Genetic diversity and phylogeny analysis of *Azolla* based on DNA amplification by arbitrary primers

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The polymerase chain reaction was used to amplify random sequences of DNA from 25 accessions of *Azolla* to evaluate the usefulness of this technique for identification and phylogenetic analysis of this aquatic fern. Accessions were selected to represent all known species within the genus *Azolla* and to encompass the world-wide distribution of the fern. Primers of 10 nucleotides with 70% G + C content were used to generate randomly amplified polymorphic DNA from the symbiotic *Azolla–Anabaena* complex. Twenty-two primers were used and each primer gave 4–10 bands of different molecular weights for each accession. Bands were scored as present or absent for each accession and variation among accessions was quantified using Nei's genetic distances. A dendrogram summarizing phenetic relationships among the 25 accessions was generated using the unweighted pair-group method with arithmetic mean. Principal component analysis was also used to evaluate genetic similarities. Three distinct groups were identified: group 1 contains five species, group 2 contains the *pinnata* species, and group 3 contains the *nilotica* species. The analysis demonstrates that the major groups of *Azolla* species can be easily distinguished from one an other and, in addition, that closely related accessions within species can be identified. We further found that using 10 primers, a phylogeny that is essentially the same as that derived from 22 primers can be constructed. Our results suggest that total DNA extracted from the *Azolla–Anabaena* symbionts is useful for classification and phylogenetic studies of *Azolla*.

Key words: *Azolla–Anabaena* symbiosis, genetic distances, polymerase chain reaction, principal component analysis.

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La réaction en chaîne d'une polymérase a été utilisée pour amplifier au hasard des séquences d'ADN de 25 accessions d'*Azolla* dans le but d'évaluer l'utilité de cette technique pour fins d'identification et d'analyse phylogénétique de cette fougère aquatique. Les accessions ont été sélectionnées de façon à représenter toutes les espèces connues à l'intérieur du genre *Azolla* et la distribution mondiale de cette fougère. Des segments d'initiation de 10 nucléotides contenant 70% de G + C ont servi à produire de l'ADN polymorphe amplifié au hasard, à partir de complexes symbiotiques *Azolla–Anabaena*. Vingt-deux segments d'initiation ont été utilisés et chacun d'eux ont donné de 4–10 bandes de poids moléculaires différents pour chaque accession. Les bandes ont été notées comme présentes ou absentes pour chaque accession et la variation entre les accessions a été quantifiée à l'aide des distances génétiques de Nei. Un dendrogramme résumant les relations phénétiques entre les 25 accessions a été dressé en recourant à la méthode des groupements par paires de valeurs indéfinies avec la moyenne arithmétique. L'analyse des composantes principales a aussi été utilisée pour évaluer les similarités génétiques. Trois groupes ont été identifiés : le premier comportant cinq espèces, le second contenant l'espèce *pinnata* et le troisième contenant l'espèce *nilotica*. L'analyse démontre que les groupes majeurs d'espèces d'*Azolla* peuvent être facilement distingués les uns des autres et, de plus, que les accessions étroitement reliées à l'intérieur d'une espèce peuvent être identifiées. Ces travaux montrent également qu'une phylogénie établie à

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l'aide de 10 fragments d'initiation est essentiellement la même que celle qui est dérivée de 22 fragments. Les résultats suggèrent que l'ADN total extrait des symbiotes *Azolla–Anabaena* est utile pour la classification et les études phylogénétiques d'*Azolla*.

Mots clés : symbiose *Azolla–Anabaena*, distances génétiques, réaction en chaîne d'une polymérase, analyse de composantes principales.

[Traduit par la rédaction]

Introduction

The aquatic fern *Azolla* Lam. grows in symbiotic association with a nitrogen-fixing cyanobacterium, *Anabaena Azollae* Stras. The symbiont inhabits leaf cavities of the host plant and the two partners maintain a stable relationship based on the exchange of nitrogen (fixed by *Anabaena*) for carbohydrates (provided by the plant). *Azolla* is used as green manure to maintain soil fertility in rice paddies and in taro and lotus fields, mainly in Southeast Asia and increasingly in some African countries and in other parts of the world (Lumpkin and Plucknett 1982; Van Hove and Diara 1987; Nierzwicki-Bauer 1990; Watanabe et al. 1977, 1989). Its high protein content also makes it useful as an animal feed. A collection of *Azolla* cultures is maintained at the International Rice Research Institute in the Philippines and at the Catholic University of Louvain in Belgium, where accessions are evaluated for their productivity and nitrogen-fixing potential under various ecological conditions. Research into the adaptation of specific accessions is hindered by the lack of an accurate system for identifying plants, even at the species level (Dunham and Fowler 1987; Stergianou and Fowler 1990).

