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FINGERPRINTING OF AZOLLA GERMPLASM

FINAL REPORT

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PROLOGUE

Two informal guidelines were presented to me by Dr. T. Lumpkin shortly after my arrival in 1987 to replace Dr. Narayanan as project director. They were that: 1) the technical workplan could be altered as I see fit in meeting the project goal, and 2) Dr. I. Watanabe's chief priority in the fingerprinting of *Azolla* accessions revolved around Section Azolla and the taxonomic confusion among accessions of the New World species. The agronomic significance of this priority lies in the advantageous field traits possessed by many strains of these species, which are preferentially used as a biofertilizer in much of Southeast Asia. I subsequently added direct genetic evaluations as a phase of research and emphasized New World *Azolla* accessions during completion of this project.

--W.J. Zimmerman

Species Code

A. filiculoides----------FI
A. rubra----------RU
A. caroliniana----------CA
A. mexicana----------ME
A. microphylla----------MI
A. nilotica----------NI
A. pinnata----------P"
A. pinnata v. *pinnata*-------PP
A. pinnata v. *imbricata*-----PI

INTRODUCTION

The ultimate goal of this project was to alleviate a major constraint to Azolla utilization as a rice biofertilizer--the inability to distinguish strains and even some species of this fern. There are seven designated species of Azolla divided by secondary reproductive features into two subgenera or sections (Table 1). Species traits in Section Azolla have been historically confined to morphological or anatomical characters, and are of little value among neotropical taxa. Varietal separation has never been achieved.

The morphological similarity of cultivated accessions has always been a major obstacle to the proper care of germplasm. Maintenance of live biomass is necessary since Azolla accessions rarely sporulate in culture collections. Fingerprinting would assist the process of selecting isolates for field use and provide a means to monitor cross-contamination and somaclonal variation. The identification of major taxa would also be facilitated.

This project was composed of two research stages (isoenzyme and DNA polymorphisms). The results described in this report are contained in published articles, review chapters, and in unpublished data being prepared for submission. Published papers are cited in the bibliography of this report.

RATIONALE OF TECHNOLOGY

Isoenzyme comparisons were completed in the first stage of fingerprinting. Specific enzymes rather than non-specific enzymes or total proteins were the chosen parameters. This choice yielded more precise genetic information in addition to the simple comparison of band migration patterns. Transfer of technology (methodology and interpretation) was also simplified by using specific enzymes. The number and relative migration ratios of gene loci were estimated whenever possible.

The original objectives of the investigation were technically met after completion of this stage. Duplicate accessions were located, unique germplasm identified, and many closely related accessions documented. The mis-identification of an array of accessions in the IRRI collection were found, and many of those isolates were consequently re-classified. Species relationships within Section Azolla were re-defined to some extent.

The project objectives were extended by the second investigative stage involving direct DNA evaluations.

Although a complex, tedious technology, DNA comparisons were a predetermined necessity since the genetics of Azolla are virtually unknown. The use of DNA restriction fragment length polymorphisms (RFLPs) in this study offered the first direct genetic information about Azolla biochemical taxonomy.

RFLP results reinforced the conclusions derived from phenetic data and located additional polymorphisms among strains which were enzymatically related. This technology will also be transferred in the near future to major centers of Azolla research in rice-growing LDCs (International Rice Research Institute-Philippines, Fujian Academy of Agricultural Sciences-China, Biological Nitrogen Fixation Resource Center-Thailand).

RESULTS AND CONCLUSIONS

Isozymes

Optimization of enzyme electrophoresis methodology was completed, then three initial objectives were achieved (Zimmerman et al., 1989a, Van Cat et al., 1989):
1) zymograms of healthy vs stressed leaf tissue, leaf vs root tissue, and leaf tissue with or without the cyanobiont were compared for discrepancies.
2) the potential of this methodology for hybrid identification in Azolla was tested.
3) accessions of all Azolla species were sampled to obtain a preliminary overview of divergence within this genus.

These objectives were followed by a mass screening of accessions. Banding patterns for strain and species identification were scored. The fingerprinting results were then interpreted from a perspective of biochemical taxonomy within Section Azolla (Zimmerman et al., 1989b).

