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Evolutionary Divergence in the *nif*H.D.K. Gene Region Among Nine Symbiotic *Anabaena azollae* and Between *Anabaena azollae* and Some Free-Living Heterocystous Cyanobacteria

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Abstract

Using cloned *nif* DNA from the free-living *Anabaena* sp. PCC7120, we examined restriction sites in the *nif*K, *nif*D and *nif*H region of *Anabaena azollae* extracted from 9 *Azolla* species representing 4 *Euazolla* and 5 *Rhizosperma*. Most of the restriction sites in the *nif*K and *nif*D genes were conserved among the endosymbiotic members of both *Azolla* sections. The restriction fragments which hybridized to the *nif*H probe were identical in the 5 endosymbionts of *Rhizosperma*, but differed from those observed in the 4 *A. azollae* extracted from *Euazolla* species. From restriction site variation in the *nif*DH region, we established that all symbiotic *Anabaena* derive from a common ancestral *Anabaena azollae* and belong to two slightly divergent evolutionary lines. No hybridization was found between DNA from the endosymbionts and a probe carrying a 1.8 kb fragment of the region separating *nif*K and *nif*DH in vegetative cells of *Anabaena* sp. PCC7120, whereas the same probe hybridized to the DNA extracted from seven free-living *Anabaena-Nostoc* strains.

Keywords: *Azolla*, *Anabaena azollae*, *nif* genes, symbiotic cyanobacteria, phylogeny

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1. Introduction

The genus *Anabaena* comprises heterosporous aquatic ferns which live in symbiotic association with the nitrogen fixing cyanobacterium, *Anabaena azollae*. The symbiont is located in a distinct cavity at the base of the dorsal lobe of the leaves and develops in synchrony with the pteridophyte (Peters and Mayne, 1974; Lumpkin and Plucknett, 1980; Peters and Calvert, 1983). Due to the rate of nitrogen fixation in the *Anabaena-Azollae* symbiosis, which rivals that of the *Rhizobium*-legume symbiosis, *Azolla* is of particular interest as a fertilizer in rice paddy soils (Lumpkin and Plucknett, 1982).

The classification of the sporophyte is based on reproductive and morphological features of the ferns. Six extant species of *Azolla* are recognized and divided into 2 sections, *Euazolla* and *Rhizosperma* (Lumpkin and Plucknett, 1980; Lumpkin and Plucknett, 1982). The section *Euazolla* is characterized by the presence of 3 floats on the megaspores of the ferns and 4 species have been recognized: *A. caroliniana, A. filiculoides, A. mexicana* and *A. microphylla.* The 2 species which have been assigned to the *Rhizosperma, A. nilotica* and *A. pinnata* var. *pinnata* or var. *imbricata*, harbour 9 floats on the megaspores. However it should be pointed out that *Azolla* ferns are often sterile, and in addition morphological variations within a given species can occur. Consequently, the sporophytes are often difficult to classify.

The symbiotic cyanobacteria associated with the 6 species of *Azolla* have been classified as a single species, *Anabaena azollae* (Lumpkin and Plucknett, 1980), since freshly isolated from the ferns, they share the following common characteristics: the vegetative cells increase in size as leaf maturation occurs (Sweet and Hills, 1971; Hill, 1975; Peters et al., 1982), their surface antigens are closely related (Gates et al., 1980; Ladha and Watanabe, 1982; Ladha and Watanabe, 1984) and the frequency of heterocysts in the mature leaves of the ferns is high (Hill, 1975; Neumüller and Bergman, 1981; Peters et al., 1982). However, the difficulty encountered in growing the endosymbionts in culture in the absence of their hosts has made it impossible, so far, to ascertain these relationships even for *Anabaena azollae* within the same species of *Azolla.*

In all nitrogen-fixing prokaryotes so far examined, biological nitrogen fixation is catalysed by the nitrogenase complex. This enzyme complex is composed of 2 components: nitrogenase (also called MoFe protein) and nitrogenase reductase (also called Fe protein) (Mortenson and Thorneley, 1979). In *Klebsiella pneumoniae*, these components are genetically determined by 3 genes which are linked in the same operon; *nif*H, *nif*D and *nif*K coding for the nitrogenase reductase, the α subunit of the nitrogenase and the β

subunit of the nitrogenase, respectively (Riedel et al., 1979). The DNA sequences of these genes have been highly conserved among nitrogen-fixing organisms (Mazur et al., 1980; Ruvkun and Ausubel, 1980). This property has been used to identify and clone the nitrogenase structural genes from a number of free-living and symbiotic diazotrophs (for reviews, see Robson et al., 1983; Elmerich, 1984).

