont population inhabiting the leaf cavity, leading to the suggestion that one major cyanobacterial population is involved in the symbiosis, while other minor populations are non-specifically associated with the fern (Tomaselli *et al.*, 1988; Plazinski *et al.*, 1990; Gebhardt & Nierzwicki-Bauer, 1991; Pabby *et al.*, 2003). Up to now the taxonomy of the cyanobacterial populations living inside the leaf cavity of *Azolla* remains undefined and largely controversial. What is well accepted is that these cyanobionts belong to the order Nostocales, while there is a continuous debate on whether they belong to *Nostoc* (Plazinski *et al.*, 1990; Gebhardt & Nierzwicki-Bauer, 1991; Pabby *et al.*, 2003), *Anabaena* (Gebhardt & Nierzwicki-Bauer, 1991; Svenning *et al.*, 2005) or *Trichormus* (Komárek & Anagnostidis, 1989), or if they constitute a separate genus (Caudales *et al.*, 1995; Baker *et al.*, 2003).

The aim of the present study was to use an integrated morphological and molecular approach for a comparative analysis of nostocacean cyanobacteria of symbiotic origin, including strains isolated from the angiosperm *Gunnera*, the gymnosperm cycads, the bryophyte *Anthoceros* and cyanobionts of the water fern *Azolla*, as well as cyanobacterial strains of free-living origins. The molecular tools employed in the present study, amplified rDNA restriction analysis (ARDRA) of the 16S rDNA+ITS (internal transcribed spacer) and phylogenetic analysis of the 16S rRNA gene, provided evidence of high genetic diversity among the cyanobacteria studied that was strongly supported by morphological analysis.

METHODS

Cyanobacterial strains and *Azolla* cyanobionts. We studied 28 cyanobacterial strains previously assigned to the genus *Nostoc*, and three cyanobiont populations directly extracted from the leaf cavity of plants belonging to three distinct species of *Azolla* (Table 1). Symbiotic *Nostoc* strains were isolated from the following host plants: seven strains from species of the angiosperm genus *Gunnera*, four strains from cycads and two from the genus *Anthoceros*. Fifteen cyanobacterial strains isolated from free-living conditions were also included in the study. Details of species and origins of the host plants, origins of cultures and references to original descriptions, are given in Table 1. For molecular analysis, free-living and symbiotic *Nostoc* strains were grown in nitrogen-free BG-11 medium (Rippka *et al.*, 1979) in continuous light (80 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ intensity) with gentle shaking, at 28 °C. For morphological studies, the individual strains were transferred to 1.5% w/v agar plates and kept at a temperature of 20 ± 2 °C and light intensity of 35 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, until examined. Cyanobionts from *Azolla* were directly collected from the host plants *Azolla pinnata* subsp. *pinnata* strain 7001, *Azolla rubra* strain ADUL2 and *Azolla caroliniana* strain 3001 grown in freshwater in the greenhouse at Stockholm University. Mature fronds of each strain of *Azolla* were collected, washed several times with distilled water and the roots were removed. The 'gentle roller'

Table 1. Cyanobacterial strains included in this study, their origin and references to literature and to GenBank/EMBL/DBJ sequence accession numbers

The accession numbers of strains for which the 16S rRNA gene sequence has been determined in this work are in bold.

Classification	Strain	Host or habitat, location	Reference (accession no.)
<i>Nostoc</i> sp.	Al1*	<i>Anthoceros laevis</i> (Bryophyta), Naples, Italy	Vagnoli <i>et al.</i> (1992) (AM711522)
<i>Nostoc</i> sp.	Al3*	<i>Anthoceros laevis</i> (Bryophyta), Lucca, Italy	Vagnoli <i>et al.</i> (1992) (AM711531)
<i>Nostoc</i> sp.	Ev1*	<i>Encephalartos villosus</i> (Gymnospermae), Botanical garden, Bologna, Italy	Vagnoli <i>et al.</i> (1992) (AM711535)
<i>Nostoc</i> sp.	De1*	<i>Dioon edule</i> (Gymnospermae), Rome, Italy	Vagnoli <i>et al.</i> (1992) (AM711534)
<i>Nostoc</i> sp.	Cr4*	<i>Cycas revoluta</i> (Gymnospermae), Florence, Italy	Vagnoli <i>et al.</i> (1992) (AM711533)
<i>Nostoc</i> sp.	Cc2*	<i>Cycas circinalis</i> (Gymnospermae), Botanical garden, Pisa, Italy	Vagnoli <i>et al.</i> (1992) (AM711532)
<i>Nostoc</i> sp.	Gm*	<i>Gunnera manicata</i> (Angiospermae), Siena, Italy	Vagnoli <i>et al.</i> (1992) (AM711536)
<i>Nostoc</i> sp.	0GU36S01*	<i>Gunnera tinctoria</i> (Angiospermae), Achill Island, Ireland	Not available (AM711526)
<i>Nostoc</i> sp.	0GU36S02*	<i>Gunnera tinctoria</i> (Angiospermae), Achill Island, Ireland	Not available (AM711527)
<i>Azolla</i> symbiont	7001	<i>Azolla pinnata</i> subsp. <i>pinnata</i> 7001, Australia	Zheng <i>et al.</i> (1999) (AM711551)
<i>Azolla</i> symbiont	ADUL2*	<i>Azolla rubra</i> ADUL2, New Zealand	Zheng <i>et al.</i> (1999) (AM711550)
<i>Azolla</i> symbiont	3001*	<i>Azolla caroliniana</i> 3001, USA	Zheng <i>et al.</i> (1999) (AM711552)
<i>Nostoc</i> sp.	996†	Free-living soil isolate, Central Africa	Not available (AM711530)
<i>Nostoc</i> sp.	9104†	<i>Gunnera tinctoria</i> (Angiospermae) Chile	Nilsson <i>et al.</i> (2000) (AM711542)
<i>Nostoc</i> sp.	8926†	<i>Gunnera hamiltonii</i> (Angiospermae), New Zealand	Nilsson <i>et al.</i> (2000) (AM711540)
<i>Nostoc</i> sp.	8901 : 1†	<i>Gunnera macrophylla</i> (Angiospermae), New Zealand	Nilsson <i>et al.</i> (2000) (AM711539)
<i>Nostoc</i> sp.	8964 : 3†	<i>Gunnera prorepens</i> (Angiospermae), New Zealand	Nilsson <i>et al.</i> (2000) (AM711541)
<i>Nostoc</i> sp.	Lukesova 1/86‡	Oak forest mineral soil layer, Netolice, Czech Republic	Hrouzek <i>et al.</i> (2005) (AM711545)
<i>Nostoc</i> sp.	Lukesova 40/93‡	Alluvial soil on granite and dolomite rocks, Ellesmere, Canada	Hrouzek <i>et al.</i> (2005) (AM711528)
<i>Nostoc</i> sp.	Lukesova 5/96‡	Clay soil, dump after coal mining, Sokolov, Czech Republic	Hrouzek <i>et al.</i> (2005) (AM711546)
<i>Nostoc muscorum</i>	Lukesova 1/87‡	Soil, wet meadow, Dlouha Ves, Czech Republic	Not available (AM711523)
<i>Nostoc muscorum</i>	Lukesova 2/91‡	Arable field, Nezamyslice, Czech Republic	Hrouzek <i>et al.</i> (2005) (AM711524)
<i>Nostoc</i> sp.	TO1S01*	Woodland, Tuscany, Italy	Not available (AM711549)
<i>Nostoc</i> sp.	TH1S01*	Rice field not flooded by water, Thailand	Not available (AM711547)
<i>Nostoc calcicola</i>	TH2S22*	Rice field not flooded by water, Thailand	Not available (AM711529)
<i>Nostoc elgonense</i>	TH3S05*	Rice field not flooded by water, Thailand	Not available (AM711548)
<i>Nostoc</i> sp.	Cam2S01*	Savannah soil of volcanic origin, Cameroon	Not available (AM711543)
<i>Nostoc</i> sp.	Mau15*	Rice field not flooded by water, Mauritania	Not available (AM711544)
<i>Nostoc</i> sp.	PCC 9426§	Rice field soil, Nile Delta, Egypt	Rippka & Herdman (1992) (AM711538)
<i>Nostoc</i> sp.	PCC 8976§	Brackish marshland, Mediterranean coast, France	Rippka & Herdman (1992) (AM711525)
<i>Nostoc</i> sp.	PCC 8112§	Laundromat discharge pond, Michigan, USA	Rippka & Herdman (1992) (AM711537)
<i>Nostoc</i> sp.	PCC 9231§	<i>Gunnera dentata</i> (Angiospermae), New Zealand	Svenning <i>et al.</i> (2005) (AY742452)
<i>Nostoc</i> sp.	PCC 9229§	<i>Gunnera monoica</i> (Angiospermae), New Zealand	Svenning <i>et al.</i> (2005) (AY742451)
<i>Nostoc</i> sp.	PCC 73102§	<i>Macrozamia</i> sp. (Gymnospermae), Australia	Rippka & Herdman (1992) (AF027655)
<i>Nostoc</i> sp.	PCC 7120§	Iowa State University, Iowa, USA	Rippka & Herdman (1992) (NC_003272)
<i>Nostoc</i> sp.	PCC9305§	<i>Anthoceros punctatus</i> (Bryophyta), California, USA	Svenning <i>et al.</i> (2005) (AY742453)
<i>Nostoc</i> sp.	8938†	<i>Gunnera dentata</i> (Angiospermae), New Zealand	Svenning <i>et al.</i> (2005) (AY742454)
<i>Nostoc</i> sp.	8941†	<i>Gunnera dentata</i> (Angiospermae), New Zealand	Svenning <i>et al.</i> (2005) (AY742448)
<i>Nostoc</i> sp.	8916†	<i>Gunnera monoica</i> (Angiospermae), New Zealand	Svenning <i>et al.</i> (2005) (AY742447)
<i>Nostoc</i> sp.	8963†	<i>Gunnera prorepens</i> (Angiospermae), New Zealand	Svenning <i>et al.</i> (2005) (AY742449)
<i>Nostoc</i> sp.†		<i>Azolla filiculoides</i> 1010, Peru	Svenning <i>et al.</i> (2005) (AY742450)
<i>Anabaena oscillarioides</i>	HINDAK 1984/43	Canada	Rajaniemi <i>et al.</i> (2005) (AJ630428)
<i>Anabaena</i> cf. <i>cylindrica</i>	XP6B	Sediment, Gulf of Finland, Porkkala, Finland	Rajaniemi <i>et al.</i> (2005) (AJ630414)
<i>Anabaena planctonica</i>	1TU36S8	Lake Tuusulanjärvi, Finland	Rajaniemi <i>et al.</i> (2005) (AJ630433)
<i>Anabaena oscillarioides</i>	BECID32	Epilithic, Gulf of Finland, Vousaari, Finland	Rajaniemi <i>et al.</i> (2005) (AJ630427)

