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Taxonomic status of *Anabaena azollae*: An overview

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Abstract

Despite the long-standing and widespread use of the symbiotic association between the aquatic fern Azolla and its cyanobacterial symbiont *Anabaena azollae* to augment nitrogen supplies in rice paddy soils, very little is known about taxonomic aspects of the symbiosis. The two partners normally remain associated throughout vegetative and reproductive development, limiting the opportunities for interchanges. We have used monoclonal antibodies and DNA/DNA hybridization techniques to show that the cyanobacterial partner is not uniform throughout the genus Azolla, and that substantial diversification has occurred. With these procedures it will be possible to characterize genotypes of the cyanobacterium and to monitor experiments aimed at synthesizing new combinations of *Azolla* species and *Anabaena azollae* strains.

Introduction

Azolla is a genus of heterosporous aquatic ferns that grow on the surfaces of fresh water ponds, lakes or streams. The genus was established by Lamarck in 1783 (Svenson, 1944) and its members are native to Asia (*e.g.*, China, Vietnam), Africa (*e.g.*, Senegal, Zaire, Sierra Leone), the Americas (*e.g.*, southern South America to Alaska) and the Antipodes. Individual species have been distributed by man and natural means to temperate as well as tropical and subtropical areas (Lumpkin and Plucknett, 1982).

Most taxonomic treatments recognize seven distinct species of Azolla on the basis of reproductive and morphological features. They are grouped into two sections (Lumpkin and Plucknett, 1982); section Euazolla (characterized by the presence of three floats on the megaspores) includes *A. caroliniana* (Willdenow, 1810), *A. filiculoides* (Lamarck, 1783), *A. mexicana* (Presl, 1845), *A. microphylla* (Karlfuss, 1824), *A. rubra* (R. Brown, 1810), and section Rhizosperma (possessing nine floats on the megaspores) includes *A. pinnata* (R. Brown, 1810) and *A. nilotica* De Caisne (Mettenius, 1867).

The most remarkable feature of Azolla is its symbiotic association with the cyanobacterium *Anabaena azollae*. The symbiosis can confer high rates of nitrogen fixation and biomass production, hence Azolla-Anabaena is an effective green manure for flooded crops and has been used as fertilizer in rice-growing for centuries in Vietnam and China.

Efforts are being made to improve the desirable agronomic attributes of this symbiotic association by creating host-symbiont combinations other than those available in natural populations. Under natural conditions particular partners remain associated throughout cycles of vegetative and reproductive growth because the same symbiont is transmitted to successive generations by filaments which are carried in the megasporocarp: there is, in effect, maternal inheritance of the Anabaena. Production of recombinant partnership involves breaking this strict association, and in order to be certain that a new cyanobacterial strain has been introduced it is essential to have rigorous methods of identification.

Several problems exist. The evidence that there are in fact different strains of *Anabaena azollae* is very limited. In the recent survey using polyclonal

antibodies the similarities among 32 isolates far outweighed the differences (Ladha and Watanabe, 1982). Isolates obtained from different fern species are morphologically similar (reviewed by Lumpkin and Plucknett, 1980), and all belong to section IV of Cyanobacteria, characterized by filamentous heterocystous trichomes with cells dividing in one plane (Rippka *et al.*, 1979). In *Anabaena azollae* the proportion of heterocysts varies from zero in homogonia found among glands and leaf primordia at the shoot apex of the host, up to 30–40% in those found in cavities of mature leaves, where the rate of nitrogen fixation is maximal. Finally, it is not possible to culture *Anabaena azollae* in the long term as a free-living form, so physiological properties are not readily determined. It follows that it is necessary to develop new techniques for rigorous characterization of *Anabaena azollae* strains.

In order to characterize *Anabaena azollae* strains, techniques including immunoassays and DNA/DNA hybridizations have recently been used (Arad *et al.*, 1985; Franche *et al.*, 1986; 1987; Franche and Cohen-Bazire, 1985; Gates *et al.*, 1980; Liu *et al.*, in press).

In this paper we present new data on the taxonomic status of *Anabaena azollae*, obtained by means of monoclonal antibodies and DNA hybridization, and show that there are indeed substantial differences between isolates from different species of *Azolla*, and that these differences can be related to the taxonomy of the host plants.

Use of cell antigens

Surface antigens of *Anabaena azollae* may be expected to play an active role in the establishment of cell-cell interactions as well as in the exchange of metabolites between Azolla and its phycobiont (Peters *et al.*, 1982). Mellor *et al.* (1981) have suggested that Azolla produces a lectin which recognizes surface determinants on the Anabaena. Conversely it has also been suggested that in the symbiosis an Anabaena lectin recognizes the host fern (Kobiler *et al.*, 1981). These speculations emphasize the potential importance of cell surface composition in the development of the symbiosis.