Molecular genetic analysis of nitrogen fixation in cyanobacteria has been initiated in the free-living *Anabaena* sp. PCC7120. Using DNA-DNA hybridization techniques, 4 *nif* genes have been identified: *nif*H, *nif*D, *nif*K and *nif*S, the latter gene being required in *Klebsiella pneumoniae* for the maturation of nitrogenase (Mazur et al., 1980; Rice et al., 1982). In the vegetative cells of *Anabaena* sp. PCC7120, the organization of these genes differs from that observed in the enterobacteria; *nif*K is separated from *nif*DH by about 11 kilobases (kb), and there is an extra copy of *nif*H (Rice et al., 1982). Golden et al. (1985) have demonstrated that the region separating *nif*K from *nif*DH in vegetative cells is excised and circularized during the maturation of heterocysts, *nif*K becoming adjacent to *nif*DH. A second rearrangement in the *nif*S region has also been observed (Haselkorn et al., 1985; Mulligan et al., 1985).

Nif gene organization has also been examined in some additional heterocystous cyanobacteria as well as in several non-heterocystous nitrogen-fixing cyanobacteria (Kallas et al., 1983; Kallas et al., 1985). All non-heterocystous cyanobacteria, including unicellular and filamentous forms, have a contiguous *nif*K,D,H cluster, whereas the heterocystous strains showed the separation of *nif*K from the contiguous *nif*DH genes in DNA from the vegetative cells.

Using probes from the free-living *Anabaena* sp. PCC7120, we have previously reported that the restriction sites within *nif*K, *nif*D and *nif*H genes of 4 symbiotic *Anabaena azollae* freshly isolated from 4 different *Euazolla* species were strongly conserved (Franche and Cohen-Bazire, 1985). In the present paper, we compare the restriction sites in the region of the nitrogenase structural genes of 5 *Rhizosperma* symbionts from different geographical origins, to those of the 4 *Euazolla*. We also tested for homology between DNA of the symbionts and a probe carrying part of the DNA region excised during heterocyst differentiation of *Anabaena* sp. PCC7120. The implications of our data for the phylogeny of the *Anabaena azollae* symbionts will be discussed.

Part of this work has been presented elsewhere (Cohen-Bazire and Franche, 1985).

Table 1. Free-living cyanobacterial strains and endosymbionts of *Azolla* species used for the hybridization studies

	Characteristics	Source or References
Cyanobacterial strains		
PCC7120	Free-living *Anabaena* sp.	(Rippka et al., 1979)
77S15, 74S26, 74S25, 74S24, 74S18	Free-living *Anabaena* sp. collected in Africa (Senegal)	(Franche and Reynaud, 1986)
79S05, 74S60	Free-living *Nostoc* sp. collected in Africa (Senegal)	(Franche and Reynaud, 1986)
Anabaena azollae var. *filiculoides*	Cultured isolate of *Anabaena azollae*	(Tel-Or et al., 1983)
Azolla species		
A. caroliniana	Collected in United States	(Franche and Cohen-Bazire, 1985)
A. filiculoides	Collected in South Africa	"
A. microphylla	Collected in Galapogos	"
A. mexicana	Collected in United States	"
A. pinnata var. *pinnata* Sn	Collected in Africa (Senegal)	P. Reynaud
A. pinnata var. *pinnata* SL2	Collected in Africa (Sierra Leone)	Diara, H.F. (UCL)
A. pinnata var. *imbricata* L	Collected in Australia	Diara, H.F. (UCL)
A. pinnata var. *imbricata* V	Collected in Thailand	Diara, H.F. (UCL)
A. pinnata var. *imbricata* Z	Collected in Africa (Zaire)	Diara, H.F. (UCL)

2. Materials and Methods

Cyanobacterial strains, Anabaena species and plasmids

Cyanobacterial strains and *Anabaena* species are listed in Table 1. Plasmids carrying *Anabaena* sp. PCC7120 *nif* genes are depicted in Fig. 1.

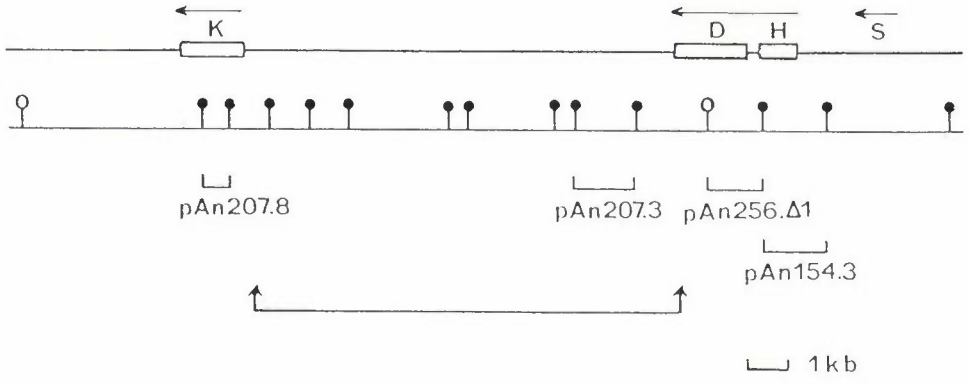

Figure 1. Physical map of the *nif* genes of *Anabaena* sp. PCC7120 according to Rice et al. (1982). Numbers below the map refer to the recombinant plasmids from which the probes used in this study were derived. Horizontal line with vertical arrows indicates the DNA region excised during heterocyst differentiation (Golden et al., 1985).
♀:*Eco*RI; ♀:*Hind*III

Media and growth conditions

Free-living cyanobacteria were grown at 30°C without shaking in BG-11 medium (Rippka et al., 1979). Cultures were illuminated with white light at an intensity of 1000 lux. Presence of contaminating bacteria was checked according to Rippka et al. (1979).

