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Phylogeny of symbiotic cyanobacteria within the genus *Nostoc* based on 16S rDNA sequence analyses

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Abstract A phylogenetic analysis of selected symbiotic *Nostoc* strain sequences and available database 16S rDNA sequences of both symbiotic and free-living cyanobacteria was carried out using maximum likelihood and Bayesian inference techniques. Most of the symbiotic strains fell into well separated clades. One clade consisted of a mixture of symbiotic and free-living isolates. This clade includes *Nostoc* sp. strain PCC 73102, the reference strain proposed for *Nostoc punctiforme*. A separate symbiotic clade with isolates exclusively from *Gunnera* species was also obtained, suggesting that not all symbiotic *Nostoc* species can be assigned to *N. punctiforme*. Moreover, isolates from *Azolla filiculoides* and one from *Gunnera dentata* were well nested within a clade comprising most of the *Anabaena* sequences. This result supports the affiliation of the *Azolla* isolates with the genus *Anabaena* and shows that strains within this genus can form symbioses with additional hosts. Furthermore, these symbiotic strains produced hormogonia, thereby verifying that hormogonia formation is not absent in *Anabaena* and cannot be used as a criterion to distinguish it from *Nostoc*.

Keywords Cyanobacteria · *Nostoc* · *Anabaena* · Symbioses · 16S rRNA gene · Sequencing · Phylogeny

The GenBank accession numbers for the cyanobacterial 16S rRNA gene sequences determined in this study are AY742447-AY742454.

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Introduction

Cyanobacteria are an ancient group of organisms that has been identified in fossils which suggest an age of 3.5 million years (Golubic and Seong-Joo 1999; Schopf 2000) and there is evidence of their symbiotic interactions with some of the first land plants, such as liverworts and hornworts (Raven 2002). Cyanobacteria are widespread throughout the world in different habitats and are major contributors to the nitrogen economy in several ecosystems. They are present as free-living organisms and in symbioses in terrestrial and aquatic environments. In marine environments, cyanobacteria are found in symbiosis with diatoms, sponges and dinoflagellates (Carpenter and Foster 2002; Janson 2002). In terrestrial environments, cyanobacteria form symbioses with a wide range of different hosts, including fungi, bryophytes (liverworts and hornworts), *Azolla* (water fern), cycads (gymnosperm) and *Gunnera* (angiosperm), (Rai et al. 2000).

According to Rippka et al. (1979) and Rippka (1988), cyanobacteria can be divided into five subsections based on phenotypic characteristics. *Nostoc*, the dominating genus in terrestrial symbiotic systems, is classified into subsection IV cluster I together with the genera *Anabaenopsis*, *Cyanospira*, *Aphanizomenon*, *Anabaena*, *Nodularia*, *Cylindrospermum*, *Cylindrospermopsis* and *Scytonema* (Wilmotte and Herdman 2001). Since the genera *Nostoc* and *Anabaena* exhibit very similar structural properties, the absence of hormogonia, the motile stage of the filaments, in *Anabaena* has been used to distinguish them (Wilmotte and Herdman 2001). Hormogonia formation, however, as a negative character is unreliable (Rippka 1988) and their formation can be dependent on growth conditions (Wright et al. 2001). According to Rikkinen et al. (2002), *Nostoc* is a monophyletic group (a clade) that consists of two subclades, *Nostoc* group A and *Nostoc* group B. Group A comprises only *Nostoc* strains from epiphytic lichen species while group B consists of *Nostoc* strains from terrestrial

lichens, a cycad root and free-living strains. *Nostoc punctiforme* has been identified as the symbiont of both fungi and plants, and many symbiotic *Nostoc* isolates are, based on morphological characterization, referred to as *N. punctiforme* (Zimmerman and Culley 1991; Zimmerman and Rosen 1992; Mollenhauer et al. 1996; Kluge et al. 2002). *Nostoc* sp. strain PCC 73120 (ATCC 29133), originally isolated from the cycad *Macrozamia* sp. (Rippka and Herdman 1992), has been proposed as the reference strain for *N. punctiforme* (Rippka and Herdman 1992; Wilmotte and Herdman 2001). The genome of *N. punctiforme* has been sequenced in its entirety (Meeks et al. 2001).

