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W.W. Zheng · M. Nilsson · B. Bergman
U. Rasmussen

Genetic diversity and classification of cyanobacteria in different *Azolla* species by the use of PCR fingerprinting

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Abstract Symbiotically associated cyanobacteria from 18 accessions within all known species in the genus *Azolla* were examined and classified by the use of polymerase chain reaction (PCR)-fingerprinting. A repetitive sequence specific for cyanobacteria, the short tandemly repeated repetitive (STRR) sequence, was used as a primer in the reaction. Cyanobacterial filaments isolated directly from the *Azolla* leaf cavity or contained within homogenised symbiotic *Azolla* tissue were used as templates. Based on the fingerprint pattern, distinct differences were demonstrated between cyanobacteria isolated from the *Euazolla* and *Rhizosperma* sections. In addition, individual fingerprints were obtained from all cyanobacteria isolated from the different *Azolla* species. The fingerprints were used to generate a phylogenetic tree. Three clusters were distinguished: one contained the four isolates from the section *Euazolla*, a second the isolate from *Azolla filiculoides*, and a third the three isolates from the section *Rhizosperma*. By the use of STRR-PCR fingerprinting, new data on the taxonomy of cyanobacteria in *Azolla* were obtained, which have been difficult to generate by other classification methods. PCR-fingerprinting may, therefore, be a valuable tool for diversity and classification studies of symbiotic cyanobateria from *Azolla* and, as co-evolution between the cyanobacteria and its corresponding host exists the method may also be useful for the taxonomy of *Azolla*.

Key words *Azolla* · Cyanobacteria-Symbiosis · Parsimony · PCR-fingerprinting · STRR

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W.W. Zheng · M. Nilsson · B. Bergman · U. Rasmussen (✉)
Department of Botany,
Stockholm University, S-10691, Stockholm, Sweden

W.W. Zheng
Biotechnology Center,
Fujian Academy of Agricultural Sciences,
Fuzhou, Fujian, 350003 P.R. China

Introduction

Cyanobacteria are prokaryotic phototrophs of great evolutionary importance, which possess an unprecedented capacity to enter into symbiotic relationships with the plant kingdom. These include plants from the divisions Bryophyta (mosses, liverworts and hornworts), Pteridophyta (aquatic ferns of the genus *Azolla*), gymnosperms of the family Cycadaceae and angiosperms of the family Gunneraceae (Rai 1990; Bergman et al. 1996). So far, the nitrogen-fixing *Azolla*-cyanobacterial symbiosis is the only one of economical importance to farming systems. The cyanobacteria, inhabiting a special cavity within the dorsal leaf lobe of *Azolla*, fix atmospheric N_2 and release the fixed nitrogen to the *Azolla* plant. In this way, the entire nitrogen requirement of *Azolla* is fulfilled. The symbiosis grows rapidly and can double its biomass in three days under optimal conditions. These unique properties have made it possible for rice production to replace part of the chemical nitrogen fertiliser with the *Azolla*-cyanobacteria symbiosis (Peters and Meeks 1989; Nierzwicki Bauer 1990; Liu and Zheng 1992). In addition, the symbiosis has for centuries been used as a green manure and as food for various animals, especially in China and Southeast Asia (Moore 1969; Lumpkin and Plucknett 1980; Peters and Meeks 1989). However, the widespread use of *Azolla* has been limited by environmental factors such as high temperature, insects and diseases (Lumpkin and Plucknett 1980). Furthermore, difficulties in classifying the *Azolla* associations and the symbiotic cyanobacteria (cyanobionts) have hindered a genetic improvement of the symbiosis. However, sexual crossing of *Azolla* plants and cross-inoculation of cyanobionts have proven to be appropriate methods to overcome these difficulties (Plazinski et al. 1988; Eskew et al. 1993).

