J. Phycol. **44,** 60–70 (2008)

DOI: 10.1111/j.1529-8817.2007.00448.x

DIVERSITY AND HOST SPECIFICITY OF *AZOLLA* CYANOBIONTS[1]

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A unique, hereditary symbiosis exists between the water fern *Azolla* and cyanobacteria that reside within a cavity in the dorsal leaf-lobe of the plant. This association has been studied extensively, and questions have frequently been raised regarding the number and diversity of cyanobionts (cyanobacterial symbionts) among the different *Azolla* strains and species. In this work, denaturating gradient gel electrophoresis (DGGE) and a clone library based on the 16S rRNA gene were used to study the genetic diversity and host specificity of the cyanobionts in 35 *Azolla* strains covering a wide taxonomic and geographic range. DNA was extracted directly from the cyanobacterial packets, isolated after enzymatic digestion of the *Azolla* leaves. Our results indicated the existence of different cyanobiont strains among *Azolla* species, and diversity within a single *Azolla* species, independent of the geographic origin of the host. Furthermore, the cyanobiont exhibited host-species specificity and showed most divergence between the two sections of genus *Azolla*, *Azolla* and *Rhizosperma*. These findings are in agreement with the recent redefinition of the taxon *Azolla cristata* within the section *Azolla*. With regard to the taxonomic status of the cyanobiont, the genus *Anabaena* of the Nostocaceae family was identified as the closest relative by this work.

Key index words: **16S rRNA; *Anabaena; Azolla;* cyanobacteria; cyanobionts; DGGE; diversity; genotypic; symbiosis**

Abbreviations: **BSA, bovine serum albumin; CI, consistency index; DGGE, denaturating gradient gel electrophoresis; ESF, European Science Foundation; GTR, general time reversible; ITS, internal transcribed spacer; OTU, operational taxonomic unit; PCC, Pasteur culture collection; RC, rescaled consistency index; RDP, ribosomal database project; RI, retention index; TBR, tree-bisection-reconnection**

Azolla (family Azollaceae) is a small aquatic fern that lives in mutualistic symbiosis with a nitrogen-fixing cyanobacterium, originally described as *Anabaena azollae* (Strasburger 1884), located inside a highly specialized cavity on the dorsal lobe of the leaves that also harbors other bacterial phyla (Lechno-Yossef and Nierzwicki-Bauer 2002). Among cyanobacteria-plant associations, the *Azolla* symbiosis is the only known permanent symbiosis: *Anabaena azollae* is present in *Azolla* during all life stages and is automatically transmitted from generation to generation, regardless of the ferns' vegetative or sexual reproduction. In contrast to other cyanobacteria-plant symbioses where the cyanobiont can be easily separated and cultured, there are no confirmed reports of successful in vitro cultivation of the cyanobionts originating from this perpetual symbiosis with *Azolla* (Lechno-Yossef and Nierzwicki-Bauer 2002).

Most authors recognize two sections in the genus *Azolla*: section *Azolla*, with four or five species (*A. caroliniana* Willd. or auct. non Willd., *A. filiculoides* Lam., *A. mexicana* Presl., *A. microphylla* Kaulf. or auct. non Kaulf., *A. rubra* R. Br.), and section Rhizosperma [*A. nilotica* Decne. ex Mett. and *A. pinnata* R. Br., this last one with two varieties, *A. pinnata* R. Br. var. *pinnata* and *A. pinnata* R. Br. var. *imbricata* (Roxb.) Bonap.] (Braun-Howland and Nierzwicki-Bauer 1990). Recently, it was proposed that the plants generally identified as *A. caroliniana*, *A. mexicana*, and *A. microphylla* belong to a unique species, *A. cristata* (Evrard and Van Hove 2004).

The taxonomy of the *Azolla* cyanobionts is still a matter of debate. Strasburger (1884) described the cyanobiont of *Azolla* as *An. azollae*. In their revised

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classification of cyanobacteria, Komárek and Anagnostidis (1989) divided *Anabaena* into two genera, *Anabaena* and *Trichormus* (see http://www.cyanodb.cz/1a.html), assigning the *Azolla* cyanobiont into the last genus, as *Trichormus azollae*. Several other authors have suggested that the cyanobiont could belong to the genus *Nostoc* (Meeks et al. 1988, Tomaselli et al. 1988, Plazinski et al. 1990, Kim et al. 1997), at least for some *Azolla* species (Pabby et al. 2003). Svenning et al. (2005) rejected this position on the basis of a conspicuous set of *Nostoc* and *Anabaena* 16S rDNA sequences and presented their own evidence for maintaining the cyanobiont in the genus *Anabaena*. Some authors have hypothesized that the cyanobiont does not belong to either *Anabaena* or *Nostoc* based on analyses of fatty acid composition, DNA fingerprints, or 16S rDNA sequences (Caudales et al. 1993, 1995, Baker et al. 2003). Others have suggested that in addition to a major nitrogen-fixing, noncultivable cyanobiont, *Azolla* harbors one or more cultivable minor symbiotic cyanobacteria (Meeks et al. 1988, Gebhardt and Nierzwicki-Bauer 1991, Kim et al. 1997). Many studies have also analyzed the diversity among the cyanobionts from diverse *Azolla* species, varieties, or ecotypes, using immunological techniques (Ladha and Watanabe 1982, Liu et al. 1986, 1989), lectin hemagglutination techniques (McCowen et al. 1987), DNA hybridization methods, DNA fingerprints (Nierzwicki-Bauer and Haselkorn 1986, Meeks et al. 1988, Plazinski et al. 1990, Van Coppenolle et al. 1995, Zheng et al. 1999), and fatty acid comparison (Caudales et al. 1995).

Our aim in this study was to investigate the diversity and host specificity of cyanobionts from individual *Azolla* species and strains by determining whether the same cyanobacterial genotype was always present in a particular species of *Azolla*, regardless of geographic origin. The genotypic diversity of cyanobionts isolated from 35 *Azolla* strains representing all eight generally recognized *Azolla* species and varieties collected at various geographic locations was examined by PCR-based 16S rRNA gene profiling using the DGGE fingerprinting method and the construction of a clone library. Phylogenetic analysis of the sequences provided evidence for the taxonomic position of the cyanobionts.