Analyses of the genetic diversity of symbiotic *Nostoc* strains using molecular methods at low taxonomic levels have revealed heterogeneity reflecting high genetic diversity and low host specificity (Costa et al. 1999, 2001; Guevara et al. 2002; Nilsson et al. 2000; Paulsrud et al. 1998; Paulsrud and Lindblad 1998; Rasmussen and Svenning 1998; West and Adams 1997). Sequence heterogeneity between strains also at a higher taxonomic level has been demonstrated using 16S-RFLP and ITS-RFLP combined with DGGE analyses of the functional *het*R gene (Rasmussen and Svenning 2001).

Within bacteria, sequence information from the gene coding for the small subunit of ribosomal RNA, 16S rDNA, is widely regarded as one of the most valid criteria for determining relationships between closely related groups, such as species or genera (Weisburg et al. 1991). The conservative nature of the gene, its universal distribution and the availability of information in public databases (GenBank, EMBL, DDBJ and RDP) make the 16S rRNA gene very useful for phylogenetic studies and taxonomy. Furthermore, its validity for phylogeny of cyanobacteria was recently documented by Oksanen et al. (2004).

The objective of the present study was to investigate the phylogenetic affiliation of different symbiotic *Nostoc* isolates, emphasizing those from the host *Gunnera*, based on their total 16S rRNA gene sequences, and to address the relationships both between these symbiotic cyanobacteria as well as to other symbiotic and free-living cyanobacteria within subsection IV.

Materials and methods

Cyanobacterial strains

Eight symbiotic *Nostoc* strains from different host species were sequenced for this study (Table 1). All strains, except for the isolate from *Azolla filiculoides*, were selected based on our previous diversity studies (Rasmussen and Svenning 1998, 2001). Strains with a PCC number were obtained from the Collection Nationale de Cultures de Microorganisms, Institute Pasteur, Paris, France. The eight cyanobacteria were grown in $BG11_0$ medium (Stainer et al. 1971) at 28°C under continuous shaking and an illumination at 18 μmol photons m^{-2} s^{-1}. *A. filiculoides* was grown in a pond in the greenhouse and the cyanobacteria were isolated after surface sterilization of the leaves by carefully removing the cyanobacteria from the leaf cavity with a needle, and then kept at −20°C until used directly in the PCR reactions as described by Zheng et al. (2002).

Morphological characterization

The growth behavior on $BG11_0$ agar plates of the individual symbiotic isolates was followed over a 3-week period and recorded using a Leitz DM RB/E microscope (Leica, Wetzlar, Germany). Once a week, filaments from the plates were examined by light microscopy using a Olympus BX60 microscope (Olympus, Tokyo, Japan).

PCR amplification and sequencing of 16S rDNA

Fragments of the 16S rRNA gene were amplified using the primer pairs fD1-r363, f359-r781, f712-r1090 and fC2-rD1 (Table 2). Amplification and sequencing were performed in four separate steps. PCR was carried out as described by Rasmussen and Svenning (2001).

Both strands of the PCR products were fluorescently labeled using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA) according to the manufacturer's manual. The sequences were detected on an Automatic ABI PRISM 310 Genetic Analyser (Applied Biosystems).

Sequence analyses and alignments

Sequences were assembled manually or using the Staden package (Staden 1996) under Linux. The dataset was aligned using Se-Al (Rambaut 1996) and exported to Nexus format (Maddison et al. 1997) for subsequent phylogenetic analysis. The dataset contained 49 taxa and 1,511 aligned positions. This included eight new cyanobacterial symbiotic sequences along with a set of sequences selected from Genbank (Table 1). The sequences from GenBank were selected based on their length and in order to obtain a representative sample of the taxonomic variation. An enlarged dataset included an additional 30 lichen symbiotic sequences (Rikkinen et al. 2002). The dataset and trees are available from TreeBase (http://www.treebase.org).