The genus *Azolla*, established by Lamark in 1783, has been grouped into two sections, *Euazolla* and *Rhizosperma*, based on the structure of their megasporocarps, and is assumed to consist of seven species. The section *Euazolla* includes the following *Azolla* spe-

cies: *A. caroliniana* Willdenow, *A. filiculoides* Lamark, *A. mexicana* Presl *A. microphylla* Kaulfuss and *A. rubra* Brown, while the section *Rhizosperma* includes *A. nilotica* Decaisne and *A. pinnata* Brown. *A. pinnata* contains two subspecies, *A. pinnata pinnata* and *A. pinnata imbricata* (Lumpkin and Plucknett 1980; Perkins et al. 1985).

The symbiotic cyanobacteria associated with the seven *Azolla* species were earlier designated as a single species, *Anabaena azollae* Strasburger (Moore 1969; Lampkin and Plucknett 1980). However, the taxonomical status of *A. azollae* has been repeatedly questioned. It has been suggested that the cyanobionts may be more closely related to the genus *Nostoc* than to the genus *Anabaena*. So far, no strict evidence have been obtained, mainly due to difficulties in growing the isolated cyanobacteria on artificial media (Meeks et al. 1988; Plazinski et al. 1990).

In recent years, different techniques have been employed to identify and group the cyanobacteria forming symbiosis with the different *Azolla* species. The use of polyclonal fluorescent antibody staining, ELISA and quantitative immunobinding assays, has revealed a high degree of similarity between cyanobacteria freshly isolated from several *Azolla* species but distinct differences occurred to the cultured isolates (Gates et al. 1980; Ladha and Watanabe 1982; Arad et al. 1985; McCowen et al. 1987). When using monoclonal antibodies, it was proposed that at least four subgroups of cyanobionts were present within the different *Azolla* species tested (Liu et al. 1989). Furthermore, fatty acid profiles of the cyanobionts from all the known species of *Azolla* revealed differences between those collected from *Azolla* plants within the section *Euazolla* and those from the section *Rhizosperma*. The fatty acid profiles obtained from the cyanobionts of *A. rubra* led to the grouping of this species into the section *Rhizosperma* (Caudales et al. 1992, 1995).

Molecular genetic techniques, such as restriction fragment length polymorphism (RFLP), have been used for analysis of the cyanobionts from the different *Azolla* species. Franche and Cohen-Bazire (1987) demonstrated that cyanobionts from *Azolla* plants within *Euazolla* and *Rhizosperma* belong to two different evolutionary lines. Moreover, two subgroups of cyanobionts could be distinguished within *Euazolla*. One consisted of cyanobionts from *A. caroliniana* and *A. filiculoides*, and the other cyanobionts from *A. microphylla* and *A. mexicana* whereas the cyanobionts of *Rhizosperma* could not be subdivided. Plazinski et al. (1990) found that the cyanobionts from *A. caroliniana, A. mexicana* and *A. microphylla* were grouped together, but differed from the cyanobiont of *A. filiculoides*. Furthermore, van Coppenolle et al. (1995) concluded that the cyanobionts from the seven species of *Azolla* could be divided into three groups. Group 1 contained the cyanobionts from the section *Euazolla*, group 2 contained the cyanobiont from *A. pinnata*, and group 3 contained the cyanobiont from *A. nilotica*. A taxonomy of the cyanobionts, which is parallel to the *Azolla* taxonomy, has been proposed (van Coppenolle et al. 1993). However, there is little direct evidence for the dichotomous classification of cyanobacteria from the seven extant species of *Azolla*.

Recently, a polymerase chain reaction (PCR)-based fingerprinting method was developed for cyanobacteria using short tandemly repeated repetitive (STRR) sequences as primers (Rasmussen and Svenning 1998). The STRR sequences consist of tandemly amplified heptanucleotides and was first described by Mazel et al. (1990) in the cyanobacterium *Calothrix*. Subsequently, the repeated element has been found in a number of cyanobacteria, all belonging to heterocystous cyanobacteria (Jackman and Mulligan 1995; Rasmussen and Svenning 1998). The presence and highly conserved status of repetative sequences in the genome of microorganisms make them methodologically important tools for identification and diversity studies (de Bruijn 1992; Lapski and Weistock 1992; Jackman and Mulligan 1995; Rouhiainen et al. 1995; Rasmussen and Svenning 1998).

