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Genetic diversity and phylogeny analysis of *Anabaena azollae* based on RFLPs detected in *Azolla-Anabaena azollae* DNA complexes using *nif* gene probes

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Abstract The cyanobacterium *Anabaena* has both symbiotic and free-living forms. The genetic diversity of *Anabaena* strains symbiotically associated with the aquatic fern *Azolla* and the evolutionary relationships among these symbionts were evaluated by means of RFLP (restriction fragment length polymorphism) experiments. Three DNA fragments corresponding to *nif* genes were cloned from the free-living cyanobacterium *Anabaena* PCC 7120 and used as probes. A mixture of *Azolla*, *Anabaena* and bacterial DNA was extracted from *Azolla* fronds and digested with two restriction enzymes. Single-copy RFLP signals were detected with two of the probes in all *Azolla Anabaena* examined. Multiple-copy RFLP signals were obtained from the third probe which corresponded to a part of the *nif* N gene. A total of 46 probe/enzyme combinations were scored as present or absent and used to calculate pairwise Nei's genetic distances among symbiotic *Anaebaena* strains. Phylogenetic trees summarizing phenetic and cladistic relationships among strains were generated according to three different evolutionary scenarios: parsimony, UPGMA and neighbour joining. All trees revealed identical phylogenetic relationships. Principal component analysis was also used to evaluate genetic similarities and revealed three groups: group one contains the cyanobacteria associated with plants from the *Azolla* section, group two contains those associated with plants from the *pinnata* species and group three contains those associated with plants from the *nilotica* species. The same groups had already been identified earlier in a random amplified polymorphic DNA (RAPD) analysis of *Azolla-Anbaena* DNA complexes, suggesting that the present *Azolla* taxonomy should be revised. We now suggest a taxonomy of *Anabaena azollae* that is parallel to such a revised *Azolla* taxonomy. An *Azolla* chloroplast DNA sequence derived from *Oryza sativa* was also used as an RFLP probe on *Azolla* DNA to confirm the presence of plant DNA in the total genomic DNA extracted from ferns with or without the symbiont. Our results also suggest that total DNA extracted from the *Azolla-Anabaena* complexes includes both plant and symbiont DNA and can be used equally well for RFLP analysis of host plant or symbiotic cyanobacteria.

Key words *Azolla-Anabaena* symbiosis · *nif* genes · Principal component analysis · Restriction fragment length polymorphism · Parsimony · Phenetic distances

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Introduction

The nitrogen-fixing cyanobacterium *Anabaena azollae* inhibits leaf cavities of the aquatic fern *Azolla*. The exchange of nitrogen from the cyanobiont for carbohydrates from the host plant forms the basis of an interesting symbiosis that has been intensively studied mainly because of the potential for *Azolla/Anabaena* to be used as organic fertilizer, especially in wetland rice soils, and as food for various animals (Lumpkin and Plucknett 1982; Van Hove et al. 1987; Van Hove 1989; Watanabe et al. 1989)

Since Strasburger (1873), the cyanobacterial endophyte of *Azolla* has been named *Anabaena azollae*, without any bearing to the *Azolla* species concerned. Research aimed to resolve the taxonomical status of *A.*

azollae have often been restricted to solely identifying the cyanobiont as belonging to *Anabaena, Nostoc* (Caudales et al. 1988; Tomaselli et al. 1988; Plazinski et al. 1990) or *Trichormus* genera (Komarek and Anagnostidis, 1989). The difficulty in culturing the symbiont on artificial medium has been the main reason preventing in-depth classification studies (Zimmerman et al. 1989c; Tang et al. 1990; Plazinski 1990).

A few immunological studies using polyclonal fluorescent antibodies have revealed a high degree of similarity between symbionts isolated from different *Azolla* species (Gates et al. 1980; Tel-Or et al. 1983). However, when they used monoclonal antibodies, Liu et al. (1989) observed a low degree of similarity between symbionts. Ladha and Watanabe (1982) used polyclonal antibodies prepared from freshly isolated symbionts and found no cross reaction with free-living *Anabaena* and other blue-green algae species. The negative reaction of antibodies obtained with cultured isolates (Tel-Or et al. 1984; Rosen et al. 1987) suggested either that antigenic modifications could have occurred in the cyanobacteria during the passage from a symbiotic stage to a cultured, free-living stage or that the cultured isolates were not true isolates from the leaf cavity. Likewise, DNA amplification fingerprinting (DAF) experiments revealed low-to-high degrees of similarity between a few freshly isolated *A. azollae*, depending on the primer used (Eskew et al. 1993). Polymorphisms in cyanobacterial amplified DNA were used by the authors to verify the maternal inheritance of *A. azollae* in sexual crosses between plants from different *Azolla* species.