Phylogenetic analyses

The dataset was analyzed using Bayesian inference of phylogeny (Rannala and Yang 1996; Lewis 2001), which is a model-based phylogenetic method. The evolutionary model used was selected by applying PAUP*(Swofford 2001) and MrModeltest (Nylander 2001), which is a scaled-down version of Modeltest (Posada and Crandall 1998). The general time-reversible model (GTR) with gamma distribution of rates and a proportion of invariable sites was selected. All model parameters were

Table 1 Cyanobacterial strains used for the phylogenetic analyses in this study. Numbers before the strain name correlate to number in brackets in Figs. 1 and 3. Strains in bold and marked * are those from which the 16S rRNA gene was sequenced in this study. Accession no. refers to sequences that came from Genbank *F* Free-living isolates, *S* symbiotic isolates

	Strain		Origin	Accession no.
1	*Anabaena variabilis*	F	Unknown	AB016520
2	*Anabaena* sp.	F	Unknown	X59559
3	*Anabaena cylindrica* strain PCC 7122	F	Pond water, England	AF091150
4	*Anabaena* sp. strain A277	F	River Peraniönjoki, Finland	AJ133160
5	*Anabaena* sp. strain A202A1	F	Lake Vesijärvi, Finland	AJ133159
6	*Anabaena* sp. strain CYA83.1	F	Lake Edlandsvatnet, Norway	AJ133158
7	*Anabaena* sp. strain 66A	F	Lake Kiikkara, Finland	AJ133157
8	*Anabaena* sp. strain 90	F	Lake Vesijärvi, Finland	AJ133156
90	*Anabaena* sp. strain 14	F	Lake Sääksjäavi, Finland	AJ133152
10	*Anabaena* sp. strain 86	F	Lake Villikkalanjäävi, Finland	AJ133151
11	*Anabaena* sp. strain PCC 7108	F	Intertidal zone, USA	AJ133162
12	*Aphanizomenon* sp. strain 202	F	Lake Vesijärvi, Finland	AJ133153
13	*Aphanizomenon* sp. strain PCC 7905	F	Lake BrielseMeer, The Netherlands	AJ133154
14	*Calothrix* sp. strain PCC 7714	F	Pool, India	AJ133164
15	*Calothrix desertica* strain PCC 7102	F	Desert sand, Chile	AF132779
16	*Calothrix* sp. strain D253	F	Unknown	X99213
17	*Chlorogloeopsis* sp.	F	Pond water, Iceland	X68780
18	*Cylindrospermum* sp. strain PCC 7417	F	Soil, greenhouse, Sweden	AJ133163
19	*Fischerella muscicola* strain PCC 7414	F	Hot spring, New Zealand	AB039003
20	*Microcystis wesenbergii*	F	Unknown	AB035553
21	*Microcystis viridis*	F	Unknown	AB035552
22	*Nodularia* sp. strain PCC 73104/1	F	Soil, Canada	AJ133184
23	*Nodularia* sp. strain BCNOD9427	F	Baltic Sea	AJ224447
24	*Nostoc* sp. strain PCC 7120	F	USA	AF317631
25	***Nostoc* sp. strain PCC 9305***	S	*Anthoceros* sp.	AY74243
26	*Nostoc commune*	F	China	Y12687
27	*Nostoc* sp. strain NIVA-CYA 194	F	Jutulsessen, Antarctica	Z82805
28	*Nostoc* sp. strain NIVA-CYA308	F	Ny Ålesund, Spitsbergen, Svalbard	Z82804
29	*Nostoc* sp. strain ATCC 53789	F	Unknown	AF062638
30	*Nostoc flagelliforme*	F	China	Y12688
31	*Nostoc* sp. strain NIVA-CYA 124	F	Lake Steinsfjorden, Norway	Z82776
32	***Nostoc* sp. strain 8963***	S	*Gunnera prorepens*, New Zealand	AY74249
33	*Nostoc* sp. strain TDI#AR94	S	*Peltigera membranacea*	AF027653
34	*Nostoc* sp. strain PCC 73102/ATCC 29133	S	*Macrozamia* sp., Australia	AF027655
35	*Nostoc* sp. strain GSV224	F	Unknown	AF062637
36	*Nostoc* sp. strain PCC 9709	S	*Peltigera membranacea*	AF027654
37	***Nostoc* sp. strain PCC 9229***	S	*Gunnera monoika*, New Zealand	AY74241
38	*Nostoc* sp. strain 152	F	Lake Sääksjäavi, Finland	AJ133161
39	***Nostoc* sp. strain PCC 9231***	S	*Gunnera dentata*, New Zealand	AY74242
40	***Nostoc* sp. strain 8938***	S	*Gunnera dentata*, New Zealand	AY74244
41	***Nostoc* sp. strain 8916***	S	*Gunnera monoika*, New Zealand	AY74247
42	***Nostoc* sp.***	S	*Azolla filiculoides* 1010, Peru	AY74240
43	***Nostoc* sp. strain 8941***	S	*Gunnera dentata*, New Zealand	AY74248
44	*Oscillatoria sancta* strain PCC 7515	F	Greenhous watertank, Sweden	AF132933
45	*Scytonema hofmanni* strain PCC 7110	F	Crystal Cave (limestone), Bermuda	AF132781
46	*Trichodesmium thiebaultii*	F	Caribbean Sea	AF091321
47	*Trichodesmium hildebrandtii*	F	Caribbean Sea	AF091322
48	*Trichodesmium contortum*	F	Caribbean Sea	AF013028
49	*Prochlorococcus* sp. strain MIT9302	F	The Sargasso Sea	AF053396

