Plant Molecular Biology **21**: 363–373, 1993.

DNA amplification fingerprinting of the *Azolla-Anabaena* symbiosis

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Received 7 May 1992; accepted in revised form 2 October 1992

Key words: eukaryotic, arbitrary oligonucleotide primer, PCR, prokaryotic, silver staining, taxonomy

Abstract

The *Azolla-Anabaena* symbiosis has been used for centuries as a nitrogen biofertilizer in rice paddies. Genetic improvement of the symbiosis has been limited by the difficulty in identifying *Azolla-Anabaena* accessions and *Anabaena azollae* strains. The recently developed technique of DNA amplification fingerprinting (DAF) was applied to this problem. DAF uses single, short, oligonucleotide primers of arbitrary sequence to direct amplification of a characteristic set of DNA products by a thermostable DNA polymerase in a thermocycling reaction. The products are separated in polyacrylamide gels and detected by silver staining. DAF could easily distinguish and positively identify accessions of *Azolla-Anabaena* with DNA extracted from the intact symbioses. The contribution of prokaryotic *Anabaena* sequences to the fingerprint of the intact symbioses, however, ranged from 0 to 77%, depending on the primer sequence. Therefore, DNA extracted from the intact symbioses would not be suitable for *Azolla* taxonomy studies. The fingerprints of *Anabaena* strains isolated by sucrose gradient centrifugation from different species of *Azolla* could be easily distinguished, and DAF patterns were used to confirm the maternal pattern of transmission of *Anabaena* in a sexual hybrid. Template DNA extracted from roots was used to produce fingerprints for *Azolla* without interference from the microsymbiont. Comparison of the patterns from the parents and a hybrid gave strong evidence confirming sexual hybridization.

Introduction

The nitrogen-fixing *Azolla-Anabaena* symbiosis has been used since at least the 11th century as a source of nitrogen for rice production [18]. *Azolla* is a genus of heterosporous, aquatic ferns and *Anabaena azollae* Strasb. is a cyanobacterial microsymbiont found inside a cavity in the dorsal leaf lobe of *Azolla*. The symbiosis grows rapidly, and can double its biomass in less than three days in nitrogen-free nutrient solution [20]. The potential of the *Azolla-Anabaena* symbiosis to replace part of the chemical nitrogen fertilizer for rice production has been well documented [20, 21]. A number of environmental factors, however, limit its widespread use. Some *Azolla* accessions are sensitive to high or low temperatures, all are limited by low phosphorous availability, and are susceptible to insect pests [21]. To overcome these factors, wild *Azolla* plants with their associated symbiotic cyanobacteria are being collected and screened for desirable agronomic traits.

Sexual crossing of *Azolla* and cross-inoculation of microsymbionts has shown that it is possible to improve the performance of the symbiosis. Hybrids between *A. filiculoides* and *A. microphylla*

showed temperature tolerance intermediate between the parents and enhanced growth during the cool season [19]. Lin et al. [8] transferred the cyanobacterial microsymbiont from the high-temperature-tolerant species *A. microphylla* to the high-temperature-sensitive species *A. filiculoides*. This resulted in increased high-temperature tolerance in *A. filiculoides* [22]. This indicates that the agronomic value of the *Azolla-Anabaena* symbiosis can be improved by genetic manipulation of both the macrosymbiont and microsymbiont. These efforts have been hindered by difficulties in identifying *Azolla-Anabaena* accessions and strains of *Anabaena azollae*.

The taxonomy of both *Azolla* and *Anabaena azollae* is poorly defined. The taxonomy of *Azolla* is based primarily on the structure of the megasporocarp [6, 17]. The genus *Azolla* is divided into two sections, Azolla and Rhizosperma. In the section Azolla, the megasporocarps have three floats and the currently recognized species are *A. caroliniana* Willdenow, *A. filiculoides* Lamarck, *A. mexicana* Presl, *A. microphylla* Kaulfuss, and *A. rubra* R. Brown. In the section Rhizosperma the megasporocarps have nine floats and the recognized species are *A. pinnata* R. Brown and *A. nilotica* De Caisne [11].

Species classification within the section Azolla is based upon details of the megasporocarp structure, including scanning electron microscopy of the perine architecture [11]. Many accessions, however, do not develop sporocarps in culture collections, making their taxonomic assignment doubtful. Different studies on the megasporocarp structure have also failed to give a clear species classification within section Azolla [6, 17].

Results using isozyme and restriction fragment length polymorphism (RFLP) analysis have given more consistent results [27–30]. Isozyme analysis of 9 enzymes showing 17 polymorphic loci indicated that *A. filiculoides* and *A. rubra* were both biochemically unique and recognizable as species, but principal component analysis did not indicate that *A. microphylla*, *A. caroliniana*, and *A. mexicana* were distinct species [28]. Zimmerman et al. [29, 30] screened 30 heterologous DNA clones and a partial genetic library of *A. mexicana* to find DNA probes which would reveal RFLP differences. RFLP analysis confirmed the overall conclusions of the earlier isozyme analysis, and could also differentiate among accessions that were enzymatically closely related.