During the past 10 years, several research groups working on the process of nitrogen fixation and its regulation have isolated nitrogenase, of *nif*, genes from nitrogen-fixing organisms, including *Anabaena.* Reports indicated that these genes were organized in clustered arrays and that similar physical arrangements of the genes existed in free-living *Anabaena* and other N_2-fixing bacteria like *Nostoc* (Meeks et al. 1988). Evolutionary studies involving *nif* gene sequences supported phylogenies that were consistent with those based on 16S rRNA sequences (Young 1992) and suggested the use of *nif* genes for studying bacterial taxonomy. Franche and Cohen-Bazire (1985, 1987), Meeks et al. (1988) and Nierzwicki and Haselkorn (1986) observed structural differences in *nif* genes isolated from *Anabaena* that had been freshly extracted from *Azolla* by the method of Peters and Mayne (1974) and from *Anabaena* strains that had been cultured *in vitro* following isolation from the fern. This either implies a fast adaptation to non-symbiotic conditions or, more probably, reflects the fact that the isolates do not correspond to the real, or at least the major, *Azolla* endophyte. In a few similar experiments, cloned *nif* genes from free-living *Anabaena* PCC 7120 were used as RFLP (restriction fragment length polymorphism) probes on cyanobacterial DNA extracted from isolates of different *Azolla* species. The resulting grouping of symbionts showed a certain degree of consistency with the actual *Azolla* taxonomic framework, but the isolated cyanobacteria were derived from only a subset of known *Azolla* species (Franche and Cohen-Bazire 1985, 1987; Franche et al. 1987; Plazinski et al.1988). Later, Plazinski et al. (1990) used homologous probes to hybridize DNA extracted from different *Anabaena* isolates. They found a large degree of genetic diversity among fresh isolates from all known *Azolla* species and reported a closer relationship between *A. azollae* and free-living *Nostoc* strains than among free-living *Anabaena* strains. They suggested that their findings provided an indirect way of classifying *Azolla* species, but they did not report any *A. azollae* classification.

In the study described here, we demonstrate that *A. azollae* DNA can be directly analyzed from total DNA extracted from *Azolla* plants using *nif* genes, or parts of *nif* genes, as probes. This overcomes the problem of isolating symbiotic cyanobacteria for molecular analysis. We also show a RFLP analysis and classification of *Anabaena* symbionts from all known *Azolla* species. The RFLP data were used to generate Nei's genetic distances and to build phylogenetic trees. Different evolutionary models were used, but they all revealed identical phylogenetic relationships. A PCA (principal component analysis) representation based on Nei's genetic distances showed the same three groups of species that we reported earlier in a RAPD (random amplified polymorphic DNA) analysis of *Azolla-Anabaena* DNA complexes (Van Coppenolle et al. 1993) and draws attention to the phylogenetic distance between strains from the two species forming the *Rhizosperma* section. A taxonomy of the symbiotic cyanobacteria that is parallel to the revised *Azolla* taxonomy suggested from earlier RAPD results, can be proposed. An *Azolla* chloroplast (cp), DNA sequence derived from *Oryza sativa* was also used as an RFLP probe to confirm that the total genomic DNA extracted from plants with or without the symbiont really containied plant DNA (rather than just cyanobacterial DNA). Our results suggest that total DNA extracted from the *Azolla-Anabaena* complexes includes both plant and symbiont DNA and can be used equally well for host plant or symbiotic cyanobacterial analysis.

Materials and methods

Plant materials and total DNA extraction

Eleven *Azolla* accessions were selected from a germ plasm collection maintained at the International Rice Research Institute (IRRI, Philippines). Accessions which represent all known *Azolla* species are listed in Table 1 according to their IRRI code numbers (Watanabe et al. 1992). Two *Anabaena*-free accessions, as well as a rice variety ('IR 36') were included in the experiments as controls. The symbiont-free plants were obtained earlier either by micromanipulation of female reproductive structures to remove the cyanobacteria (FI 1050) or by treatment with antibiotics (PI 97). A description of how the plants were grown and of the DNA extraction procedure has been previously reported (Van Coppenolle et al. 1993)

Table 1 *Azolla* accessions used for RFLP analysis of *A. azollae* with one chloroplast and three *nif* gene probes[a]

Accession no. (IRRI code)	Species	Code[b]	Section
1052	*filiculoides*	FI	*Azolla*
1050 + N[c]	*filiculoides*	FI	*Azola*
2007	*mexicana*	ME	*Azolla*
3504	*caroliniana*	CA	*Azolla*
4059	*microphylla*	MI	*Azolla*
6502	*rubra*	RU	*Azolla*
97 + N[c]	*pinnata* var *imb.*	PI	*Rhizosperma*
535	*pinnara* var *imb.*	PI	*Rhizosperma*
7004	*pinnata* var *pinn.*	PP	*Rhizosperma*
5002	*nilotica*	NI	*Rhizosperma*
5501	*nilotica*	NI	*Rhizosperma*

[a] Abbreviations: var *imb.* = variety *imbricata*; var *pinn.* = variety *pinnata*
[b] Species code according to Van Hove et al. (1987)
[c] *Anabaena*-free as recorded in Watanabe et al. (1992)

Hybridization of probes

Three DNA fragments corresponding to *nif* genes or parts of *nif* genes were kindly provided by R. Haselkorn (University of Chicago, USA). They were isolated from the free-living *Anabaena* strain, PCC 7120, inserted into Bluescript plasmids and cloned into *E. coli* (Rice et al. 1982). These were then used as probes in RFLP studies of *A. azollae*, in *Azolla-Anabaena* DNA complexes. These three fragments are present in a single-copy form in *Anabaena* PCC 7120. The first two fragments were identified respectively as 1.4 *nif* (1.4 kb long, part of the *nif*N gene) and 1.9 *nif* (1.9 kb long, *nif*X, *nif*W genes, ORF 1 and 2, and Hesa). Both fragments were present in subclones of pAn 281. The third fragment was present in clone pAn 207.1 (3.0 kb long, *nif*E, part of *nif*N, and part of *nif*K). A plot map representation for the three fragments is shown in Fig. 1 (Rice et al. 1982). A chloroplast sequence was also used as a probe to confirm the presence of plant genomic DNA in the total DNA extracted from the symbiotic cyanobacteria/plant association. Pairs of specific primers were designed from a chloroplastic DNA sequence of *O. sativa* (G. Second, personal communication). Primers 10229204 (5′ CATATTCGTGAAGCAGA AAC 3′, forward) and 10229205 (5′ ACGGTTCGAATCCGTATA GC 3′, reverse), which gave a 750-bp polymerase chain reaction (PCR) product in rice, were used to amplify total DNA of several *Azolla* accessions. In almost all cases, a single product of approximately the expected length occurred, but in the case of accession 1050 + N, an additional PCR fragment of 500 bp was revealed. This fragment was later called AC-1 and used as an RFLP probe.