The STRR-PCR fingerprinting method was previously used to study the diversity of symbiotic cyanobacteria living in symbiosis with the angiosperm *Gunnera* (Rasmussen and Svenning 1998). Here we describe the use of the same STRR-PCR fingerprinting method for diversity studies and the characterisation of cyanobionts from extant *Azolla* species. PCR was performed on cyanobacterial filaments freshly picked from the *Azolla* leaf cavities as well as on homogenised symbiotic tissues. In addition, the generated PCR fingerprint patterns provided taxonomic information for the classification of cyanobacteria living in symbiosis with *Azolla*.

Materials and methods

Plant materials and culture conditions

Sixteen *Azolla* accessions were kindly provided by the National *Azolla* Research Center, Fujian Academy of Agricultural Sciences, Fuzhou, China, and the three *A. rubra* from Prof. C. van Hove, Catholic University of Louvain, Louvain-la-Neuve, Belgium. The accessions used, which represent all seven known *Azolla* species and one cyanobacteria-free *Azolla* accession (used as a control), are listed in Table 1. In addition, free-living cultures of *Anabaena azollae* (Newton's isolate), *Nostoc* PCC 7120, and two symbiotic isolates, *Nostoc* PCC 7422 (isolated from cycads) and *Nostoc* PCC 9229 (isolated from *Gunnera*), were also included. All *Azolla* accessions, except the cyanobacteria-free *Azolla*, were cultivated in a greenhouse at a temperature of 19–22°C, 85% humidity and a 15-h light and 9-h dark cycle. The light intensity was 57 µmol photons$\cdot m^{-2}\cdot s^{-1}$. The cyanobacteria-free *Azolla* was cultured under sterile conditions in liquid medium (Bai et al. 1979).

Isolation of intact cyanobacterial filaments

Two different procedures were used for the isolation of intact filaments: (1) using a fine needle the cyanobacterial filaments were picked directly from the *Azolla* leaf cavities under a Nicon MSZ-U stereomicroscope and the filaments were washed in sterile Milli Q water four times and kept at –20°C before use; (2) fresh *Azolla* fragments, consisting of 3–4 branches, were rinsed two-times in Milli Q water before being homogenised with a pestle in an Ep-

Table 1 *Azolla* accessions used

Azolla species/strain	Accession code	Collection origin	Source[b]
A. caroliniana	3001[a]	USA	NARC
A. caroliniana	3219	Brazil	NARC
A. caroliniana	3525	Rwanda	NARC
A. filiculoides	1001[a]	Germany	NARC
A. filiculoides	1010	Peru	NARC
A. filiculoides	1025	Colombia	NARC
A. mexicana	2002	Guyana	NARC
A. mexicana	2012[a]	Mexico	NARC
A. mexicana	2027	Colombia	NARC
A. microphylla	4018[a]	USA	NARC
A. microphylla	4060	Philippines	NARC
A. nilotica	5001[a]	Sudan	NARC
A. pinnata imbricata	436	Sri Lanca	NARC
A. pinnata imbricata	566[a]	China	NARC
A. pinnata pinnata	7001[a]	Australia	NARC
A. rubra	ADUL2	New Zealand	C.v.H.
A. rubra	ADUL163[a]	Australia	C.v.H.
A. rubra	ADUL200	New Zealand	C.v.H.
Anabaena-free *Azolla*		China	NARC
Anabaena azollae (Newton's isolate)			J.C.M.
Nostoc	PCC 7120		CNCM
Nostoc	PCC 7422	Sweden	CNCM
Nostoc	PCC 9229	New Zealand	CNCM

[a] *Azolla* accessions used in Figs. 1 and 2
[b] NARC- National *Azolla* Research Center, China
CNCM- Collection Nationale de Cultures de Microorganismes, Institute Pasteur, Paris, France
J.C.M.- Meeks, Department of Microbiology, University of California, Davis, USA
C.v.H.- van Hove, Department of Biology, Catholic University of Louvain, Louvain-1a-Neuve, Belgium

pendorf tube containing 500 µl of TE buffer and 2% PVP w/v (PVP-40, Sigma, MO, USA). The macerated material was centrifuged for 5 min at 10000 rpm and the pellet re-suspended in 500 µl of the TE/PVP buffer and centrifuged. This washing step was repeated four times. During centrifugation, the cyanobacteria were separated from the host tissue, forming a dark-green pellet. The pellet was used as a template in the subsequent PCR or stored at –20°C until used.