estimated in the Bayesian inference analysis, which was performed using MrBayes (Huelsenbeck and Ronquist 2001a, b).

Four separate runs, each of 1,000,000 Markov Chain Monte Carlo generations, were performed, and trees were sampled from the chain every ten generations. Trees sampled after reaching chain stationarity (the ''burn-in'') were used in a majority-rule consensus tree calculated in PAUP*. The enlarged dataset including data from Rikkinen et al. (2002) was analyzed similarly.

In order to obtain a tree with optimized branch lengths, the tree sampled in MrBayes with best-likelihood score was selected for further maximum-likelihood (ML) analysis in PAUP*. *Prochlorococcus* was used to root the trees.

Results and discussion

The genus *Nostoc*

Most of the *Nostoc* strains analyzed in this study are found within a large and well-supported clade that includes both free-living strains and symbiotic strains:

Table 2 Primers used for partial amplification of 16S rDNA

Primer	Sequence (5′-3′)	Reference
fD1	AGA GTT TGA TCC TGG CTC AG	Weisburg et al. (1991)
r363	AAT ACC GCG TGA GGG AGG AAG GC	This work
f359	GGG GAA TYT TCC GCA ATG GG	Nübel et al. (1997)
r781	GAC TAC TGG GGT ATC TAA TCC CAT T	Nübel at al. (1997)
f712	ACC CCA GTA GTC CTA GCC GT	This work
r1090	GTT TGT CAC CGG CAG TCT CT	This work
fC2	CGC AAC CCT CGT TTT TAG TT	This work
rD1	AAG GAG GTG ATC CAG CC	Weisburg et al. (1991)

Nostoc sp. strain PCC 9305, *Nostoc* sp. strain 8963, *Nostoc* sp. (33, Table 1), *Nostoc* sp. strain PCC 9229, *Nostoc* sp. strain PCC 9709 and *Nostoc* sp. strain PCC 73102, the reference strain for *N. punctiforme* (Fig. 1, clade II). Some other *Nostoc* strains, however, fall outside of clade II among strains classified as other genera, mainly *Anabaena* (Fig. 1, clade IV). When combined with the sequences of Rikkinen et al. (2002), their entire group B falls within our clade II, which means that this clade is fully congruent with their results. The clade consists of both free-living and symbiotic strains, and support is strong (1.00) for this ecologically heterogeneous mix of cyanobacteria from different environments (Table 1). Included in this clade are the three species *N. flagelliforme*, *N. commune* and *N. punctiforme*, which occupy different ecological niches. The three symbiotic cyanobacteria sequenced in this study and located in clade II are isolates from different hosts. *Nostoc* sp. strain PCC 9305 is from the liverwort *Anthoceros* sp., *Nostoc* sp. strain 8963 is from *Gunnera prorepens* and *Nostoc* sp. strain PCC 9229 is from *G. monoika* (Table 1). The colony morphology of the symbiotic cyanobacteria shows differences with regard to color, hormogonia formation and colony structure when grown on agar medium (Fig. 2). *Nostoc* sp. strain PCC 9305 and *Nostoc* sp. strain 8963 have well defined col-