Only one species of *A. azollae* is currently recognized [12]. Taxonomic studies have been limited because it has not been possible to isolate and culture *A. azollae* [7]. It has only recently become possible to distinguish among strains of the cyanobacterial microsymbiont [14]. *A. azollae* remains associated with *Azolla* throughout vegetative and reproductive growth, and is passed between generations through the megasporocarp [12]. Plazinski *et al.* [13–16] developed DNA probes which could differentiate among several strains of *A. azollae* and proposed a tentative phylogenetic tree for *A. azollae*. The *A. azollae* strains isolated from *A. caroliniana*, *A. mexicana*, and *A. microphylla* were grouped together, but were separated from *A. filiculoides* strains. This parallels the relatedness of the macrosymbionts [29, 30].

A novel tool for DNA fingerprinting has recently been proposed [2, 24, 26]. The strategy is based on amplification of characteristic DNA fragments by a thermostable DNA polymerase directed by a single oligonucleotide primer of arbitrary sequence in a thermocycling reaction. DNA amplification fingerprinting (DAF) uses very short primers (≥ 5 nucleotides in length) to generate complex fingerprint patterns when amplification products are separated by polyacrylamide gel electrophoresis and detected by silver staining [1, 2]. DAF has been very useful for distinguishing among closely related genotypes of both eukaryotic and prokaryotic organisms [3]. We have established that DAF produces unique reproducible fingerprints for the intact *Azolla-Anabaena* symbiosis, and have defined the contribution of both the micro- and macrosymbiont to the fingerprints. Application of DAF for confirming sexual hybridization and maternal transmission of the *A. azollae* strain was also demonstrated.

Materials and methods

Azolla-Anabaena *cultures*

The *Azolla-Anabaena* symbiosis accessions used in this study are listed in Table 1. The *Anabaena azollae* strains used were freshly isolated from these accessions. The *A. azollae* strains are identified in the text by a two letter abbreviation of the *Azolla* species and the accession code from which they were isolated (Table 1). The terminal 0.5 mm of several fronds were excised and surface sterilized by the method of Peters and Mayne [10]. The sterilized tissue was transferred to sterile IRRI nutrient solution [20] supplemented with 5 mM KNO_3 and incubated for at least one month. Cultures where the nutrient solution remained clear were propagated in N-free nutrient solution and used for DNA extraction. *Azolla* plants were grown in plastic Petri dishes in a growth chamber at 25 °C with a photosynthetic photon flux density of 75 μmol m^{-2} s^{-1}, with a 12 h light period.

Table 1. List of *Azolla-Anabaena* accessions, isolated *Anabaena* strains, and primer sequences.

Azolla-Anabaena accession	Isolated *Anabaena* strains	Source[a]
A. caroliniana WT	*A. azollae* CA WT	G. Peters
A. caroliniana C_1	–	G. Peters
A. pinnata P_1	*A. azollae* PI P_1	G. Peters
A. filiculoides 301	*A. azollae* FI 301	NARC
A. microphylla 357	*A. azollae* MI 357	NARC
Hybrid Rong Ping C_4	*A. azollae* RP C_4	NARC
Primer code[b]	**Sequence (5′–3′)**	
7.7a	CGAGCTG	
8.6i	GTTACGCC	
8.6j	CCTGGAGG	
8.6l	GTAACCCC	
8.7b	GCTGGTGG	
8.7d	CCGAGCTG	
8.9a	CGCGGCCA	
10.6e	GTGACGTAGG	

[a] G. Peters, Virginia Commonwealth University, Richmond; NARC, National Azolla Research Center, Fuzhou, Fujian, People's Republic of China.

[b] The nomenclature for individual oligonucleotide primers gives the length, approximate G + C content, and an arbitrary letter assigned to a specific sequence. For example, 8.7b refers to an octamer, with 70–79% G + C.

DNA extraction

DNA was extracted from the intact *Azolla-Anabaena* symbiosis or from *Azolla* roots by the method of Dellaporta *et al.* [5]. *Anabaena* cells were isolated from *Azolla* leaves by the gentle roller technique and purified by sucrose step gradient centrifugation [10]. *Azolla-Anabaena* symbiosis plants were floated in a minimum volume of IRRI nutrient solution (10 ml) supplemented with 0.5 g of water insoluble polyvinylpyrrolidone. A sterile flask cap was used as a rolling pin to gently express the *Anabaena* cells into the solution. The suspension was filtered through 4 and then 8 layers of cheesecloth. The cells were spun down and resuspended in water before layering onto a sucrose step density gradient. The interface at the top of the 2.5 M sucrose layer was microscopically examined and found to be essentially free of contamination with plant cells or chloroplasts. The *Anabaena* cells were incubated with lysozyme (200 μg/ml) for 10 min at 37 °C and then Triton X-100 was added to 0.01% to lyse bacterial cells that are known to be coinhabitants of the *Azolla* leaf cavity [12]. The *Anabaena* cells were pelleted, washed, and resuspended in 500 μl DNA extraction buffer. The cells were then lysed by vortexing for 2–3 minutes with 0.1 g of glass chips. Subsequent steps followed the procedure of Dellaporta *et al.* [5]. Before amplification the template DNA was reprecipitated and dissolved in deionized water to remove EDTA which interferes with amplification.