The present taxonomic framework shows the *Azolla* genus divided into two sections: *Azolla* and *Rhizosperma*. There are seven species: two in the *Rhizosperma* section (*A. nilotica* and *A. pinnata*) and five in the *Azolla* section (*A. caroliniana*, *A. mexicana*, *A. microphylla*, *A. filiculoides*, and *A. rubra*). The *A. pinnata* species itself is divided into two varieties: var. *pinnata* and var. *imbricata*. This classification system is based primarily on the morphology of a very limited number of reproductive and vegetative structures. Moreover, reproductive organs are rarely found, and vegetative organs that show a high phenotypic plasticity depending on different environmental conditions have never been critically evaluated for their nature and variability (Dunham and Fowler 1987). Cytological studies of *Azolla* strains have also contributed to our understanding of the taxonomy of this genus, but there is no sufficient variation in chromosome size and number to allow for high-resolution discrimination of species, especially within the *Azolla* section (Stergianou and Fowler 1990).

Recent applications of molecular technology, including isozyme characterization and RFLP analysis, suggest some rearrangements in the *Azolla* taxonomy, particularly in the *Azolla* section. Zimmerman et al. (1989*a*, 1989*b*, 1991) analyzed 17 polymorphic isozyme loci and 30 RFLP markers and found evidences for a regrouping of three species in the *Azolla* section, namely *A. caroliniana*, *A. mexicana*, and *A. microphylla*. Despite the usefulness of RFLP analysis, the procedure is still time consuming, labor intensive, and expensive, and the use of radioisotopes is usually required. Thus, the applicability of the technique is limited if large numbers of accessions are to be evaluated or if analysis is required in laboratories with limited facilities. Furthermore, most of the polymorphism shown by RFLP is interspecific and the intraspecific resolution remains low for some species (Zimmerman et al. 1991). The ability to rapidly and economically survey a larger number of loci using the polymerase chain reaction (PCR) increases the probability that more informative sequences may be discovered, simplifying the procedure required to identify an accession, and increasing the resolution of the result.

PCR has been shown to be an extremely efficient means of revealing polymorphism that can be used for classification and taxonomic studies. Williams et al. (1990) illustrated the usefulness of PCR with the use of arbitrary primers of random sequence to amplify genomic DNA. Analysis of the amplified PCR products (200–4000 bp) shows a few to many bands on agarose gels. These bands are called random amplified polymorphic DNAs or RAPDs. Since the primers used in these reactions are short (normally 10 nucleotides in length) and do not require any DNA sequence information, large numbers of RAPD bands can be obtained easily, rapidly, and inexpensively. These bands can be scored as present or absent in each accession and can then be used to evaluate genetic similarities. Similar works using PCR on bacteria, plants, and animals have been published (Williams et al. 1990; Hu and Quiros 1991; Welsh et al. 1991; Halward et al. 1992).

In this report, we demonstrate the use of RAPDs to study phylogenetic relationships among accessions of the aquatic fern *Azolla*. We found a high frequency of polymorphism among accessions and species. This information was used to construct a dendrogram and a principal component analysis (PCA) representation which indicated that the genus *Azolla* (Azollaceae) contains three distinct groups: group 1 contains the entire *Azolla* section, consisting of the currently named species; group 2 contains the cluster of *pinnata* species within the *Rhizosperma* section; and group 3 contains the cluster of *nilotica* species, also within the *Rhizosperma* section.

Materials and methods

Plant material

Azolla accessions from all continents where the plant is found were selected from the germplasm collection at the International Rice Research Institute (IRRI, Philippines). They are listed in Table 1 according to their IRRI code numbers (Watanabe et al. 1992). A total of 25 accessions were selected, 13 accessions from the *Rhizosperma* section and 12 accessions from the *Azolla* section. Two *Anabaena*-free accessions were also included in the experiments. These plants were obtained by micromanipulation of female reproductive structures to remove the cyanobacteria, which are exclusively associated with these structures as the starting point of the symbiosis (Lumpkin and Plucknett 1982; Peters and Meeks 1989). All plants containing *Anabaena* symbionts were grown in brown bottles with 450 mL of nitrogen-free culture medium under light, temperature, and humidity con-