The growth of *Azolla* species and the extraction of the symbionts from the ferns were performed as previously reported (Franche and Cohen-Bazire, 1985).

Preparation of DNA

Plasmid DNA containing *nif* fragments of *Anabaena* sp. PCC7120, and chromosomal DNA of vegetative cells from symbiotic or free-living cyanobacteria were prepared as previously described (Franche and Cohen-Bazire, 1985).

Restriction endonuclease digestions and DNA electrophoresis

Restriction enzymes from Genofit were used as described by Maniatis et al. (1982). Restriction fragments were routinely separated in 0.7% (w/v) agarose horizontal gels using Tris-borate buffer (Maniatis et al., 1982). DNA fragments of small size (0.2 to 1 kb) were applied to 1.5% agarose gels to

allow a better resolution of the bands (Maniatis et al., 1982). Lambda phage DNA fragments were used as molecular weight standards.

Hybridization procedures

Plasmid DNA or fragments isolated by electroelution were labelled with (α ^{32}P)dCTP (400 Ci/mmol, Amersham International) by nick translation (Rigby et al., 1977). The specific activity of the labelled DNA was approximately 10^8 cpm/μg of DNA.

DNA digests separated by electrophoresis were transferred to nitrocellulose filters (Millipore HAWP, 0.45μm pore size) by the method of Southern (1975). Hybridizations with the heat denaturated probe were carried out at 65°or 60°C for 16 hr as previously reported (Franche and Cohen-Bazire, 1985), or at 42°C in 10% formamide as described by Sibold et al. (1985).

Estimation of sequence divergence

An estimation of DNA sequence divergence in and around the *nif*DH structural genes of the 9 symbiotic *Anabaena azollae* was obtained from the pairwise comparisons of hybridization patterns obtained with each strain, after digestion with *Hind*III, *Eco*RI or *Pvu*II and hybridization with pAn256.Δ1 (*nif*D) or pAn154.3(*nif*H) probe. In a manner similar to that described by Stanley et al. (1985) who examined the interrelationships of 10 *Rhizobium japonicum* strains, we employed the equation 6b established by Upholt (1977). This equation is applicable to DNA of moderate complexity ($1-100\times10^6$ daltons) in which the divergence of base substitution is defined as follows:

$$P = 1 - \left[-F + \sqrt{\frac{F^2 + 8F}{2}}\right]^{1/n}$$

where n is the number of nucleotides recognized by the endonuclease and F the fraction of conserved hybridizing fragments. Data from *Hind*III, *Eco*RI and *Pvu*II digestions, with *nif*D and *nif*H probes were additive and pooled.

3. Results

Comparison of restriction sites in the structural nif genes of symbionts from Rhizosperma and Euazolla

Although the cloning vector pBR322 carrying *nif*K,D or H genes of *Anabaena* PCC7120 was a suitable probe in hybridization studies with DNA of *Anabaena azollae* from *Euazolla* species (Franche and Cohen-Bazire, 1985),

the vector itself produced a strong hybridization signal with DNA of the symbionts extracted from the ferns of the *Rhizosperma* section. Therefore, all hybridization experiments with the *Rhizosperma* endosymbionts were carried out with *Anabaena* PCC7120 *nif* genes separated from the vector by hydrolysis with the appropriate restriction enzyme followed by gel electrophoresis and electroelution.

The DNA of all 5 endosymbionts of *Rhizosperma* (see Table 1) was digested with *Eco*RI, *Hind*III and *Pvu*II prior to hybridization with the *nif* probes. Three symbionts of *Rhizosperma* (*A. pinnata* var. *pinnata* SL2 and var. *imbricata* L and Z) and 2 of *Euazolla* (*A. caroliniana* and *A. mexicana*) were chosen for a more extensive analysis, their DNA being digested by 7 additional restriction enzymes (*Ava*I, *Ava*II, *Bgl*II, *Hae*II, *Hinc*II, *Hpa*II and *Pst*I).

Restriction sites identified by the nifK probe

The 0.7 kilobase (kb) *Hind*III fragment of pAn207.8 carrying part of the *nif*K gene of *Anabaena* PCC7120 (see Fig. 1) was used as the *nif*K probe. The respective *Eco*RI, *Hind*III and *Pvu*II restriction fragments of DNA from all 5 symbionts of *Rhizosperma* hybridizing to the *nif*K probe were of identical size (*Eco*RI:0.9 and 2 kb: *Hind*III:0.35 and 0.5 kb; *Pvu*II 25 kb). The two small fragments hybridizing to the *nif*K probe were only detected by using the following modification of the hybridization procedure: the DNA was concentrated 3-fold (to yield 5μg per slot) prior to hydrolysis with *Hind*III, electrophoresis was carried out in a 1.5% agarose gel and the labelled probe was of a higher specific activity (2×10^8 cpm/μg of DNA). The same experimental protocol permitted the detection of these small *Hind*III fragments in the DNA of the 4 *A. azollae* of *Euazolla* (Table 1), in contrast to previous results (Franche and Cohen-Bazire, 1985).