Enzymatic digestion and electrophoretic separation of DNA fragment

Approximately 2.5–5 µg of extracted DNA was digested with 10–20 U either of *Eco*RI or *Hin*dIII restriction enzyme according to Sambrook et al. (1989). Digested DNA fragments were separated by electrophoresis in 0.9% agarose gels at 1 V/cm and alkali transferred onto nylon membranes (HybondTM − N +) according to the manufacturer's specifications (Amersham, Buckinghamshire, UK).

PCR amplification of *nif* gene probes

Nif gene clones were amplified by PCR according to Saiki et al. (1988), prior to labelling and hybridization, in order to avoid any cross-hybridization interference with plasmid DNA (such as we found occurring in earlier similar studies). An amount of 50–75 ng of inserted *nif* gene template was PCR-amplified in a total volume of 50 µl, with 80 ng of each primer (forward and reverse), 175 µ*M* of each dNTP (Boeringher), 10 m*M* TRIS-HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 100 µg/ml gelatine and 2 units of Ampli *Taq* polymerase (Perkin-Elmer). After an initial denaturation step at 94 °C for 3 min, 35 amplification cycles for 1 min at 94 °C (denaturation step), 1 min at 36 °C (annealing step) and 2 min at 72 °C (extension step) were performed in a DNA Thermo Cycler (Perkin-Elmer/Cetus 480), followed by 1 single extension cycle for 7 min at 72 °C. Amplified *nif* fragment sizes were verified by electrophoresis on agarose gel. DNAs were then precipitated and the pellets dissolved ini 50 µl TE (10 m*M* TRIS, 1 m*M* EDTA, pH 8.0).

Radioactive labelling of probes and Southern hybridization

An amount of 150–200 ng of PCR-amplified probe was radiolabelled with [^{32}P] dCTP prior to an overnight hybridization at 65 °C as prescribed by Sambrook et al. (1989). Southern blots were then washed at 65 °C for 30 min in 2 × SSC, 0.1% SDS; then for 20 min in 1 × SSC, 0.1% SDS; and finally for 15 min in 0.5 × SSC, 0.1% SDS before exposure for 3–5 days at −80 °C with a Kodak X-OMAT K autoradiogram film. Blots were subsequently reused for further hybridization with other probes after removal of the hybridized probe as described by the manufacturer.

Genetic distances and statistical analysis

An NTSYS-pc version 1.70 (Numerical taxonomy and multivariate analysis system) computer software (Rohlf 1992) was used to calculate Nei's genetic distances (index 72) based on the proportion of shared bands (Nei and Li 1987) in pair-wise comparisons between accessions. Dendrograms were then built according to the unweighted pair group method with arithmetic mean (UPGMA). A Phylogeny Inference Package, Phylip version 3.4, was also used to build a dendrogram from the same genetic distances based on the neighbour joining (NJ) method. A cladogram based on RFLP character data was also constructed using a PAUP version 3.0 (phylogeny analysis using parsimony) McIntosh software package (Swofford 1990) for further comparison with the results based on distance methods.. Eventually, PCA was performed on Nei's distance matrix with a Data Desk Macintosh computer program.

Results

A total of 46 probe/enzyme combinations were scored in our hybridization of *A. azollae* DNA along with three

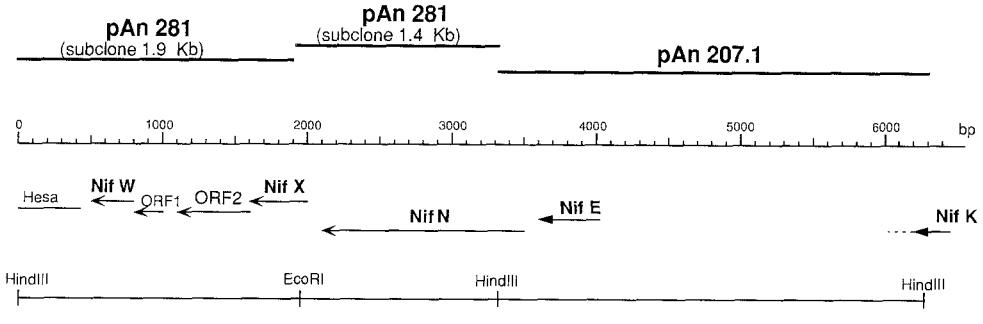


Fig. 1 Plot map representation of inserted fragments from two subclones of pAn281 and clone pAn 207.1 that were used as probes in RFLP experiments on *Azolla-Anabaena* DNA complexes (modified from Rice et al. 1982)

cyanobacterial *nif* gene probes. Probes 207 *nif* and 1.9 *nif* gave single-copy signals with *Eco*RI and *Hin*dIII, while probe 1.4 *nif*, which is located just besides 1.9 *nif* in *Anabaena* PCC 7120, gave multicopy signals. In the following paragraphs, we mostly refer to the cyanobacterial strains by the species code of their respective *Azolla* host (see Table 1), e.g. strains from *A. caroliniana* 3504, *A. mexicana* 2007 and *A. microphylla* 4059 will be called CA 3504, ME 2007 and MI 4059, respectively.