TABLE 1. *Azolla* accessions used for RAPDs analysis

Accession No. (IRRI code)	Country of origin	Species	Section	ID[a]
1	Philippines	*pinnata* var. *imbricata*	*Rhizosperma*	1
5	Thailand	*pinnata* var. *imbricata*	*Rhizosperma*	2
18	Vietnam	*pinnata* var. *imbricata*	*Rhizosperma*	3
19	Vietnam	*pinnata* var. *imbricata*	*Rhizosperma*	4
23	India	*pinnata* var. *imbricata*	*Rhizosperma*	5
5001	Sudan	*nilotica*	*Rhizosperma*	6
5002	Sudan	*nilotica*	*Rhizosperma*	7
5501	Burundi	*nilotica*	*Rhizosperma*	8
7001	Australia	*pinnata* var. *pinnata*	*Rhizosperma*	9
7512	Zaire	*pinnata* var. *pinnata*	*Rhizosperma*	10
7516	Ivory Coast	*pinnata* var. *pinnata*	*Rhizosperma*	11
7529	Madagascar	*pinnata* var. *pinnata*	*Rhizosperma*	12
7530	Rwanda	*pinnata* var. *pinnata*	*Rhizosperma*	13
2001	U.S.A.	*mexicana*	*Azolla*	14
2026	Brazil	*mexicana*	*Azolla*	15
3002	U.S.A.	*caroliniana*	*Azolla*	16
3017	Brazil	*caroliniana*	*Azolla*	17
4018	Paraguay	*microphylla*	*Azolla*	18
4021	Galapagos	*microphylla*	*Azolla*	19
4025(AF)[b]	Paraguay[c]	*microphylla*	*Azolla*	20
6501	New Zealand	*rubra*	*Azolla*	21
6502	Australia	*rubra*	*Azolla*	22
1001	Germany	*filiculoides*	*Azolla*	23
1032(AF)	Germany[c]	*filiculoides*	*Azolla*	24
1034	China	*filiculoides*	*Azolla*	25

[a]Identification numbers (ID) used in Figs. 1–3.
[b]AF, *Anabaena*-free.
[c]*Anabaena*-free created in the Philippines.

TABLE 2. Primers used for random amplification of *Azolla–Anabaena* DNA[a]

Primer code	5'–3' sequence	RAPD markers detected	Species[b]
A06	GGTCCCTGAC	9	
A09	GGGTAACGCC	17	PI, NI, RU
A13	CAGCACCCAC	13	RU
B03	CATCCCCCTG	18	RU
B05	TGCGCCCTTC	14	
B06	TGCTCTGCCC	14	NI
B07	GGTGACGCAG	18	
B08	GTCCACACGG	35	RU
B10	CTGCTGGGAC	24	
B13	TTCCCCCGCT	24	PI, NI, FI
B14	TCCGCTCTGG	14	
B19	ACCCCCGAAG	26	NI
C05	GATGACCGCC	25	
C07	GTCCCGACGA	15	
C08	TGGACCGGTG	22	All except FI
C09	CTCACCGTCC	34	RU
C17	TTCCCCCCAG	15	All except NI
C19	GTTGCCAGCC	28	FI
D01	ACCGCGAAGG	29	
D02	GGACCCAACC	33	PI, PP
D03	GTCGCCGTCA	37	
D07	TTGGCACGGG	22	
Total		486	

[a]A set of 22 primers of 10 nucleotides length and of 70% G + C content was selected from an Operon primer kit (Operon Technologies, Inc.).
[b]Species where individual accessions can be identified (FI, *A. filiculoides*; NI, *A. nilotica*; PI, *A. pinnata* var. *imbricata*; PP, *A. pinnata* var. *pinnata*; RU, *A. rubra*). *Azolla mexicana*, *A. caroliniana*, and *A. microphylla* accessions were identified with almost each primer.

trol, according to the method described by Watanabe et al. (1977). Culture medium was replaced every 5 days to prevent algal growth. Doubling growth rate was 3–5 days, depending on the accession. A minimum of 10–12 days was needed to grow enough plant material for DNA extraction (see below).

Anabaena-free accessions were supplemented with a nitrogen source (ammonium nitrate) and the medium was replaced every 3 days. The growth rate of these plants was lower and it took 15–20 days before plant material could be harvested for DNA extraction.

Total DNA extraction

The procedure for total DNA extraction from *Azolla* was based on that of Zimmerman et al. (1991) but with modifications. Approximately 4 g of fresh plant material (whole plant) was harvested, carefully washed with a sterilizing solution (0.12% sodium hypochlorite), rinsed four to five times with deionized water, and dried with absorbent paper. Then it was ground into a fine powder in liquid nitrogen using a mortar and pestle. A first extraction step was performed with 3 mL of preheated (65°C) 2.5× CTAB extraction buffer (2.5% CTAB (w/v), 50 mM Tris (pH 8.0), 10 mM EDTA, 1.75 M NaCl). A purification step with chloroform–isoamylalcohol (24:1) followed before an extraction with 1/5 volume of a preheated (65°C) 10% CTAB solution (10% CTAB (w/v), 0.7 M NaCl). The chloroform–isoamylalcohol step was repeated and the DNA was then precipitated with 100% ethanol (4°C) at −70°C for 40–45 min. Centrifugation at 4°C gave crude DNA pellets that were resuspended into 350 µL of TE (10 mM Tris, 1mM EDTA, pH 8.0). Each DNA solution was combined in one 1.5-mL Eppendorf tube for an RNAase treatment followed by a second extraction step with 1/2 volume of 2.5× CTAB buffer (65°C) and 1/10 volume of 5 M NaCl solution. Another chloroform–isoamylalcohol extraction was performed, but this time tubes were cen-