DNA amplification

Amplification was done in a 25 μl reaction volume with 5 ng of template DNA, 0.25 μg of oligonucleotide primer, 0.2 units of a truncated DNA polymerase from *Thermus aquaticus* (Stoffel fragment, Perkin-Elmer/Cetus, Norwalk, CT)

in reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$ containing 200 μM of each dNTP). Primer sequences are given in Table 1. The reaction mix was overlaid with 2 drops of mineral oil and amplified in an Ericomp thermocycler (Ericomp, San Diego, CA) for 35 cycles (1 s at 96 °C and 1 s at 30 °C, with heating and cooling rates of 23 °C per minute and 14 °C per minute respectively). DNA concentration after the amplification reaction was typically in the range of 100–130 ng/μl. Approximately 30 ng DNA was loaded for electrophoresis. Amplification products were separated by electrophoresis in either 4.8% or 6% polyacrylamide with an acrylamide/piperazine diacrylamide ratio of 20:1. DNA products were detected by silver staining [1]. Initial template and amplified DNA concentrations were determined by fluorometry (Hoefer Scientific Instruments, San Francisco, CA).

Fingerprint interpretation

Amplification reactions were done at least in duplicate. Using optimized amplification conditions the generated fingerprints were reproducible between different amplifications and gel runs. Different DNA preparations from the same source produced identical fingerprint patterns.

All bands both of weak and strong intensity, within the well resolved portion of the gel, were scored and compared. In the 6% and 4.8% polyacrylamide gels only products of less than 500 nucleotides and 1000 nucleotides were considered, respectively. Positions of bands were measured from the bottom of the loading wells for band comparison.

Results

Intact symbioses

Initial experiments examined the possibility of using DAF to distinguish and identify accessions of the *Azolla-Anabaena* symbiosis in culture collections. DNA extracts containing both prokaryotic *Anabaena* DNA and eukaryotic *Azolla* DNA were prepared from intact symbioses because this was the easiest approach and most suitable for use in maintaining a large culture collection. Four primers; 8.7b, 8.7d, 8.9a, and 10.6e were used to direct amplification (Table 1). All four primers yielded fingerprints which easily distinguished among *A. caroliniana* WT, *A. microphylla* 357, and *A. pinnata* P_1 (data not shown). Primer 8.7b was selected for further study because it showed the greatest proportion of monomorphic bands, and primer 10.6e because it showed the greatest proportion of polymorphic bands.

Using primer 8.7b, 52% (12 out of 23) of the *A. microphylla* 357 bands (less than 500 nucleotides in length) were monomorphic with *A. caroliniana* WT bands (Fig. 1). In contrast, only 9% of the *A. microphylla* 357 bands were monomorphic with *A. pinnata* P_1. With primer 10.6e, again several *A. microphylla* 357 bands (26%) were monomorphic with *A. caroliniana*, but only 5% were monomorphic with *A. pinnata* P_1. This result was not surprising as *A. pinnata* belongs to

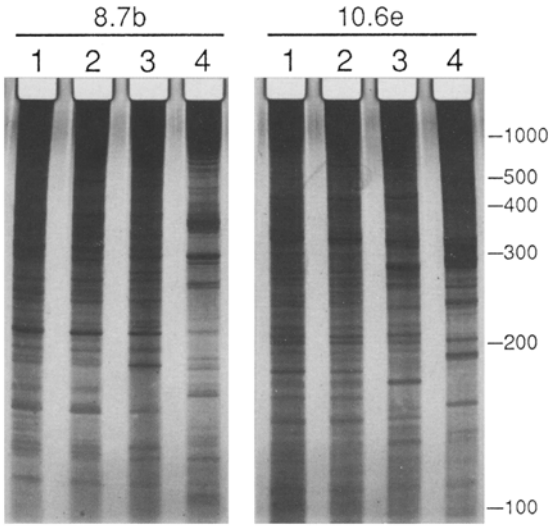

Fig. 1. Silver-stained polyacrylamide gel of DNA amplification products generated from intact *Azolla-Anabaena* symbiosis DNA templates with primer 8.7b or 10.6e. DNA reference size markers (nucleotides) are given on the right margin. Accession identification: 1, *A. caroliniana* WT; 2, *A. caroliniana* C_1; 3, *A. microphylla* 357; 4, *A. pinnata* P_1.

the Rhizosperma section of the *Azolla* genus and *A. microphylla* and *A. caroliniana* both belong in the Azolla section.

The fingerprints for *A. caroliniana* C_1 and *A. caroliniana* WT were 100% monomorphic with primer 8.7b and 96% monomorphic with primer 10.6e as only one polymorphic product (250 nucleotides in length) was seen. This was the expected result as these were duplicate cultures obtained from the same source. Culture *A. caroliniana* WT, however, had been obtained 8 years earlier, and had been used as the wild-type parent in a mutation induction project. This indicates that DAF fingerprints were reproducible and stable over relatively long periods of time.