Healthy leaves yielded more extensive information in their zymograms than did stressed leaves. Phosphorus deficiency was chosen since it is a common field stress and the most important limiting nutrient for Azolla. A reduced staining intensity of enzymes or the lack of visualization of loci was often observed in the zymograms of P-deficient fronds. The presence of the cyanobiont in leaves did not influence banding patterns (comparing FI ± Anabaena azollae). Our grinding procedure left most cyanobacterial cells intact in the enzyme extract (and A. azollae composes only a small fraction of the total biomass).

Root zymograms of PI were found to be unlike those of leaves for two of four tested enzymes. Additionally, the presence

of phenolics and other root compounds interfered with the resolution of zymograms. The general conclusion was that no advantage was obtained in utilizing root tissue as a symbiont-free alternative to leaf tissue because of: 1) the lack of contribution of the cyanobiont to leaf banding patterns, 2) the presence of substances which interfere with root enzyme visualization, and 3) the extra effort entailed in harvesting and grinding root tissue.

Hybrids of FI x MI were successfully confirmed by observing the patterns of certain multi-loci enzymes such as PGM or SKDH. New hybrids (including those from crosses of ME x MI) continue to be confirmed at IRRI by enzyme electrophoresis.

The preliminary overview found ≥15 loci in the tested 11-enzyme system. Three enzymes are illustrated in Fig. 1., and all enzymes used during this study are listed in Table 2. The exact number of loci in some of the multi-loci enzymes (SKDH, MDH, G6PDH) was uncertain, and may not be certified until segregation studies are possible. Locus 1 of PGI was not consistently stainable. Locus 2 of PGM had a higher relative migration (R_f value) for accessions of PI and MI than for those of other species (Fig. 1c & 1d).

A greater diversity was exhibited in Section Azolla (five designated species) than in Section Rhizosperma (two species). Within Section Azolla, two similar groupings of accessions were noticed. One was FI-RU, but further investigation (as described later) proved that the RU accessions of the initial comparisons were most likely FI. The other was CA-ME-MI.

The subsequent mass screening of accessions within this section was combined with data garnered from trichome morphology prior to final species analyses. Twelve enzymes were stained. Nine contained 17 polymorphic loci and 126 allelomorphic characters; three (ALD, G6PDH, F1,6DP) appeared to be monomorphic. At least two loci each were present in SKDH and 6PGD.

FI accessions were enzymatically distinctive (e.g., IDH) and also identified by prominent one-celled leaf trichomes. True RU accessions were found only in Australia and New Zealand, and possessed barely discernable trichomes. FI and RU are both known to be heat-sensitive species with a particular degree of cold tolerance indicated by RU (unpublished observations).

The Japanese RU was concluded to actually be FI. Probable duplicated entries (e.g., 2010/2022, 1005/1006/SWD, 1010/1016) and other enzymatically identical accessions (6007/6008, 6003/6005/6006, 2016/2018) were reported. Unique strains were recorded, both those positively identified to species such as FI and RU and others of the CA-ME-MI cluster (e.g., 2001, 2009, 2021, UCD 60 [probably IRRI 2005], 3001, 3008, 3016, 4033).

MI (from Paraguay and the Galapagos Islands) were all unique by their faster PGM-2 locus. However, a principal component analysis showed no discernible phenetic distance separating MI from the other species of the CA-ME-MI cluster (Fig. 2). The strains of these species commonly have two-celled leaf trichomes.

Incorrect labelling was found among many isolates of the 1000 series (FI) in the IRRI collection. Colombian strains within the 1014-1027 series, among others, have now been re-labelled as ME (2010-2022). An earlier series of growth experiments under high temperature had indicated that these strains were heat-tolerant (IRRI, 1986). Biomass production was similar to MI 4018 and unlike heat-sensitive FI. Possible natural hybrids in this series were noted for further genetic analysis.

The success of isoenzyme fingerprinting was multifold: 1) documentation of accessions, 2) the first evaluations of gene loci in Azolla, 3) species separation and possible re-evaluation, 4) confirmation of manipulated hybrids, and 5) identification of enzyme markers possibly linked to species or accessional traits such as cold tolerance (in RU 6501-6503; illustrated in Zimmerman et al., 1989a), heat tolerance (in MI or the Colombian ME), or other agronomically desireable features.