The hybridization patterns obtained with the DNA of the 3 symbionts of *Rhizosperma* and the 2 of *Euazolla* studied more extensively were also identical, the hybridizing fragments in kb being: *Ava*I,30; *Ava*II,2.6; *Bgl*II,20; *Hae*II, 4.9; *Hinc*II,0.95; *Hpa*II.1 and 1.37; *Pst*I,25. These results, together with those of Franche and Cohen-Bazire (1985), demonstrate a high degree of conservation in the *nif*K region of all endosymbionts examined, regardless of whether their hosts are assignable to *Rhizosperma* or *Euazolla*. Except for the *Eco*RI fragment of 2 kb in symbionts of *Rhizosperma* and 1 of 2.7 kb in those of *Euazolla*, no differences in the sizes of the respective hybridizing fragments could be detected with the restriction enzymes employed.

Restriction sites identified by the nifD probe

The 1.1 kb *Hind*III *Eco*RI fragment of pAn256.Δ1 (see Fig. 1) was used as the labelled *nif*D probe. The hybridization patterns of DNA from all 5 symbionts of *Rhizosperma* to the *nif*D probe were identical, the sizes of the hybridizing fragments in kb being: *Eco*RI,1.55; *Eco*RI/*Hind*III,1.1; *Hind*III,2.6; *Pvu*II, 26. They were also identical to those obtained by Franche and Cohen-Bazire (1985) in a hybridization study with the same probe and DNA of the 4 symbionts of *Euazolla* described in Table 1.

DNA of the 3 symbionts of *Rhizosperma* (*A. pinnata* var. *pinnata* SL2, and var. *imbricata* L and Z) and of the 2 of *Euazolla* (*A. caroliniana* and *A. mexicana*) digested with the 7 other restriction enzymes also gave identical hybridizing fragments with the exception of the *Hae*II digests. The restriction fragments of the latter that hybridized to the *nif*D probe were 17 kb for the 3 symbionts of *Rhizosperma* and 2.8 kb for the 2 of *Euazolla*. The sizes of the various hybridizing restriction fragments are given in Fig. 2a and 2b, which show the hybridization patterns for 1 representative endosymbiont of *Rhizosperma* (*A. pinnata* var. *imbricata* Z) and 1 of *Euazolla* (*A. mexicana*).

Restriction sites identified by the nifH probe

The 1.8 kb *Hind*III fragment of pAn154.3 carrying part of *nif*D (0.1 kb), the entirety of *nif*H (0.9 kb) and non-*nif* DNA (0.8 kb) (Fig. 1) was used as the labelled *nif*H probe.

The *Eco*RI and *Pvu*II restriction fragments hybridizing to the *nif*H probe were respectively identical for DNA of all 5 endosymbionts of *Rhizosperma* and corresponded in size to those presented in Table 2 for the 3 endosymbionts of this section studied in more detail.

DNA of the latter 3 symbionts also gave identical hybridization patterns after hydolysis with the other 7 restriction enzymes (Table 2) which were considerably different from those obtained with the DNA of the 2 symbionts of *Euazolla* (*A. caroliniana* and *A. mexicana*) which in fact showed slight differences among themselves for certain restriction endonucleases (see results for *Bgl*II, *Eco*RI, *Hae*II and *Pst*I, Table 2). This heterogeneity in the *nif*H region of the symbionts of *Euazolla* was already noted previously (Franche and Cohen-Bazire, 1985). Whereas the *Hind*III and the *Pvu*II restriction sites were identical in all 4 symbionts examined, the *Eco*RI sites were only conserved in *A. caroliniana* and *A. filiculoides* and differed from those observed in *A. microphylla* and *A. mexicana*.