Qualitative differences were recently demonstrated in antigenic structures between fresh and cultured isolates of *Anabaena azollae* (Arad *et al.*, 1985; Gates *et al.*, 1980; Ladha and Watanabe, 1982). Despite some inconsistency, two conclusions could be drawn: (i) using polyclonal antibodies one can differentiate the *A. azollae* of Euazolla from that of Rhizosperma and (ii) laboratory cultured isolates of *A. azollae* have different antigenic properties from those isolated freshly from the fern.

Recently, in the National Azolla Research Centre (Fuzhou, People's Republic of China), 13 hybridoma cell lines secreting monoclonal antibody against *Anabaena azollae* have been established (Liu *et al.*, 1987). Among the 13 monoclonal antibodies (MAbs), eleven MAbs reacted with all *A. azollae* representing seven species of Azolla, indicating that they were detecting a common antigen. One MAb reacted with *A. azollae* from Euazolla but not with *A. azollae* from Rhizosperma (a subgroup-specific monoclonal antibody), whereas the other reacted with only *A. azollae* from *A. pinnata* (a species-specific MAb). None of the MAbs reacted with two free-living N_2-fixing cyanobacteria *Anabaena azotica* and Tolypothrix. The authors concluded that there are at least four subgroups of *A. azollae* in *Azolla* species. This is the first report showing that monoclonal antibodies could be used to discriminate *Anabaena azollae* strains.

Use of DNA/DNA hybridization technique

In order to characterize *Anabaena azollae* strains several laboratories have begun to use a technique of recombinant DNA research (Franche and Cohen-Bazire, 1985, 1987; Franche *et al.*, 1986; J. Meeks, personal communication).

In nitrogen-fixing prokaryotes, including *Anabaena azollae*, biological nitrogen fixation is catalysed by the nitrogenase enzyme complex. This complex contains two components: the nitrogenase (called MoFe protein) and the nitrogenase reductase (called Fe protein) (Mortenson and Thorneley, 1979). In the free-living *Anabaena* sp. PCC7120 these components are genetically determined by three genes; *nif* H, *nif* D and *nif* K (Mazur *et al.*, 1980; Rice *et al.*, 1982). Another *nif* gene (*nif* S), is clustered with the structural nitrogenase genes and is required for the maturation of the nitrogenase complex (Rice *et al.*, 1982).

In vegetative cells of *Anabaena* sp. PCC7120, *nif*

K is separated from *nif* DH by an 11 kilobase (kb) DNA fragment (Rice *et al.*, 1982). Golden *et al.* (1985) have recently demonstrated that this intervening region is excised and circularized during the differentiation of heterocysts, resulting in subsequent linking of the *nif* K and *nif* DH genes. Lammers *et al.* (1986) have shown that the 11 kb DNA element is excised by site-specific recombination between directly repeated 11 base pair (bp) sequences at each of its ends, and encodes a gene, *xis* A required for its own excision. A second rearrangement which leads to the excision of a circular DNA element from vegetative-cell DNA is also observed near *nif* S (Haselkorn, 1986).

Using DNA probes from the free-living *Anabaena* sp. PCC7120 Franche and Cohen-Bazire (1985) have previously reported that the restriction sites within *nif* K, *nif* D and *nif* H genes of four symbiotic *Anabaena azollae* (freshly isolated from four different *Euazolla* species), were strongly conserved. The same authors also compared the restriction sites in the region of the nitrogenase structural genes of five Rhizosperma endosymbionts to those of the four Euazolla (Franche and Cohen-Bazire, 1987). They presented evidence that symbiotic Anabaena strains derive from a common ancestral *Anabaena azollae* and belong to two slightly divergent evolutionary lines. However, from the taxonomic point of view the *nif* genes that were investigated were not satisfactory, since the nitrogenase structural genes have been strongly conserved during evolution, presumably because of stringent structural requirements in the protein (Hennecke *et al.*, 1985).

In our hybridization studies fifteen different Azolla isolates representing all known species have so far been used (Table 1), in conjunction with a wide variety of heterologous gene clones as the DNA probes. The probes fell into two groups; one including DNA fragments which contain nitrogenase genes isolated from free-living *Anabaena* sp. PCC7120, and the second containing non-nitrogenase genes including the rRNA genes and the ribulose biphosphate carboxylase genes from *Anacystis nidulans* (Table 2). The susceptibility of *Anabaena azollae* DNA to 25 different endonucleases was first determined and led to the selection of four restriction enzymes (*e.g.*, *Eco* RI, *Hin*dIII, *Bgl* II and *Kpn* I) which were routinely used.