Hybridization with fragment 207 *nif*

Fragment 207 *nif* gave very simple and interesting profiles at the species level. As expected, symbiont-free strain PI 97 + N, FI 1050 + N and the rice variety DNA did not show any hybridization signal (Fig. 2)

When *Azolla-Anabaena* DNA was digested with *Hin*dIII, identical hybridization patterns were observed for all strains except the two NI strains: in the former, 2 bands at 1.9 and 1.0 kb were observed; the NI strains (5002 and 5501) showed bands at 5.5, 2.8, 2.3 and 1.0 kb.

Digestion with *Eco*RI revealed identical patterns for the CA-ME-MI-FI-RU strains (respectively 3504, 2007, 4059, 1052, 6502): bands at 3.0 and 1.6 kb. Strains of the NI species (5002 and 5501) also showed a band at 1.6 kb, but an additional band at 2.85 kb was observed as well. Strains PI 535 and PP 7004 showed only 1 band at 1.5 kb.

The results were consistent when ten strains from each *Azolla* species were compared. For two *Azolla* species (sp. *nilotica* and *rubra*), only strains from three accessions (rather than ten) were available (results not shown).

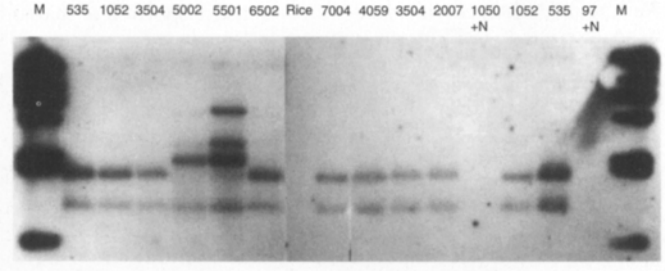


Fig. 2 RFLPs detected with probe corresponding to inserted fragment from clone pAn 207.1 in *Azolla-Anabaena azollae* DNA complexes digested with restriction endonuclease *Hin*dIII. *M* molecular weight market Lambda phage digested with *Hin*dIII, from *top* to *bottom* (kb): 23.1, 9.6, 6.6, 4.4, 2.3, 2.0 and 0.5

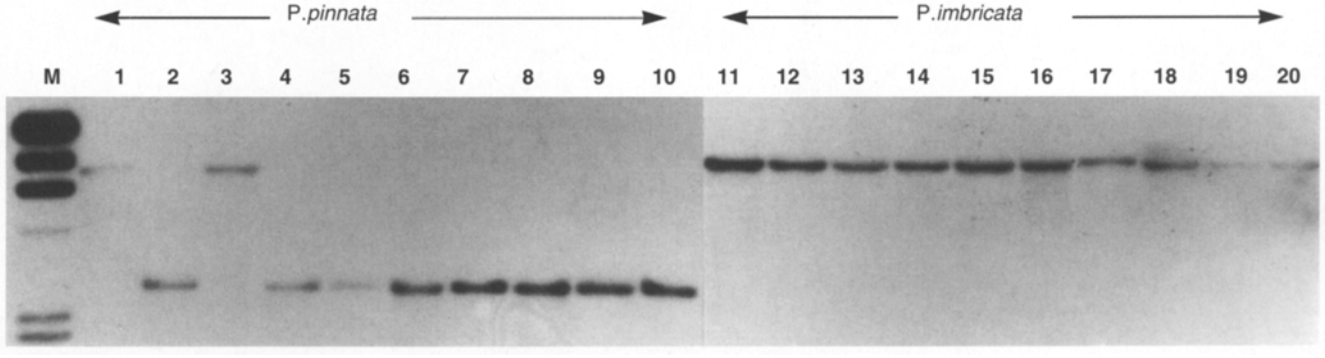
Hybridization with fragment 1.9 *nif*

Interesting results were obtained when DNAs of the *Azolla-Anabaena* DNA complexes were digested by *Hin*dIII and probed with the 1.9-kb *nif* fragment. Profiles for accessions from the *Rhizosperma* section, PP 7004, PI 535, NI (5002 and 5501), showed 3 bands: 3.0 kb (strong), 2.1 kb (weaker) and 0.5 kb (strong). Profiles of FI 1052, MI 4059, CA 3504, ME 2007 and RU 6502 shared 2 bands at 2.5 and 0.5 kb. A weak band at 3.2 kb also appeared in the case of the strains from the CA-ME-MI *Azolla* species.

Digestion of *Azolla-Anabaena* DNA complexes with *Eco*RI gave very simple profiles. All strains except PI 535 shared 2 bands at 3.0 kb (strong) and 1.2 kb (weak). Strains from the NI species showed 1 additional band at 1.7 kb. Strain PI 535 showed a band at 1.2 kb (weak) and 7.5 kb(strong). As expected, the symbiont-free strains PI 97 + N, FI 1050 + N and the rice variety did not show any hybridization signal.

Results were also consistent for all ten strains of each species tested, or for strains from three accessions in the case of *A. nilotica* and *A. rubra* (results not shown). A polymorphism between strains from *A. pinnata* var '*imbricata*' and those from *A. pinnata* var '*pinnata*' was confirmed for the enzyme/probe combination *Eco*RI/1.9 *nif*. In addition, we discovered that two *Azolla-Anabaena azollae* DNA complexes from var '*pinnata*' showed an hybridization signal corresponding to that obtained from the *Azolla-Anabaena azollae* DNA complexes from var '*imbricata*' (Fig. 3).