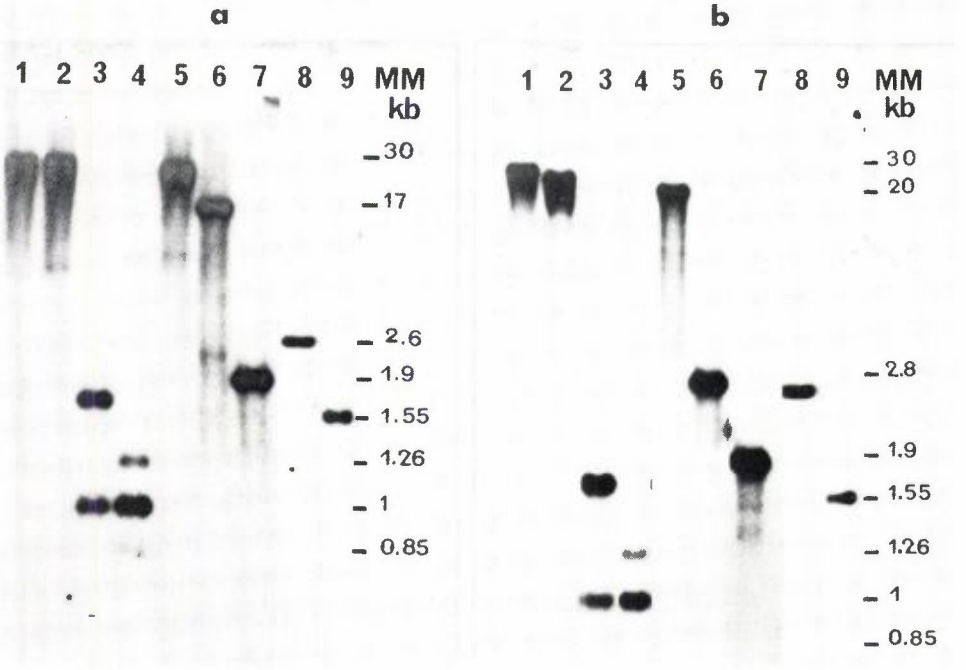

Figure 2. Autoradiogram obtained after hybridization of the ^{32}P-labelled *Anabaena* PCC7120 *nif*D probe to DNA extracted from the endosymbiotic *A. pinnata* var. *imbricata* Z (a) and *A. mexicana* (b). DNA was digested with *Ava*I (1), *Pst*I (2), *Ava*II (3), *Hinc*II (4), *Bgl*II (5), *Hae*II (6), *Hpa*II (7), *Hind*III (8) and *Eco*RI (9). Hybridizations were performed at 65°C as described in the methods.

Estimation of DNA sequence divergence in nifD-H region of endosymbiotic Anabaena azollae

The results presented in this communication and a comparison with previously published work (Franche and Cohen-Bazire, 1985) indicated clearly that certain restriction sites in the *nif*D-H region of the symbionts of *Rhizosperma* and those of *Euazolla* are conserved whereas others are not. Therefore, we decided to determine the percentage of DNA sequence divergence from the Upholt equation (1977) (for details see Methods), using the data obtained for all 9 symbionts for the restriction fragments of *Eco*RI, *Hind*III and *Pvu*II digests hybridized to the *nif*D and *nif*H probes. The results are presented in Table 3. When no common hybridizing fragment had been observed in the DNA of a given pair of symbionts compared in the analysis, the divergence was noted as being greater than the maximum calculable value. When all hybridizing fragments in the pairwise comparison were identical, the divergence was considered to be inferior to the minimum calculable value.

Table 2. Sizes of restriction endonuclease fragments of *A. azollae* DNA hybridizing to the *Anabaena* PCC7120 *nif*H probe

Restriction endonuclease	Rhizosperma section	Euazolla section	
	A. pinnata var. *pinnata* SL2 *A. pinnata* var *imbricata* L,Z	*A. mexicana*	*A. caroliniana*
*Ava*I	30	30-12	30-12
*Pst*I	30	26	26-12.5
*Ava*II	8-7.5-1.1	5.5-4-2.5-4.8-1.25-1.2	5.5-4-2.5-1.8-1.25-1.2
*Hinc*II	2-1.8-1.1	2-1.8-1.1	2-1.8-1.1
*Bgl*II	25	20	14-5.5
*Hae*II	12.5-11.5	24-10-2.45	24-10
*Hpa*II	2-1.8-1.3	3-2-1.19	3-2-1.19
*Hind*III	10-2.6	3-2.6-2.1	3-2.6-2.1
*Eco*RI	7.5-5	12.5-6.5-1.9	18-16-1.9
*Eco*RI/*Hind*III	5-1.6	2.1-1.6-1.4	2.1-1.6-1.4
*Pvu*II	12	26-24-12	26-24-12

The sizes of restriction endonuclease fragments are indicated in kilobases

Table 3. Matrix of percent DNA sequence divergence in the *nif*DH region of nine symbiotic *Anabaena azollae* and 2 free-living *Anabaena* strains

Strain	*A. azollae carolinana*	*A. azollae filiculoides*	*A. azollae mexicana*	*A. azollae microphylla*	*A. azollae pinnata* Sn	*A. azollae pinnata* SL2	*A. azollae pinnata* L	*A. azollae pinnata* V	*A. azollae pinnata* Z	PCC7120	Cultured *A. azollae filiculoides*
A. azollae carolinana											
A. azollae filiculoides	<0.48										
A. azollae mexicana	1	1									
A. azollae microphylla	1	1	0.48								
A. azollae pinnata Sn	5	5	5	5							
A. azollae pinnata SL2	5	5	5	5	<0.7						
A. azollae pinnata L	5	5	5	5	<0.7	<0.7					
A. azollae pinnata V	5	5	5	5	<0.7	<0.7	<0.7	<0.7			
A. azollae pinnata Z	5	5	5	5	<0.7	<0.7	<0.7	<0.7			
Anabaena PCC7120	12.7	12.7	12.7	12.7	11	11	11	11			
Cultured *A. azollae filiculoides*	>12.7	>12.7	>12.7	>12.7	>11	>11	>11	>11	>11	>11	

Values were calculated as described in the Methods using the combined hybridization data with *Anabaena* PCC7120 *nif*D and *nif*H probes and digests of *Eco*RI, *Hind*III and *Pvu*II.