DNA Polymorphisms

Optimization of procedures was again one of the initial priorities. The first step was to overcome two overlapping technical problems:

1) Consistently successful extraction and purification of Azolla genomic DNA. Azolla possesses all of the disadvantages of ferns (high levels of polysaccharides and other impurities) and aquatic plants (low tissue density and DNA yield) which influence attempts at DNA extraction.

2) Transfer of technology. Techniques had to be developed which were relatively inexpensive, even if time-consuming, so that their transfer to LDC centers could be maximized. A key

modification was a serial purification-precipitation technique (Zimmerman, unpublished) used in place of ultracentrifugation.

Numerous techniques for DNA extraction and purification were tested and modified until the one above was selected. Periodic Azolla harvests were necessary because of the low DNA yields and the occasional arrival of new germplasm for study. One compromise made for the sake of data generation was the continued use of radioactive isotopes (^{32}P) to label probes for DNA-DNA hybridizations. This choice of probe labelling will be replaced by enhanced chemiluminescence (ECL) techniques during collaborations continued beyond the scope of this particular project.

The second step involved the collection and testing of multiple gene probes in an attempt to find as many useful probes for Azolla as possible. As mentioned in a preliminary report, this exhaustive preparatory work was essential due to the lack of genetic information on Azolla. The intent was to garner as much genetic knowledge as possible using known heterologous probes as well as tabulating polymorphisms via random homologous genomic probes.

Thirty heterologous probes, mostly from sources in the United States, were tested under varying stringencies. Their origins were from maize, pea, lentil, squash, rice, soybean, Clarkia, barley, tomato, and lettuce. In addition to acquiring several genes known to be highly conserved, others coding for specific plant enzymes (e.g., glutamine synthetase, phosphoglucoisomerase) were tested. Data which correlated with isoenzyme results would logically be preferred if any of the enzyme-coding genes were sufficiently conservative to cross-hybridize to Azolla DNA. The bacteriophage M13 was tested as a potential probe to detect hypervariable minisatillite DNA.

A genomic DNA library (fragment sizes from 0.5 - 2 kb) was also made from an ME strain and partially screened. Clones were particularly screened for inserts with middle repetitive DNA sequences useful to illustrate polymorphisms. Another homologous probe from MI 431 (FAAS) was donated by the Plant Cell Biology Group (courtesy of J. Plazinski) of the Australian National University, Canberra.

The most useful probes to date have been: 1) heterologous genes for rDNA (pHA-2, one complete repeat unit), chloroplast DNA (e.g., phytochrome), actin (pSAC3), and ubiquitin (recently obtained and to be chiefly utilized in future

work), and 2) homologous genomic clones (e.g., pA5Ha1, pA1HAb3, pAM7).

The RFLP data reinforced the species relationships documented in the first research stage and represented by the hierarchial phenogram in Fig. 3. Hybridization patterns in several cases distinguished P" (and sometimes PI from PP), the true RU, NI, and FI. The CA-ME-MI cluster remained highly coherent. None of the last three species were elucidated as a separate identity by genetic fingerprints, as was the case of MI with certain zymograms.

Distinctively polymorphic accessions, which had been previously observed to be enzymatically unique, included 2001, 3001, 6502, and 6503. New strains to be added to this list included 66, ENG, 3015, 4030, 4072, 5001, and 7001. Based on preliminary information, there is a high liklihood of additional unique accessions being identified as RFLP research is continued.

Two random homologous probes (pA5Ha1 and pA1HAb3) yielded RFLPs useful for defining Azolla species relationships. The uniqueness of NI and P" was depicted by their respective banding patterns. The relatedness of the two varieties of P" was shown (PI 5, 72, PP 7001, 7002). Likewise, the genetic affinity of the amorphous CA-ME-MI group was again illustrated. The measure of genetic diversity was represented by the polymorphisms of the exceptional strains listed in the previous paragraph. An example of a slight affinity of FI and RU was depicted by their identical banding patterns from hybridizations with pA5Ha1 (Fig. 4).