Oligonucleotide primer and PCR amplifications

The STRR oligonucleotide primer employed has the following sequence: 3′-CCTRACCCCTRACC-5′. The primer was synthesised by CyberGene AB, Sweden. All PCR reactions were carried out in a 25-µl volume containing 50 pmol of primer, 1.25 mM of deoxynucleotide triphosphate, 1 µl of cyanobacterial filaments (about 1000–2500 cells), and 1 U of DNA polymerase (DynaZyme™, Oy, Espoo, Finland). The buffer supplied with the enzyme was used according to the manufacturer's directions. For the cyanobacterial filaments prepared according to procedure (1) additional compounds were added: 3.75 µl of 10% BSA, 1 µl of 2.5% Tween 20 and 2 µl of 5 mM $MgCl_2$. The PCR cycles were: 1 cycle at 95°C for 6 min; 35 cycles at 94°C for 1 min, 56°C for 1 min, and 65°C for 5 min; 1 cycle at 65°C for 16 min and a final step at 4°C. The DNA amplification was performed in a DNA Thermal Cycler 480 (Perkin Elmer Cetus, Calif., USA). After the reaction was completed, 10 µl of amplified DNA was separated on 1.5% Meta Phor agarose (FMC BioProducts, ME., USA), stained with ethidium bromide and recorded using a Kodak DC120 digital camera and Kodak Digital Science 1D Image Analysis software. All PCR reactions were repeated at least four times.

Phylogenetic analysis

Eight cyanobacterial isolates, representing all extant species of *Azolla*, were included in the phylogenetic analysis. The *A. azollae* (Newton's strain), *Nostoc* PCC 7120, *Nostoc* PCC 7422, and *Nostoc* PCC 9229, were used as an outgroup. A total of 15 characters based on the generated fingerprint patterns were employed in the cladistic analysis using the PAUP (Phylogenetic Analysis using Parsimony) computer program (Swofford 1993).

Results

Cyanobacteria isolated from a total of 18 *Azolla* accessions, representing the seven *Azolla* species (see Table 1), were used in the STRR-PCR fingerprinting and a distinct banding pattern was obtained from all the isolates (see Figs. 1, 2). Two to three strains from each *Azolla* species, with the exception of *A. nilotica*, were included. The same fingerprint pattern was obtained from the individual strains within each species, representing different geographic origins (data not shown). The results are reproducible and, in addition, the same fingerprint pattern was obtained from cyanobacteria isolated from individual *Azolla* species over the time period from March to August 1998.

Diversity between cyanobacterial isolates from the sections *Euazolla* and *Rhizosperma*

The generated PCR fingerprint patterns, obtained from cyanobacterial filaments picked out directly from the *Azolla* leaf cavities, are shown in Fig. 1. In the following, the isolated cyanobacteria are referred to by the host *Azolla* from which they were collected. Our results demonstrated distinct differences between the cyanobionts from the sections *Euazolla* and *Rhizosperma*. Cyanobionts within the five *Azolla* species from the section *Euazolla: A. rubra*, *A. caroliniana*, *A. filiculoides*, *A. mexicana* and *A. microphylla*, were found to resemble one another due to the presence of two high-molecular-weight PCR products sized at 2100 and 1850 bp, respectively. Additionally, a cluster of four intensely stained bands sized at 1010, 915, 870, and 820 bp were characteristic for isolates from *Euazolla*. Furthermore, two