The data in Table 3 clearly indicate that *A. azollae* extracted from *Euazolla* are different from, but related to, those of *Rhizosperma*, the DNA sequence divergence in the *nif*DH region between the 2 groups being about 5%. The divergence among *A. pinnata* var. *pinnata* and *A. pinnata* var. *imbricata* was found to be inferior to the minimum calculable value. A divergence ranging from less than 0.5% up to 1% was found among *A. azollae* within the *Euazolla* section.

The Upholt equation was also applied to obtain an estimate of the divergence between the *nif*DH region of the *Azolla* symbionts and that of the free-living *Anabaena* PCC7120 and *A. azollae* var. *filiculoides* of Tel-Or et al. (1983) using published data on the restriction sites (Rice et al., 1982; Franche and Cohen-Bazire, 1985). Since hybridization results performed with *Pvu*II digests were not available for the 2 free-living *Anabaena* strains, the DNA sequence divergence was calculated by comparing only the sites of the hybridizing *Eco*RI and *Hind*III fragments. A common *Hind*III fragment of 2.6 kb is found in the *nif*H region of *Anabaena* PCC7120 (Rice et al., 1982) and all of the *Azolla* symbionts of this study. Thus the DNA sequence divergence for this region is about 12%. As previously reported (Franche and Cohen-Bazire, 1985), there are no common *Eco*RI or *Hind*III sites in the *nif*DH region of the cultured *A. azollae* var. *filiculoides* and that of the *Euazolla* symbionts. The sequence divergence among these organisms is therefore greater than 12%, the maximum calculable value. On the basis of the results presented in Table 3, a dendogram for the relationships between the symbiotic *A. azollae* and the free-living *Anabaena* strains was constructed (see Fig. 3).

Hybridization with the Anabaena PCC7120 DNA carried on pAn207.3

The 1.8 kb *Hind*III fragment of pAn207.3 (coding for unknown functions) located on the 11 kb DNA segment of *Anabaena* PCC7120 which is excised during heterocyst differentiation (Rice et al., 1982; Golden et al., 1985) (see Fig. 1) was used as the labelled probe for hybridization studies with *Eco*RI and *Hind*III digests of the 5 symbionts of *Rhizosperma*, the 4 of *Euazolla* (Table 1) and, as a control, with DNA of *Anabaena* PCC7120 hydrolysed with the same restriction enzymes. The latter hybridized strongly with the probe (see Fig. 4a, lane 1). In contrast, DNA hybridization with this probe was not observed for any DNA of the *Azolla* symbionts, regardless of whether the hybridization was performed in an aqueous phase at 65°C, 60°C, 55°C,

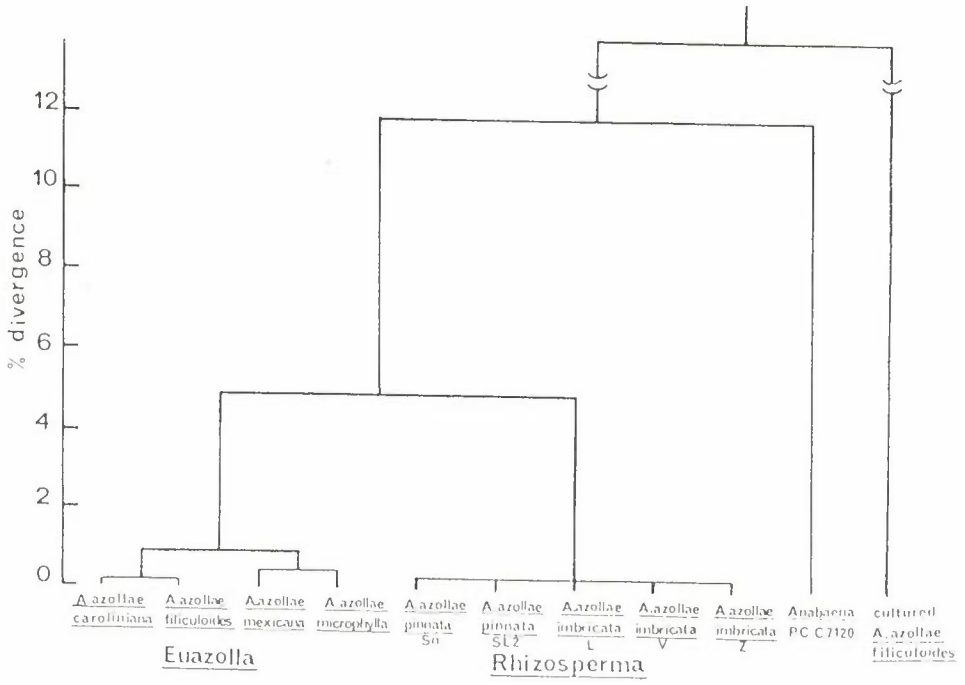

Figure 3. Dendogram representing the phylogenetic relationships between the symbiotic *Anabaena azollae* from the *Euazolla* and *Rhizosperma* sections. The dendogram was constructed from data presented in Table 2. The break line with the cultured isolate of *Anabaena azollae* var. *filiculoides* indicates that the divergence was too great to be calculated.