MI strains collected in Paraguay over ten years ago were not genetically identical to others collected in 1988 by Drs. Watanabe and Zimmerman. In the case of hybridizations of the rDNA probe pHA-2 to EcoRI-digested Azolla DNA, original accessions (4001, 4003, 4009, 4021, 4028 were tested) lacked two bands (approx. 5.5 and 3.5 kb) found in the newer accessions. These recent strains, mostly taken from the same semi-arid region (Chaco Province) as the first survey, possessed vigorous growth and environmental adaptability in growth chamber and greenhouse culture. Field tests with one of the new Paraguayan strains (4064) have been very favorable under IRRI conditions.

Certain bands from the pHA2 hybridizations also helped distinguish the cold tolerant RU 6500 series. The presence or absence of an approx. 3.2 kb band in the BamHI fingerprint separated certain heat-tolerant CA-ME-MI accessions from the

main cluster. The intensity of the band when present might also be a determinant to subdivide this group even further. Further and more extensive trials are necessary in this regard.

The pAm7 MI homologous probe was useful by its discriminatory (+/-) effect with Azolla. The Australian researchers (Plazinski et al., 1990) had claimed that this probe was species-specific for MI. Their results were based on dot-blot hybridizations with a few Azolla strains of different species. Our hybridizations with dozens of Azolla accessions showed that the discrimination was not species-specific (Fig. 5). Successful hybridizations, however, have so far only been achieved with accessions from the CA-ME-MI cluster. On the other hand, accessions of this group also formed the majority of strains which did not hybridize to pAm7. Strains of FI, NI, PI, and PP accessions did not hybridize, but their sample numbers were small. Furthermore, results were unclear for several strains (including RU 6502 and PI 72), so one cannot state unequivocally at this time that this probe is specific to a select subgroup of taxa of CA-ME-MI. Genetic information pertaining to species separation with pAm7 offered no additional insight over the other probes.

A very interesting, and as yet inadequately explained, coincidence involves the nature of the gene in pAm7. Actin gene sequences apparently are contained in this insert. The banding patterns of DNA from accessions which hybridize to pAm7 were identical to those obtained from pSAC3 (actin gene from soybean). Also, pSAC3 hybridizations at moderate stringency (6x SSPE, 60 ^{o}C) resulted in the same +/- banding results as pAm7 hybridized at a higher stringency (68 ^{o}C). Since actin is ubiquitous and essential to plants, we assume that lower hybridization and wash stringencies would result in positive hybridizations for all Azolla accessions.

The overall success from the DNA RFLP work come from:
1) the elucidation of more unique Azolla accessions,
2) initial definition of genetic differences between PI and PP,
3) evidence of a slightly closer taxonomic relationship of RU to FI than to the other species of Section Azolla,
4) more evidence confirming the separate status of New World FI from CA-ME-MI,
5) the lack of separation within CA-ME-MI and the need for re-classification of these species.

CA-ME-MI could arguably be designated as one species, possibly derived from extensive hybridization of two or more

species over time. This process is very common among ferns, although often leading to polyploidy which is not the case for most Azolla taxa. This taxonomic suggestion still requires more empirical evidence, but is much less speculative with our cumulative findings.

The answers found in our enzyme and RFLP results are exceeded by the number of new questions derived from our data. The enhancement of Azolla utilization in ricefields lies in future research not only in agronomy but in the areas of population biology, molecular biology, and evolutionary biology. Major prerequisites to advancing Azolla genetics are successful utilization of molecular markers, the sporulation trigger, and sexual hybridizations.

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RELATED AZOLLA PUBLICATIONS RECEIVING AID GRANT SUPPORT

Roger, P., W.J. Zimmerman, and T.A. Lumpkin. Microbiological management of wetland ricefields. In: B. Metting (ed.), Soil Microbial Technologies, Marcel Dekker, Inc., New York (in press)

Zimmerman, W.J., T.A. Lumpkin, and I. Watanabe. Biochemical taxonomy of Azolla Lam. In: Isotopic Studies of Nitrogen Fixation and Nitrogen Cycling by Blue-Green Algae and Azolla (Proceedings of the Research Programme), International Atomic Energy Agency (Joint FAO/IAEA Division), Vienna (in press)