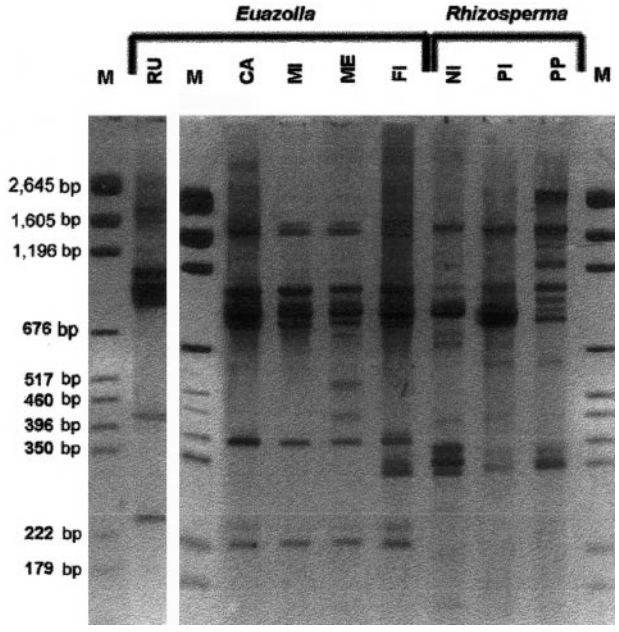

Fig. 1 PCR fingerprint patterns generated from cyanobacteria freshly isolated from the leaf cavities of different *Azolla* spezies (*RU- A. rubra*, CA- *A. caroliniana*, *MI- A. microphylla*, *ME- A. mexicana*, *FI- A. filiculoides*, *NI- A. nilotica*, *PI- A. pinnata imbricata*, *PP- A. pinnata pinnata*). *Lanes M* are DNA molecular-weight standards in bp

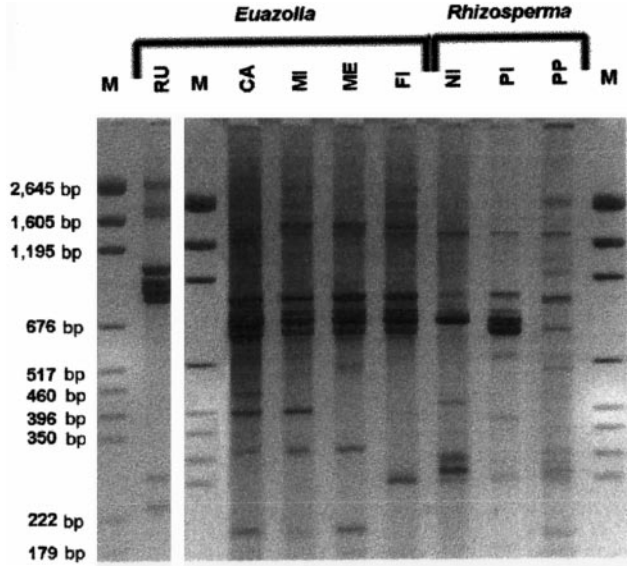

Fig. 2 PCR fingerprint patterns generated from homogenised symbiotic tissues of different *Azolla* species. (*RU- A. rubra*, *CA- A. caroliniana*, *MI- A. microphylla*, *ME- A. mexicana*, *FI- A. filiculoides*, *NI- A. nilotica*, *PI- A. pinnata imbricata*, *PP- A. pinnata pinnata*). *Lanes M* are DNA molecular-weight standards in bp

bands at 385 and 230 bp were found in all isolates. The cyanobionts from *Azolla* species within the section *Rhizosperma: A. nilotica*, *A. pinnata imbricata* and *A. pinnata pinnata*, had only one high-molecular-weight PCR product, sized at 1850 bp. Furthermore, the four clustered bands between 1010 and 820 bp showed pronounced differences in their intensity compared to the isolates from *Euazolla*. *A. nilotica* was identified by generating only one intense band at 870 bp; the bands at 1010 and 915 bp were faint and the band at 820 bp was missing. In *A. pinnata imbricata*, only two of the four bands showed a high intensity at 870 and 820 bp, respectively. The cyanobiont of *A. pinnata pinnata* was characterised by two intense bands at 1010 and 820 bp.