Isolated Anabaena azollae

Unique fingerprints were obtained using DNA prepared from *A. azollae* freshly isolated from three different species of *Azolla* (Fig. 2). Out of 30 clear bands in the *A. azollae* MI 357 pattern generated with primer 8.7b, 27% appeared monomorphic with *A. azollae* FI 301 and 17% appeared monomorphic with *A. azollae* PI P_1. Similarly, 25% of the *A. azollae* MI 357 bands generated with primer 10.6e were monomorphic with *A. azollae* FI 301 and 17% with *A. azollae* PI P_1.

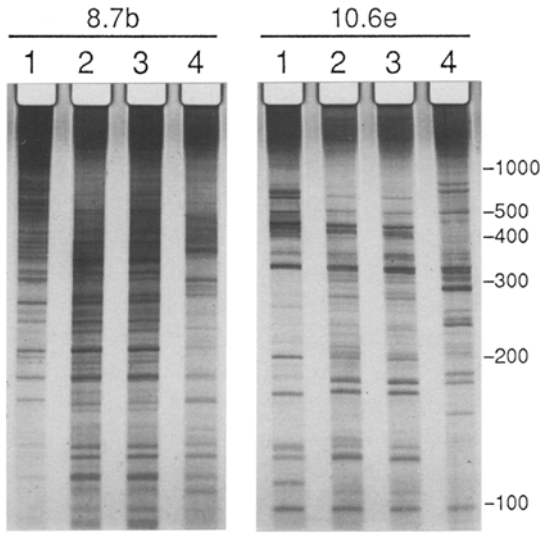

Fig. 2. DNA amplification products generated from isolated *Anabaena* DNA templates with primer 8.7b or 10.6e. DNA reference size markers (nucleotides) are given on the right margin. Strain identification: 1, *A. azollae* FI 301; 2, *A. azollae* RP C_4; 3, *A. azollae* MI 357; 4, *A. azollae* PI P_1.

Azolla-Anabaena accession Rong Ping C_4 is a sexual hybrid between *A. filiculoides* 301, and *A. microphylla* 357, maternal parent [23]. Maternal transmission of the microsymbiont is clearly illustrated by the identical fingerprints with primer 8.7b for the *Anabaena* isolates from Rong Ping C_4 and *A. microphylla* 357 (Fig. 2). The fingerprints for these two *Anabaena* isolates with primer 10.6e also showed a high proportion of monomorphic bands. However, three clear polymorphic products of 290, 240, and 220 nucleotides in length were detected.

There are several potential sources for these observed polymorphisms. Because primer 10.6e preferentially amplified *Azolla* DNA sequences (see below), it seems most likely that these polymorphisms represent minor contamination by *Azolla* DNA. Although unlikely, we cannot rule out that the observed polymorphic products could result from the contribution of DNA from bacteria or minor cyanobacterial co-symbionts which have been reported to be present in the leaf cavity [7, 12]. Precautions to minimize the presence of bacterial DNA were taken by using surface sterilized *Azolla* cultures, purification of *Anabaena* cells by sucrose step-gradient centrifugation, and lysozyme treatment of isolated *A. azollae* cells. Gerhardt and Nierzwicki-Bauer [7] could not detect the presence of minor cyanobacterial symbiont DNA in preparations from freshly isolated *A. azollae* cells using conventional DNA hybridization techniques, even with extensive exposure times during autoradiography. Thus it seems unlikely that minor cyanobacterial co-symbionts would make a large contribution to the fingerprints.

Contribution of Anabaena

When amplified with primer 8.7b, almost all of the bands from the intact symbiosis fingerprint for *A.*

microphylla 357 were monomorphic with bands in the *A. azollae* MI 357 pattern (compare lanes 3, 8.7b, of Fig. 1 and Fig. 2). Only two products of about 210 and 220 nucleotides in length were absent in the *A. azollae* MI 357 pattern. This indicates that *Anabaena* DNA sequences were heavily represented in the fingerprint of the intact symbiosis. In contrast, the fingerprints generated from these two templates with primer 10.6e were distinctly different (compare lanes 3, 10.6e, of Fig. 1 and Fig. 2).

To examine further the contribution of the *Anabaena* DNA to the fingerprint of the intact symbioses, DNA prepared from isolated *Anabaena*, intact symbioses, and the roots of *A. caroliniana* WT was amplified using primers 8.7b and 10.6e. All of the bands in the intact symbiosis pattern were monomorphic with either an *Anabaena* band or with an *Azolla* root band (Fig. 3). Although several of the *Anabaena* or *Azolla* products were not detected in the fingerprint of the intact symbiosis, there were no unique new bands. Thus the contribution of either *Anabaena* or *Azolla* to the fingerprint of the intact symbiosis could be expressed as a percentage of the intact symbiosis bands (Table 2).