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APPENDIX

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Table 1. Taxonomy and distribution of extant *Azolla*

Section	No. of floats per megasporocarp	Species	Major natural range
Azolla	3	*A. filiculoides* Lamarck	Western North America; Central America; South America
		A. rubra R. Brown	Western Pacific
		A. caroliniana Willdenow	Eastern North America Central America, South America
		A. mexicana Presl	Central and Western North America; Central America
		A. microphylla Kaulfuss	Tropical and Subtropical America
Rhizosperma	9	*A. pinnata* R. Brown	East, South and Equatorial Asia; Australia; Sub-Saharan to Southern Africa
		A. nilotica DeCaisne	Upper Nile and Sudan; Equatorial and Southern Africa

Table 2. *Azolla* enzymes elucidated by electrophoresis.

Enzymes

Aldolase (EC 4.1.2.13; ALD)
Aspartate aminotransferase (EC 2.6.1.1; AAT)*
Fructose-1,6-diphosphatase (EC 3.1.3.11; F1,6DP)
Glucose 6-phosphate dehydrogenase (EC 1.1.1.49; G6PDH)
Glutamate dehydrogenase (EC 1.4.1.2; GDH)
Glutamine synthetase (EC 6.3.1.2; GS)
$NAPD^+$-dependent glyceraldehye-3-phosphate dehydrogenase (EC 1.2.1.12; G3PDH)
Isocitrate dehydrogenase (EC 1.1.1.42; IDH)
Malate dehydrogenase (EC 1.1.1.37; MDH)
Malic enzyme (EC 1.1.1.40; ME)
Phosphoglucoisomerase (EC 5.3.1.9; PGI)
Phosphoglucomutase (EC 2.7.5.1; PGM)
6-phosphogluconate dehydrogenase (EC 1.1.1.44; 6PGD)
Shikimate dehydrogenase (EC 1.1.1.25; SKDH)
Triosephosphate isomerase (EC 5.3.1.1; TPI)*
Xanthine dehydrogenase (EC 1.2.1.37;XDH)

*AAT and TPI are elucidated exclusively on starch gels because of the improved staining intensities.

FIGURE LEGENDS

Fig. 1. (A-C) Enzyme comparisons of *Azolla* accessions, four of section Rhizosperma and five of section Azolla. Accession numbers (left to right) are 2, 17, 72, TDP17, 2021, 2002, 3015, 4024, 6008.
A) PGI,
B) 6PGD,
C) PGM (with the addition of ADUL 15 and 5001 of *A. nilotica* and 1001 of *A. filiculoides* at the far right),
D) PGM of 12 accessions of *A. microphylla*.
(from Zimmerman et al., 1989a)