Table 1. cont.

Classification	Strain	Host or habitat, location	Reference (accession no.)
<i>Trichormus variabilis</i>	HINDAK 2001/4	Soil, Dombay Valley, Caucasus Mountains, Russia	Rajaniemi <i>et al.</i> (2005) (AJ630456)
<i>Trichormus azollae</i>	KOM BAI/1983	India	Rajaniemi <i>et al.</i> (2005) (AJ630454)
<i>Nostoc</i> sp.	1TU14S8	Lake Tuusulanjärvi, Finland	Rajaniemi <i>et al.</i> (2005) (AJ630453)
<i>Anabaena augstumalis</i>	JAHNKE/4a	Rostock, Germany	Rajaniemi <i>et al.</i> (2005) (AJ630458)
<i>Anabaena</i> cf. <i>cylindrica</i>	133	Lake Arresø, Denmark	Gugger <i>et al.</i> (2002) (AJ293110)
<i>Nostoc muscorum</i>	CENA 18	Wetland sediment, Japurá River, Amazon basin, Brazil	Fiore <i>et al.</i> (2005) (AY218827)
<i>Nostoc calcicola</i>	III (Lukesova 9/89)‡	Field, České Budějovice, Czech Republic	Rajaniemi <i>et al.</i> (2005) (AJ630447)
<i>Nostoc edaphicum</i>	X (Lukesova 11/88)‡	Field, Chelcice, Czech Republic	Rajaniemi <i>et al.</i> (2005) (AJ630449)
<i>Nostoc ellipsosporum</i>	V (Lukesova 51/91)‡	Field, Nezamyslice, Czech Republic	Rajaniemi <i>et al.</i> (2005) (AJ630450)
<i>Nostoc muscorum</i>	I (Lukesova 14/86)‡	Field, Dlouha Ves, Czech Republic	Rajaniemi <i>et al.</i> (2005) (AJ630451)
<i>Nodularia harveyana</i>	Lukesova 18/94‡	Saline meadow, Sedlec, Czech Republic	Not available (AM711554)
<i>Nodularia</i> sp.	Lukesova 1/91‡	Salty sand, oil mining area, West Siberia, Russia	Not available (AM711553)
<i>Cylindrospermopsis raciborskii</i>	Solomon Dam		(AF067819)
<i>Cylindrospermopsis raciborskii</i>	AWT205		(AF092504)
<i>Chroococcidiopsis thermalis</i>	PCC 7203		Rippka & Herdman (1992) (AB039005)

*Strains kept in the CNR-CCC, the Cyanobacterial Culture Collection of the CNR - Institute for Ecosystem Study, Sesto Fiorentino, Italy.

†Strains kept in the Culture Collection of the Department of Botany, Stockholm University, Sweden.

‡Strains kept in the Culture Collection of Soil Algae and Cyanobacteria of the Institute of Soil Biology, České Budějovice, Czech Republic.

§Strains kept in the PCC (Pasteur Culture Collection of Cyanobacteria), Institut Pasteur, Paris, France.

procedure (Peters & Mayne, 1974) was utilized to isolate filaments of symbiotic cyanobacteria from within leaf cavities.

DNA extraction. DNA was extracted from biomass of freshly grown cyanobacterial cultures and from filament bundles isolated from *Azolla* leaves as described by Hrouzek *et al.* (2005). After ethanol precipitation, DNA was resuspended in 50 or 100 µl TE buffer (10 mM Tris/HCl pH 7.5, 1 mM EDTA) and stored at -20°C .