MATERIALS AND METHODS

Host strains. Thirty-five *Azolla* strains from the International Rice Research Institute (IRRI, Los Banos, Philippines) germplasm collection, maintained as duplicates at the Catholic University of Louvain (Louvain-La-Neuve, Belgium), were included in this study. The classification of the genus *Azolla* (Table 1) at the species and variety levels was according to the IRRI system (Watanabe et al. 1992). The culture medium (Hoagland and Arnon 1950, where nitrates were replaced by chlorides) was renewed every week. The daily light intensity was 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$, for 16 h, and 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 8 h. The culture medium temperature varied between 20°C and 24°C.

Isolation of Azolla *cyanobacterial packets.* Half a gram of plant material was collected for each *Azolla* strain, after removal of roots, and the biomass was thoroughly surface-cleaned by rinsing for 10 min in Triton X-100 detergent (0.1% v/v; Promega, Charbonnières, France) with shaking. The detergent solution was discarded, and the plants were rinsed with distilled water. The fronds were then transferred in 5 mL enzymatic solution (2.5% Cellulase Onozuka RS, 0.125% Pectolyase Y-23 in phosphate buffer [12.1 mM Na_2HPO_4 and 87.9 mM KH_2PO_4 solution, pH = 6]) and degassed by vacuum for 30 min to 1 h while kept at the bottom of the tube by means of a metal grid and a weight. The metal grid and weight were removed, and the fronds were incubated in the enzymatic solution for another 6–9 h at 32°C with continuous shaking (70 rpm), resulting in their complete digestion (Peters et al. 1978). Several washes of the remaining material with distilled water followed before the final collection of the intact cyanobacterial packets (*Azolla* leaf cavities) by pipette under the dissecting microscope.

Genomic DNA extraction. DNA was extracted from approximately 50 cyanobacterial packets isolated from each strain using the Wizard® Magnetic DNA Purification System for Food (Promega, Madison, WI, USA) with the following modifications of the manufacturer's protocol: the starting material (50 cyanobacterial packets/sample) was diluted in 100 μL lysis buffer A (supplied in the Promega kit) before all other reagents were added. Proteinase K (20 $mg \cdot mL^{-1}$) was included in the solution at step 2 (addition of lysis buffer A and RNase to the sample) with subsequent incubation of the mixture at 55°C for 1 h.

PCR amplification and DGGE analysis of the 16S rRNA gene. The first PCR amplification was carried out on extracted genomic DNA from each strain. Cyanobacterial-specific primers CYA359F (5′-GGGGAATTTTCCGCAATGGG-3′) (Nübel et al. 1997) and 23S30R (5′-CTTCGCCTCTGTTGTCCTAGGT-3′) (Taton et al. 2003) were used to amplify the fragment of ~1,700 base pairs (bp), consisting of the 16S rRNA genes plus the internal transcribed spacer (ITS) region. The 50 μL PCR reaction volume contained 200 μM dNTPs mix (MBI Fermentas, Vilnius, Lithuania), 0.5 μM each primer, 1 U of Super *Taq* Plus DNA polymerase (HT Biotechnology, Cambridge, UK), 10 $mg \cdot mL^{-1}$ BSA, and 6% glycerol. Buffer supplied with the enzyme was used as recommended by the manufacturer. DNA was amplified in an iCycler apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA), with the following temperature profile: one denaturation step at 94°C for 10 min, 25 cycles of 94°C for 45 s, 54°C for 45 s, and 68°C for 2 min, followed by one incubation step at 68°C for 7 min. The amplified products were visualized on 1% agarose gel. Approximately 0.5 μL of the PCR products was used as template for a seminested PCR amplification, with the following temperature profile: one denaturation step at 94°C for 5 min, 35 cycles of 94°C for 1 min, 60°C for 1 min, and 68°C for 1 min, followed by one incubation step at 68°C for 7 min. The PCR mixture was the same as above with the exception of the reverse primer. This time we used two reverse primers (Boutte et al. 2006) in two different reactions: CYA781R (a) 5′-GACTACTGGGGTATCTAATCCCAT T-3′, targeting filamentous species; and (b) 5′-GACTACAGGGGTATCTAATCCCTT T-3′, targeting nonfilamentous cyanobacteria. After visualization of the amplified fragment on 1% agarose gels, the PCR products were used for DGGE analysis on the Dcode Universal Mutation Detection System (Bio-Rad Laboratories Inc.) as described by Boutte et al. (2006). Then 20 μL of each PCR product was applied directly onto a 6% (w/v) polyacrylamide gel in 1× TAE buffer (40 mM Tris-base, 20 mM acetic acid, and 10 mM EDTA [pH 8.0]) with a linear 45% to 55% denaturation gradient (100% denaturation solution was defined as 7 M urea and 40% [v/v] formamide). The gels were run at a constant temperature of

TABLE 1. *Azolla* species and strains and their geographic origin.

Azolla species	Strain (IRRI[a] code)	Country of origin	Collection site	Cyanobacterial reference in the present study
filiculoides	1007	USA	Nevada	Fi1007
filiculoides	1043	Brazil	Palmital Goia	Fi1043
filiculoides	1046	UK	Hampshire	Fi1046
filiculoides	1534	China	Hangzhou	Fi1534
filiculoides	Japan[b]	Japan	Unavailable	FiJapan
filiculoides	Unknown[c]	Unavailable	Unavailable	FiUnknown
caroliniana	3004	Uruguay	Treinta y tres	Ca3004
caroliniana	3006	Brazil	Manaus	Ca3006
caroliniana	3515	Surinam	Coronie	Ca3515
caroliniana	3518	Brazil	Unavailable	Ca3518
caroliniana	3525	Unavailable	Unavailable	Ca3525
rubra	6503	New Zealand	Road Lumsdem- Kingston	Ru6503
microphylla	4001	Paraguay		Mi4001
microphylla	4064	Paraguay	86 km Ruta Trans-Chaco	Mi4064
microphylla	4072	Paraguay	Boquerón	Mi4072
microphylla	4504	Ecuador	Galápagos	Mi4504
microphylla	4510	Paraguay	Unavailable	Mi4510
mexicana	2002	Guyana	Unavailable	Me2002
mexicana	2003	Guyana	Unavailable	Me2003
mexicana	2007	USA	Ohio	Me2007
mexicana	2008	Colombia	Cali	Me2008
mexicana	2009	Brazil	Paraná	Me2009
mexicana	2011	Unavailable	Unavailable	Me2011
nilotica	5001	Sudan	Kosti	Ni5001
pinnata pinnata	7001	Australia	Kakadu Northern Park	PP7001
pinnata pinnata	7522	Burkina Faso	Mare aux hippopotames	PP7522
pinnata pinnata	7524	Australia	Perth	PP7524
pinnata pinnata	7526	Rwanda	Kirirambogo	PP7526
pinnata pinnata	7528	Mali	Bagineda	PP7528
pinnata imbricata	2	Malaysia	Bumbong Lima	PI2
pinnata imbricata	6	Thailand	Bangkok	PI6
pinnata imbricata	49	China	Fuzhou	PI49
pinnata imbricata	64	Sri Lanka	Hunnasgiriya	PI64
pinnata imbricata	74	Indonesia	Bogor	PI74
pinnata imbricata	534	Sri Lanka	Hunnasgiriya	PI534

[a]International Rice Research Institute, Los Banos, Philippines.
[b]Non-IRRI.
[c]Non-IRRI, unknown origin.