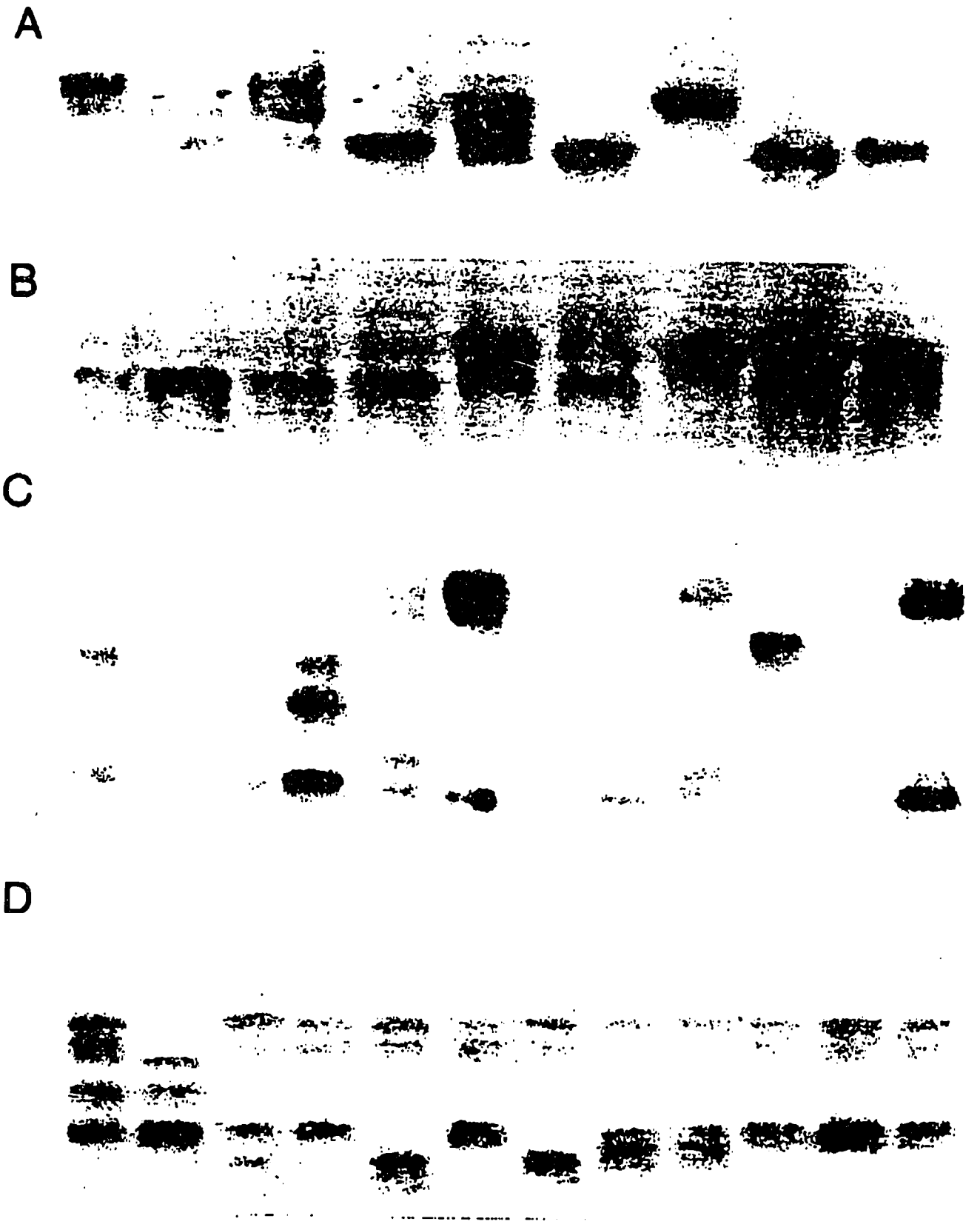
Fig. 2. Principal component analysis of data matrix. Projection of 57 populations of *Azolla*, section Azolla, onto the first and third principal components. AFIL = FI, ARUB = RU, AMIC = MI, AMEX-ACAR = ME-CA.

Fig. 3. Phenogram of *Azolla* species, section Azolla, as derived from cluster and PCA analyses. Cophenetic correlation coefficient = 0.901. Code descriptions as in Fig. 2.

Fig. 4. Southern blot analyses with hybridizations using the heterologous pHA-2 ribosomal probe and the random homologous pA5Hal probe (same membrane, separate hybridizations of genomic *Azolla* DNA digested with *Eco*RV).
Codes: A) LDN, B) 1016, C) 2012, D) 1006, E) 2019, F) 6003, G) 6005, H) 6007, I) 6502, J) 6503, K) 4062, L) FLA.

Fig. 5. Southern blot analyses with hybridization to the homologous pAm7 probe (genomic DNA digested with *Hin*dIII).
Codes: A) 2010, B) 2012, C) 2019, D) 2020, E) 2002, F) 2004, G) 2001, H) 2004, I) 2007, J) 2020, K) 3502.

Figure 1.