Fig. 3 RFLPs detected with probe corresponding to an inserted fragment of clone pAn 281 (subclone 1.9 *nif*) in *Azolla-Anabaena azollae* DNA complexes extracted from *A. pinnata* var '*pinnata*' as compared to those extracted from *A. pinnata* var '*imbricata*' after digestion with *Eco*RI. *M* molecular weight marker Lambda phage digested with *Hin*dIII, from *top* to *bottom* (kb): 23.1, 9.6, 6.6, 4.4, 2.3 and 2.0. Accession numbers (IRRI codes): *lane 1* 7534 (Philippines), *2* 7005 (Australia), *3* 7511 (Guinea-Bissau), *4* 7527 (Zaire), *5* 7002 (Australia), *6* 7533 (Australia), *7* 7523 (Australia), *8* 7524 (Australia), *9* 7512 (Zaire), *10* 7004 (Australia), *11* 17 (Vietnam), *12* 24 (Philippines), *13* 33 (China), *14* 84 (Japan), *15* 90 (Philippines), *16* 508 (Australia), *17* 515 (Thailand), *18* 534 (Sri Lanka), *19* 540 (China), *20* 541 (China)



Hybridization with fragment 1.4 *nif*

With both *Hin*dIII and *Eco*RI restriction enzymes, RFLP profiles obtained by hybridization with fragment 1.4 *nif* were always difficult to analyze and revealed multiple-copy signals. Five to seven bands were commonly observed in individual profiles. Some of these bands appeared to be very close to each other in molecular weight. As expected, symbiont-free strains PI 97 + N, FI 1050 + N and the rice variety did not give any hybridization signal.

Digestion with *Hin*dIII revealed identical patterns for the CA-ME-MI strains: bands at 14.0, 7.0, 5.0, 3.5 and 2.5 kb. The same pattern was observed for FI 1052 but with 2 additional bands at 9.4 and 2.3 kb. Strain RU 6502 gave the following pattern: bands at 9.4, 7.0, 1.9, 3.0 and 2.5 kb. A different pattern was obtained with strains from species NI (5002 and 5501): bands at 10.5, 5.5, 2.8, 3.2, 0.7 and 0.6 kb. The patterns of strains PP 7004 and PI 535 gave some identical signals at 8.0, 5.5, 4.0 and 2.8 kb. Strain PI 535 showed 2 more bands at 3.7 and 1.0 kb.

Digestion with *Eco*RI gave identical patterns for the CA-ME-MI strains (3504, 2007 and 4059): bands at 10.0, 8.5, 4.5, 2.0 and 1.5 kb. Strains FI 1052, also in the *Azolla* section, showed a similar profile: bands at 8.5, 4.5, 3.5 and 1.5 kb. The following pattern was observed for accessions from the *Rhizosperma* section. Strains PP 7004 had bands at 10.8, 6.8, 5.3, 4.5 and 1.5 kb. The same pattern was detected for PI 535 except for the band at 6.8 kb, and it had 2 additional bands at 7.5 and 3.5 kb. Patterns of strains of NI species (5002 and 5501) and strain RU 6502 were very messy and did not allow us to score bands unambiguously, except for a band at 1.5 kb.

A confirmation of the results was obtained using the only available blots of *Azolla-Anabaena azollae* DNA complexes extracted from ten accessions of *A. microphylla* (result not shown).

Hybridization with amplified chloroplast DNA fragment

The *Azolla* PCR fragment obtained by amplification with rice chloroplast specific primers gave single-copy signals upon hybridization of *Azolla-Anabaena* DNA complexes and of rice 'IR 36' DNA digested either by *Hin*dIII or *Eco*RI (Fig. 4). A signal which was identical to the one obtained with other *A. pinnata* accessions was detected with *Anabaena*-free accession PI 97 + N. A similar observation was obtained with *Anabaena*-free accession FI 1050 + N and symbiotic *filiculoides* accessions. This confirmed the presence of plant genomic DNA in the total DNA extracted from symbiotic *Azolla* plants and the stability of the chloroplast genome between *Azolla* and rice. Experiments with other *Anabaena*-free *Azolla* accessions and other restriction enzymes (*Pvu*I) confirmed the first results (data not shown).

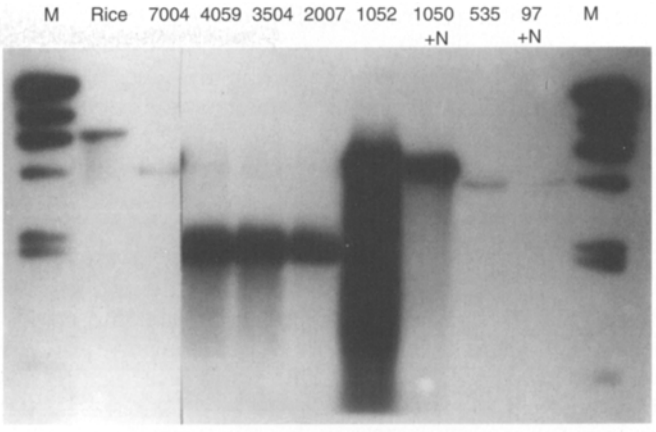


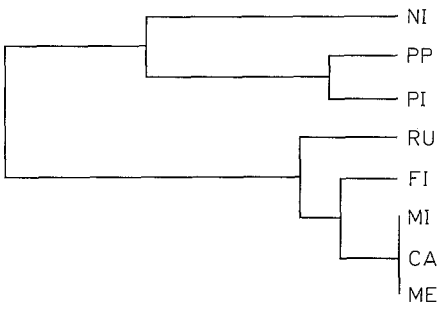
Fig. 4 RFLPs detected with chloroplastic probe AC-1 in *Azolla-Anabaena* DNA complexes digested with *Eco*RI. The AC-1 fragment was isolated from PCR amplification of *Azolla* total DNA with forward and reverse primers built from a rice chloroplastic sequence. *M* molecular weight marker Lamba phage digested with *Hin*dIII, from *top* to *bottom* (kb): 23.1, 9.6, 6.6, 4.4, 2.3, 2.0 and 0.5