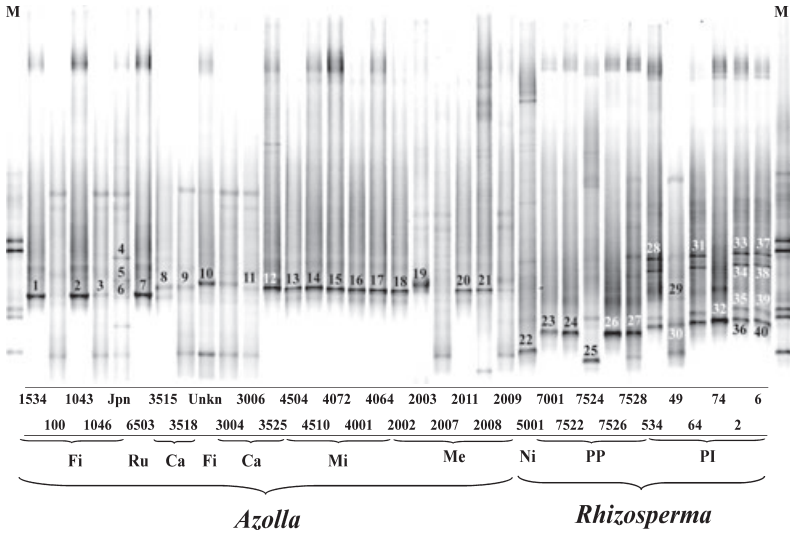

FIG. 1. DGGE analysis of the 16S rRNA gene PCR products from the cyanobionts of all *Azolla* strains. The bands that were excised and sequenced are indicated by numbers adjacent to each band. Marker (M). Host origin: *A. filiculoides* (Fi), *A. caroliniana* (Ca), *A. mexicana* (Me), *A. microphylla* (Mi), *A. rubra* (Ru), *A. nilotica* (Ni), *A. p. pinnata* (PP), *A. p. imbricata* (PI). Strain numbers are indicated; unknown (Unkn), Japan (Jpn).

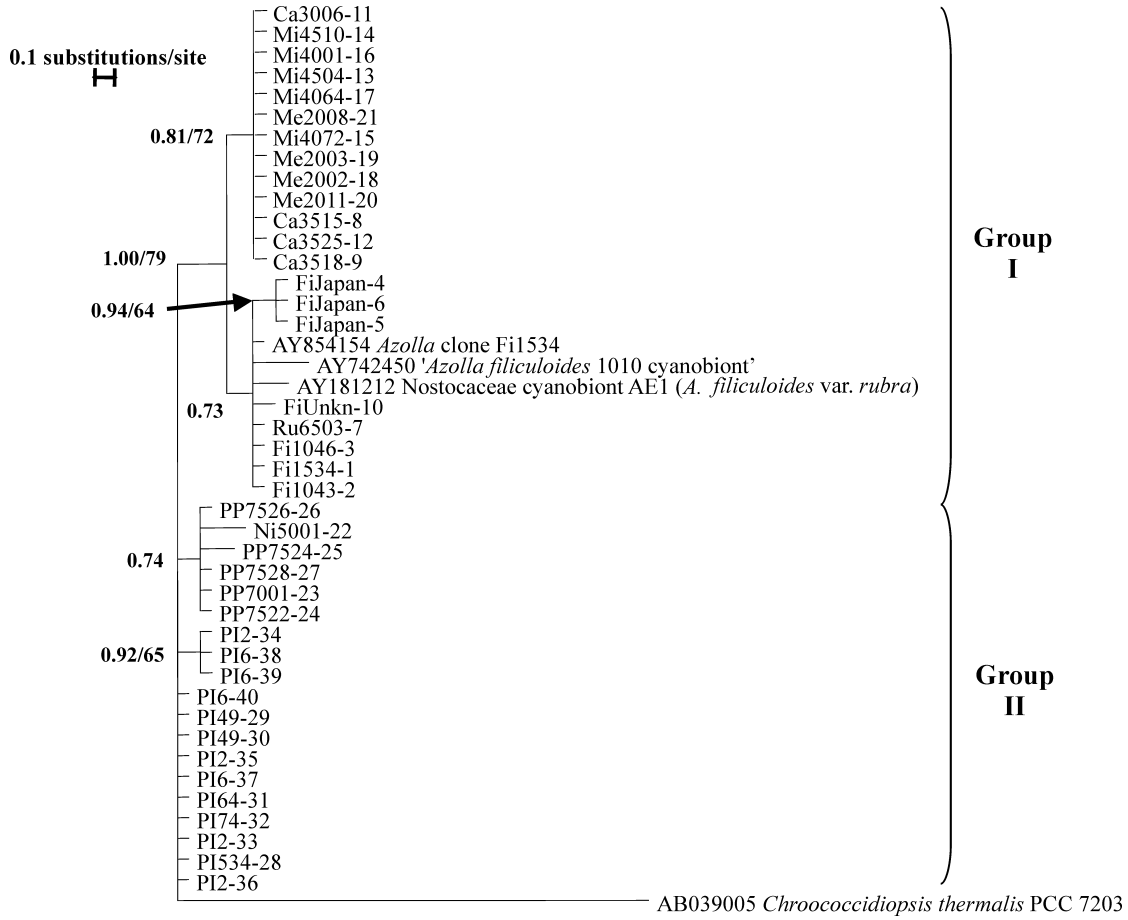

FIG. 2. Phylogenetic tree produced with both Bayesian and maximum-parsimony algorithms. Bayesian posterior probabilities >0.5 are indicated before the slash, and bootstrap values ≥50% after the slash, beside the nodes concerned. The scale bar corresponds to 0.1 nucleotide substitutions. Sequences are identified by the names of the corresponding *Azolla* host strain, and the excised DGGE band number follows after the dash (abbreviations and numbers are according to Table 1 and Fig. 1).