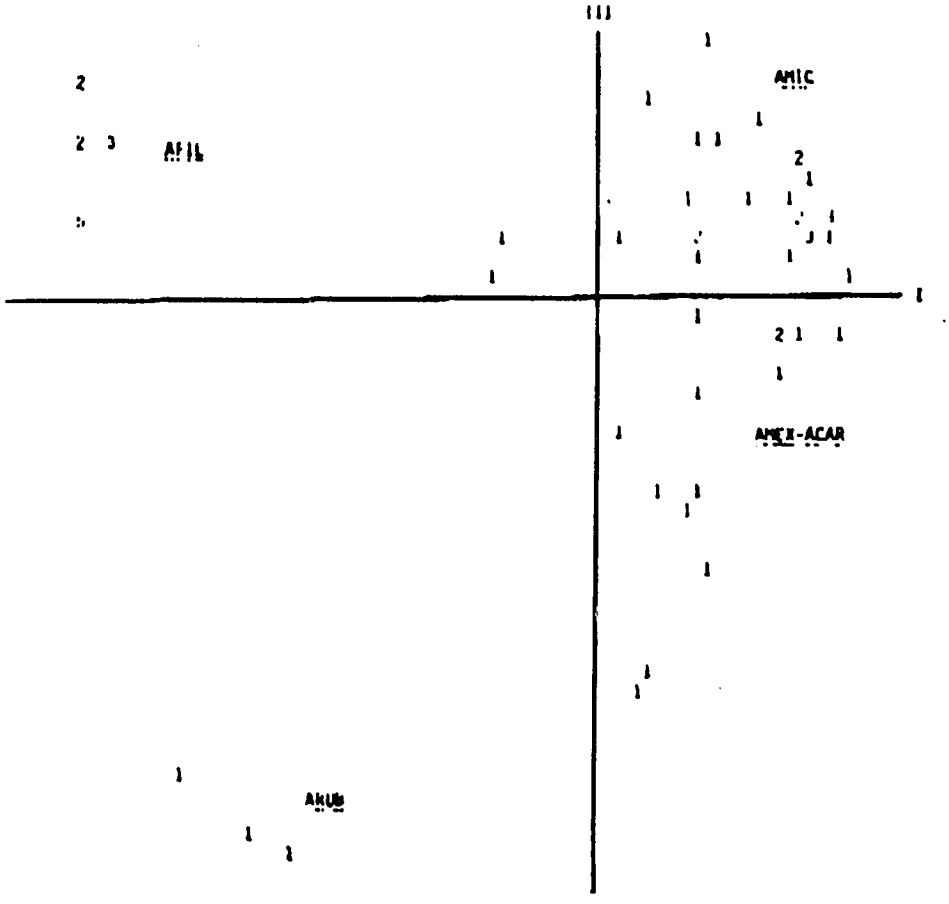


Figure 2.

Figure 3.