50°C or at 42°C in 10% (v) formamide with non-stringent washings (Sibold et al., 1985). The negative hybridization results under the latter conditions for 2 representative symbionts are shown in Fig. 4a, lanes 2 and 3. Hybridization was not observed even when the DNA of the symbionts was concentrated 3-fold with ethanol prior to digestion with *Eco*RI and *Hind*III, subjected to electrophoresis in 1.5% agarose gels and hybridized under non-stringent conditions to the same probe labelled to twice the specific activity ($2–10^8$ cpm/μg DNA).

The same type of experiment was also performed with DNA extracted from 7 free-living *Anabaena* or *Nostoc* strains (listed in Table 1), in order to examine whether the lack of hybridization observed in the symbionts reflects a divergence unique to the *A. azollae* or whether it is also characteristic for other heterocystous cyanobacteria. Hybridizing bands of a size range from 1.15–4.8 kb started to be recognizable in DNA of most of the free-

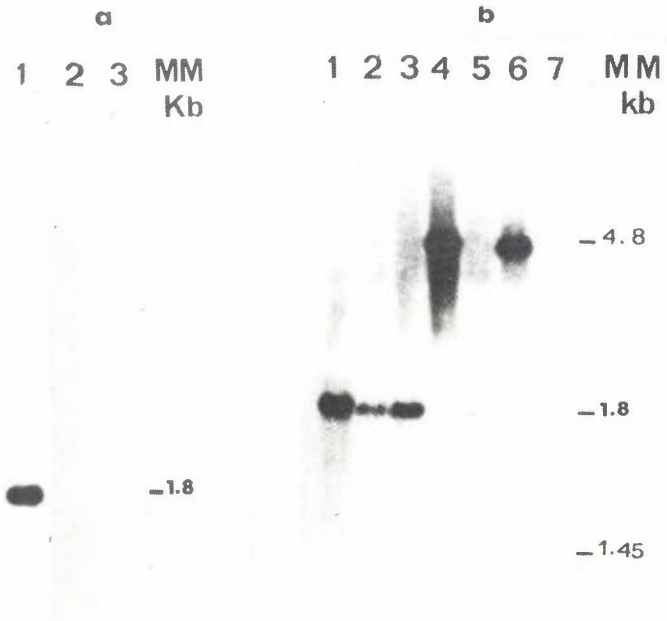

Figure 4a and b. Autoradiograms obtained after hybridization to the ^{32}P-labelled *Anabaena* PCC7120 pAn207.3 probe to DNA digested with *Hind*III. Figure 4a: lane 1, DNA was extracted from vegetative cells of *Anabaena* PCC7120; lane 2, DNA from *Anabaena azollae pinnata* var. *imbricata* Z; lane 3, DNA from the symbiont of *A. mexicana*. Figure 4b: DNA from free-living tropical *Anabaena/Nostoc* strains; lane 1, *Anabaena* 74S18, lane 2, *Anabaena* 74S24; lane 3, *Anabaena* 74S25; lane 4, *Anabaena* 74S26; lane 5, *Anabaena* 74S60, lane 6 *Nostoc* 79S05; lane 7, *Anabaena* 77S15.

living *Anabaena/Nostoc* strains after hybridization in an aqueous phase at 60°C and were clearly visible in 6 of the 7 strains examined and very faintly in *Anabaena* 77S15,when the hybridizations had been performed under the same conditions at 55°C (see Fig. 4b, lanes 1–7). Thus, the high degree of divergence in (or the complete absence of) the DNA sequence corresponding to the 1.8 kb *Hind*III probe of pAn207.3 seems to be a characteristic of the *Azolla* symbionts. However, as shown by the necessity to use less stringent conditions to reveal hybridization to this probe with DNA of the 7 *Anabaena/Nostoc* strains (in contrast to hybridizations performed with DNA of the same strains using *nif*K,D,H probes of *Anabaena* PCC7120, which were positive even at 65°C, unpublished results), it is clear that the corresponding DNA segment is also less conserved than the structural *nif* genes in the DNA of these cyanobacteria.

4. Discussion

Our results together with those of a previous study (Franche and Cohen-Bazire, 1985) demonstrate a high degree of conservation of the restriction sites in and around the *nif*K and D genes (including those identified by a large number of different restriction enzymes) in the 9 *Azolla* symbionts examined, whereas significant differences were observed in the *nif*H region.

In contrast, Hennecke et al. (1985) concluded from a comparative study on the DNA sequences of *nif*DH and K of different nitrogen-fixing organisms that the homologies in the *nif*H region were normally higher than those in *nif*K and *nif*D. This discrepancy can, however be explained: both the *nif*K and *nif*D probes used in our hybridization studies are small internal fragments of the respective *Anabaena* PCC7120 *nif* genes and are thus not representative of the entire gene. The *nif*H probe, however carried the entire *Anabaena* PCC7120 *nif* gene and some non-*nif* DNA; the latter may also have contained different restriction sites. Furthermore, a second copy of *nif*H, homologous but not identical with that adjacent to *nif*D (Haselkorn and Robinson, unpublished) has been described for *Anabaena* PCC7120 and has also been suggested to occur in *Azolla* symbionts (Franche and Cohen-Bazire, 1985). Its presence could obscure the interpretation of hybridization results, since without a more detailed analysis it is impossible to discriminate between the 2 *nif*H copies, one of which for example, could be more conserved than the other.

