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Classification of *Azolla* spp., section Azolla

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Summary

Azolla accessions (section Azolla) from the germplasm collections of the International Rice Research Institute and Washington State University were fingerprinted and classified by enzyme electrophoresis and leaf trichome morphology. *A. filiculoides* was enzymatically distinctive and also reliably identified by its prominent one-celled trichomes. Neotropical accessions labelled as *A. filiculoides* proved to be members of other species. Two groups of isolates were designated *A. rubra,* but those from Japan were identified as *A. filiculoides.* The *A. rubra* of Australia-New Zealand was biochemically unique and possessed less protuberant trichomes than *A. filiculoides. A. microphylla, A. mexicana,* and *A. caroliniana* were phenetically similar, but *A. microphylla* was identifiable from the others in the banding patterns of certain enzymes. *A. mexicana* and *A. caroliniana* were closely related enzymatically. The two-celled leaf trichomes of these three species were similar in size and shape.

Introduction

Azolla Lam. is an aquatic fern which lives symbiotically with the nitrogen-fixing cyanobacterium *Anabaena azollae* Strasb. As a consequence of this diazotrophic association, *Azolla* is utilized as a nitrogen biofertilizer with irrigated rice crops in some regions. One major limitation to research has been the inability to properly identify ecotypes and species of this pteridophyte. A definitive classification does not exist.

Specific identification by vegetative features (Svenson, 1944) is often imprecise because of the plasticity of this genus. Leaf trichome morphology is helpful in some instances (Van Oostroom, 1948; Lumpkin & Plucknett, 1982). Identification of *Azolla* species by reproductive structures (Perkins et al., 1985) is difficult since accessions in germplasm collections rarely sporulate. None of these methods serve to describe ecotypes or subspecies.

This problem is particularly evident in section (subgenus) Azolla, which contains five of the seven extant species. These taxa differ from those of section Rhizosperma in the number of float corpuscles (accessory reproductive structures homologous to massulae) per megasporocarp, type of glochidia on microsporic massulae, and the branching patterns of fronds. Four of the five species are indigenous New World taxa and three have broad geographic ranges (Table 1).

The intent of this study was two-fold. The first objective was to initiate fingerprinting of accessions for indexing purposes. The second was to gain insight on the enzyme characteristics which typify each species or subspecies, and to provide information for classification of species by their chemotaxonomic affinities. The utility of this scheme was complemented by trichome data.

Materials and methods

Enzyme electrophoresis

Fifty-seven accessions of *Azolla* from the germplasm collections of the International Rice Research Institute (IRRI) and Washington State University were characterized. *Azolla* growth conditions, leaf enzyme extractions, electrophoretic protocol, and staining techniques have been previously described (Zimmerman et al., 1988). Starch gels were used in place of polyacrylamide gels to increase band staining intensities for two enzymes, triosephosphate isomerase and aspartate aminotransferase.

Twelve enzymes were stained-aldolase (ALD, EC 4.1.2.13), aspartate aminotransferase (AAT, EC 2.6.1.1), fructose-1,6-diphosphatase (F1,6DP, EC 3.1.3.11), $NAPD^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (G3PDH, EC 1.2.1.12), isocitrate dehydrogenase (IDH, EC 1.1.1.42), phosphoglucoisomerase (PGI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 2.7.5.1), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), shikimate dehydrogenase (SKDH, EC 1.1.1.25), triosephosphate isomerase (TPI, EC 5.3.1.1), xanthine dehydrogenase (XDH, EC 1.2.1.37), and an unnamed (negative-staining, non-substrate-specific) dehydrogenase.

Electron microscopy

Fronds from 35 accessions were fixed for two hours in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). The fixed tissue was rinsed three times, ten minutes each time, in phosphate buffer and postfixed for two hours in 1% osmium tetroxide at 4° C. Following two distilled water rinses of five minutes each, material was dehydrated in ethanol to 100%. Frond tissue from each accession was then dried in a Bomar 1500 critical point dryer using carbon dioxide, mounted, and sputter coated with gold (Technics Hummer sputter coater). Trichomes on dorsal lobes of prepared frond samples were then examined with a Hitachi S5-70 scanning electron microscope (SEM).