60°C at 45 V for 16 h. DGGE gels were run twice to test the reproducibility of the results. Staining with GelStar nucleic acid stain (Biowhittaker Molecular Applications, Madison, WI, USA) allowed the visualization of the gels on the Fluor-S Max Multimager (Bio-Rad Laboratories Inc.). Normalized gel images were produced with the software Fingerprinting II (Bio-Rad Laboratories Inc.), by using the markers as reference (Fig. 1). Major bands were excised from the DGGE gel on a standard UV trans-illuminator, and the DNA eluted overnight in 100 µL TE (10 mM Tris-Cl [pH 7.5], 1 mM EDTA [pH 8.0]) at 4°C. One reamplification step was done with primers CYA359F and CYA784R (5′-GACTACAGGGGTATCTAATCCC-3′) (Boutte et al. 2006) to ensure the purity of the products, which were subsequently sequenced commercially (Genome Express, Meylan, France). Sequences were repeated in one or both directions whenever necessary to increase the sequence quality.

Cloning. A cloning library was produced for the DNA extracted from the cyanobiont of *A. filiculoides* strain 1534. Primers CYA359F and 23S30R were used in the PCR conditions already described. PCR products were purified with the Quantum Prep PCR Kleen Spin Columns (Bio-Rad Laboratories Inc.) and ligated into the pCR2.1 vector of the TOPO TA cloning kit following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). They were further transformed into competent TOP10 *Escherichia coli* cells. Plasmid DNA was extracted with the Quantum Prep Plasmid Miniprep kit (Bio-Rad Laboratories Inc.) and sequenced with the primers 359F and 16S1494R (5′-GTACGGCTACCTTGTTACGAC-3′) (Taton et al. 2003).

Phylogenetic analysis. Editing of the resolved sequence chromatograms from DGGE bands and the clone library was carried out using Trev 1.5 (Bonfield et al. 2002) to visualize the sequences, and BioEdit (Hall 1999) for editing. All sequences were aligned with the ClustalX program (Thompson et al. 1997) and analyzed with BLAST (Altschul et al. 1997), widely available on Internet, while the clone sequences were also analyzed with RDPII 9.41 (http://rdp.cme.msu.edu/). Phylogenetic analysis of the sequences was carried out with PAUP 4.0b10 for Windows (Swofford 2003) and MrBayes 3.1 (Huelsenbeck and Ronquist 2001). Modeltest 3.7 (Posada and Crandall 1998) and PAUP were employed to compute the evolutionary model best fitting the data by the Akaike information criterion, for use in phylogenetic analyses. Distance, maximum-likelihood (ML), maximum-parsimony (MP) optimality criteria, and the Markov chain Monte Carlo (MCMC) algorithm were used, and the criterion showing best fit was selected to construct the phylogenetic trees (Figs. 2 and 3), which were visualized and edited with TreeView 1.6.6 (Page 1996).