The DNA hybridization patterns between *Anabaena azollae* isolates extracted from different *Azolla* species representing both sections Euazolla and Rhizosperma using single or combined *nif* probes from the free-living *Anabaena* sp. PCC7120 were compared. Most of the restriction sites within and around the *nif* H and *nif* S genes were different among the endosymbionts of the section Euazolla and Rhizosperma. Slight differences in the hybridization patterns were observed among *Anabaena azollae* isolates of the four species of Euazolla and among a few strains of *A. pinnata*. As

Table 1. Azolla species used for extracting cyanobacterial endosymbiont

Azolla species	Collection site	Origin or reference
Euazolla		
1. *A. caroliniana*	United States	Franche and Cohen-Bazire (1985)
2. *A. filiculoides* 2	United States	H.F. Diara
3. *A. filiculoides* (Shepparton)	Australia	W. Shaw
4. *A. filiculoides* (Snowy River)	Australia	W. Shaw
5. *A. filiculoides* (Canberra)	Australia	W. Shaw
6. *A. filiculoides*	East Germany	W. Shaw
7. *A. filiculoides*	China	C-C. Liu
8. *A. microphylla*	China	C-C. Liu
9. *A. microphylla*	Galapagos	Franche and Cohen-Bazire (1985)
10. *A. mexicana*	United States	Franche and Cohen-Bazire (1985)
Rhizosperma		
11. *A. pinnata* var. *pinnata* Sn	Africa	Franche and Cohen-Bazire (1987)
12. *A. pinnata* var. *imbricata* Ind	India	P. Reynaud
13. *A. pinnata* var. *imbricata* ImA	Africa	P. Reynaud
14. *A. pinnata* var. *imbricata* (Darwin)	Australia	W. Shaw
15. *A. pinnata* var. *imbricata* (Townsville)	Australia	W. Shaw

Table 2. DNA probes used for DNA/DNA hybridization study

Probe	Characteristics	Source/reference
Anabaena sp. PCC7120		
pAn154.3	1.8 kb *Hin* dIII fragment containing *nif* H	Rice *et al.* (1982)
pAn154.1	6.0 kb *Hin* dIII fragment containing *nif* S	Rice *et al.* (1982)
pAn207.3	1.8 kb *Hin* dIII subfragment of the 11 kb region being excised during heterotocyst formation	Rice *et al.* (1982)
Anacystis nidulans 6301		
pAn4	6.5 kb *Pst* I fragment of entire *rrn* A operon (16S, 23S and 5S rRNA genes)	Tomioka and Sugiura (1983)
pANP1155	2.3 kb *Pst* I fragment of the genes for the large (LS) and small (SS) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (*ruBisCo*)	Shinozaki and Sugiura (1985)

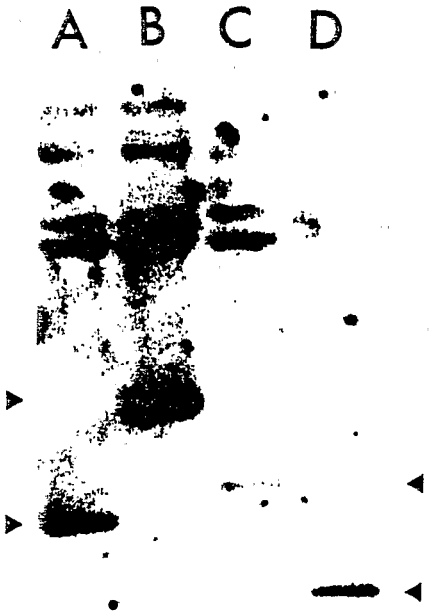

Fig. 1. Autoradiogram of ^{32}P-labelled RuBisCo probe hybridized to *A. azollae* DNA extracted from: (**A**) *A. filiculoides* (East Germany), (**B**) *A. filiculoides* (Shepparton), (**C**) *A. pinnata* var. *pinnata* and (**D**) *A. caroliniana*. The DNA was digested with the endonuclease *Eco* RI. *Arrows* indicate unique hybridizing bands that distinguish between *A. azollae* strains.

previously reported (Franche and Cohen-Bazire, 1987) no hybridization was found between DNA from the *A. azollae* symbionts and the probe pAn207.3, carrying a part of the 11 kb region that separates *nif* K from *nif* DH in vegetative cells of *Anabaena* sp. PCC7120. This observation is in agreement with a contiguous *nif* H, D, K organization in *A. azollae*, as suggested by mRNA studies (Nierzwicki-Bauer and Haselkorn, 1985) and by hybridization studies (J. Meeks, personal communication).