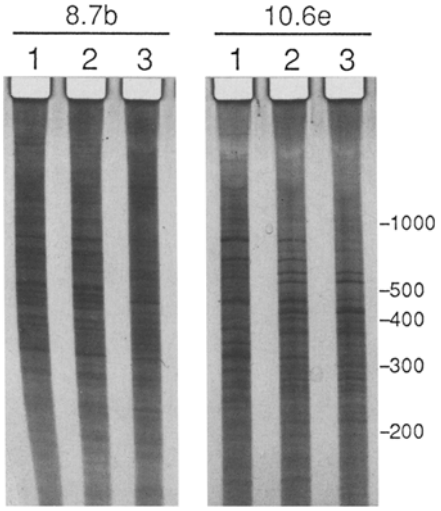

Fig. 3. DNA amplification products generated from DNA templates: 1, isolated *A. azollae* CA WT; 2, intact *A. caroliniana* WT symbiosis; 3, roots of *A. caroliniana* WT, with primer 8.7b or 10.6e. DNA reference size markers (nucleotides) are given on the right margin.

Table 2. Contribution of *Anabaena* and *Azolla* to the DNA amplification fingerprint of intact *Azolla-Anabaena* symbioses with oligonucleotide primers differing in length or sequence.

Intact symbiosis template	Primer	% monomorphic bands[a]	
		Anabaena	*Azolla*
A. caroliniana WT	8.7b	77	23
	10.6e	29	71
A. pinnata P_1	7.7a	0	100
	8.6i	59	41
	8.6l	50	50
	8.7j	36	64

[a] Proportion of bands in the fingerprint of the intact symbiosis that were monomorphic with bands in the fingerprints of the individual symbiosis components.

With primer 8.7b, *Anabaena* bands again dominated the fingerprint of the intact *A. caroliniana* WT symbiosis (Fig. 3, Table 2). In contrast, with primer 10.6e the intact *A. caroliniana* WT symbiosis DNA fingerprint was dominated by root DNA bands. Similar results were obtained with *A. pinnata* P_1 (data not shown). These results suggested that shorter primers would preferentially amplify DNA sequences from the prokaryotic microsymbiont.

To test this hypothesis, the three DNA templates from the *A. pinnata* P_1 symbiosis were amplified using three additional octamer primers and one heptamer primer. Among the four octamer primers tested, *Anabaena* products accounted for 36 to 77% of the bands in the intact symbiosis fingerprint (Fig. 3 and 4, Table 2). With the heptamer 7.7a, however, none of the intact symbiosis bands were monomorphic with an *Anabaena* band (Fig. 4, Table 2). Primer sequence was apparently more important than primer length for determining whether prokaryotic or eukaryotic bands were amplified from the DNA mixture.

Verification of sexual crosses

Fingerprints produced from root DNA showed that Rong Ping C_4 was a sexual hybrid between

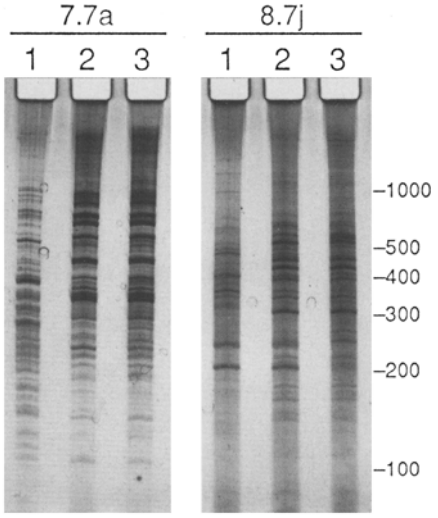

Fig. 4. DNA amplification products generated from DNA templates: 1, isolated *A. azollae* PI P1; 2, intact *A. pinnata* P1 symbiosis; and 3, roots of *A. pinnata* P1, with primer 7.7a or 8.7j. DNA reference size markers (nucleotides) are given on the right margin.

A. filiculoides 301 and *A. microphylla* 357 (Fig. 5). The fingerprint of Rong Ping C_4 was easily distinguishable from either of the individual parents. However, the contribution of each parent to the hybrid fingerprint was clearly observed. In the fingerprints generated with primer 8.7b, three bands corresponding to products of 310, 250, and 220 nucleotides in length were monomorphic between *A. filiculoides* 301 and *A. microphylla* 357. All three monomorphic bands were also present in the hybrid fingerprint. Five bands corresponding to products of 520, 480, 440, 420, and 170 nucleotides in length were clearly inherited from *A. filiculoides* 301, and two bands corresponding to products of 150 and 140 nucleotides in length were inherited from *A. microphylla* 357. There was also one clear example of an amplification product of about 370 nucleotides in length in the *A. microphylla* 357 fingerprint which was not inherited by Rong Ping C_4. This uninherited band probably represents a heterozygous chromosomal DNA sequence. Because *A. microphylla* 357 was the female parent in this cross, a chloroplast or mitochondrial sequence would almost certainly have been inherited in the hybrid progeny.

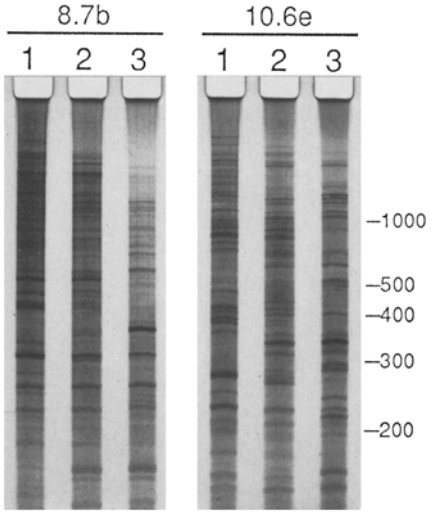

Fig. 5. DNA amplification products generated from DNA templates extracted from the roots of: 1, *A. filiculoides* 301; 2, hybrid Rong Ping C4; and 3, *A. microphylla* 357, with primer 8.7b or 10.6e. DNA reference size markers (nucleotides) are given on the right margin.