The dendogram of the phylogenetic relationships among the symbionts (Fig. 3), constructed on the DNA sequence divergence in the *nif*DH region, leads to the following conclusions. (1) Although symbionts from *Euazolla* and *Rhizosperma* belong to two different evolutionary lines, they probably arose from a common ancestral *Anabaena azollae*, since they show only limited DNA sequence divergence. (2) Within *Euazolla* two groups can be distinguished, one representing the seemingly identical *A. caroliniana* and *A. filiculoides* and the other by *A. microphylla* and *A. mexicana*. Although the latter species show some divergence among themselves, they are clearly different from the members of the first groups. (3) The symbionts of *Rhizosperma* could not be further subdivided. This probably shows that the sensitivity of the analysis based on restriction and hybridization patterns in only one region of the genome is not sufficient to distinguish between varieties of a given *Azolla* species.

It is clear that the proposed dendogram of the phylogenetic relationship among the symbionts needs to be be confirmed by additional studies, par-

ticularly since the nitrogenase structural genes have been strongly conserved during evolution due to the structural requirements of the proteins (Hennecke et al., 1985). However we are confident that our proposal may be valid since Hennecke et al. (1985) demonstrated that the divergence of the *nif* genes sequenced is consistent with the phylogeny of the corresponding nitrogen-fixing organisms established by the cataloguing of the 16s rRNA.

The divergence observed between *Euazolla* and *Rhizosperma* endosymbionts correlates remarkably well with the different geographical origin of the host ferns. Prior to the dispersal by man, *Azolla* species were endemic in the following areas (Lumpkin and Plucknett, 1980): *A. caroliniana*, in the eastern United States and the Carribean; *A. filiculoides*, in southern South America northwards into western North America including Alaska, *A. mexicana*, in northern South America through western North America; *A. microphylla*, in tropical and subtropical America; *A. pinnata*, in most of Asia and the coasts of tropical Africa. *A. pinnata* var. *imbricata* is found in India, Southeast Asia, the Philippines, China and Japan; *A. pinnata* var. *pinnata* is endemic to Australia, New Guinea, Africa and Madagascar (Sweet and Hills, 1971). From the study of fossilized *Azolla* it is assumed that the geographical separation of *A. pinnata* populations may have occurred as the result of glaciation about 3 million years ago during the pleistocene (Sweet and Hills, 1971).

Our *nif* hybridization studies did not reveal any differences between the endosymbionts of *Azolla caroliniana* and *Azolla filiculoides*. However, as pointed out by Lumpkin and Plucknett (1982), the hosts are sometimes difficult to distinguish since the immature fronds of *A. caroliniana* can be mistaken for those typical of *A. filiculoides*; only the mature plants seem to be sufficiently different: *A. caroliniana* forms multiple nearly horizontal layers whereas *A. filiculoides* grows nearly vertically above the water surface. The fronds of the hosts of *Anabaena azollae caroliniana* and *Anabaena azollae filiculoides* used for our studies were morphologically identical and, from the growth characteristics of the mature plants, both hosts could have been identified as *Azolla caroliniana*. Thus, we cannot exclude the possibility that the hybridization studies were carried out on two isolates of *Anabaena azollae caroliniana*. In contrast, the hosts of *Anabaena azollae pinnata* var. *pinnata* and *Anabaena azollae pinnata* var. *imbricata* are relatively easy to distinguish, the fronds of the former being pinnate in appearance and those

of the latter being more circular. Nevertheless, their symbionts seemed to be identical with respect to their *nif* regions.

The lack of, or the low degree of homology between the probe of pAn207.3 and DNA of the symbionts and the 7 free-living *Anabaena/Nostoc* strains might reflect a low degree of importance of the functions encoded by this DNA segment. In *Anabaena* PCC7120 the fragment corresponding to this probe is located on the 11 kb segment that separates *nif*K from *nif*DH in DNA of the vegetative cells of this organism which is excised in the course of heterocyst differentiation (Golden et al., 1985). Recently, a DNA fragment of 17 kb containing the 11 kb DNA segment of *Anabaena* PCC7120 has been cloned in pBR322, maintained in *E. coli*, and the excision process was examined using lac-fusion (Lammers et al., 1986). The results suggested that the DNA region located close to *nif*D and carried on pAn207.3 is not essential for the DNA rearrangement, in contrast to an open reading frame located next to *nif*K (coding for a 39 kilodalton protein). If excision is also obligatory for heterocyst differentiation in the *Azolla* symbionts, one could possibly expect a higher degree of conservation between the latter gene of *Anabaena* PCC7120 and the symbionts than for the probe of pAn207.3.

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