Polymorphisms were detected between accessions from the *Azolla* and *Rhizosperma* sections, but also between accessions from *A. pinnata* and *A. nilotica* species (data not shown). In the *Azolla* section, no polymorphism with either *Hin*dIII or *Eco*RI was found among accessions from the CA-ME-MI species. However, when DNAs were digested with *Hin*dIII, a polymorphism was detected between accessions from the two *A. pinnata* varieties, PP 7004 and PI 535.

Statistical analysis and phylogeny reconstruction

Genetic distances calculated from similarity coefficients were used to build a dendrogram based on a UPGMA phylogeny reconstruction (cophenetic correlation coefficient: 0.978), which assumes a molecular clock model of evolution (Fig. 5). It revealed a clustering similar to

Fig. 5 Dendrogram based on Nei's genetic distances demonstrating relationships among *A. azollae* strains from all known *Azolla* species; each species being composed of all their analyzed accessions listed in Table 1. NTSYS-pc software was used to calculate genetic distances based on RFLP data obtained with three *nif* probes and to build a dendrogram according to the unweighted pair group method with arithmetic mean (UPGMA). See Table 1 for abbreviations of *Azolla* species



the one obtained from published RAPD data (Van Coppenolle et al. 1993).

A NJ method based on the same distances' data but without the assumption of a molecular clock evolutionary theory, was also used to build a dendrogram. This one showed an almost identical clustering (Fig. 6). The cophenetic correlation coefficient calculated in this case was 0.895.

Parsimony analysis (PA) based directly on the RFLP data as character data, without the assumption of a molecular clock evolutionary model, gave a mid-rooted cladogram that revealed an identical clustering to the one obtained with both UPGMA and NJ (Fig. 7).

Principal component analysis based on the RFLP distance data revealed, in a scatter-plot of the two first principal components, the presence of three groups observed earlier with RAPD results from the *Azolla-Anabaena* DNA complexes (Fig. 8). Group 1 contained strains from the *Azolla* section species; group 2 contained strains from the *A. pinnata* species and group 3 contained strains from the *A. nilotica* species.

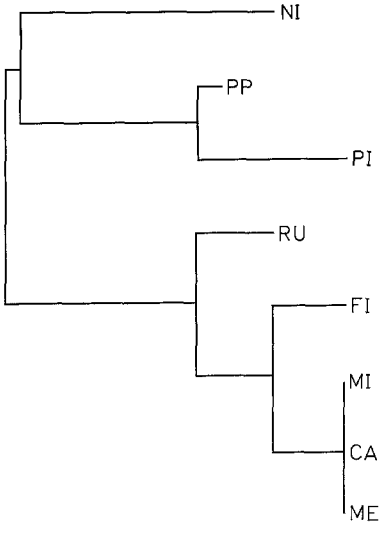


Fig. 6 Dendogram based on Nei's genetic distances among *A. azollae* strains from all *Azolla* species built according to the neighbor joining (NJ) method; each species being composed of all their analyzed accessions listed in Table 1. The Phylip software package was used to generate a dendrogram based on the same genetic distances that were used to build a UPGMA dendrogram in Fig. 2. See Table 1 for abbreviations of *Azolla* species

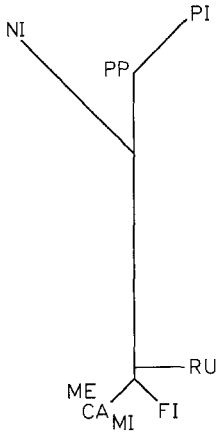


Fig. 7 Most parsimonious phylogenetic tree built from RFLP data obtained with three *nif* gene probes on *A. azollae* from all known *Azolla* species; each species being composed of all their analyzed accessions listed in Table 1. Parsimony analysis was done with a PAUP version 3.0 software to generate a mid-rooted cladogram representing phylogenetic relationships among *A. azollae*. See Table 1 for abbreviations of *Azolla* species

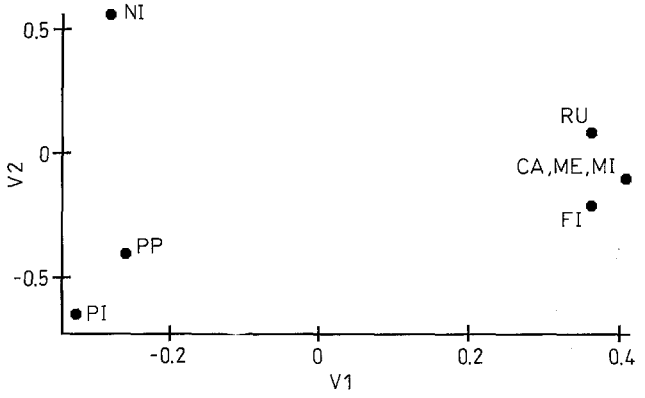


Fig. 8 Scatter plot of the first two axes analyzed by principal component analysis (PCA) using Nei's genetic distances for *A. azollae* strains from all known *Azolla* species; each species being composed of all their analysed accessions listed in Table 1. Genetic distances were calculated from RFLP data (using the NTSYS-pc software) using three *nif* gene probes. Data Desk was used to perform PCA based on the genetic distances. The first two axes represent 97.4% of the total variance. See Table 1 for abbreviations of *Azolla* species