Amplification of the 16S rRNA gene+ITS. All cultures of the cyanobacterial strains studied were unialgal, but several of them were not axenic. Therefore, a cyanobacteria-specific protocol was used for the amplification of the 16S rRNA gene+ITS region, using primers 16S27F and 23S30R, the latter being specific for cyanobacteria (Taton *et al.*, 2003). The 50 µl PCR volume contained 1 µl DyNAzyme EXT (1 U µl⁻¹) (Finnzymes), 5 µl 10 × DyNAzyme EXT buffer containing 50 mM MgCl₂, 5 µl dNTP mixture containing 2 mM concentration of each dNTP, 2.5 µl (10 pmol µl⁻¹) of each primer and 5 µl BSA (10 mg ml⁻¹). The mixture was sterilized under UV light and 1 µl DNA template was added for amplification to take place according to the following PCR program: 94 °C for 5 min, 10 cycles of 94 °C for 45 s, 57 °C for 45 s and 2 min at 72 °C, 25 cycles of 94 °C for 45 s, 54 °C for 45 s and 2 min at 72 °C, and extension at 72 °C for 7 min. PCR products were checked on 1% w/v agarose gel.

Fingerprint analysis (ARDRA). The PCR products (approx. 2 kbp) were digested separately with restriction endonucleases *Mbo*I, *Hpa*II

and *Hha*I (Fermentas). Digestions were carried out according to the manufacturer's instructions in 10 µl total volume. The restriction fragments obtained were separated on a 2.8% w/v MetaPhor agarose gel (Lonza) for 4.5 h at 150 V and 15 °C. Analysis of electrophoretic profiles was carried out on digital images using the software BioNumerics version 2.5 (Applied Maths). For each strain, a composite profile obtained by assembling three normalized restriction profiles, one for each restriction endonuclease, was included in the analysis. DNA molecular weight marker XIV (Roche) was run in several positions of each gel for electrophoretic profile normalization. Gel profiles were manually edited to check for the correct assignment of the bands to band classes. The unweighted pair-group method using arithmetic averages (UPGMA) was performed on the normalized composite profiles. Fragments shorter than 100 bp were not considered in the analysis.

Sequencing and analysis of the 16S rRNA. Aliquots of PCR products from the 16S rRNA gene+ITS region were sent for full-length sequencing to Cogenics (Grenoble, France). Sequencing primers were 16S979F, 16S544R and 16S1092R (Hrouzek *et al.*, 2005). The chromatograms were analysed by the gauntlet PHRED/PHRAP/CONSED (Gordon, 2004) and consensus sequences imported in ARB (Ludwig *et al.*, 2004), which was used for all subsequent analytical steps. Sequences were aligned with the ARB database of public domain sequences and trimmed at a position homologous to the end of the *Escherichia coli* 16S rRNA gene sequence. The alignment was used to generate phylogenetic trees based on three different algorithm options

integrated in ARB: neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML). A selection of publicly available sequences belonging to the genera *Anabaena*, *Cylindrospermopsis*, *Nodularia*, *Nostoc* and *Trichormus* (Table 1) was added to the sequences of the strains studied in this work; *Chroococcidiopsis thermalis* PCC 7203 was used to root all trees. NJ and MP analysis were bootstrapped with 500 and 100 replicates, respectively; due to software constraints, ML analysis was not subjected to bootstrap.

Morphological study. The morphological study was performed on a total of 17 cultured strains (Table 2), including ten symbiotic and seven free-living ones. All examinations were performed on cultures growing on agar plates. In addition, basic morphometric measurements were performed on three *Azolla* cyanobionts, which were directly mounted on slides after collection from the leaf cavity; the observation of such natural samples did not allow the evaluation of hormogonia formation and life cycle changes. Morphology of the isolates was studied in young, exponentially growing and old cultures of 3, 10 and 30 days, respectively. Cyanobacteria were examined under an Olympus CX40 light microscope equipped with a digital camera. The morphology of colonies, vegetative filaments, hormogonia and of the mucilaginous sheaths of filaments and the different cell types (vegetative cells, heterocytes and akinetes) were studied. Cell parameters were measured using the DP-SOFT software (Olympus); 50–300 measurements were taken for each parameter to describe the trait variability. Vegetative filaments and hormogonia were classified according to their length and shape into the following categories: long filaments (>50 vegetative cells), short filaments (<50 vegetative cells), coiled, wavy or straight filaments. The morphology of the mucilaginous sheaths was classified into two main categories:

diffusive (where no sharp edges of microcolony could be observed) and enveloping filament (delicate, not laminated sheath expanding 2–5 µm around the filament). The collected data were analysed with the software Statistica for Windows (StatSoft). Cluster analysis was carried out for similarities in selected morphological characters of studied strains [width of filaments, presence of akinete-like cells (AKLC), length of filament and proportion of terminal cells] by using the same software. Morphological data previously obtained following the same protocol (Hrouzek *et al.*, 2005) were included in the cluster analysis. To calculate distances in the cluster analysis, the Euclidean distance and complete linkage parameters were used.

RESULTS AND DISCUSSION

Phenotypic analysis

To allow for a wider account of *Nostoc* morphology, data obtained in this work were considered along with data from 14 other free-living and symbiotic *Nostoc* strains described by Hrouzek *et al.* (2005). In all seven studied isolates from *Gunnera*, the apoheterocytic formation of akinetes and the formation of motile hormogonia observed corresponded with recent descriptions of the development and the morphological structures of the genera *Nostoc* or *Trichormus* (Komárek & Anagnostidis, 1989; Rippka *et al.*, 1979). However, their morphometric evaluation showed a remarkable degree of heterogeneity in several characters. Data are summarized in Table 2 and Figs 1–3.

Table 2. Morphological features of cyanobacterial strains included in this study

TH, Shape of terminal heterocyte; Horm., presence of hormogonia; AKLC, presence of akinete-like cells; Fattened, fattened cells similar to AKLC; ND, not determined. Dimensions given as length × width: max.(mean)min.