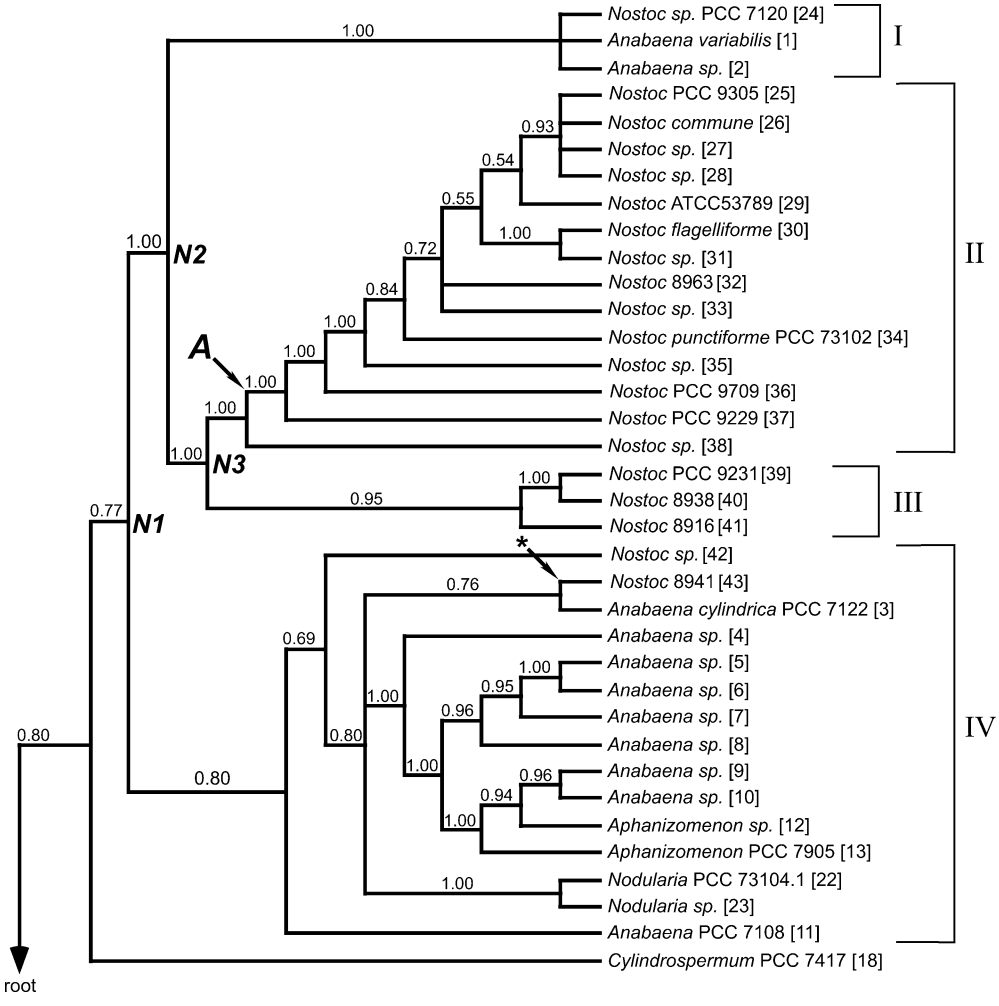

Fig. 1 Majority rule consensus (50%) of the trees sampled in the Bayesian analysis. Estimates of clade probabilities are indicated *above the branches*. The *arrow* pointing to the root excludes 18 outgroup sequences. *Numbers within square brackets* refer to sequence numbers in Table 1. The *arrow 'A'* indicates where group A of Rikkinen et al. (2002) was found in the extended analyses. The *arrow marked with an asterisk* indicates where the *Calothrix* sequences were positioned in the extended analyses