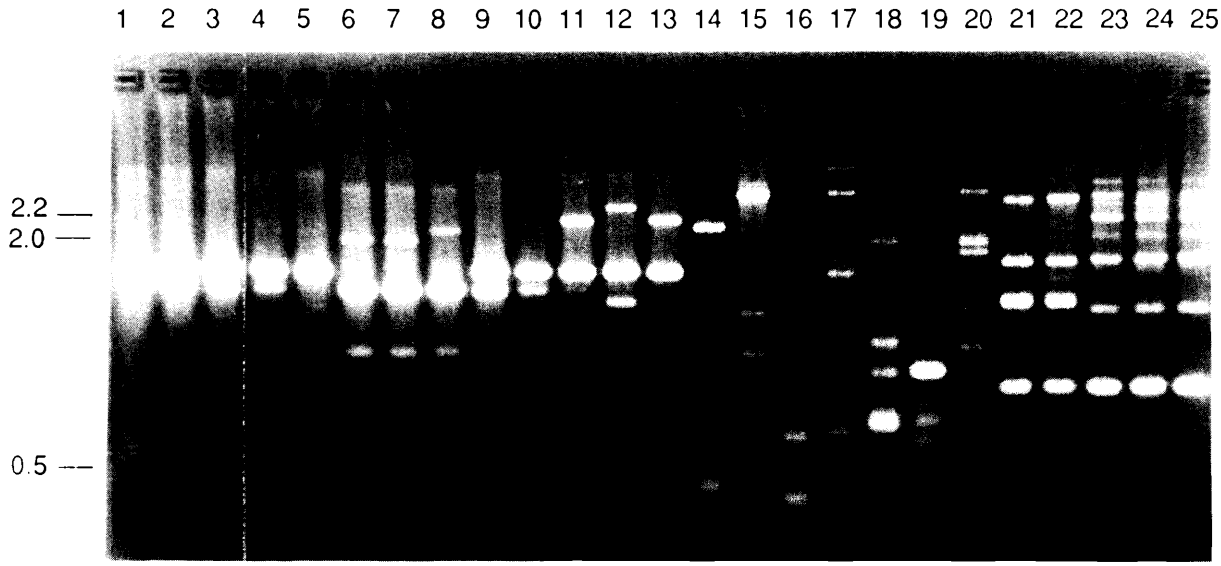

FIG. 1. DNA polymorphism of 25 *Azolla* accessions obtained by amplification of total DNA from *Azolla–Anabaena* symbionts using random primer C09 (5'CTCACCGTCC3'). Lane numbers 1–25 correspond to the numbers in the last column of Table 1.

trifuged at 13 800 × *g* for 10 min. An ethanol precipitation followed and the final DNA pellet was resuspended in 300 µL of TE. For few accessions that gave very sticky DNA solutions, a further phenol–chloroform purification step was performed according to Sambrook et al. (1989).

DNA quality was checked by gel electrophoresis and the concentration was estimated by dot-blot method (Sambrook et al. 1989, p. E6) on plastic Petri dishes.

PCR amplification procedure and electrophoretic analysis of RAPD products

All pipetman, tips, and Eppendorf tubes were UV treated for 5 min before use (Sarkar and Sommers 1990).

Table 2 lists the 22 primers selected from an Operon primer kit (Operon Technologies, Inc.). Their length is 10 nucleotides and they were randomly chosen among those with 70% G + C content. Amplifications with primers of high G + C content have been shown to reveal high levels of PCR product (Williams et al. 1990).

Total PCR volumes were 25 µL, containing 20 ng of total genomic *Azolla–Anabaena* DNA, 100 µM of each dNTP (Boeringher), 0.5 µM of primer, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 100 µg/mL gelatine, and 1 unit of *Taq* DNA polymerase (Boeringher). One drop of light mineral oil was added to prevent evaporation. Forty-five amplification cycles for 1 min at 94°C (denaturation step), 1 min at 36°C (annealing step), and 2 min at 72°C (extension step) were performed in a DNA Thermo Cycler (Perkin-Elmer/Cetus 480), followed by one single extension cycle for 7 min at 72°C.

PCR products were stored at 4°C before separation by electrophoresis in 2% agarose gels (1% Nusieve, 1% Ultrapure) at 25–30 V for 10–14 h. Gels were stained 30–40 min in ethidium bromide solution (1 µg/mL) and observed under UV light.

Genetic distances and statistical analysis

A Macintosh computer program, HyperRFLP, developed by Wang and Tanksley (1989) was used to calculate Nei's genetic distances based on the proportion of shared bands (Nei and Li 1987) in pairwise comparisons between accessions and to build a dendrogram according to the unweighted pair group method with arithmetic mean (UPGMA). The Nei's distance matrix was also used for PCA with a Data Desk Macintosh computer program.