Genus	Strain	Dimensions (µm)		TH	Shape of filaments	Horm.	AKLC	Mucilaginous envelope
		Vegetative cells	Akinetes					
<i>Nostoc</i> sp.	All	6.9(4.4)2.7 × 4.9(4.2)3.2	8.2(6.7)5.0 × 7.0(6.2)5.1	Oval	Long wavy	+	–	Diffusive
<i>Nostoc</i> sp.	De1	6.3(4.0)2.3 × 5.4(4.4)3.4	8.0(7.0)5.9 × 4.9(4.3)3.6	Oval	Long wavy	+	–	Diffusive
<i>Nostoc</i> sp.	Cr4	8.2(5.5)3.2 × 5.4(4.4)3.3	9.5(6.4)3.7 × 6.2(5.1)3.8	Oval	Long wavy	+	–	Diffusive
<i>Nostoc</i> sp.	Gm	7.5(4.6)3.0 × 4.1(2.9)1.8	7.4(6.7)5.9 × 3.8(3.7)3.6	Oval	Long wavy	+	+	Diffusive
<i>Nostoc</i> sp.	9104	6.8(3.9)1.9 × 4.3(3.5)2.7	7.8(4.5)2.2 × 5.0(4.0)3.1	Oval	Long wavy	+	+	Diffusive
<i>Nostoc</i> sp.	8901:1	6.9(4.7)2.8 × 5.2(3.9)2.5	9.2(6.6)4.5 × 6.3(4.3)3.3	No	Long wavy	–	Fattened	Diffusive
<i>Nostoc</i> sp.	8964:3	6.8(4.1)1.8 × 7.0(4.5)2.8	8.2(6.0)4.3 × 5.6(4.8)3.8	No	Long wavy	–	–	Diffusive
<i>Nostoc muscorum</i>	Lukešová 1/87	7.3(4.6)2.9 × 5.8(4.5)3.5	8.1(6.4)4.5 × 6.3(5.0)3.9	No	Long wavy	+	–	Diffusive
<i>Nostoc</i> sp.	T01S01	7.6(5.0)2.9 × 4.1(3.4)2.7	8.4(8.2)8.1 × 4.8(4.6)4.4	Conic	Long wavy	+	–	Diffusive
<i>Nostoc</i> sp.	TH1S01	6.9(4.9)3.3 × 5.9(5.0)3.9	7.8(5.8)4.3 × 6.3(5.3)4.5	Oval	Long wavy	–	–	Diffusive
<i>Nostoc</i> sp.	Cam2S01	5.8(3.7)2.3 × 4.7(3.8)2.9	5.9(4.5)3.0 × 4.8(3.6)2.7	Conic	Short wavy	–	–	Diffusive
<i>Nostoc</i> sp.	PCC 8976	7.4(3.9)2.3 × 3.9(3.9)2.5	9.1(7.3)6.0 × 5.8(4.9)4.2	Oval	Long wavy	–	–	Diffusive
<i>Nostoc</i> sp.	PCC 8112	6.7(4.2)2.8 × 5.4(3.9)3.1	7.2(6.4)4.6 × 4.7(4.0)3.4	Oval	Long wavy	–	–	Diffusive
<i>Nostoc</i> sp.	OGU36S01	6.8(4.6)2.7 × 4.6(3.7)2.5	7.4(6.0)4.7 × 5.7(5.1)4.2	Conic	Irreg. coiled	+	+	Enveloping
<i>Nostoc</i> sp.	OGU36S02	6.6(4.3)2.9 × 4.5(3.5)2.4	7.3(6.6)5.1 × 7.0(5.1)3.9	Conic	Irreg. coiled	+	+	Enveloping
<i>Nostoc</i> sp.	8926	8.6(5.4)3.1 × 4.3(3.6)2.9	7.9(7.3)6.1 × 4.5(4.1)3.8	Conic	Short wavy	+	+	Enveloping
<i>Nostoc</i> sp.	996	5.1(3.1)1.7 × 4.1(2.8)1.9	6.7(5.4)4.4 × 3.8(3.4)3.1	Oval	Irreg. coiled	–	–	Firm
<i>Azolla</i> symbiont	7001	15.0(10.8)5.5 × 11.5(8.9)6.6	ND	No	Long wavy	ND	–	ND
<i>Azolla</i> symbiont	ADUL2	15.2(8.7)5.1 × 7.5(6.1)4.6	ND	No	Long wavy	ND	–	ND
<i>Azolla</i> symbiont	3001	18.9(8.7)5.5 × 7.6(5.9)4.7	ND	No	Long wavy	ND	–	ND

Fig. 1. Cluster analysis of selected morphological data of symbiotic and free-living cyanobacterial strains performed with the method of single linkage, Euclidean distance. Width of filaments, presence of AKLC, length of filaments and proportion of terminal cells were taken into consideration. Morphological data relative to strains Lukesova 5/96, 40/93, 1/86, 2/91, Al3, Mau15, Ev1, TH3SO5, Cc2 and TH2S22 had been obtained previously (Hrouzek *et al.*, 2005). symb., symbiont.

Under the cultivation conditions applied, hormogonia could be observed in all but two symbiotic *Nostoc* strains: *Gunnera* cyanobionts 8901:1 and 8964:3 (Table 2 and Hrouzek *et al.*, 2005). The cyanobacterial strains *Nostoc* sp. 8926 (isolated from *Gunnera hamiltonii*), OGU36S01, OGU36S02 and 9104 (isolated from *Gunnera tinctoria*) and Gm (isolated from *Gunnera manicata*) showed abundant formation of typical straight hormogonia with conical end-cells (Fig. 2d, f); formation of akinete-like cells (AKLC) was also observed (Fig. 2e, p). However, strains 8964:3 and 8901:1, isolated from *Gunnera prorepens* and *Gunnera macrophylla*, respectively, showed a clearly distinct morphology, forming long vegetative filaments consisting of oval cells embedded in diffusive mucilage (Fig. 2a–c). Moreover, no hormogonia were observed and AKLC were completely missing from strain 8964:3, while atypical fattened cells, similar to AKLC, were observed in strain 8901:1. Granulated akinetes in long chains appeared during the differentiation of the filaments. Such morphology is typical of the *N. muscorum* group as defined by Hrouzek *et al.* (2005). The division of *Nostoc* strains on the basis of the morphological characters observed is supported by the cluster analysis of selected morphological data shown in Fig. 1 (width of filaments, presence of AKLC, length of filaments and proportion of terminal cells were taken into consideration). For this cluster analysis, previously obtained morphometric data were also used (Hrouzek *et al.*, 2005).

Apart from the *Azolla* cyanobionts and the *Gunnera* symbiont strain 8901:1, which were situated at the base of the UPGMA dendrogram of morphological data, three main branches could be seen in the cluster analysis shown in Fig. 1. Mainly strains with long vegetative filaments, clearly defined granulated akinetes (Fig. 2b) and hormogonia similar to vegetative filaments were located in branch A. Except for *Gunnera* strain 8964:3, symbionts of cycads

strains De and Cr4 (Fig. 2m–o), *Anthoceros* symbiont strain Al1 (Fig. 2h) and the free-living strains TH1SO1 (Fig. 3a) and TH3SO5 were also members of this branch. This morphological group also included the free-living *N. muscorum* strains Lukesova 1/87 and 2/91 (Fig. 2c) and the cycad symbiont strain Cc2, which had been characterized previously (Hrouzek *et al.*, 2005). Branches B and C contained most of the *Gunnera* symbionts included in the present study: strains 9104 (Fig. 2f), 8926 (Fig. 2d, e), Gm (Fig. 2l), OGU36S01 (Fig. 2p) and OGU36S02. These groups corresponded to the morphological group of *N. calcicola*.

Despite the identification of common characters, the group of *N. calcicola* is still very heterogeneous, as demonstrated by the fact that strains similar to *N. calcicola* were separated into two clusters, B and C.

Strains characterized by microcolony formation (*Nostoc* sp. 996, Lukesova 5/96 and 40/93), a character not included in the calculation of the dendrogram, were present in group C and strains growing in thin films (*N. calcicola* Lukesova 1/86, TH2S22, TO1SO1 and Mau15) were present in all clusters. In two of the film-forming strains (TH2S22 and TH3SO5), similarity to the *Trichormus* description is evident, which can be distinguished from *Nostoc* solely by the thin-film growth (Komárek & Anagnostidis, 1989).

Morphological analysis of the cyanobionts from the *Azolla* leaf cavities was performed on cyanobacterial filaments collected directly from the leaf cavity, since the cyanobionts are uncultivable (Lechno-Yossef & Nierzwicki-Bauer, 2002). Filaments consisted of significantly wider cells (8–15 µm) than those observed in all other strains studied. The cell enlargement could be a consequence of the symbiotic condition (Rai *et al.*, 2000), and thus the measured dimensions cannot be considered necessarily stable in hypothetical *in vitro* cultivation conditions.