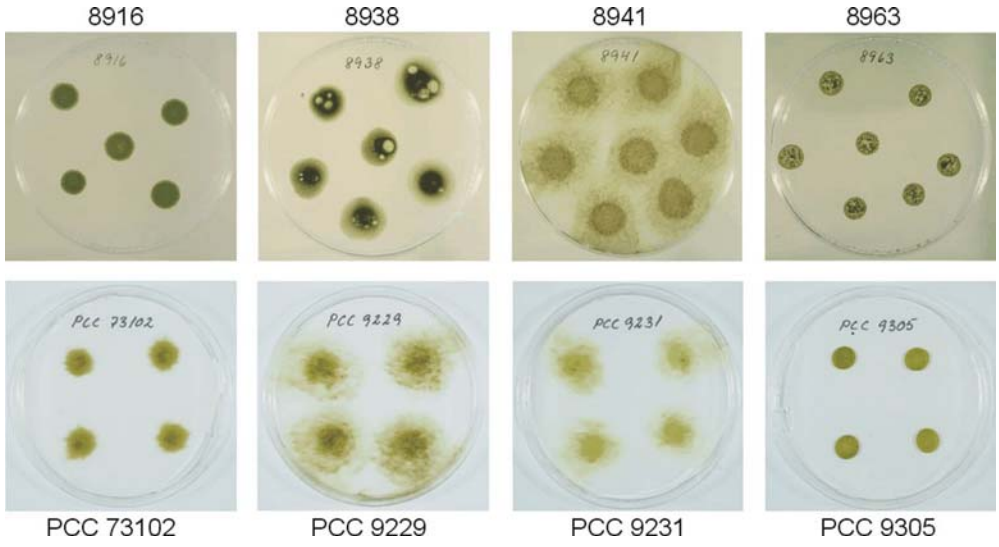

Fig. 2 Growth morphology of symbiotic cyanobacterial colonies after 3 weeks on agar medium

onies without visible hormogonia and differ from strain *Nostoc* sp. strain PCC 9229 and *N. punctiforme* PCC 73102, which show hormogonia after 3 weeks on agar plates (Fig. 2). However, after prolonged growth, all of the symbiotic isolates tested showed hormogonia formation. The presence of hormogonia was recorded by carefully examination under light microscopy. The lack of heterocysts in the filaments, the smaller cell size, and the motility of the filaments were used as criteria to distinguish them from the vegetative filaments (data not shown).

None of the symbiotic cyanobacteria analysed in this study fall within the highly specific group of epiphytic lichen, *Nostoc* group A found by Rikkinen et al. (2002). The group is recovered in our extended analysis with strong support (0.98) (Fig. 1, position indicated as A).

The single *Nostoc* strain 152 [38] of Lyra et al. (2001), isolated from a freshwater lake, is sister to the *Nostoc* group A and clade II. One step further down the tree, clade III is sister to the clade joining group A and clade II plus *Nostoc* sp. strain 152 (Fig. 1). Clade III forms a separate symbiotic clade consisting of *Nostoc* sp. strain PCC 9231, *Nostoc* sp. strain 8938 and *Nostoc* sp. strain 8916. *Nostoc* sp. strain 8938 and *Nostoc* sp. strain 8916 appeared as dark green, compact, slimy colonies without hormogonia formation after 3 weeks growth on agar plates, while *Nostoc* sp. strain PCC 9231 formed light green colonies with hormogonia formation (Fig. 2). *Nostoc* sp. strains 9231 and 8938 were isolated from *Gunnera dentata* and strain 8916 from *G. monoika*; hence, this is the only clade showing phylogenetic host specificity. Host specificity has hitherto not been documented for *Nostoc*, and this well supported clade III indicates that specificity can be present. This is in contrast to the broad host spectrum (lack of specificity) documented for *N. punctiforme* (Enderlin and Meeks 1983; Johansson and Bergman 1994; Mollenhauer et al. 1996). Hence, the symbiotic strains within clade III cannot be considered as the same species as *N. punctiforme*. Moreover, some symbiotic *Nostoc* isolates, strain 8941 from *G. dentata* and the isolate from *A. filiculoides* (42, Table 1) are additionally found more affiliated with *Anabaena* than with *Nostoc*, based on this complete 16S rRNA gene analysis. The estimated branch length (Fig. 3) indicates a considerable amount of divergence between symbiotic strains. This might not be expected if all symbiotic *Nostoc* belonged to the same species and genus.

The problems linked to traditional phenotypic classification and to the lack of molecular data are well known for systematics of cyanobacteria. The consensus tree presented in this study is well resolved and many clades have high support values. These values represent estimates of the probability that the clades are correct (Lewis 2001) and are not necessarily comparable to bootstrap values (Cummings et al. 2003). A tree with branch lengths optimized under ML is shown in Fig. 3. The ML tree shows the same major relationships as the Bayesian tree, but it also indicates the relative amount of evolution that is estimated to have occurred between the clades.