Examination of fingerprints produced with primer 10.6e gave similar results. Three amplification products of 200, 190, and 130 nucleotides in length were inherited from *A. filiculoides* 301 and five amplification products of 600, 360, 340, 320, and 220 nucleotides in length were inherited from *A. microphylla* 357.

Discussion

Maintenance of culture collections

A simple technique for positive identification of *Azolla-Anabaena* accessions would be a valuable tool for maintenance of large culture collections. Accessions must be maintained vegetatively, and with each transfer, the probability that a culture has been mislabeled increases. Also, it is desirable to eliminate duplications to control costs. DAF offers the possibility that DNA can be extracted from a new accession and stored for confirming its identity at a later time.

At the beginning of this work there was no information available to indicate whether *Anabaena* DNA sequences would contribute to the

fingerprint if DNA was extracted directly from the intact symbiosis. Zimmerman *et al.* [27] had shown that the contribution of *Anabaena* to isozyme patterns was negligible. Techniques such as extraction of DNA from roots, to obtain *Azolla* DNA without *Anabaena* DNA, require additional labor and would be less suitable for maintaining culture collections. For these reasons, the possibility of using DNA extracted from intact *Azolla-Anabaena* symbioses for identification of accessions was tested.

To determine if DAF could produce unique fingerprints which could differentiate among *Azolla-Anabaena* accessions, *A. caroliniana* WT and *A. microphylla* 357 were compared to *A. pinnata* with the arbitrary primers 8.7b and 10.6e. *A. caroliniana* and *A. microphylla* are closely related and may in fact belong to the same species [28, 29, 30]. In contrast, *A. pinnata* belongs to the other section of the genus *Azolla* (Rhizosperma). Both primers easily distinguished between *A. caroliniana* WT and *A. microphylla* 357. Also, as expected the proportion of monomorphic bands between these two accessions was 5–6 fold higher than between *A. pinnata* P1 and *A. microphylla* 357. This suggests that DAF may be applicable in assessing the relatedness among *Azolla-Anabaena* accessions.

The second critical function of an identification technique in maintenance of a culture collection would be to eliminate duplications. Basically this requires that the patterns must be highly reproducible and stable over time. To test DAF in this respect, a duplicate culture of one accession was obtained from the original source after a lapse of 8 years. *A. caroliniana* WT had been used as the wild type parent in a mutation breeding project that produced a mutant which is partially tolerant to Propanil, the most common rice herbicide (H. Brunner and D.L. Eskew, unpublished results). At the start of that project a single apex was dissected out and propagated vegetatively to produce a uniform population. *A. caroliniana* C_1 was obtained as a duplicate culture. The fingerprints produced with primer 8.7b from these two templates were 100% monomorphic, and with primer 10.6e they were 96% monomorphic. A minor amount of polymorphism between these cultures could be expected as both were subjected to 75–100 subculturings during the 8-year period. From these studies we can conclude that these two cultures are indeed duplicates, and that DAF is a suitable technique for establishing the identity of *Azolla-Anabaena* accessions.

Anabaena *strain identification*

DAF is a major advance in the identification of *A. azollae* strains. It has only recently been possible to distinguish among strains of *A. azollae* [12, 13]. Liu *et al.* [9] used monoclonal antibodies to confirm that the *A. azollae* strain from *A. microphylla* had been transferred to *A. filiculoides*. After screening 13 hybridoma lines they were able to find one monoclonal antibody that reacted with only one of the *Anabaena* strains. To confirm this transfer by RFLP analysis, Plazinski *et al.* [15] screened 25 restriction enzymes, 40 genomic clones of *A. azollae* MI, and 120 genomic clones of *A. azollae* FI. In contrast, DAF was able to distinguish easily between *A. azollae* strains using either of the two primers tested.

Azolla *taxonomy*

DAF easily distinguished between closely related accessions of the *Azolla-Anabaena* symbiosis, and the proportion of monomorphic bands was higher between closely related species than between distantly related species (Fig. 1). These results suggested that DAF could help to resolve the taxonomy in the Azolla section of the genus *Azolla*. While DNA templates prepared from intact symbioses were acceptable for identification of accessions in culture collections, the question of the contribution of *Anabaena* DNA sequences to the *Azolla-Anabaena* fingerprint required a thorough examination before these templates could be used for taxonomic purposes. Our DAF analyses have shown that representation *Anabaena* DNA sequences in the *Azolla-Anabaena* fingerprint was variable and depended on primer sequence.