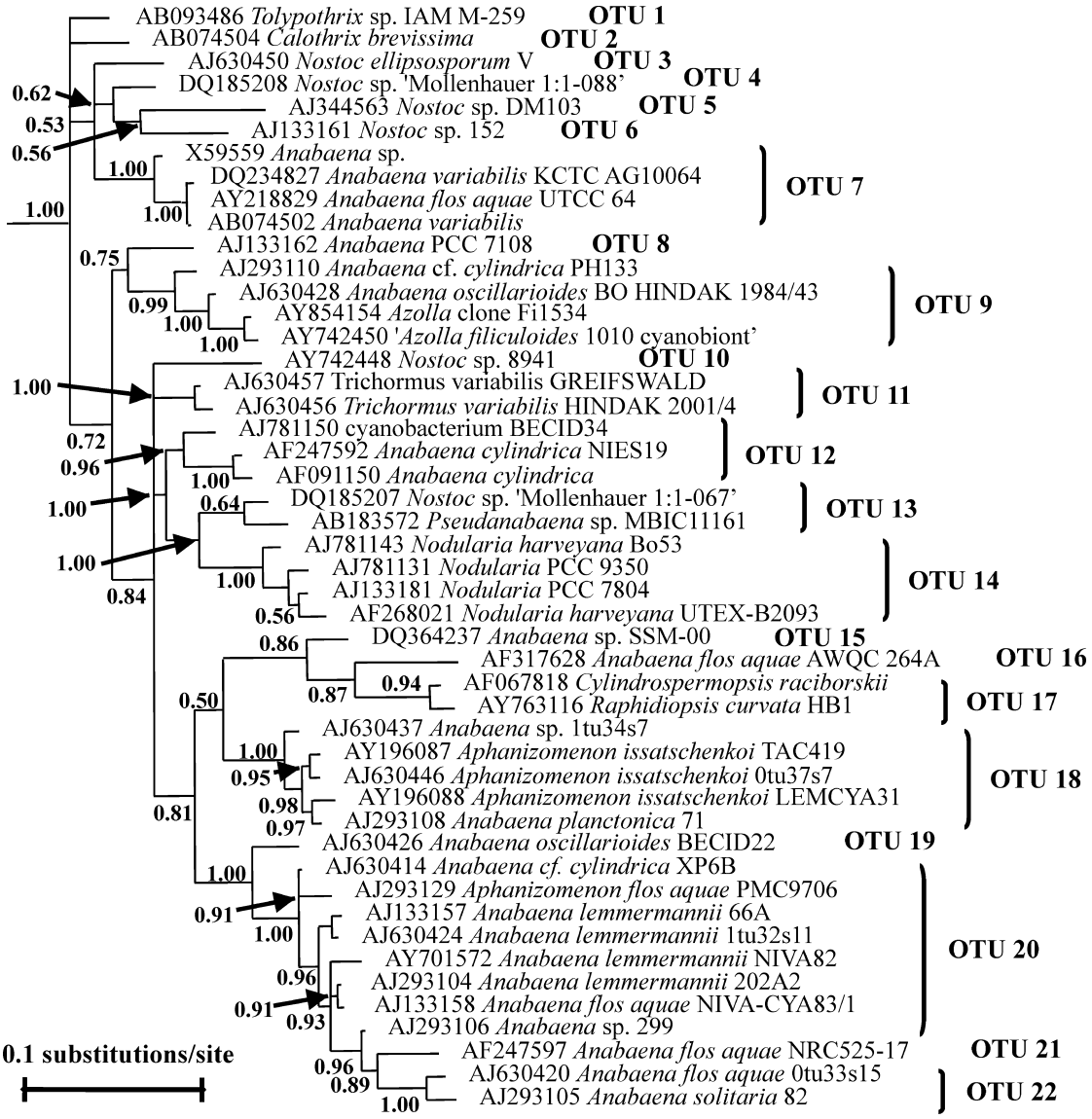

FIG. 3. Bayesian majority-rule consensus tree based on a 1,101 bp fragment of the 16S rRNA gene sequence of a representative *Azolla filiculoides* 1534 clone sequence (AY854154 *Azolla* cyanobiont Fi1534) and 66 sequences (grouped into 22 operational taxonomic units [OTUs]). Bayesian posterior probabilities >0.5 are indicated beside the nodes concerned. The scale bar corresponds to 0.1 nucleotide substitutions.

Aligned partial 16S rRNA gene sequences corresponding to *E. coli* sequence positions 387 to 750 (Neefs et al. 1990) from all 43 sequences obtained from DGGE bands were used for the parsimony analysis in Figure 2, with the outgroup taxon *Chroococcidiopsis thermalis* PCC 7203. Bootstrap method with heuristic search was performed, and 30,000 bootstrap replicates were used to calculate a statistical confidence for the final 50% majority-rule consensus tree. All 363 characters were included in the analysis, and equal weights were assumed. Gaps were treated as missing data. Starting trees were obtained via stepwise addition, sequence addition was simple, and one tree was held at each step. Branch-swapping algorithm was the tree-bisection-reconnection (TBR), branches were collapsed when maximum branch length was zero, and topological constraints were not enforced. The general time-reversible nucleotide substitution model with the gamma distribution parameter (GTR+G) was additionally used for Bayesian inference of phylogeny. Three million generations were performed for four chains, and 3,000 initial unstable burn-in cycles were removed, producing a majority-rule consensus tree.

The clone sequence from *A. filiculoides* 1534 was used in an extensive search on the Ribosomal Database project II (RDPII, Cole et al. 2003) to obtain adequate taxa representation for evolutionary analysis of the cyanobiont. The command SequenceMatch on the RDPII was used to find its 20 closest relatives, and 20 subsequent searches were run, for each sequence obtained. The resulting 190 sequences were downloaded as a Fasta alignment into BioEdit. Small sequences were eliminated from the data set, and 1,101 positions, where all sequences had data, were selected (*E. coli* positions 359–1,460). We checked with BLAST that no significant matches (>97% sequence similarity) had been missed by the RDPII analysis,

resulting in a total of 138 sequences. A distance matrix was constructed with Phylip 3.66 (Felsenstein 1993) using the program DNAdist with the Jukes and Cantor correction. The software program DOTUR (Schloss and Handelsman 2005) was used at a threshold of 1% to select representatives from closely related sequences (Table S1, see supplementary material), producing a final alignment of 62 sequences. The outgroup (*Merismopedia* sp. [EF088332]; uncultured cyanobacterium [AB154318]; *Synechococcus* sp. PCC 9005 [AF216950]; uncultured *Chroococcales* [AY945292]; *Synechococcus* sp. PCC 7920 [AF216948]; *Synechococcus* sp. PCC 7918 [AF216947]; *Cyanobium* sp. PCC 6904 [AF216944]) was manually added, based on conclusions of cyanobacterial phylogenetic relationships by Tomitani et al. 2006. Bayesian inference of phylogeny was applied by implementing the (GTR+G) model with calculated invariable sites. Four chains were performed for 5 million generations, and a total of 180,004 trees were sampled for calculating a majority-rule consensus tree (Fig. 3).

The alignment was also used for the construction of a distance matrix with Phylip, using the program DNAdist with the Jukes and Cantor correction. This matrix was used to define the OTUs with the software DOTUR at a level of 97.8% of 16S rRNA similarity (Fig. 3).

Nucleotide sequence accession numbers. Nucleotide sequence accession numbers of selected 16S rRNA gene sequence data obtained in this study are AY854154–AY854166. Detailed organism names and accession numbers of submitted sequences are listed in Table S2 (in the supplementary material).

RESULTS

DGGE profiles of PCR-amplified 16S rRNA gene fragments. DGGE analysis of the approximately 422 bp region amplified from the 16S rRNA gene of the cyanobionts produced a diverse fingerprint pattern for the various *Azolla* species and between individual strains within a species (Fig. 1). The only exception was the cyanobionts of the diverse strains of *A. microphylla,* which all produced one distinctive band with identical mobility (Fig. 1). The same fingerprint pattern was generated using the two different reverse primers (a) or (b) in all experiments (data not shown). Figure 1 represents the results of DGGE analysis of PCR products amplified using primer (b).

In section *Azolla*, four different patterns were observed for *A. filiculoides*. Cyanobionts of *A. rubra* strain 6503 produced the same fingerprint as *A. filiculoides* strains 1534 and 1043. Similar bands were observed for *A. filiculoide*s strains 1007 and 1046, although these bands differed from those of other *A. filiculoides* cyanobionts examined. The fingerprint of the cyanobionts originating from *A. filiculoides* strain Japan also differed slightly. The fingerprint of *A. filiculoides* strain unknown cyanobionts was similar to that of *A. caroliniana* strain 3004. The cyanobionts from the other four *A. caroliniana* strains produced rather similar profiles, with slight variations in mobility. The five patterns for *A. microphylla* cyanobionts were identical to each other and similar to those of *A. mexicana* 2002 and 2011. *A. mexicana* strain 2008 cyanobionts had an additional faint band lower in the gel. We observed two other patterns for *A. mexicana*, characterizing the cyanobiont from strain 2003 and those from strains 2007 and 2009, respectively.

The profile of the cyanobionts of *A. nilotica* strain 5001 was distinct from all other members of the *Rhizosperma* section. Fingerprints of the cyanobionts of *A. pinnata* var. *pinnata* strains 7001, 7522, and 7526 were very similar to each other and to *A. pinnata* var. *pinnata* strain 7528. The latter also had additional bands (Fig. 1). The cyanobionts of *A. pinnata* var. *pinnata* strain 7524 produced a quite different banding pattern, yet still shared some bands with strain 7528. The profiles of isolates from *A. pinnata* var. *imbricata* strains 2, 6, 64, and 534 produced four almost identical bands. *A. pinnata* var. *imbricata* strains 49 and 74 cyanobionts showed different patterns than the other four but had at least one band in common with each of the other four.