Nostoc–Anabaena

Overall, the phylogenetic analysis resulted in a mix of *Nostoc* strains within the genus *Anabaena* and vice versa. At the present time, well-defined criteria for separation of these two genera are not clear. A small clade of three strains (*Nostoc* sp. strain PCC 7120, *Anabaena variabilis* and *Anabaena* sp.), clade I, was found to be a sister to the *Nostoc* clades II and III. Clade I consists of three free-living isolates, and the generic mix highlights the problem of assigning generic names. *Nostoc* sp. strain PCC 7120 was originally named *Nostoc muscorum*, then classified as *Anabaena* (Rippka et al. 1979) and, based on DNA–DNA hybridization data (Lachance 1981) and hybridization pattern with repetitive (STRR) DNA sequences (Mazel et al. 1990), it was later renamed *Nostoc* sp. strain PCC 7120. Moreover, two symbiotic *Nostoc*

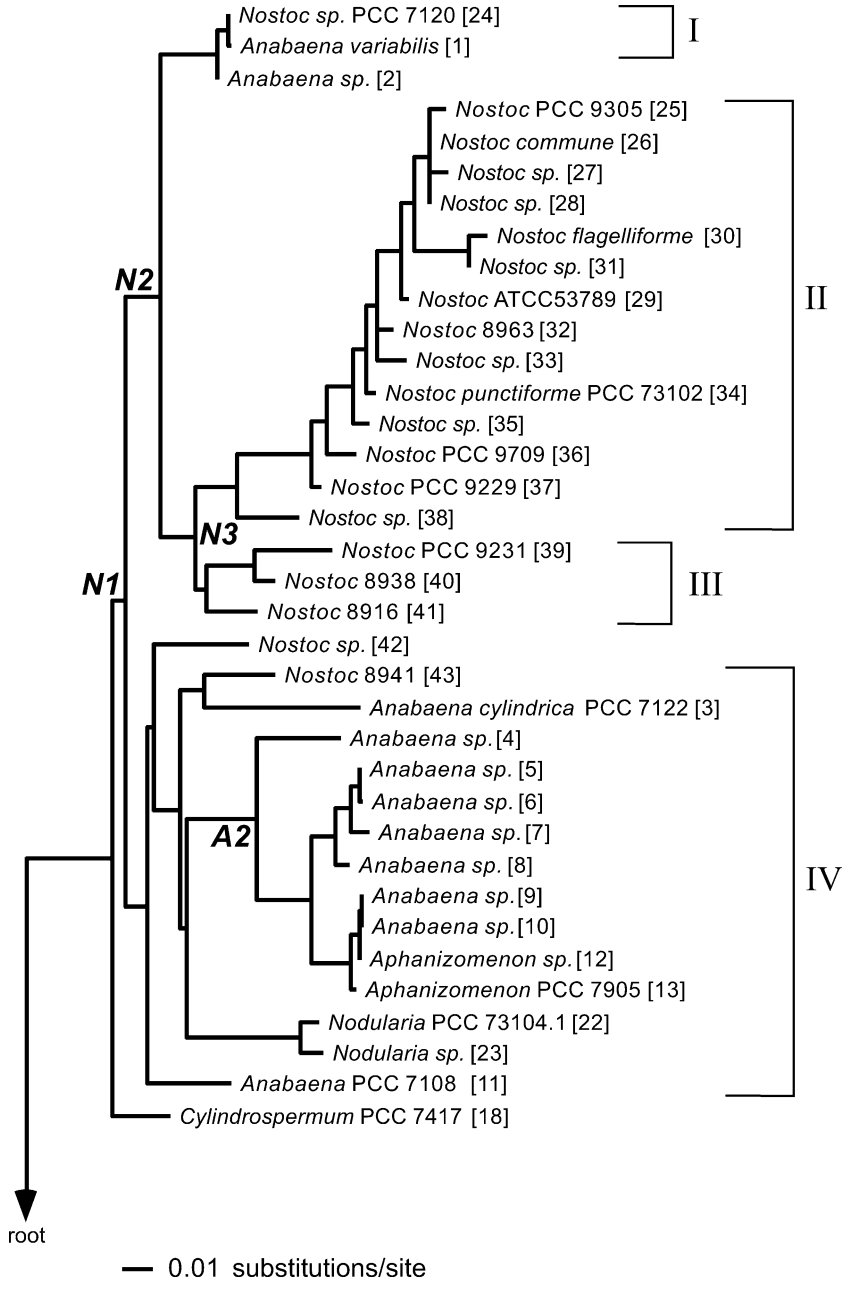

Fig. 3 Maximum-likelihood tree with branches drawn proportional to the inferred amount of change. The tree was obtained using the general time-reversible model with gamma distribution of rates and a proportion of invariable sites (GTR + G + I). The *arrow* pointing to the root excludes 18 outgroup sequences. *Numbers within square brackets* refer to sequence numbers in Table 1