Results

Electrophoretic analysis of RAPD products

Twenty-two primers were used to amplify random sequences from total *Azolla* or *Azolla–Anabaena* DNA extracted from 25 *Azolla* accessions. Analysis of the PCR products by gel electrophoresis revealed that each primer gave 4–10 DNA bands per accession. The migration distances of the bands from different accessions may or may not be the same. Each band in the gel was scored as one RAPD marker. Therefore, the total number of markers revealed by a single primer was generally larger than 10 when all accessions were compared (Table 2). Some primers generated up to 37 RAPD markers. Clear amplification patterns such as the one shown in Fig. 1 were obtained. Experiments were replicated and only reproducible RAPD markers were scored for further analysis. Differences between the *Rhizosperma* and *Azolla* sections and between *A. nilotica* and *A. pinnata* species were readily observed in most amplification patterns. A single primer was usually sufficient to distinguish species among sections, especially in the *Rhizosperma* section and the *rubra* and *filiculoides* species in the *Azolla* section. Three primers were usually required to distinguish among individual accessions within a species. However, an identical pattern was always obtained for *nilotica* accessions 5001 and 5002, indicating the presence of a duplicate or two very closely related accessions. More polymorphism was observed from RAPD patterns of var. *pinnata* than from var. *imbricata*. In the *Azolla* section, more polymorphism was observed among accessions within *A. caroliniana*, *A. mexicana*, and *A. microphylla* than within *A. rubra* and *A. filiculoides*.

The banding patterns generated from the two *Anabaena*-free strains were unpredictable. It was expected that these strains would contain a subset of the bands observed in *Azolla–Anabaena* symbionts. However, in one case,

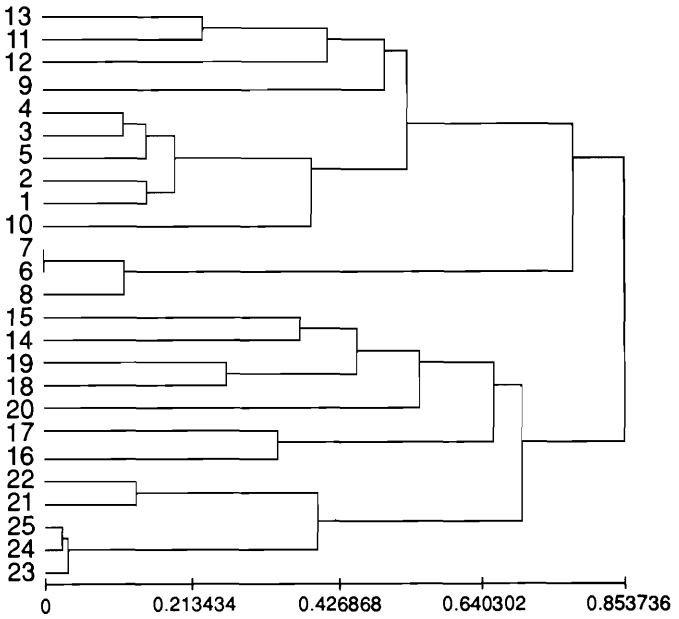

FIG. 2. Dendrogram based on Nei's genetic distances demonstrating relationships among 25 *Azolla* accessions. The HyperRFLP program was used to calculate genetic distances based on RAPD data from 22 primers and to build the dendrogram according to the unweighted pair-group method with arithmetic mean (UPGMA). Code numbers of *Azolla* accessions are listed in Table 1.

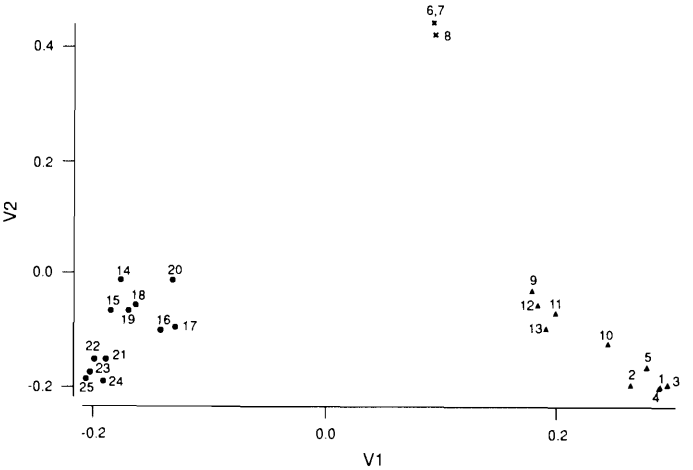

FIG. 3. Scatter plot of the first two axes analyzed by principal component analysis (PCA) using Nei's genetic distances for 25 *Azolla* accessions. Genetic distances were calculated from RAPD data (using the HyperRFLP program) using 22 random primers. Data Desk was used to perform PCA based on the genetic distances. The first two axes represent 74.4% of total variance. Code numbers for *Azolla* accessions are listed in Table 1.