Fig. 2. Light microscope pictures of symbiotic *Nostoc* strains. (a) Vegetative filaments of *Nostoc* sp. 8901 : 1, symbiont of *Gunnera macrophylla*. (b) Akinete formation in *Nostoc* sp. 8964 : 3, symbiont of *Gunnera prorepens*. (c) Vegetative filaments and akinetes of *N. muscorum* Lukesova 1/87. (d) Hormogonia with pointed end-cells of *Nostoc* sp. 8926, symbiont of *Gunnera hamiltonii*. (e) AKLC of *Nostoc* sp. 8926. (f) Hormogonium of *Nostoc* sp. 9104, symbiont of *Gunnera tinctoria*. (g) Akinete formation of free-living strain *Nostoc* sp. 996. (h) Vegetative filaments of *Nostoc* sp. Al1, symbiont of *Anthoceros laevis*. (i) Akinete formation in the free-living strain *Nostoc* sp. Cam2SO1. (j, k) Hormogonia and vegetative filaments in *Nostoc* sp. Al3, symbiont of *Anthoceros laevis*. (l) Formation of AKLC in *Nostoc* sp. Gm, symbiont of *Gunnera manicata*. (m) Vegetative filaments of *Nostoc* sp. Cr4, symbiont of *Cycas revoluta*. (n) *Nostoc* sp. De, symbiont of *Dioon edule*. (o) Hormogonia formation in *Nostoc* sp. Cr4, symbiont of *Cycas revoluta*. (p) AKLC in *Nostoc* sp. OGU36S02, symbiont of *Gunnera tinctoria*. Bars, 10 μ m.

However, since the *Azolla* cyanobionts might actually be incapable of growing under free-living conditions, the observed morphology might represent their stable and sole morphology. A morphological characterization had previously been performed on *Azolla* cyanobionts (Hill, 1975; Pabby *et al.*, 2003). In all populations examined, only vegetative filaments with elliptic heterocytes were observed (Fig. 3c–e). No akinetes or hormogonia were observed within the cavity at the time of isolation. In the cluster analysis shown in Fig. 1, the separation of *Azolla* cyanobionts into two branches could be explained by the fact that filaments isolated from *Azolla pinnata* subsp. *pinnata* strain 7001 were significantly wider (8.9 μ m on average) than those from *Azolla rubra* strain ADUL2 and from *Azolla caroliniana* strain 3001 (6.1 and 5.9 μ m on

Fig. 3. Light microscope pictures of *Azolla* cyanobionts and free-living cyanobacteria. (a) Unusual akinete formation in free-living strain *Nostoc* sp. TH1SO1. (b) Vegetative filaments of *Nostoc* sp. TH2S22. (c) *Azolla rubra* cyanobiont isolate ADUL2. (d) Cyanobiont of *Azolla caroliniana*, isolate 3001. (e) *Azolla pinnata* cyanobiont isolate 7001. Bars, 10 μ m.

average, respectively). However, when considering the other morphological characters, all these isolates were definitely similar. The separation of these isolates in two branches could even be an artefact due to the clustering algorithm, since the *Azolla* cyanobionts did not fall into any other cluster and were placed at the base of the dendrogram.

Amplified rDNA restriction analysis (ARDRA)

As a first step to investigate their genetic diversity, 22 *Nostoc* sp. strains, two *Nodularia* sp. strains and three cyanobionts of *Azolla* were subjected to ARDRA applied to the 16S rRNA gene + ITS region. Supplementary Fig. S1, showing the UPGMA cluster analysis of the aligned ARDRA profiles, is available with the online version of this paper. The RFLP of the ITS region alone had been previously demonstrated to discriminate cyanobacterial strains at the intra- or interspecific level (Scheldeman *et al.*, 1999).

Our approach revealed that the cyanobacterial filaments isolated from *Azolla* shared highly similar ARDRA profiles and formed a branch separated from all other strains studied. Two *Nodularia* sp. strains clustered in a deep branching group, loosely related to the three free-living *Nostoc* sp. strains Mau 15, TH3S05 and TH1S01. All the remaining *Nostoc* sp. strains were part of a unique large cluster with an overall similarity around 62 %, inside which three main subgroups could be identified with similarity values ranging between 69 and 76 %.

The results indicated a wide variability among *Nostoc* isolates and only strain De, isolated from the cycad *Dioon edule*, and the free-living soil isolate Lukesova 1/87 showed 100 % similarity of ARDRA profiles. In all other strains, similarity of ARDRA profiles was lower than 95 %. Since ARDRA was not informative enough in resolving the similarity level, the precise relationships among the strains

were then investigated by analysis of the 16S rRNA sequence.

Phylogenetic analysis

The 16S rRNA gene of 28 *Nostoc* sp. strains, three cyanobionts of *Azolla* and two strains of *Nodularia* was sequenced. The 27 cyanobacterial sequences retrieved from the public domain, listed in Table 1, were added to the phylogenetic analysis. As a whole, this selection of sequences included representatives of the clusters obtained in a phylogenetic analysis of 81 *Nostoc* strains by Hrouzek *et al.* (2005) that covered the presently known 16S rRNA

gene diversity range of soil and symbiotic *Nostoc*. The phylogenetic trees were built using nearly complete 16S rRNA gene sequences longer than 1363 bp, apart from the partial sequence of strain *Nostoc* sp. 996, which was only 905 bp long. NJ, MP and ML trees had nearly identical topologies (data not shown) and the bootstrap values, available for NJ and MP analyses, were largely congruent. This indicated that the phylogenetic relationships among the studied strains inferred from our analysis were largely independent of method-related biases. Therefore, only the NJ tree with the indication of bootstrap values obtained with the NJ and MP approaches is presented in Fig. 4.

Fig. 4. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of heterocystous cyanobacteria, with the indication of retrieved stable clusters. Sequences obtained in this study are indicated in bold. An asterisk indicates sequences from symbiotic cyanobacteria. Numbers at nodes indicate bootstrap values obtained from distance and parsimony analyses, respectively; apart from one cluster discussed in the text, bootstrap values were reported only at nodes where at least one value was higher than 50 %. GenBank sequence accession numbers are given in parentheses.

The smallest clusters of highly similar sequences were all supported by high bootstrap values, indicating that they represented stable monophyletic assemblages of strains. Inferred evolutionary patterns leading to these monophyletic units were sometimes less robust, as indicated by nodes connecting stable clusters, which were not always supported by bootstrapping. At greater evolutionary distance, three larger strain assemblages, supported by relatively high bootstrap values, could also be identified.

A large, rather compact group of 19 strains, indicated as cluster I in Fig. 4, showed bootstrap support of 81–58 %. Apart from *Nostoc* sp. strain Al1, a 98–100 % bootstrap value was granted to the group of the remaining 18 strains. This latter, strongly supported group harboured 12 symbiotic strains mainly isolated from *Gunnera* species, and the four free-living isolates Lukesova 1/86, 40/93, 5/96 and 1TU14S8. Included in this cluster were also *N. calcicola* III and *N. edaphicum* X, the morphology of which had been previously described (Hrouzek *et al.*, 2003). The clustering together of *N. calcicola* and *N. edaphicum* was supported by the study of Rajaniemi *et al.* (2005) using *rbclX*, *rpoB* and the 16S rRNA gene as genetic markers. This group also contained *Nostoc* sp. PCC 73102, the reference strain for DNA–DNA hybridization *Nostoc* cluster 1 (Lachance, 1981; Rippka & Herdman, 1992). Clustering of *Nostoc* sp. PCC 73102 with *Gunnera* and lichen symbionts has been previously shown in other studies (Svenning *et al.*, 2005; Rikkinen *et al.*, 2002). *Nostoc* sp. strain Al1, isolated from *Anthoceros laevis*, was placed relatively apart in cluster I; it was also distinct from the other members in the cluster analysis of morphological data shown in Fig. 1, where it belonged to branch A, while other cluster members were located in branches B or C. Even if *Nostoc* sp. strain Al1 formed a separate branch, its contiguity to other cluster I members was shown by all three methods used for the phylogenetic analysis in the present study.