Discussion

The presence of prokaryotic DNA in the total DNA extracted from symbiotic *Azolla* plants was confirmed by positive hybridization signals obtained with *nif* genes used as RFLP probes. The absence of any signal with *Anabaena*-free plants reinforces the observation. The absence of cyanobiont in these plants was confirmed earlier by light microscopy analysis of young fronds. An interesting observation was made in total DNA of *Anabaena*-free accession FI 1032 + N when a positive hybridization signal appeared with all *nif* gene probes. It confirmed the unexpected presence of prokaryotic DNA (result not shown). A microscope observation of the accession revealed the presence of *A. azollae* cells, but relative to that of other *filiculoides* accessions, there were undoubtedly fewer *Anabaena* cells. The low amount of cyanobionts observed per frond in 1032 + N, roughly estimated to be as low as one-third or one-quarter of the original symbiotic association, probably indicates that the artificial creation of the symbiont-free accession (the same method as for accession FI 1050) was not totally successful and that the two partners of the former symbiosis were able to maintain an incomplete association. Further investigations are needed to fully characterize this interesting accession, but the example serves to demonstrate the usefulness of our approach.

According to the literature, controversies about the *in vitro* culturing of cyanobacterial symbionts from *Azolla* plants exist. Several authors have described their attempts and methods used to extract and cultivate

isolates (Bai Ke-Zhi et al. 1979; Berliner and Fisher 1987; Rosen et al. 1987; Caudales et al. 1988; and see Gebhardt and Nierzwicki-Bauer 1991 for a complementary review). Newton and Herman (1979) described an isolation procedure based on the enzymatic digestion of plant material, which lead to an in vitro culture of isolates. They claimed to have found a method allowing *Azolla* symbionts to be cultured artificially, but many researchers were unable to confirm the identity of their isolates (Gates et al. 1980; Franche and Cohen-Bazire 1987; Meeks et al. 1988; Ladha and Watanabe 1982). Moreover, the necessity of undertaking a massive digestion of plant material in order to culture a single strain of cyanobacteria reinforces the suggestion that most of the symbionts living in leaf cavities are not able to grow on artificial medium. Zimmerman et al. (1989c) later compared morphologies, lectin binding and zymograms of cultured isolates obtained from different laboratories and found that the origin of cultured symbionts was questionable and that no assurance could be given that they were indeed true isolates. They also found the isolates to belong either to *Nostoc* or to *Anabaena* genera and that a classification of these isolaltes did not follow the current taxonomy framework of their *Azolla* hosts. The implications of these observations are that at the present time the symbionts living in *Azolla* leaf cavities should be studied without a culturing stage, which would tend to select mutants or minor symbionts that are not necessarily representative of the whole cyanobacterial population. On another hand, Gebhardt and Nierzwicki-Bauer (1991) have suggested the possibility that several strains of cyanobacteria inhabit leaf cavities of *Azolla* plants. A review of the literature, however, uncovers very few reports confirming such a hypothesis. Plazinski et al. (1990) revealed the possibility of having more than one *Anabaena* strain in one *Azolla* leaf cavity, but Lin et al. (1989) suggested that in natural environment this would be very unlikely and that each *Azolla* species would more likely be inhabited by its own specific strain.

Our results did not allow us to confirm either hypothesis, as we only used a small number of probes that were restricted to a small portion of the cyanobacterial genome. However, by directly studying the symbionts without passing through an isolation step, we were able to survey the entire population of prokaryotic cells. Also, we did not find any polymorphism between strains from the CA-ME-MI *Azolla* species, suggesting either that there is only one cyanobacterial strain for the diversity of host plants or that, if there is more than one strain, their nitrogenase genes (at least what we surveyed) are perfectly conserved. Franche and Cohen-Bazire (1987) and Plazinski et al. (1988) obtained RFLP data on isolates using other *nif* gene probes and their inference of a phylogeny revealed a clustering of cyanobacteria, with one cluster associated with CA-ME-MI-FI plants and the other PI-PP plants. Our results are very consistent with theirs and go further in that we found an additional evolutionary line, i.e. the *Anabaena* strains from the *A. nilotica* species, and we clearly distinguish the cyanobacterial strains associated with CA-ME-MI plants from those associated with FI and RU plants. The former was suggested by Plazinski et al. (1990) in their RFLP experiments on isolates using genomic probes created from isolated symbionts. They reported that isolates obtained from each *Azolla* species could be identified, but they did not show any classification nor any phylogeny reconstruction. The fact that their probe came from an isolate explained the high frequency of signal that they observed. On the basis of overall observations, the existence of a single cyanobacterial strain inhabiting the CA-ME-MI *Azolla* species can be proposed and if the evolutionary history of the plant and symbionts is found to be parallel, then another scheme for *Azolla* taxonomy is required. Specific RFLP analysis of *Azolla* plant DNA will shed light on the issue (in preparation). Recently, Caudales et al. (1995) suggested a hypothesis of coevolution between the *Azolla* plant and its cyanobiont on the basis of a comparison of fatty acid analysis results obtained from freshly isolated cyanobionts with the molecular and physiological results of *Azolla* plants obtained by Zimmerman et al. (1991). The comparison is interesting, but the suggestion of coevolution does not fully agree with ours. A clustering of cyanobionts from seven *Azolla* species differs from the one we found, mainly in that the cyanobionts from RU strains cluster with a group containing those from NI, PI and PP straints. The fact that on one hand RU *Azolla* species have never been classified with those from the *Rhizosperma* section, i.e. NI, PI and PP *Azolla* species, and on another hand that FI and RU *Azolla* species have been classified together on the basis of morphology (Saunders and Fowler 1993), isozymes analysis (Zimmerman et al. 1989a, 1991), RFLPs (Plazinski et al. 1990; Zimmerman et al. 1989b) and RAPDs (Van Coppenolle et al. 1993) indicate that these fatty acid results would be unlikely to suggest a scenario of coevolution as far as all known species are concerned.