Williams *et al.* [25] performed an analogous comparison by mixing eukaryotic DNA from *Glycine soja* and prokaryotic DNA of the cyanobacteria PCC6803. Fingerprints generated with a decamer primer indicated that the prokaryotic DNA was not amplified even when it was present in a 460-fold molar excess. They suggested that better primer-template matches occurred in the more complex *G. soja* genome than in the simpler cyanobacterial genome and that this suppressed the amplification of prokaryotic sequences. They also calculated that a shorter primer of approximately 7.5 nucleotides would amplify more bands from a prokaryotic template. Although our results with the octamer 8.7b and the decamer 10.6e were in agreement with their suggestions, examination of one heptamer and three additional octamer primers showed that primer sequence was more important than length (Table 2).

The mathematical probability of finding perfect matches for a decamer primer in the more complex genome of a eukaryote is indeed higher than in the simpler prokaryotic genome, but Caetano-Anollés *et al.* [4] have shown that the 8 bases at the 3′ end of a primer condition the pattern produced and that the additional bases at the 5′ end of a decamer have only a limited effect. Also, since the primer is present in a large excess there is no reason to assume that primer competition would result in the exclusion of prokaryotic products using decamer primers. Only a few nanograms of DNA are required to perform DAF, and sufficient DNA for several amplifications was extracted from 0.1 g fresh weight of *Azolla* roots. This amount of root material can be harvested from a single 150 mm × 20 mm Petri dish culture of *Azolla*. Thus, there is no reason to avoid the use of pure *Azolla* DNA for taxonomic studies.

Sexual hybridization

Because there are few reliable morphological markers to differentiate among *Azolla* accessions, it has been necessary to use isozyme or RFLP analyses to confirm sexual hybridization. Comparison of the DAF fingerprints using root DNA as the template provided convincing evidence that Rong Ping C_4 was a sexual hybrid between *A. filiculoides* 301 and *A. microphylla* 357 (Fig. 5). Thus DAF can also be used for this purpose to great advantage.

Interpretation of the differences in the fingerprints between the parents and the hybrid is complex because DAF markers are inherited codominantly [25]. The contribution of organelle DNA to DAF fingerprints has not yet been demonstrated, but it is likely that organelle DNA is present and amplified. Thus, it is not possible to interpret all the bands observed in Fig. 5. The presence of several bands from the fingerprints of each of the parents in the pattern for the hybrid, however, provides strong evidence that sexual hybridization has occurred.

Conclusions

DAF offers several advantages over isozyme and RFLP analyses for identification of accessions of the *Azolla-Anabaena* symbiosis. Isozyme analysis requires live healthy cultures, and that the identity of these cultures is reliable. RFLP work requires 1000-fold larger amounts of DNA, requires much more preliminary work to identify specific probes, and requires the use of radioisotopes for optimum sensitivity. In contrast, DNA preparations for DAF can be stored indefinitely, and with little preliminary work it was possible to identify and differentiate accessions of *Azolla* and also strains of *Anabaena*. The primers used in this work were selected on the basis that they worked well with many different organisms, and presumably would work equally well with a wider range of *Azolla* or *Anabaena* strains.

DAF [2] and other related techniques [24, 26] are similar in many aspects. Although the amplification reaction conditions are significantly different, all use single, short primers of arbitrary sequence to amplify discrete fragments of template DNA. However, DAF uses shorter primers, polyacrylamide gel electrophoresis and silver staining to produce, separate and detect a more complex array of DNA products. Because of this,

DAF is able to produce a more informative fingerprint for each genotype examined. The additional bands present in a silver stained DAF gel may be more difficult to interpret in genetic studies, but are clearly more informative for establishing the unambiguous identity of an accession. Another advantage is that the plastic backed polyacrylamide gels can be stored indefinitely after they are dried, and the bands can be dissected out, reamplified, and used as hybridization probes [4].

A greater number of *Azolla-Anabaena* accessions will have to be examined and more primers screened, before the full potential of the DAF technique can be assessed. The current results clearly indicate that DAF should help to accelerate progress in improving this agronomically important symbiosis.

Acknowledgements

The authors thank G. Peters and Cang Lin for providing the *Azolla* accessions. Research was supported in part by the Ivan Racheff Endowment of the University of Tennessee.