The second supported group (bootstrap values 79–96 %), named cluster II in Fig. 4, contained ten *Nostoc* sp. strains, six of which originated from symbiotic associations, three from *Gunnera* and three from cycads, and four free-living strains. Very high bootstrap values supported the existence of two subclusters within cluster II. Subcluster IIa harboured the two *N. muscorum* strains Lukesova 1/87 and 2/91, *N. muscorum* I and strains De and 8938, isolated from *Dioon* and *Gunnera dentata*, respectively. Subcluster IIb contained strains Cc2 and Cr4, isolated from *Cycas*, and strains PCC 9231 and 8964:3, isolated from *G. dentata* and *G. prorepens*, respectively, together with the free-living strain TO1S01.

Cluster III (Fig. 4), composed of *Nostoc* sp. strain 8916, from *Gunnera monoica*, and *Nostoc* sp. Mau15, a free-living isolate from a rice field, had low bootstrap support (36–52 %), but was nevertheless considered because it was generated in all three phylogenetic analyses, with a deep branching position in the tree. This deep branching position of strain 8916 was shown by Svenning *et al.*

(2005), where the strain was linked to *Nostoc* strains PCC 9231 and 8938. However, by the inclusion of strain *Nostoc* sp. Mau15 and of more strains related to *Nostoc* sp. PCC 9231 and 8938, we could demonstrate that *Nostoc* strains PCC 9231 and 8938 are members of the newly described cluster II, the well-defined group of *N. muscorum*, while the sequence of strain 8916 fell outside this group and is related to the sequence of strain Mau15.

Reference strain PCC 7120, a typical free-living strain and a representative of *Nostoc* cluster 3 based on DNA–DNA hybridization (Lachance, 1981), was found to be quite distant from strains of symbiotic origin and located near the root of the large branch including clusters I, II and III.

A large cluster IV (Fig. 4) corresponded to the monophyletic radiation of clusters 1–4, described in details by Rajaniemi *et al.* (2005), and included the genera *Anabaena*, *Trichormus*, *Cylindrospermopsis* and *Nodularia*. On the basis of bootstrap values, several subclusters could be identified inside cluster IV. Strongly supported by bootstrap analysis, subcluster IVa included the sequences retrieved from four cyanobionts of *Azolla* plants: three sequences were obtained in the present study, while one (GenBank accession no. AY742450) had been previously published by Svenning *et al.* (2005). Compact clustering of the *Azolla* cyanobionts is consistently supported by ARDRA of the 16S rRNA gene+ITS and 16S rRNA gene sequence analysis. This is in complete agreement with a large survey of the cyanobionts of 35 strains of all species of *Azolla*, shown to be very closely related to each other, with a maximal sequence divergence of 2.3 % (Papaefthimiou *et al.*, 2008). In addition, the sequence of *Nostoc* sp. ‘*Azolla* cyanobiont’ (GenBank accession no. AY742450), as well as cyanobiont populations isolated from *Azolla pinnata* subsp. *pinnata* 7001, have been included in both that study and the present one. High bootstrap support obtained in all studies confirmed that the cluster containing the four sequences of *Azolla* cyanobionts represents the cyanobacterial taxon that evolved in the symbiotic association with all species of *Azolla*. The nearest sequences to the *Azolla* cyanobionts analysed in this study were *Anabaena oscillarioides* HINDAK 1984/43 and *Anabaena cylindrica* 133, isolated from freshwater lakes, in accord with a phylogenetic analysis performed by Papaefthimiou *et al.* (2008). *Trichormus* is one of the genera to which the *Azolla* cyanobionts were assigned in the past (Komárek & Anagnostidis, 1989; Baker *et al.*, 2003); however, the present study showed that species of *Trichormus* were unrelated to the *Azolla* symbionts, which is also in agreement with data obtained by Papaefthimiou *et al.* (2008). The sequences of the *Azolla* cyanobionts did not enter any of the *Nostoc* clusters previously defined by Hrouzek *et al.* (2005), thus ruling out the possibility that they belong to the genus *Nostoc* (data not shown). *A. oscillarioides* HINDAK 1984/43 is consistently connected to *Cylindrospermopsis raciborskii* in our analysis as well as in the work by Rajaniemi *et al.* (2005). The same authors described a cluster (cluster 3) formed by *A. oscillarioides*

HINDAK 1984/43 and *A. cf. cylindrica* 133, which could well correspond to our subcluster IVa with the inclusion of the less related strain 133.

The highly stable subcluster IVb held three free-living *Nostoc* strains of different origins, which were clustered together only on the basis of sequence relatedness; this cluster deserves a complete characterization and possibly the addition of more strains before a supported interpretation is possible.

Sequences in subcluster IVc corresponded to most of the planktonic *Anabaena* strains included in the study by Rajaniemi *et al.* (2005), with the addition of some benthic ones, and were not closely connected to any of the new sequences introduced in our study.

Nostoc sp. strain 8941 is the only cyanobiont belonging to cluster IV that did not originate from the *Azolla* symbiosis; a preferential connection of this strain to any other single or subcluster member of cluster IV is not supported by the phylogenetic analysis. Given that it lacks tightly related companions, the analysis of a larger number of cyanobionts from the *Gunnera* symbiosis and from other symbioses involving cyanobacteria could give more robust support to the phylogenetic distribution of non-*Azolla* cyanobionts.

Nostoc strain Cam2S01, *N. muscorum* CENA 18 (Fiore *et al.*, 2005) and the free-living strain 996 all came from soil or sediments in tropical areas; they were loosely but constantly related in all phylogenetic analyses. A larger number of strains would be necessary to understand whether they represent a new phylogenetic unit. The same is true for *Nostoc ellipsoforum* V and *Nostoc* sp. TH2S22, which were always found together and, in MP and ML analysis, also related to the previously cited group of tropical soil strains. Our study included five strains isolated from rice fields in different locations; interestingly, these strains, while coming from habitats where the presence of *Azolla* is rather common, did not cluster together, nor did they show any appreciable relatedness to cluster IV, where the *Azolla* cyanobionts were located. This supports the perpetual nature of the *Azolla* symbiosis, able to exclude all other cyanobacteria living in the nearby environment.