Diversity among the cyanobionts collected from the section *Euazolla*

Based on the banding patterns of the five cyanobacterial isolates, the *Euazolla* section was divided into four groups (Fig. 1). Group 1, contained the isolates from *A. caroliniana* and *A. microphylla*, which showed identical fingerprint patterns. Group 2, contained the cyanobionts from *A. rubra*, which were distinguished from the four other isolates in the section by one extra band at 2640 bp. Group 3, contained the cyanobionts from *A. mexicana* which showed two specific bands at 530 and 450 bp. Group 4, contained the isolate from *A. filiculoides* which generated two bands at 355 and 340 bp, distinct from the other representatives within the section *Euazolla*.

Diversity among the cyanobionts collected from the section *Rhizosperma*

The three cyanobacterial isolates within the *Rhizosperma* section generated individual and unique banding patterns (Fig. 1). The isolate from *A. nilotica* showed three specific bands at 385, 355 and 340 bp, while the isolate from *A. pinnata imbricata* was distinguished by missing the two bands at 385 and 340 bp. The isolates from *A. pinnata pinnata* showed two additional bands at 2640 and 1250 bp, but lacked the band at 340 bp.

Fingerprinting of the cyanobacterial filaments from homogenised symbiotic tissue

To simplify the procedure, STRR- PCR was performed on homogenised symbiotic tissue. As seen in Fig. 2, the generated fingerprint patterns from all the tested isolates were nearly identical to the banding pattern generated from using cyanobacterial filaments picked out directly from the leaf cavities (Fig. 1). The differences were an additional band at 520 bp present in *A. caroliniana* and *A. microphylla* and the lack of the band at 396 bp in *A. filiculoides.* To ensure that the fingerprint patterns obtained were of cyanobacterial origin, and not from the bacteria present in the leaf cavity or from the host, a cyanobacterial-free *Azolla* was included as a control. As seen in Fig. 3 (lane 1) no PCR products were obtained.

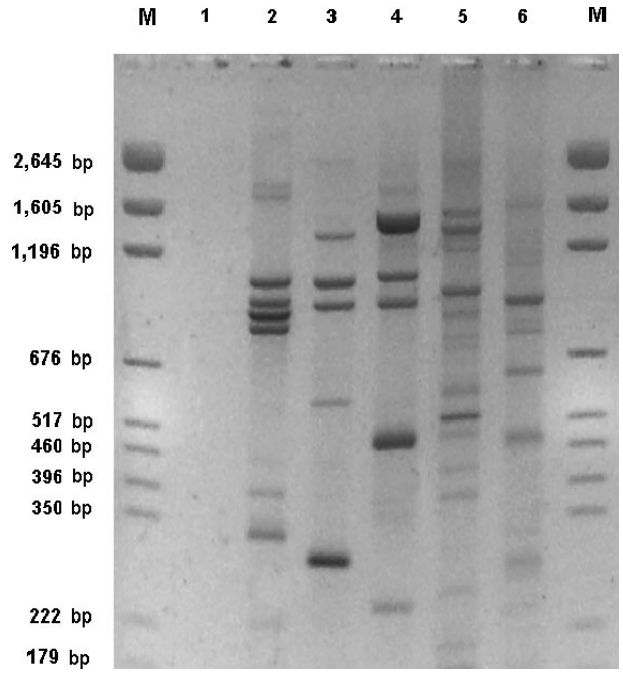

Fig. 3 PCR fingerprint patterns obtained from cyanobacterial-free *Azolla* (*lane 1*), cyanobacteria isolated from *A. filoculoides* (*lane 2*), *Anabaena azollae*, Newton's isolate (*lane 3*), *Nostoc* PCC 7120 (*lane 4*), *Nostoc* PCC 7422 (*lane 5*), and *Nostoc* PCC 9229 (*lane 6*). *Lanes M* are DNA molecular-weight standards in bp