References

1. Bassam BJ, Caetano-Anollés G, Gresshoff PM: Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 196: 80–83 (1991).
2. Caetano-Anollés G, Bassam BJ, Gresshoff PM: DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Bio/technology 9: 553–557 (1991).
3. Caetano-Anollés G, Bassam BJ, Gresshoff PM: DNA amplification fingerprinting: a strategy for genome analysis. Plant Mol Biol Rep 9: 292–305 (1991).
4. Caetano-Anollés G, Bassam BJ, Gresshoff PM: Primer -template interactions during DNA amplification fingerprinting with arbitrary oligonucleotides. Mol Gen Genet (in press).
5. Dellaporta SL, Wood J, Hicks JB: A plant DNA minipreparation: Version II. Plant Mol Biol Rep 1: 19–21 (1983).
6. Dunham DG, Fowler K: Taxonomy and species recognition in *Azolla* Lam. In: *Azolla* Utilization, pp. 7–16. International Rice Research Institute, Los Baños, Philippines (1987).
7. Gebhardt JS, Nierzwicki-Bauer SA: Identification of a common cyanobacterial symbiont associated with *Azolla* spp. through molecular and morphological characterization of free-living and symbiotic cyanobacteria. Appl Environ Microbiol 57: 2141–2146 (1991).
8. Lin C, Liu Z, Zheng D, Tang L, Watanabe I: Re-establishment of symbiosis to *Anabaena*-free *Azolla*. Sci China (Ser B) 32: 551–559.
9. Liu CC, Chen Y, Tang L, Zheng Q, Song T, Chen M, Li Y, Lin T: Study on preparation and application of monoclonal antibodies to *Anabaena azollae*. Sci China (Ser B) 32: 562–569 (1989).
10. Peters GA, Mayne BC: The *Azolla, Anabaena azollae* relationship. I. Initial characterization of the association. Plant Physiol 53: 813–819 (1974).
11. Peters GA, Meeks JC: The *Azolla-Anabaena* symbiosis: basic biology. Annu Rev Plant Physiol Plant Mol Biol 40: 193–210 (1989).
12. Plazinski J: The *Azolla-Anabaena* symbiosis. In: Gresshoff PM (ed) The Molecular Biology of Symbiotic Nitrogen Fixation, pp. 51–75. CRC Press, Boca Raton, FL (1990).
13. Plazinski J, Croft L, Taylor R, Zheng Q, Rolfe BG, Gunning BES: Indigenous plasmids in *Anabaena azollae*: their taxonomic distribution and existence of regions of homology with symbiotic genes of *Rhizobium*. Can J Microbiol 37: 171–181 (1991).
14. Plazinski J, Franche C, Liu CC, Lin T, Shaw W, Gunning BES, Rolfe BG: Taxonomic status of *Anabaena azollae*: An overview. Plant Soil 108: 185–190 (1988).
15. Plazinski J, Zheng Q, Taylor R, Rolfe BG, Gunning BES: Use of DNA/DNA hybridization techniques to authenticate the production of new *Azolla-Anabaena* symbiotic associations. FEMS Microbiol Lett 65: 199–204 (1989).
16. Plazinski J, Zheng Q, Taylor R, Croft L, Rolfe BG, Gunning BES: DNA probes show genetic variation in cyanobacteria symbionts of the *Azolla* fern and a closer relationship to free-living *Nostoc* strains than to free-living *Anabaena* strains. Appl Environ Microbiol 56: 1263–1270 (1990).
17. Tan BC, Payawal P, Watanabe I, Lacdan N, Ramirez C: Modern taxonomy of *Azolla*: a review. Phil Agric 69: 4491–516 (1986).
18. Tuan DT, Thuyet TQ: Use of *Azolla* in rice production in Vietnam. In: Nitrogen and Rice, pp. 395–405. International Rice Research Institute, Los Baños, Philippines (1979).
19. Van Cat D, Watanabe I, Zimmerman WJ, Lumpkin TA, de Waha Baillonville T: Sexual hybridization among *Azolla* species. Can J Bot 67: 3482–3485 (1989).
20. Watanabe I, Espinas CR, Berja NS, Alimagno BV: Utilization of the *Azolla-Anabaena* complex as a nitrogen fertilizer for rice. IRRI Research Paper Series, International Rice Research Institute 11: 1–6 (1977).
21. Watanabe I, Lin C, Ramirez C, Lapis MT, Santiago-Ventura T, Liu CC: Physiology and agronomy of *Azolla-*

Anabaena symbiosis. In: Skinner FA *et al.* (eds) Nitrogen Fixation with Non-legumes, pp. 57–62. Kluwer Academic Publishers, Dordrecht (1989).

22. Watanabe I, Lin C, Santiago-Ventura T: Response to high temperature of the *Azolla-Anabaena* association, determined in both the fern and in the cyanobacterium. New Phytol 111: 625–630 (1989).
23. Wei WX, Jin GY, Zhang N: Preliminary report of *Azolla* hybridization studies (in Chinese). Bull Fujian Acad Agric Sci 1: 73–79 (1986).
24. Welsh J, McClelland M: Fingerprinting genomes using PCR with arbitrary primers. Nucl Acids Res 18: 7213–7218 (1990).
25. Williams JGK, Hanafey MK, Rafalski JA, Tingey SV: Genetic analysis using RAPD markers. In: Recombinant DNA Volume. Meth in Enzymol, in press (1992).
26. Williams JGK, Kubilek AR, Livak KJ, Rafalski JA, Tingey SV: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl Acids Res 18: 6531–6535 (1990).
27. Zimmerman WJ, Lumpkin TA, Watanabe I: Isozyme differentiation of *Azolla* Lam. Euphytica 42: 163–170 (1989).
28. Zimmerman WJ, Lumpkin TA, Watanabe I: Classification of *Azolla* spp., Section Azolla. Euphytica 43: 223–232 (1989).
29. Zimmerman WJ, Watanabe I, Lumpkin TA: The *Anabaena-Azolla* symbiosis: Diversity and relatedness of neotropical host taxa. Plant Soil 137: 167–170. (1991).
30. Zimmerman WJ, Watanabe I, Ventura T, Payawal P, Lumpkin TA: Aspects of the genetic and botanical status of neotropical *Azolla* species. New Phytol 119: 561–566. (1991).