A. microphylla, accession 4025, contained some new bands not observed in accessions of the same species containing *Anabaena*. On the other hand, the banding pattern of *A. filiculoides*, accession 1032, appeared quite similar to *Anabaena*-containing *A. filiculoides* accessions. Despite these differences, similarity analysis and PCA revealed that both *Anabaena*-free accessions had banding patterns which clearly placed them within their species clusters (Figs. 2 and 3).

Phenetic distances and statistical analysis

A distance matrix method was applied to the *Azolla* RAPD data. A total of 486 RAPD markers were scored, an average of 20 markers per primer. RAPD markers were treated as separate units and were recorded as present or absent for each accession. The HyperRFLP program was used to generate a distance matrix based on Nei's genetic distances (data not shown). A dendrogram was built according to the UPGMA method (Felsenstein 1988; Li and Graur 1991) (Fig. 2). This result indicated a clear distinction between the two sections of the *Azolla* genus and between a *pinnata* species cluster and a *nilotica* species cluster in the *Rhizosperma* section. Distances between an *Azolla* section group, a *nilotica* species group, and a *pinnata* species group were calculated from the distance average between accessions from each group. They revealed an approximately equal distance between the three groups: 0.88 between *A. nilotica* species and *Azolla* section species; 0.84 between *A. pinnata* species and *Azolla* section species; and 0.76 between *A. nilotica* and *A. pinnata* species.

In the *Azolla* section group, *A. rubra* and *A. filiculoides* species could be grouped together, relatively distant (0.70) from an *A. mexicana*, *A. caroliniana*, and *A. microphylla* species group.

In the *A. pinnata* var. *pinnata* species group, accession 7512 (Zaire, ID No. 10, see Table 1) was found to be more genetically similar to the Asian *A. pinnata* var. *imbricata* accessions than to other var. *pinnata* accessions. Accessions 7001 (Australia, ID No. 9) and 7529 (Madagascar, ID No. 12) were found to be unambiguously related genetically to other members of the var. *pinnata* even though they are geographically distant. Distances among var. *imbricata* accessions were found to be smaller than that among var. *pinnata* (see Fig. 2).

This result was then compared with that of a PCA based on the same genetic distance matrix. A representation of that PCA is shown in Fig. 3, a scatter plot where 74.4% of the total variance of the accessions was explained by the first two axes. The three groups that were suggested with the UPGMA (Fig. 2) appeared more clearly distinct. The analysis clearly shows that the two sections are separated by the first axis (where most of the variance (55.4%) is expressed) and that the *nilotica* group is separated from the *pinnata* species group by the second axis. The graphical representation shows relatively small intragroup distances. A clear separation into two subgroups nevertheless appeared between *pinnata* and *imbricata* varieties of the *pinnata* species group, with accession 7512 (Zaire, ID No. 10) falling in between and to a lower extent in the *Azolla* section group. Both the PCA and the UPGMA analysis also showed that *A. rubra* and *A. filiculoides* are very closely related genetically like, to a lower extent, the CA–ME–MI (*A. caroliniana*, *A. mexicana*, and *A. microphylla*) species (Figs. 2 and 3). A PCA representation for species of the *Azolla* section confirmed that result (data not shown).

Primer efficiency

We identified the least number of primers needed to classify the 25 *Azolla* accessions by comparing scatter plots of PCA that were derived from distances generated from different numbers of primer data sets selected at