The deepest nodes of the phylogenetic tree, although with only partial support by the bootstrap analyses, separated the 60 sequences in two large clades. A clade that included clusters I, II and III, and is equivalent to cluster 5 of Rajaniemi *et al.* (2005), corresponded to the typical *Nostoc* classification, with representatives of the species *N. muscorum*, *N. calcicola*, *N. edaphicum* and *N. ellipsoforum* and strains related to *N. commune*. A life stage with the abundant presence of motile hormogonia is widespread among its members. This large cluster included all non-*Azolla*-related nostocacean symbiotic isolates, except the *Gunnera* isolate *Nostoc* strain 8941. Symbiotic strains (except 8901:1 and Al1) belonging to cluster I contained inside this larger clade showed morphological characters resembling the descriptions of *N. calcicola* and *N.*

edaphicum (morphological groups B and C in Fig. 1) previously given by Hrouzek *et al.* (2005). The compact cluster II had a comparable occurrence of symbiotic strains, with morphological traits mostly resembling those of the species *N. muscorum* (group A based on morphology, shown in Fig. 1). We suggest that the frequent occurrence of symbiotic strains within clusters I and II could be explained by the intensive production of hormogonia, found in 11 out of 13 cyanobionts and in many free-living strains.

A small cluster of three strains related to *Trichormus azollae* strain Kom BAI/1983 was found to have a deep rooting inside this large branch with NJ and ML phylogenies, but not with MP, where it remained as a third independent branch at the initial subdivision of the entire tree (data not shown). Strain Kom BAI/1983, which has been isolated and cultured from *Azolla*, was in fact definitely unrelated to uncultured, directly isolated filaments of *Azolla* symbionts. Since the other two strains of this small cluster originated from rice fields, one could suppose that strain Kom BAI/1983 was also originally a free-living cyanobacterium in rice fields, isolated by chance from the external surface of the water fern. This would delineate a cluster of these cyanobacteria, even if sequences of other rice field isolates are found in different positions along the tree (Table 1 and Fig. 4). A specifically focussed study on rice field cyanobacterial populations seems to be required to understand the real identity of the *T. azollae* cluster.

The lowest clade of the phylogenetic tree shown in Fig. 4 corresponded to the wide radiation including the genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia* and possibly *Anabaenopsis* and *Cyanospira*, discussed in details for the genera *Anabaena* and *Aphanizomenon* by Rajaniemi *et al.* (2005); clusters 1–4 described in that study specifically correspond to this large branch. The directly isolated uncultured *Azolla* cyanobionts are thus phylogenetically related to the *Anabaena* radiation while, interestingly, according to literature, *Azolla* cyanobionts show *Nostoc*-like apoheterocytic akinete formation (Anagnostidis & Kómarek, 1988), differing from paraheterocytic akinete formation typically found in species of *Anabaena*.

The deep branching pattern of the phylogenetic tree indicated that, with only one exceptional case deserving more accurate consideration (strain 8941), two distinct patterns of evolution of the symbiotic behaviour could be hypothesized for the nostocacean cyanobacteria, one leading to symbioses of *Nostoc* species with a wide variety of plants, the other leading to the association of a unique cyanobacterial type with the water fern *Azolla*. The former type of association has evolved into a present-day remarkable genetic and morphological diversity of the cyanobionts, coupled with a lack of clear-cut differentiation from free-living cyanobacterial counterparts, mostly belonging to typical soil species of *Nostoc*. The latter association, contrastingly, led to the evolution of a compact and homogeneous cluster, separated from the neighbouring

free-living cyanobacterial genera. This association is particularly strict and selective and up to now it has not been possible to cultivate the cyanobionts separately. What caused the two evolutionary patterns to differentiate remains unclear; however, we can only notice that, while symbioses of *Nostoc* species involve terrestrial plants, the *Azolla* symbiosis has evolved in an aquatic environment.

ACKNOWLEDGEMENTS

We thank gratefully M. C. Margheri for having made cyanobacterial strains of the ISE culture collection available and for critical discussion. C. Sili is thanked for help with microscopy and for critical discussion. D. P. was the recipient of a short-term travel grant to visit S.V.'s lab in the mainframe of the ESF Scientific Programme on Nitrogen Fixing Cyanobacteria – CYANOFIX. Visit exchanges between S.V. and P.H. were supported by a bilateral exchange agreement between the Academy of Sciences of the Czech Republic and the National Research Council of Italy. P.H. was supported by grants MSM 6007665801 (Ecological, evolutionary and experimental biological approaches to the study of the origin and significance of biodiversity) and MSM 6007665808 (Physical biology – New approaches in biological research).

REFERENCES

- Anagnostidis, K. & Kómarek, J. (1988). Modern approach to the classification system of cyanophytes 3 – *Oscillatoriales*. *Arch Hydrobiol Suppl* 80, 327–472.
- Baker, J. A., Entsch, B. & McKay, D. B. (2003). The cyanobiont in an *Azolla* fern is neither *Anabaena* nor *Nostoc*. *FEMS Microbiol Lett* 229, 43–47.
- Caudales, R., Wells, J. M., Antoine, A. D. & Butterfield, J. E. (1995). Fatty acid composition of symbiotic cyanobacteria from different host plant (*Azolla*) species: evidence for co-evolution of host and symbiont. *Int J Syst Bacteriol* 45, 364–370.
- Costa, J.-L., Paulsrud, P. & Lindblad, P. (1999). Cyanobiont diversity within coralloid roots of selected cycad species. *FEMS Microbiol Ecol* 28, 85–91.
- Costa, J. L., Paulsrud, P. & Lindblad, P. (2001). Genetic diversity of *Nostoc* symbionts endophytically associated with two bryophyte species. *Appl Environ Microbiol* 67, 4393–4396.
- Fiore, M. de F., Neilan, B. A., Copp, J. N., Rodrigues, J. L. M., Tsai, S. M., Lee, H. & Trevors, J. T. (2005). Characterization of nitrogen-fixing cyanobacteria in the Brazilian Amazon floodplain. *Water Res* 39, 5017–5026.
- Gebhardt, J. S. & Nierzwicki-Bauer, S. A. (1991). Identification of a common cyanobacterial symbiont associated with *Azolla* spp. through molecular and morphological characterization of free-living and symbiotic cyanobacteria. *Appl Environ Microbiol* 57, 2141–2146.
- Gordon, D. (2004). Viewing and editing assembled sequences using Consed. In *Current Protocols in Bioinformatics*, pp. 11.2.1–11.2.43. Edited by A. D. Baxevanis & D. B. Davison. New York: Wiley.
- Guevara, R., Armesto, J. J. & Caru, M. (2002). Genetic diversity of *Nostoc* microsymbionts from *Gunnera tinctoria* revealed by PCR-STR fingerprinting. *Microb Ecol* 44, 127–136.
- Gugger, M., Lyra, C., Henriksen, P., Couté, A., Humbert, J.-F. & Sivonen, K. (2002). Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. *Int J Syst Evol Microbiol* 52, 1867–1880.
- Henson, B. J., Watson, L. E. & Barnum, S. R. (2002). Molecular differentiation of the heterocystous cyanobacteria, *Nostoc* and *Anabaena*, based on complete *nifD* sequences. *Curr Microbiol* 45, 161–164.
- Henson, B. J., Hesselbrock, S. M., Watson, L. E. & Barnum, S. R. (2004). Molecular phylogeny of the heterocystous cyanobacteria (subsections IV and V) based on *nifD*. *Int J Syst Evol Microbiol* 54, 493–497.
- Hill, D. J. (1975). The pattern of development of *Anabaena* in the *Azolla*-*Anabaena* symbiosis. *Planta* 122, 179–184.
- Hrouzek, P., Simek, M. & Komárek, J. (2003). Nitrogenase activity (acetylene reduction activity) and diversity of six soil *Nostoc* strains. *Arch Hydrobiol Suppl* 146, 87–101.
- Hrouzek, P., Ventura, S., Lukešová, A., Mugnai, M. A., Turicchia, S. & Komárek, J. (2005). Diversity of soil *Nostoc* strains: phylogenetic and phenotypic variability. *Arch Hydrobiol Suppl* 159, 251–264.
- Komárek, J. & Anagnostidis, K. (1989). Modern approach to the classification system of cyanophytes 4 – *Nostocales*. *Arch Hydrobiol Suppl* 82, 247–345.
- Lachance, M.-A. (1981). Genetic relatedness of heterocystous cyanobacteria by deoxyribonucleic acid-deoxyribonucleic acid reassociation. *Int J Syst Bacteriol* 31, 139–147.
- Lazaroff, N. (1966). Photoinduction and photoreversal of the Nostocacean developmental cycle. *J Phycol* 2, 7–17.
- Lazaroff, N. & Vishniac, W. (1961). The effect of light on the developmental cycle of *Nostoc muscorum*, a filamentous blue-green alga. *J Gen Microbiol* 25, 365–374.
- Lechno-Yossef, S. & Nierzwicki-Bauer, S. A. (2002). *Azolla*-*Anabaena* symbiosis. In *Cyanobacteria in Symbiosis*, pp. 153–178. Edited by A. N. Rai, B. Bergman & U. Rasmussen. Dordrecht: Kluwer Academic.
- Lohtander, K., Oksanen, I. & Rikkinen, J. (2003). Genetic diversity of green algal and cyanobacterial photobionts in *Nephroma* (Peltigerales). *Lichenologist* 35, 325–339.
- Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* 32, 1363–1371.
- Mollenhauer, D. (1970). Beiträge zur Kenntnis der Gattung *Nostoc*. *Abh Senckenb Naturf Ges* 524, 1–80.
- Nilsson, M., Bergman, B. & Rasmussen, U. (2000). Cyanobacterial diversity in geographically related and distant host plants of the genus *Gunnera*. *Arch Microbiol* 173, 97–102.
- Pabby, A., Prasanna, R., Nayak, S. & Singh, P. K. (2003). Physiological characterization of the cultured and freshly isolated endosymbionts from different species of *Azolla*. *Plant Physiol Biochem* 41, 73–79.
- Papaefthimiou, D., Van Hove, C., Lejeune, A., Rasmussen, U. & Wilmotte, A. (2008). Diversity and host specificity of genus *Azolla* cyanobionts. *J Phycol* (in press).
- Peters, G. A. & Mayne, B. C. (1974). The *Azolla*, *Anabaena azollae* relationship. I. Initial characterisation of the association. *Plant Physiol* 53, 813–819.
- Plazinski, J., Zheng, Q., Taylor, R., Croft, L., Rolfe, B. G. & Gunning, B. E. S. (1990). DNA probes show genetic variation in cyanobacterial symbionts of the *Azolla* fern and a closer relationship to free-living *Nostoc* strains than to free-living *Anabaena* strains. *Appl Environ Microbiol* 56, 1263–1270.
- Rai, A. N., Söderbäck, E. & Bergman, B. (2000). Tansley review no. 116. Cyanobacterium–plant symbioses. *New Phytol* 147, 449–481.