Sequence and phylogenetic analyses of the DGGE bands. All the major bands generated on the DGGE profiles were excised and sequenced as indicated in Figure 1. In general, the sequences were highly conserved. However, the 363 bp fragment used in the phylogenetic analysis, which included the V3 and V4 variable regions of the 16S rRNA gene, revealed nucleotide polymorphisms at 14 positions (Table 2), of which two were synapomorphies consistent with the assignment of the cyanobionts to the different host sections/species. In particular, all analyzed cyanobionts from the *Azolla* section had G477 and T582, while cyanobionts from the *Rhizosperma* section had C477 and C582 (Table 2). A further distinction within these sections existed at position 453, where all *Azolla* section cyanobionts had A453, in contrast to all *A. pinnata* var. *pinnata* and *A. nilotica*, which had C453, while all *A. pinnata* var. *imbricata* had G453. *Rhizosperma* cyanobionts and all *A. caroliniana*, *A. mexicana,* and *A. microphylla* had A721, while all *A. filiculoides* (including Nostocaceae cyanobiont AE1, isolated from *A. filiculoides* var. *rubra*, Baker et al. 2003) and *A. rubra* had G721 (Table 2). *A. caroliniana*, *A. mexicana,* and *A. microphylla* isolates had A610, while G610 was common to all *A. filiculoides*, *A. rubra*, and isolates of the *Rhizosperma* section. There were also nine autapomorphies.

The polymorphisms corresponded to band mobilities in the DGGE gel, except in a few cases. Band 19 from *A. mexicana* (strain 2003) migrated to a slightly higher position on the gel than band 18 (strain 2002), yet the sequences of their 363 bp 16S rRNA gene fragment were identical. Likewise, bands 29 and 30 (from *A. pinnata* var. *imbricata* strain 49) were excised at different positions from the same lane, yet were identical in sequence. The four bands of isolates from *A. pinnata* var. *pinnata* strains 2 and 6 produced only two different sequences, while bands 34, 38, and 39 had the same sequence synapomorphy, but only bands 34 and 38 had exactly the same mobility in the gel.

TABLE 2. Alignment of polymorphic positions for the sequences from the present study and Nostocaceae cyanobiont AE1 (3). The *Azolla* sections are separated by a horizontal line. Asterisks indicate synapomorphies between the sections. The nucleotide positions on top correspond to *Escherichia coli* 16S rRNA gene standard numbering (Neefs et al. 1990). Cyanobiont abbreviations and DGGE band numbers are according to Table 1 and Figure 1.

	Nucleotide position													
Cyanobiont (DGGE band number)	420	427	453	477	576	582	610	621	629	635	680	684	703	721
PI (28,29,30,31,32,33,35,36,37,40)	C	C	G	C	G	C	G	A	A	A	C	T	G	A
PI (34,38,39)	T	C	G	C	G	C	G	A	A	A	C	T	G	A
PP (23,24,26,27)	C	C	C	C	G	C	G	A	A	A	C	T	G	A
PP (25)	C	T	C	C	G	C	G	A	A	A	C	T	G	A
Ni5001 (22)	C	C	C	C	G	C	G	A	G	G	T	T	G	A
FiJapan (4,5,6)	C	C	A	G	G	T	G	A	A	A	C	C	G	G
Fi (1,2,3)	C	C	A	G	G	T	G	A	A	A	C	T	G	G
Fi (clone)	C	C	A	G	G	T	G	A	A	A	C	T	G	G
FiUnknown (10)	C	C	A	G	A	T	G	A	A	A	C	T	G	G
Ru6503 (7)	C	C	A	G	G	T	G	A	A	A	C	T	G	G
Nostocaceae cyanobiont AE1	C	C	A	G	G	T	G	C	A	A	C	T	C	G
Ca (8,9,11,12)	C	C	A	G	G	T	A	A	A	A	C	T	G	A
Me (18,19,20,21)	C	C	A	G	G	T	A	A	A	A	C	T	G	A
Mi (13,14,15,16,17)	C	C	A	G	G	T	A	A	A	A	C	T	G	A
Synapomorphies				*		*								

To estimate the taxonomic relatedness of the cyanobionts, MP analysis was performed for the 40 cyanobacterial 16S rRNA gene fragments retrieved from the DGGE bands, the representative clone sequence obtained for this study (AY854154 *Azolla* cyanobiont Fi1534), and two published sequences from *Azolla* cyanobionts (AY742450 from *A. filiculoides* 1010 of Peru, Svenning et al. 2005; AY181212 from *A. filiculoides* var. *rubra* of Australia, Baker et al. 2003). *Chroococcidiopsis thermalis* PCC 7203 (AB039005) was used as the outgroup (Fig. 2). High parsimony scores, 0.8571 consistency index (CI), 0.8941 retention index (RI), and 0.7664 rescaled consistency index (RC) indicated low homoplasy in this data set. The analysis was complemented by Bayesian posterior analysis, where likelihood scores had been preestimated. The dendrogram showed two major groups. Group I was clearly resolved in the tree with a high support by both algorithms and included all the sequences from cyanobionts of section *Azolla*. Two clusters diverged; one contained identical sequences from all the cyanobionts from *A. caroliana*, *A. microphylla*, and *A. mexicana*, while the *A. filiculoides* cyanobionts showed more internal diversity and formed a separate cluster with 0.73 posterior probability. Inside the *A. filiculoides* cluster, the three sequences isolated from *A. filiculoides* strain from Japan formed a distinct branch with 64% bootstrap and 0.94 posterior probability supports. The *A. filiculoides* cyanobiont clone obtained in this study (AY854154, Fi1543,) had the same branch length as Fi1043-2, Fi1046-3, Fi1534-1, and Ru6503-7. Based on minute differences, FiUnkn-10, the cyanobiont AY181212 isolated from *A. filiculoides* var. *rubra*, and AY742450 from *A. filiculoides* 1010 branched off from this group. All other sequences remained as a basal aggregate (group II) containing all sequences from *Rhizosperma* strains. Ten sequences from *A. pinnata imbricata* formed its basis, from which a group of three *A. pinnata imbricata* cyanobiont sequences (PI2-34, PI6-38, and PI6-39) with 65% bootstrap and 0.92 posterior probability, a group with cyanobionts from all *A. pinnata pinnata*, and the only strain of *A. nilotica* (0.74 posterior probability) slightly diverged.