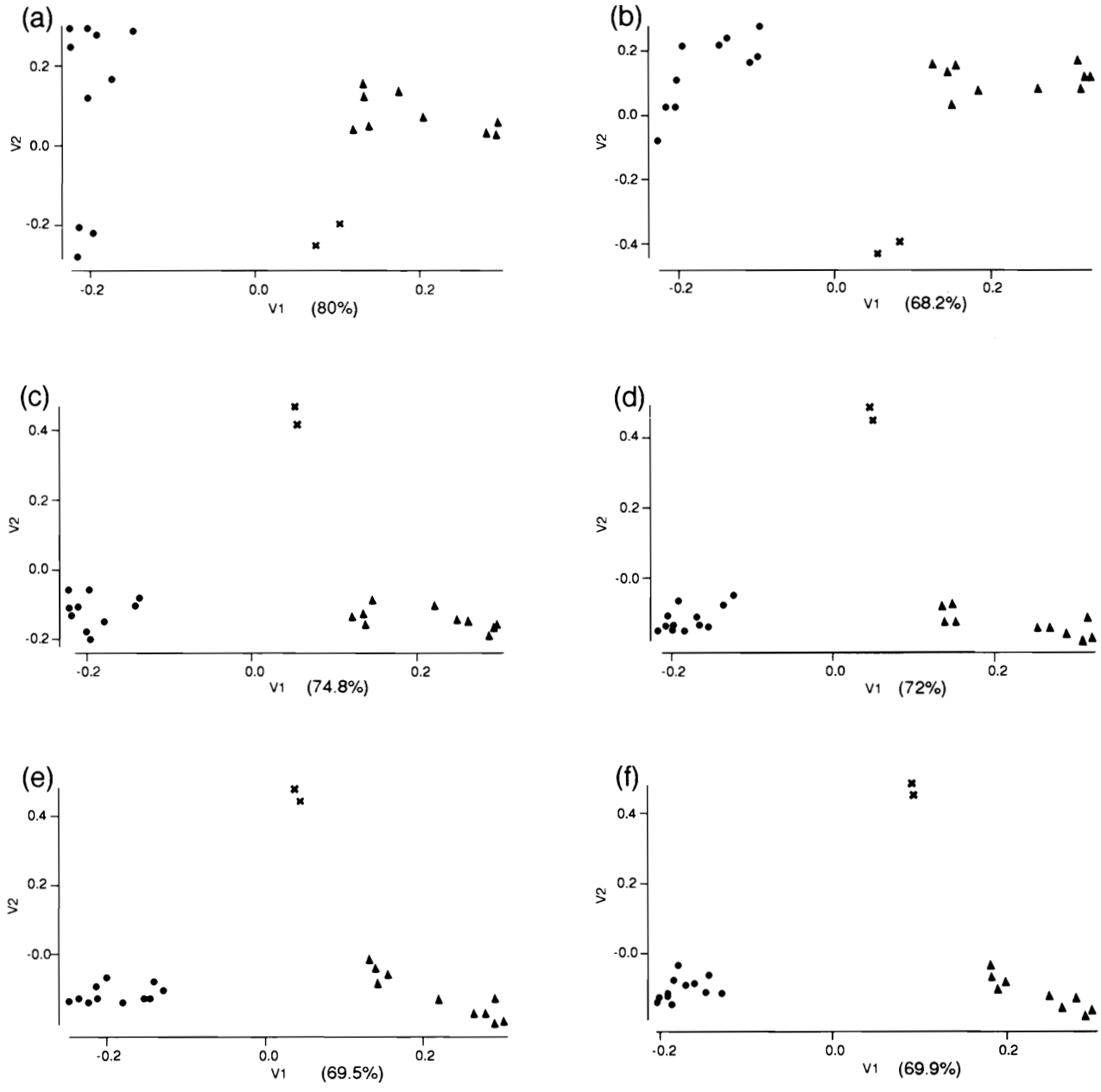

FIG. 4. Scatter plots illustrating the effect of size of data set generated by RAPD experiments on resolution of principal component analysis (PCA) of 25 *Azolla* accessions representing all known species in the genus. PCA based on Nei's genetic distances was used to evaluate the similarity of classification schemes using data sets consisting of (*a*) 3 primers, (*b*) 5 primers, (*c*) 7 primers, (*d*) 10 primers, (*e*) 15 primers, and (*f*) 20 primers. Variance expressed is indicated below each figure. Primers are fully described in Table 2. For a description of *Azolla* accessions from each group, refer to Fig. 3. The *pinnata* species accessions are represented by triangles, the *nilotica* species accessions by crosses, and the *Azolla* section species accessions by circles.

random. We started with data analysis from 3 primers and randomly added primers one at a time until all 22 were used. Figure 4 shows representations in two dimensions for 3, 5, 7, 10, 15, and 20 primers. The percentage of variance expressed in the first two dimensions is indicated under each plot. The figure clearly shows that starting from a random selection of 10 primers representing 186 RAPD markers, the classification of *Azolla* accessions is essentially the same as that observed with 22 primers (Fig. 3). With 3–10 primers, the different groups we found are already formed, but their spatial distribution differs from that with 22 primers.

Discussion

With the recent application of molecular techniques, such as RFLP analysis and PCR, polymorphism at the DNA level, which is very useful for classification and taxonomic studies, is revealed. Williams et al. (1990) and Welsh and McClelland (1990) further extended the efficiency of PCR by using arbitrary primers of random sequence to amplify polymorphic sequences from genomic DNA. Caetano-Anolles et al. (1991) described a technique called DNA amplification fingerprinting, or DAF, in which they used very short primers (approximately five nucleotides) and short amplification times (1 s for

each step). Separation of the amplified PCR products by polyacrylamide gels and silver staining allowed them to distinguish more bands and perform fine-tuned fingerprinting analysis. Although this special method is very promising, amplification patterns are more difficult and expensive to analyze and artifacts are more likely to be encountered.

We used the RAPD procedure because it allowed us to obtain a large amount of information with a simple, straightforward and economical analysis using regular agarose gels and an ethidium bromide staining procedure. Using only 22 primers to amplify the symbiotic complex of *Azolla–Anabaena* DNA, we were able to scan 486 RAPD markers. This data set provided a broader survey of the genome than was previously available (17 polymorphic isozyme loci and 30 RFLP markers) (Zimmerman 1989*a*, 1989*b*, 1991) and therefore offers improved resolution in classifying *Azolla* accessions. We observed large genetic distances between the different groups, suggesting that we were able to detect relatively more polymorphism by random amplification than was detected using isozymes and a limited number of RFLP probes. However, because different accessions were included in the different analyses, a direct comparison of the efficiency of the different methods awaits a future study.