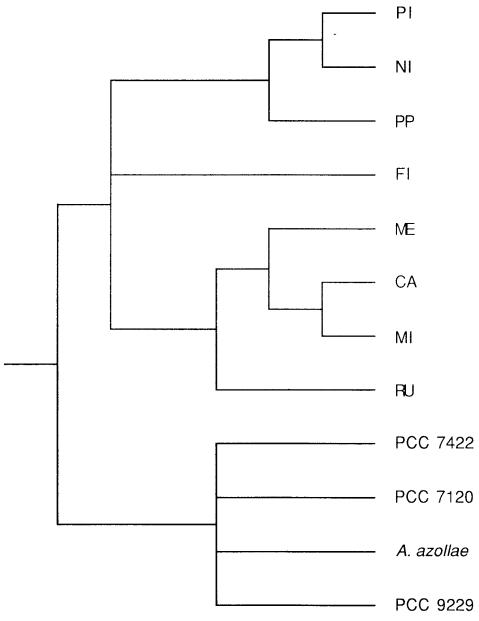

Fig. 4 Dendogram denerated with the PAUP computer program based on the PCR fingerprint patterns from cyanobacteria isolated from different *Azolla* species (*RU- A. rubra*, *CA- A. caroliniana*, *MI- A. microphylla*, *ME- A. mexicana*, *FI- A. filiculoides*, *NI- A. nilotica*, *PI- A. pinnata imbricata*, *PP- A. pinnata pinnata*) and the free-living cultures *Nostoc* PCC 7422, *Nostoc* PCC 7120, *A. azollae* (Newton's isolate), and *Nostoc* PCC 9229

Phylogeny reconstruction

A dendrogram (Fig. 4) was created based on the generated fingerprints of the individual cyanobacterial isolates. Four additional cyanobacteria, *A. azollae* (Newton's isolate), *Nostoc* PCC 7120, *Nostoc* PCC 7422 and *Nostoc* PCC 9229, were used as an outgroup (Fig. 3, lanes 3–6). Three clusters were obtained: one contained isolates collected from the *Rhizosperma* section, the second the isolate from *A. filiculoides*, and the third isolates from the section *Euazolla*. Within the section *Rhizosperma, A. pinnata imbricata* and *A. nilotica* were found to be more closely related to each other than to *A. pinnata pinnata*. Within section *Euazolla*, the isolates from *A. caroliniana*, *A. microphylla* and *A. mexicana* were separated from *A. rubra*. However, *A. caroliniana* and *A. microphylla* were found to be more closely related to each other than to *A. mexicana*.

Discussion

The cyanobacteria are intimately associated with their host plant, *Azolla* (Peters and Meeks 1989), such that continuity of the symbiosis has been maintained during the phylogenetic history of the *Azolla* plants. Each taxon of the extant *Azolla* cyanobionts may be considered as the result of an interaction between individual cyanobacterial strains and their corresponding *Azolla* host. Several attempts have been made to classify the cyanobacteria inhabiting different *Azolla* species by using serological methods, fatty acid profiling, RFLP analysis or PCR with arbitrary primers (Franche and Cohen-Bazire 1987; Plazinski et al. 1988, 1990; Eskew et al. 1993; Caudales et al. 1995; van Coppenolle et al. 1995). So far, the different techniques used have not distinguished more than four cyanobacterial groups. However, in this study we have demonstrated that the STRR-PCR fingerprinting method is suitable for the classification of cyanobacteria inhabiting the leaf cavity of all extant *Azolla* species. Furthermore, the method was shown to be applicable directly on homogenised symbiotic tissue which provides a simple and efficient method for the analysis of larger collections of closely related, or widely divergent, *Azolla* species. Specific fingerprint patterns were obtained from the cyanobionts associated with the different *Azolla* species and these data provided taxonomic information for the classification of the cyanobionts.

All the cyanobacterial isolates from the *Euazolla* section were characterised by a specific banding pattern which was distinct from that of the *Rhizosperma* section. The clustering of the cyanobacteria into two sections was consistent with results obtained previously (Franche and Cohen-Bazire 1987; Caudales et al. 1995; van Coppenolle et al. 1995). In addition, the presence of identical-sized fragments (the four clustered bands) in cyanobacteria isolated from all species of *Azolla*, indicates that both lineages once shared a common and original monophyletic unit. The isolates from the *Euazolla* section have apparently retained the whole tetramer of bands,

while cyanobacteria of *Azolla* from the *Rhizosperma* section have reduced the band intensities, or even lost bands during evolution.