Clone library sequences and phylogenetic analysis. Cloning analysis of the cyanobionts from *A. filiculoides* strain 1534 produced 28 quasi-identical sequences. We detected small inconsistencies between the clone sequences. The sequence chosen to represent the clone library (AY854154 *Azolla* cyanobiont Fi1534) was found in 16 of the 28 clones. Single nucleotide variations in the 12 remaining sequences were not present in more than one clone and were absent from the *Azolla* cyanobiont sequences used in the phylogenetic analysis (Fig. 3). All clones were highly similar (98.8%–100% similarity) to the other cyanobionts of *A. filiculoides* strains, including the Nostocaceae cyanobiont AE1 (3) as well as *A. rubra* strain 6503.

Phylogenetic analysis of the representative clone sequence (AY854154 *Azolla* cyanobiont Fi1534) produced the phylogenetic tree shown in Figure 3. The operational taxonomic units (OTUs) were superimposed on the topology. Some 22 OTUs were detected at a threshold of 97.8% similarity. The two symbiotic sequences from *A. filiculoides* (our clone and the strain from 1010) were located in the same OTU as *Anabaena oscillarioides* BO HINDAK 1984/43 (99.3% sequence similarity) and *Anabaena* cf. *cylindrica* PH133 (98.1% sequence similarity). This relationship coincided with a posterior probability value of 1.00 for the same grouping in the tree. A posterior probability of 0.75 supported the grouping of this OTU with another branch containing the strain *Anabaena* sp. PCC 7108,

originally isolated from the intertidal zone of Moss Beach in California, USA (Lyra et al. 2001). The closest related *Nostoc* (with 96.5% similarity) is strain 8941, also a symbiont from *Gunnera dentata*, New Zealand (Svenning et al. 2005).

DISCUSSION

The study of the genetic diversity of the cyanobionts from *Azolla* has been complicated in the past by the impossibility of culturing them in vitro and the difficulty of sorting out the true cyanobionts from cyanobacteria living at the surface of the fern. Our study is the first attempt to circumvent the latter problem. We used a highly specific approach for studying the diversity of the exclusively cyanobacterial fraction from the microbial population residing inside the leaf cavities through the combination of a thorough surface washing followed by enzymatic digestion of all plant leaf material, thus releasing the intact cyanobacterial packets, and PCR amplification of extracted DNA with cyanobacteria-specific primers.

The two reverse primers, which target filamentous (a) and unicellular (b) cyanobacteria, produced the same DGGE profiles, indicating that the unicellular primer (b) acted as a degenerate primer, thus annealing to the heterocystous filamentous cyanobacterial population occurring abundantly inside the leaf cavity. Therefore, the resulting DGGE pattern could be considered as specific for the cyanobionts (Boutte et al. 2006), while the complexity of the profile was an approximate measure of the cyanobacterial diversity in the sample (van Wintzingerode et al. 1997). In general, one strong band per profile was visible (Fig. 1) and it is possible to sequence the DGGE bands after excision and reamplification. Interestingly, the observed base substitutions (Table 2) of the DGGE band sequences showed the specificity of the cyanobionts to their hosts and followed the classification of the *Azolla* sections and species recognized by most authors (Braun-Howland and Nierzwicki-Bauer 1990), except for *A. caroliniana*, *A. mexicana*, and *A. microphylla*. This pattern was also evident in the parsimony analysis (Fig. 2) of the sequences generated from the excised DGGE bands (Fig. 1). Therefore, the 363 characters (containing the 16S rRNA variable domains V3 and V4) used for the construction of the phylogenetic tree in Figure 2 allowed for accurate distinction of these different groups of cyanobionts and showed good correlation with their host classification. Indeed, all the sequences obtained from the cyanobionts of section *Azolla* formed a distinct group from the *Rhizosperma* section. This division of sequences was strongly supported in the tree topology by the Bayesian analysis; however, the parsimony bootstrap supports were not very high due to the small number of polymorphisms. In addition, all sequences from all the *Azolla* identified in the IRRI collection as *A. caroliniana*, *A. mexicana*, and *A. microphylla* were closely related to each other (Fig. 2), corroborating with the suggestion of Van Coppenolle et al. (1995) that the same cyanobiont strain inhabits these three *Azolla* species. These results are also coherent with the fact that all these *Azolla* actually belong to the same species, *A. cristata* (Evrard and Van Hove 2004). However, our results concerning the cyanobionts from the strains identified in the IRRI collection as *A. caroliniana*, *A. mexicana*, and *A. microphylla* do not fit those presented by Reid et al. (2006) for the corresponding *Azolla* hosts. These authors studied the phylogenetic relationships in the genus *Azolla* on the basis of DNA sequences from three noncoding regions—two were plastid derived, and one was derived from the ITSs of the nuclear rRNA genes (Reid et al. 2006). Their analysis did not distinguish *A. microphylla* from *A. mexicana*, yet they considered *A. caroliniana* as a separate species. Their taxonomic conclusions are confusing, and synonymies presented in their table 1 do not correspond either to the data from Saunders and Fowler (1993) or to those from Evrard and Van Hove (2004), as erroneously indicated in the legend of the table. *A. caroliniana* Willd. and *A. microphylla* Kaulf. were described as the currently recognized species even though it is to *A. caroliniana* auct. non-Willd. and to *A. microphylla* auct. non-Kaulf to which most authors refer. The ''*A. caroliniana*'' clade was said to contain only samples identified as *A. caroliniana* sensu Svenson, whereas most authors consider *A. caroliniana* as sensu Mettenius.