Based on the UPGMA and PCA, three major groups were found: the *Azolla* section, the *A. nilotica* species cluster within the *Rhizosperma* section, and the *A. pinnata* species cluster, also within the *Rhizosperma* section. These three groups were almost equidistant to each other (see Results and Fig. 3) in terms of genetic distance. This could suggest an extension of the present taxonomy where the *Azolla* genus is divided into two sections: *Azolla* and *Rhizosperma* (Lumpkin and Plucknett 1982; Dunham and Fowler 1987; Van Hove et al. 1987; Watanabe et al. 1989; Stergianou and Fowler 1990). Although the *A. nilotica* and *A. pinnata* species clusters are both classified into the *Rhizosperma* section according to the classical taxonomy, the two species have usually been recognized morphologically from one another, confirming the distant relationship between the two species. In a recent study by Stergianou and Fowler (1990), it was shown that *A. nilotica* has a different number of chromosomes from all other species, implying a large genetic distance between this and other species. Our results on the DNA level, along with the morphological and the cytological data suggested, that *Azolla* may be divided into three groups. Whether or not *Azolla* should be classified into three groups, as suggested by this study, or should persist as two sections, remains to be seen since no comparative study of all known classification criteria of *Azolla* have been carried out. Further experiments will clarify this speculation.

Although it is known that the CA–ME-MI species in the *Azolla* section are closely related, the relationship between *A. rubra* and *A. filiculoides* species has been confirmed. Our data show that the genetic distance between *A. rubra* and *A. filiculoides* species is small and that the two species are equally distant from CA–ME–MI species (see Results and Fig. 2).

One accession from the *Rhizosperma* section (accession 7512 from Zaire) is not closely related to the other *A. pinnata* var. *pinnata* accessions (Figs. 2 and 3). This suggests that the accession from Zaire is an intermediate form of the two varieties of *pinnata* species or it could simply be a mislabelling. Further studies of other *pinnata* var. *pinnata* accessions from Zaire as well as from other African countries and Australia should clarify this point.

No correlation between *Anabaena*-free accessions and particular RAPD patterns was found. Considering the large number of markers investigated and that our molecular classification on the species level is essentially the same as that classified by morphological analysis, we believe that *Anabaena* is not critical in our RAPD experiments. Plazinski et al. (1990*b*) showed an RFLP-based classification of *Anabaena* isolated from different species of *Azolla*. The authors were able to distinguish very easily between *Anabaena* strains from all *Azolla* species. They suggested that the cyanobacteria isolated from *A. nilotica* species formed a distinct group along with the two other major groups corresponding to *Azolla* and *Rhizosperma* sections. Furthermore, studies in China revealed that spontaneous changes of algal partners were very unlikely in nature, although successful when performed in the laboratory (Lin et al. 1989). We suggest that the evolution of both partners has probably been parallel and that the contribution of *Anabaena* is likely to be in the same phylogenetic direction as the *Azolla* partner it inhabits. Therefore symbiotic *Anabaena* DNA is probably not complicating the classification of *Azolla* accessions using molecular markers.

Several authors (Wallace and Gates 1986; Forni et al. 1989; Nierzwicki-Bauer and Aulfinger 1990; Plazinski et al. 1990*a*) reported the presence of eubacterial symbionts also residing inside *Azolla* leaf cavities. A portion of the DNA extracted from the whole *Azolla* plant could have a bacterial origin but in relatively small amounts, compared with the host plant, suggesting a minor or no contribution to RAPD results. Further studies are needed to provide more evidences on this issue.

The fact that the main groups in the dendrogram derived from molecular data reflect classification based on other criteria indicates that those groups are natural. As verified in the matrix of distances, there is a good homogeneity in the distance estimated between any accession of one section and any accession of another section. This holds true for the comparison between species of the same section and indicates little or no genetic exchange between species.

The use of both UPGMA and PCA to analyze our data provides strong support for our classification scheme, since both analyses gave consistent results. The two methods weigh the data differently. While PCA gives a better view of group relationships, UPGMA shows the exact minimum distances found in pairwise comparisons.

Our work on *Azolla* showed that using only 3 primers, individual accessions could be routinely identified and that using 10 primers enough polymorphism was revealed to consistently classify the 25 *Azolla* accessions selected for this study. When only 10 primers were used, an average of 200 markers were detected and therefore a large amount of polymorphism was revealed. While we cannot conclude that 10 random primers are sufficient for evaluating the genetic diversity of any system, this study suggests that 10 would be a good starting point

when initiating a similar study with other organisms, especially where wild species are involved.

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