strains, the isolate from *A. filiculoides* (42, Table 1) and strain 8941 from *G. dentata* fall well nested within a clade that comprises most of the *Anabaena* sequences (Figs. 1, 3, clade IV) used in these analyses. Even though clade IV is not especially well supported (0.80), it is consistently recovered in the analyses. The cyanobacterium from the water fern *Azolla* has been named *Anabaena azollae* based on its affiliation with the genus *Anabaena* (Moor 1969; Lumpkin and Plucknett 1980; Wilmotte and Herdman 2001). Based on RFLP analysis of the 16S rRNA gene, Plazensky et al. (1990) suggested that it be included in the genus *Nostoc*. According to Baker et al. (2003), the isolate from *A. filiculoides* could not be affiliated with either *Anabaena* or *Nostoc*. However, it should be noted that only a small fragment (approximately 600 bp) was sequenced in the work by Baker et al. (2003).

Our results, based on sequence analysis of the entire 16S rRNA gene of the cyanobacteria from *A. filiculoides*, supports classification of the "*Azollae*" strain in the genus *Anabaena* rather than *Nostoc* and thus correlates with the phylogenetic tree presented by Wright et al. (2001). Surprisingly, *Nostoc* sp. strain 8941, isolated from *G. dentata,* is found within clade IV while the two other cyanobacteria isolated from *G. dentata* are positioned in clade III. Therefore, there may be other cyanobacterial representatives from host genera other than *Azolla* within *Anabaena* clade IV. Moreover, these

results highlight the problems of using the presence of hormogonia as a morphological criterion to distinguish between *Anabaena* and *Nostoc* since both of the cyanobacteria in the *Azolla* symbiosis and *Nostoc* strain 8941 form hormogonia (Fig. 2). The use of such "key characters" has been widespread in organism classifications and has often been shown to be inconsistent with phylogeny. Classification of organisms into species and genera are made mandatory when naming by the codes of nomenclature. Hence, it is necessary to consider into which genera the strains should be classified. Due to restrictions in the current nomenclatural systems, a genus cannot affiliate within another genus. Therefore, when phylogenetic analyses show that genera intermix, re-circumscriptions of genera with name changes of species are the rule. It seems reasonable in such situations to rank well-supported clades as genera while trying to minimize name changes.

There are several candidate clades to which the name *Nostoc* might be attached. A possible good candidate is clade N3 (Fig. 1), which is well supported and would comprise clades II and III (this study) and *Nostoc* groups A and B (Rikkinen et al. 2002). According to Wilmotte and Herdman (2001), this is the most "solid" *Nostoc* clade. However, a more prudent approach suggests clade N2, even though a few strains that are now named *Anabaena* would then be included in *Nostoc* (clade I). Clade I includes *Nostoc* sp. strain PCC 7120, which was shifted between *Nostoc* and *Anabaena* as mentioned earlier. Otherwise, clade I, which is sister to clades II and III, would need another genus name, probably an entirely new one. However, the reassignment of strain PCC 7120 to *Nostoc* has recently been questioned by Tamas et al. (2000) in an analysis of a portion of the *nif*H gene. Although *Nostoc* strains are included in clade IV, node N1 is excluded as a node for *Nostoc*. We suggest N3 as the most reasonable node for the name *Nostoc*.

Assigning a clade for *Anabaena* poses more of a problem since other genera are intermixed and there is less support. It seems clear at least, that *Aphanizomenon* is deeply nested within *Anabaena*, which becomes paraphyletic if *Aphanizomenon* is maintained. Our analyses are in accordance with those of Gugger et al. (2002) suggesting that *Anabaena* and *Aphanizomenon* belong to the same genus (Fig. 1, clade IV). However, given the data at hand and the size of our samples within clade IV, it seems premature to make strong suggestions of how to apply genus rank in this clade.

This study has specifically addressed symbiotic cyanobacteria which, based on the total 16S rRNA gene sequence analyses, are placed in well-separated clades. The combination of symbiotic and free-living cyanobacteria in clade II suggests the possibility that some of the free-living isolates have symbiotic competence. Few studies have been undertaken to address this issue. In the study by West and Adams (1997), free-living isolates from the soil surrounding *Blasia* could not be identified in the symbiotic tissue but showed symbiotic competence when tested by reconstitution experiments in the laboratory. Further studies should clarify the phenotypic and genotypic characteristics of the strains and species within this clade and may possibly lead to an updated nomenclature.

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