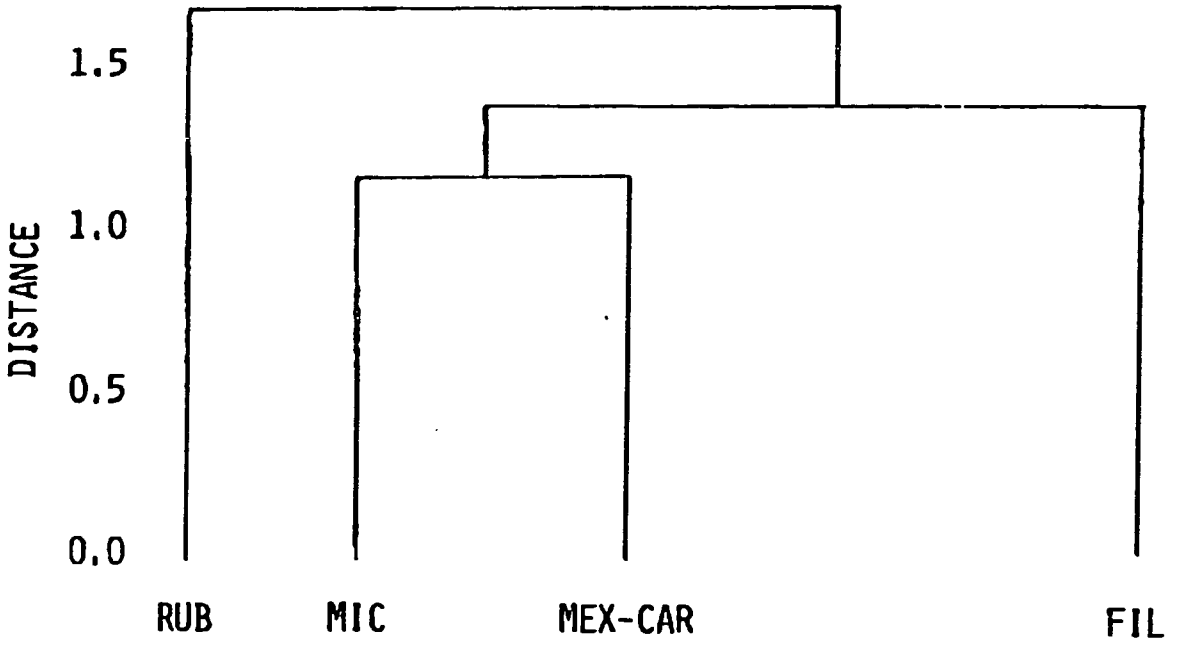


Figure 4.

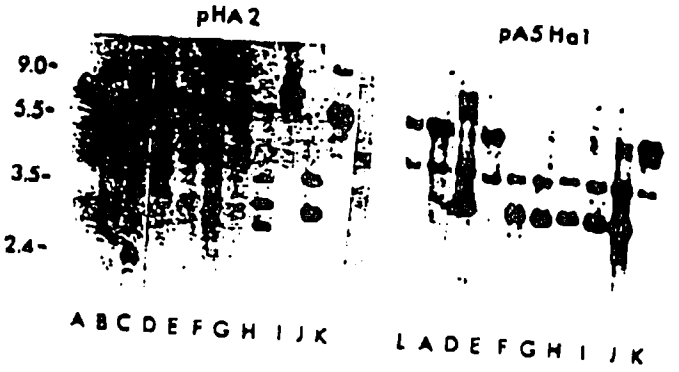
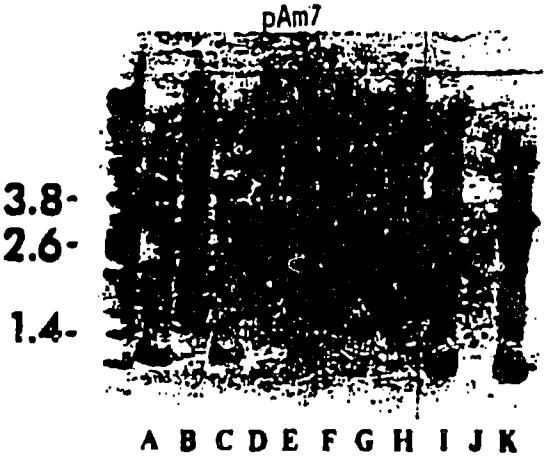


Figure 5.



TIME SCHEME
August, 1986 - February, 1987
Start date of grant, but no commencement of research*

February, 1987
Start date of new project director

February, 1987 - March, 1987
Outfitting of laboratory for research

April, 1987 - August, 1988
Enzyme research

August, 1988 - February, 1989
Leave of absence by project director

February, 1989 - August, 1990
DNA research; includes one-year no-cost extension

*Dr. Narayanan, original project director, had accepted permanent employment elsewhere; position advertisement was conducted and miscellaneous funds allocated.

TIME PERCENTAGES of participants and explanation of time breaks

I. Watanabe (5%)
T. Lumpkin (1%)
W. Zimmerman (100%)**

**Prior to leave of absence (to learn molecular protocols for Azolla) a percentage of time dealt with such non-project duties such as manuscript and proposal reviews (in the name of T. Lumpkin), a review chapter, and grant proposals with T. Lumpkin for Azolla research.

DISSEMINATION OF TECHNIQUES AND RESULTS

Technical training of Ben Padre (technician, Soil Microbiology Department, International Rice Research Institute [IRRI]) at Washington State University. March-Aug., 1987.

Third Research Coordination Meeting on "Isotopic Studies on Nitrogen Fixation and Nitrogen Cycling of Blue-Green Algae and Azolla". Food and Agricultural Organization of the United Nations (FAO)/International Atomic Energy Agency (IAEA)/Swedish International Development Agency (SIDA). Fujian Academy of Agricultural Sciences (FAAS), Fuzhou, Fujian, People's Republic of China. Nov. 2-6, 1987.

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