An interesting observation on strains from *A. pinnata* var 'pinnata' and strains from *A. pinnata* var '*imbricata*' confirms our results. A polymorphism was observed with probe/enzyme combination 1.9 *nif*/*Eco*RI, but two PP strains from the Philippines and Guinea-Bissau, respectively, showed hybridization signals corresponding to those of PI strains. However, the identical strain from the Philippines is recorded as PI collected from a mixed population in a germ plasm collection also maintained at the Catholic University of Louvain, Belgium. Our result confirms the *imbricata* origin and the non-ambiguous polymorphism demonstrates the homogeneity of the *Azolla* accession. There is less passport information about the origin of the accession from Guinea-Bissau, but based on our results we suggest a possible mislabelling. Further investigation should clarify this point.

Several studies on the leaf cavities of *Azolla* have reported the presence of bacteria other than *A. azollae* (called bactobionts) symbiotically associated with the

fern (Peters and Mayne 1974; Petro and Gates 1987; Carrapiço 1991). The presence of nitrogenases in only a subset of these bactobionts was recently revealed (Lindblad et al. 1991), but no information on coding genes is available. Similar, if not identical, bacteria were also reported in the leaf cavities of cyanobiont-free *Azolla* plants obtained by micromanipulation of female reproductive structures or megasporocarps (Nierzwicki-Bauer and Aulfinger 1990). Other studies indicate that *Anabaena*-free *Azolla* accessions obtained from treatment with antibiotics (i.e. accession 97 + N) still carry bacterial cells (Forni et al. 1991). Furthermore, the presence of bacterial cells reported in *Azolla* male reproductive structures or microsporocarps (Forni et al. 1990) indicate that they are likely to remain in *Anabaena*-free *Azolla* plants obtained by micromanipulation on the megasporocarps (i.e. accession 1050 + N). In our experiments, none of the true *Anabaena*-free accessions showed hybridization signals in all probe/enzyme combinations. This probably reflects the low amount of bacterial DNA in our extractions and Southern blots rather than a situation where no hybridization is occurring due to the total lack of bacterial DNA. As only a subset of bactobionts is reported to carry genes coding for nitrogenases, the amount of cyanobiont DNA in the case of symbiotic plants probably overshadows the amount of bacterial DNA that could be targeted with our *nif* probes. Therefore, we believe that our results reflect the analysis of *Anabaena azollae*. The presence of bacterial DNA in our *Azolla-Anabaena azollae* DNA complexes would be relatively easy to verify by DNA hybridization as long as specific probes from *Azolla* bactobionts are available, as suggested by Gebhardt and Nierzwicki-Baur (1991). Although *nif* gene sequences from those bacteria has not been reported until now, we can predict that the characterization of these nitrogenase genes will be very revealing when available. The difficulty and maybe the impossibility of isolating sole cyanobionts without any bacterial contaminant (Gebhardt and Nierzwicki-Bauer 1991; Caudales et al. 1992) reinforces our strategy of independently analyzing all partners of the symbiosis in total DNA extracted from a whole *Azolla* plant.

Phylogenetic relationship between *Azolla* symbionts were obtained using three different methods: parsimony, neighbour joining and UPGMA. Although the initial RFLP dataset analyzed was not very large, results of the tree topologies were consistent in each case. This indicates a good reliability of the estimates, as suggested by Kim (1993). Based on the use of *nif* genes, we found evidence for the elaboration of an *A. azollae* classification. However, we believe that more evidence is likely to be found as the whole genome is studied. The part we investigated is likely to be highly conserved because of its importance in the nitrogen fixation process and its regulation.

The existence of multiple-copy *nif* genes has been mentioned in the literature (Haselkorn 1986; Meeks et al. 1988). The fact that we detected a multiple-copy signal with probe 1.4 *nif* introduces a possible source of deviation in our phylogeny reconstruction since we assumed a relatively similar rate of divergence for all our RFLP markers. Duplicated genes are known to create discrepancies between the phylogenies of genes and organisms through paralogy (Young, 1992). However, when taken independently, all probes gave a tree topology or clustering which was identical to the one obtained when the results of all three probes were taken together (data not shown). This suggests that orthology is likely to explain the evolutionary behaviour of *nif* N genes in *A. azollae*, although gene duplication was detected. An interesting observation can be made at this point when comparing probes 1.4 *nif* and 207 *nif*. *Nif*N (probe 1.4 *nif*) and *nif*E (probe 207 *nif*) genes are known to cluster in several cyanobacterial free-living organisms, and their respective products are required in the biosynthesis of the FeMo-cofactor (Dean and Jacobson 1992). In this study we did not investigate the duplication of *nif*N in *A. azollae*, but further research could be undertaken to study the biosynthesis of the FeMo-cofactor in this organism.

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