Azolla from the section *Euazolla*, with the exception of *A. rubra*, are believed to be indigenous to the New World (Lumpkin and Plucknett 1980). As the isolates from *A. caroliniana* and *A. microphylla* show an identical fingerprint, we suggest that a single, or closely related, cyanobacterial strain(s) inhabit both host plants. The isolate from *A. mexicana* was distinguished by a specific banding pattern. This data is in contrast to results obtained by van Coppenolle et al. (1995), who grouped *A. mexicana* together with *A. caroliniana* and *A. microphylla*. The unique fingerprint of the cyanobiont from *A. mexicana* may, in respect of co-evolution, also reflect on the taxonomy of the *Azolla* plant and supports the validity of *A. mexicana* being a true species within the genus, as proposed by Perkins et al. (1985). The position of *A. rubra* in the *Azolla* taxonomy has been controversial for years, but has recently been recognised as a true species when analysing the perine architecture of its megasporocarp (Perkins et al. 1985) and by the use of zymograms (Zimmerman et al. 1989). The genetic diversity seen in our analyses, between the isolate from *A. rubra* and those from the other *Azolla* species from the New World, also suggests that *A. rubra* should be treated as a valid taxon at the species level.

Cyanobionts from *Azolla* in the section *Rhizosperma*, which are reported to be indigenous to the Old World (Lumpkin and Plucknett 1980), were in the present study grouped together in one evolutionary line. However, their corresponding hosts have a divergent geographical and ecological distribution with *A. nilotica* originating from Africa, *A. pinnata imbricata* from Asia and *A. pinnata pinnata* from Australia. The cyanobacterial isolates shared PCR products of equal mobility, but their overall fingerprints were quite distinct. In the generated phylogenetic tree, *A. nilotica* and *A. pinata imbricata* were grouped together. This unexpected result may be due to the low number of characters used to create the phylogenetic tree, or to the fact that only one sample of *A. nilotica* was included. Previously, *A. nilotica* has been separated from the other *Azolla* species due to a high degree of morphylogical and cytological differences between *A. nilotica* (2n=52) and the six other species of *Azolla* (2n=44) (Stergianou and Fowler 1990). Based on RFLP, Plazinski et al. (1990) concluded that the cyanobiont from *A. nilotica* was genetically divergent from those in both *Euazolla* and *Rhizosperma*. Saunders and Fowler (1993) also concluded, from an analysis of the morphology and cytology of *A. nilotica*, that it should be distinguished as a separate group. *A. pinnata imbricata* and *A. pinnata pinnata* were, due to the distinctiveness and consistency in their generated PCR banding patterns, recognised as valid taxons separated from those of other *Azolla* species. Their hosts have, however, in classic taxonomy been treated as subspecies of *A. pinnata* (Moore 1969; Lumpkin and Plucknett 1980; van Coppenolle 1995).

The cyanobacterial isolate from *A. filiculoides* shared bands with the isolates from both *Euazolla* and *Rhizosperma* sections. This led to a positioning of *A. filiculoides* between the two sections. Interestingly, according to Sculthorpe (1967), *A. filiculoides* was originally native in Europe but probably died out during the last Ice Age. In the 19th century, it was re-introduced into Western Europe and has also been widely distributed in Asia, Africa and Australia. Hence, the cyanobacterium associated with *A. filiculoides* may be a co-evolutionary resultant of the geographical, ecological and environmental evolutionary pressure experienced in both the Old and the New World.

It is interesting to note that the groupings of isolates recognised here are almost parallel to that of their corresponding host plants in the revised *Azolla* taxonomy tree produced by van Coppenolle et al. (1993), and thereby support the hypothesis of co-evolution between *Azolla* and its cyanobionts (Caudales et al. 1995).

In conclusion, the fingerprinting method used in this study has, for the first time, been able to classify all cyanobionts from the seven *Azolla* species. Distinct banding patterns were obtained from all cyanobionts and the subsequent classification follows the taxonomy of their host *Azolla* and supports the hypothesis of co-evolution between *Azolla* and its cyanobionts.

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