- Rai, H., Bergman, B. & Rasmussen, U. (2002). *Cyanobacteria in Symbiosis*. Dordrecht: Kluwer Academic.
- Rajaniemi, P., Hrouzek, P., Kastovská, K., Willame, R., Rantala, A., Hoffman, L., Komárek, J. & Sivonen, K. (2005). Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *Int J Syst Evol Microbiol* **55**, 11–26.
- Rasmussen, U. & Nilsson, M. (2002). Cyanobacterial diversity and specificity in plant symbioses. In *Cyanobacteria in Symbiosis*, pp. 313–328. Edited by A. N. Rai, B. Bergman & U. Rasmussen. Dordrecht: Kluwer Academic.
- Rasmussen, U. & Svenning, M. M. (1998). Fingerprinting of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repetitive sequences. *Appl Environ Microbiol* **64**, 265–272.
- Rikkinen, J., Oksanen, I. & Lohtander, K. (2002). Lichen guilds share related cyanobacterial symbionts. *Science* **297**, 357.
- Rippka, R. & Herdman, M. (1992). *Catalogue of strains. Pasteur culture collection of cyanobacterial strains in axenic culture*. Paris, France: Institute Pasteur.
- Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. (1979). Generic assignments, strain histories, and properties of pure cultures of cyanobacteria. *J Gen Microbiol* **111**, 1–61.
- Rippka, R., Castenholz, R. W. & Herdman, M. (2001). Subsection IV. (Formerly *Nostocales* Castenholz 1989b sensu Rippka, Deruelles, Waterbury, Herdman and Stanier 1979). In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 562–589. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. New York: Springer.
- Scheldeman, P., Baurain, D., Bouhy, R., Scott, M., Mühling, M., Whitton, B. A., Belay, A. & Wilmotte, A. (1999). *Arthrospira* ('*Spirulina*') strains from four continents are resolved into only two clusters, based on amplified ribosomal DNA restriction analysis of the internally transcribed spacer. *FEMS Microbiol Lett* **172**, 213–222.
- Strasburger, E. (1884). Die Controversen der indirecten Keimtheilung. *Arch Mikrob Anat* **23**, 301 (in German).
- Svenning, M. M., Eriksson, T. & Rasmussen, U. (2005). Phylogeny of symbiotic cyanobacteria within the genus *Nostoc* based on 16S rDNA sequence analyses. *Arch Microbiol* **183**, 19–26.
- Taton, A., Grubisic, S., Brambilla, E., De Wit, R. & Wilmotte, A. (2003). Cyanobacterial diversity in natural and artificial microbial mats of Lake Fryxell (McMurdo Dry Valleys, Antarctica): a morphological and molecular approach. *Appl Environ Microbiol* **69**, 5157–5169.
- Tomaselli, L., Margheri, M. C., Giovannetti, L., Sili, C. & Carlozzi, P. (1988). The taxonomy of *Azolla* spp. cyanobionts. *Ann Microbiol* **38**, 157–161.
- Vagnoli, L., Margheri, M. C., Allotta, G. & Materassi, R. (1992). Morphological and physiological properties of symbiotic cyanobacteria. *New Phytol* **120**, 243–249.
- van Hove, C. & Lejeune, A. (2002). Applied aspects of *Azolla-Anabaena* symbiosis. In *Cyanobacteria in Symbiosis*, pp. 179–193. Edited by A. N. Rai, B. Bergman & U. Rasmussen. Dordrecht: Kluwer Academic.
- West, N. J. & Adams, D. G. (1997). Phenotypic and genotypic comparison of symbiotic and free-living cyanobacteria. *Appl Environ Microbiol* **63**, 4479–4484.
- Zheng, W. W., Nilsson, M., Bergman, B. & Rasmussen, U. (1999). Genetic diversity and classification of cyanobacteria in different *Azolla* species by the use of PCR fingerprinting. *Theor Appl Genet* **99**, 1187–1193.
- Zheng, W., Song, T., Bao, X., Bergman, B. & Rasmussen, U. (2002). High cyanobacterial diversity in coralloid roots of cycads revealed by PCR fingerprinting. *FEMS Microbiol Ecol* **40**, 215–222.