DNA polymorphism was observed among Anabaena endosymbionts when a 2.3 kb *Pst* I fragment of the RuBisCO gene cluster was used (Fig. 1). The use of rRNA cloned genes as hybridization probe also revealed polymorphism within these genes isolated from different symbiotic Anabaena (Fig. 2). Subclones of these genes, while used as DNA probes, were found to be very useful in differentiating amongst endosymbionts extracted from different isolates of the same *Azolla* species (*e.g.*, when isolated from different *A. pinnata* and *A. filiculoides* strains).

On the basis of our present data, combined with results presented by Franche and Cohen-Bazire (1985, 1987), we were able to draw a simple phylogenetic tree of symbiotic Anabaena strains (Fig. 3). At least nine different strains of *Anabaena azoll-*

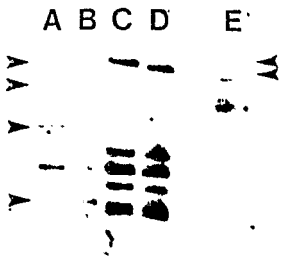

Fig. 2. Autoradiogram of ^{32}P-labelled rRNA probe hybridized to *A. azollae* DNA extracted from (**A**) *A. caroliniana*, (**B**) *A. pinnata* var. *pinnata*, (**C**) *A. filiculoides* (Shepparton), (**D**) *A. filiculoides* (East Germany) and (**E**) *Anabaena flos-aquae* (free-living algae). The DNA was digested with *Hin* dIII. *Arrows* indicate unique hybridizing bands.

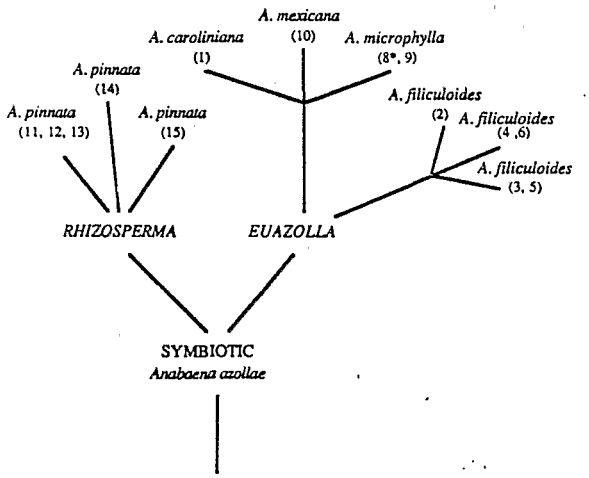

Fig. 3. Schematic presentation of the relationship between symbiotic Anabaena strains isolated from different Azolla isolates, as deduced from hybridization patterns. Strain numbers correspond to the numbers used in Table 1.
*According to the present hybridization studies strain number 8 (*Azolla microphylla*, China) seems to be similar to the strain no. 9 (see Table 1), however, more data is required before final conclusion can be drawn.

ae are associated with Azolla plants. Among five isolates of *A. filiculoides*, we were able to distinguish three different genotypes of *Anabaena azollae*; and among five isolates of *Azolla pinnata*, three different hybridization patterns were observed in the *Anabaena azollae* DNA digests. Interestingly, all the Australian Azolla strains so far studied appear to contain a symbiont which has phylogenetically diverged from the Azolla strains collected on the other continents.

Conclusions

In recent years molecular biology has provided important information in the taxonomic study of Azolla endosymbionts. At the present time it seems that Southern blot hybridization analysis using heterologous DNA probes gives finer discrimination with respect to the taxonomy of symbiotic Anabaena than do immunological analyses. However, the use of monoclonal antibody techniques to study surface antigens of *A. azollae* strains will also provide a powerful tool, alone or in combination with molecular techniques in future characterization of symbiotic Anabaena.

The current results distinguish at lest nine different strains of *Anabaena azollae* among symbiotic cyanobacteria associated with Azolla. The results are repeatable, and we have obtained reproducible blot patterns over several years. Most interestingly, using the 'fingerprinting' method one can now distinguish between Anabaena strains extracted from different biotypes of the same *Azolla filiculoides* species; several strains of *Anabaena azollae* were also identified in the species *A. pinnata*. The study of more intra-specific isolates of Azolla, in particular with *Azolla caroliniana*, *A. mexicana* and *A. microphylla*, together with the use of more test probes, will be necessary to confirm that the symbiotic cyanobacterium is represented by many different genotypes. The symbiotic relationship between Azolla and Anabaena could therefore be as host, and/or strain-specific as in the case of the Rhizobium-legume symbiosis.

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