Our results on the cyanobionts from *A. rubra* and *A. filiculoides* fit with standard opinion concerning the close relationships between their hosts. *A. rubra* is considered by most authors as a subspecies, a variety, or even as a synonym of *A. filiculoides*. In addition, the *A. filiculoides* and *A. rubra* strains used in our study were collected from very different locations (Table 1), but some of their cyanobionts had identical sequences. *A. filiculoides* and *A. rubra* were characterized as separate species within section *Azolla* by Reid et al. (2006). The cyanobionts of *A. nilotica* differed from those collected from *A. pinnata pinnata* strains, but they are associated within the same subgroup (Fig. 2), thus displaying their close relationship (98.6%–99.7% sequence similarity). Three polymorphic sites were observed in the sequence from the *A. nilotica* cyanobionts (Table 2). However, these polymorphic sites cannot be considered a trait of *A. nilotica* species since only a single strain was included in this study. Interestingly, this result is supported by the study of Van Coppenolle et al. (1995) in which unique RFLP patterns were produced for cyanobionts of *A. nilotica* species. All *A. pinnata* var. *pinnata* cyanobiont sequences were gathered on a branch clearly separated from the *A. pinnata* var. *imbricata* isolates (Fig. 2). Such specificity of the cyanobionts to their *Azolla* host has also been demonstrated

by McCowen et al. 1987, who used lectins isolated from the fern that showed selectivity to the cyanobionts collected from the same *Azolla* species.

Multiple bands were observed in the fingerprints of some isolates from *A. filiculoides*, *A. caroliniana*, *A. mexicana*, and *A. pinnata* var. *imbricata* (Fig. 1), possibly indicating the presence of more than one 16S rRNA gene type or the presence of several operons of the 16S rRNA with minor sequence differences. This complexity could also be attributed to the existence of one major cyanobacterial species and the coexistence of minor species as suggested in previous studies (Meeks et al. 1988, Gebhardt and Nierzwicki-Bauer 1991, Kim et al. 1997). Another explanation might be methodological errors made by the base-calling program, PCR-introduced errors, or sequencing errors (van Wintzingerode et al. 1997). However, in several cases we observed bands that migrated to different positions yet had the same sequence (e.g., DGGE bands 4, 5, 6 from *A. filiculoides* strain from Japan), which is not an uncommon experience with DGGE gels (Jackson et al. 2000, Boutte et al. 2006). In addition, all cyanobiont sequences retrieved in the current study were closely related, with maximal sequence divergence of 2.3% observed between the cyanobionts of *A. nilotica* and both *A. filiculoides* strains from Japan and unknown, indicating that one major cyanobacterial taxon inhabits all *Azolla* (Stackebrandt and Göbel 1994). All of the observed sequence variations in the *Azolla* cyanobionts might have been inherited from one common ancestor inhabiting one particular host species. If the 16S rRNA gene fragment reflects the evolution of the whole genome, differences in this marker would indicate that adaptive mutations have happened elsewhere in the genome. Such microdiversity has been observed for other microorganisms. Free-living *Prochlorococcus* spp. inhabiting the same oceanic region have 16S rRNA sequence differences that directly correspond to physiological differences (Moore et al. 1998).

Overall, this study confirmed the existence of different cyanobacterial strains or ecotypes inhabiting the fern species, a diversity visible even at the *Azolla* strain level for the isolates from *A. pinnata* var. *imbricata* (PI2 and PI6, Fig. 2). Our data agree with the suggestion by Plazinski et al. (1990) that different ecotypes of cyanobacteria exist within the same host species and even strain and are complemented by the morphological taxonomy of *A. pinnata* of Saunders and Fowler (1992), which recognizes three subspecies differing in their geographic origin. However, geographic distribution (Table 1) did not seem to correlate with cyanobiont sequence variation, which did agree with their host taxonomy. This fact can easily be explained by the highly conserved nature of the ribosomal gene used in the present study, in combination with the hereditary nature of the symbiotic association between *Azolla* and the cyanobionts.

The estimation of the phylogenetic position of the *Azolla* cyanobiont was carried out using the Bayesian inference of phylogeny and illustrated in the phylogenetic tree in Figure 3 that depicts the relationships of the two *Azolla* cyanobiont sequences included with their most closely related strains. The molecular taxonomy of the genera *Anabaena, Nostoc*, and *Trichormus* remains under debate, due to the polyphyletic distribution of the 16S rDNA sequences of strains assigned to these genera and to the fact that there is little agreement between morphology and molecular characteristics (Rajaniemi et al. 2005). Our cyanobionts clearly belong to the OTU 9, which also contains two free-living *Anabaena* strains. *An. oscillarioides* strain BO HINDAK 1984/43 was isolated from a plankton sample collected in South Indian Lakes, Canada, by Hedy Kling in 1984 (Rajaniemi et al. 2005). The strain *An.* cf. *cylindrica* PH133 was isolated in 1993 from Lake Arreso, Denmark (Gugger et al. 2002). Therefore, the ecological origins differ, but the sequence similarity with our clone is 99.3%. It is known that strains with >97% similarity can have quite different physiologies and genomes (e.g., *Prochlorococcus* MED4 and MIT9313, Rocap et al. 2002), and therefore, the high sequence similarities do not necessarily help to draw taxonomic limits (Stackebrandt and Göbel 1994).

Overall, the cyanobionts studied here have more genotypic affinity to strains belonging to the genus *Anabaena* than to *Trichormus*. Therefore, between the two valid names attributed to the cyanobiont, *An. azollae* and *T. azollae*, the first one seems preferable. Furthermore, the observed diversity of the cyanobionts provides evidence of their specificity for their host, even at the species level, a probable result of symbiotic coevolution. Moreover, our data suggest that one cyanobacterial taxon originally infected an ancestor of the *Azolla* species, resulting in the formation of a single lineage (shown by the very high bootstrap value [99%] for the two cyanobiont sequences from *Azolla* in Fig. 3). This symbiosis ultimately became obligatory for the cyanobionts.

A travel grant supporting the present project was provided to the first author by the ESF CYANOFIX program. We would like to thank Dr. Krzysztof Waleron and Dr. Beatriz Díez-Moreno for their help and advice. Arnaud Bataille kindly performed part of the cloning, and Stana Grubisic assisted with the sequencing operations. The DGGE marker was kindly provided by P. Rajaniemi (University of Helsinki, Finland), and primer 1092R sequence by S. Ventura (CNR-ISE, Italy). A. Wilmotte is Research Associate of the Belgian Fund for Scientific Research of Belgium (FNRS).

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Supplementary Material

The following supplementary material is available for this article:

Table S1. Table showing operational taxonomic units (OTUs) produced at a threshold of 1% similarity for selecting representatives from selected sequences used in the phylogenetic analysis in Figure 3. Several occurrences of the 16S rRNA gene within the genome of a strain are indicated by a numeral next to the accession number.

Table S2. *Azolla* strains, clone, and DGGE band numbers with the corresponding GenBank accession numbers of the sequenced cyanobionts.

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