Table 1. Taxonomy and distribution of extant *Azolla* (modified after Watanabe, 1982)

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

Classification

Allelomorphic data were analyzed with the UPGMA average linkage clustering method, and a phenogram produced from a similarity matrix (Sneath & Sokal, 1973). Principal component analysis (PCA) of the covariance data matrix was calculated to clarify phenetic relationships which were distorted at higher clustering levels in the forced hierarchy of the phenogram (Sneath & Sokal, 1973). Identification of species by leaf trichome morphology was completed using the trichome key of Lumpkin & Plucknett (1982).

Results and discussion

The number of observed isozymes reflects the fact that *Azolla* is a heterosporous diploid fern (n = 22 or 24) in a monotypic family within Salviniales, unlike the numerous homosporous polyploid pteridophytes of Filicales (Moore, 1969; Wagner & Wagner, 1980). Nine enzymes contained a total of 17 polymorphic loci and 126 allelomorphic characters; three enzymes (ALD, G3PDH, F1,6DP) appeared to be monomorphic. At least two loci each were present for SKDH and 6PGD. Allelomorphic frequencies for nine putative loci in 32 accessions are listed in Table 2. Preceding the tabulation of those frequencies, strains of erroneous classification were eliminated and *A. rubra* was reorganized (as explained in the following sections).

Fingerprinting

Benefits of fingerprinting *Azolla* germplasm include verification of duplicates, monitoring of any somaclonal change, and prevention against accidental mislabelling or cross-contamination. To assist in cataloguing isolates, a system was developed which partially discriminated among the five species in section Azolla by simple visual comparisons of relative band migrations. The R_f values of PGM-2 were a reliable indicator for *A. microphylla* and *A. rubra* (Fig. 1). IDH served the same role for conspecific accessions of *A. filiculoides* (Fig. 2).

Zymogram results revealed the incorrect labelling of some accessions, particularly those assigned to the 1000 series of the IRRI accession code (tentative *A. filiculoides* designations). Several tropical South American strains within the 1013–1027 series fingerprinted as another species or even as possible hybrids between *A. microphylla* and *A. mexicana* or *A. caroliniana*. At this time, however, there is no further proof to support a concept of natural hybridization. Accession numbers within that series have now been changed.

Mislabelled Colombian accessions had already been suspect, and had even been previously identified by the collector as *A. caroliniana* (Zimmerman, 1984). Brazilian strains were likewise under question. In addition to isoenzyme evaluations, SEM observations showed that the fronds of these accessions possessed two-celled leaf trichomes (e.g., Fig. 3a), which are not characteristic of *A. filiculoides*. This species and *A. rubra* are distinguished by their one-celled leaf trichomes (Fig. 3b–d), unlike the other taxa of section Azolla which possess trichomes of at least two cells (Fig. 3e–g).

The duplicated entry of certain strains under separate accession numbers was also confirmed (e.g., 1014/1027, 1005/1006/SWD, 1010/1016). Conversely, slightly dissimilar enzyme results were found for two cultures of a purportedly single strain – 3503 from the IRRI collection and the same accession maintained at WSU under its original germplasm code of ADUL 43. This accession had been obtained earlier by both laboratories from the original collection kept by Prof. C. Van Hove (Université Catholique de Louvain).

Accessions 2001 (*A. mexicana*) and 3001 (*A. caroliniana*), considered as the 'typical' specimens of their species by IRRI (i.e., their life histories had been followed and confirmed), exhibited atypical allozyme/allelomorphic characteristics for their species. Accession 3001 was very similar enzymatically to 1026 (both were collected in the United States). One-celled leaf trichomes were found in both isolates (Fig. 3h, i), but they have also been documented with occasional two-celled trichomes in mature fronds (unpublished results). Neither exhibited enzyme patterns characteristic of *A. filiculoides*.

Table 2. Frequency of electromorphic alleles from 32 accessions of *Azolla* species, section Azolla

Locus	Allele	Designated Species				
		filiculoides	*caroliniana*	*microphylla*	*mexicana*	*rubra*
PGI-1	a	1.00	1.00	0.64	0.67	
	b			0.27	0.33	
	c			0.09		
	d					1.00
PGM-1	a	0.42				
	b			0.20		
	c	0.58				
	d		0.67	0.20	0.60	
	e		0.33		0.20	
	f			0.10		
	g					0.33
	h					0.67
	i			0.50	0.20	
PGM-2	a	0.50				
	b	0.50				
	c					1.00
	d				0.17	
	e				0.17	
	f		1.00		0.50	
	g				0.17	
	h			0.09		
	i			0.82		
	j			0.09		
IDH	a		0.86	0.82	0.50	
	b		0.14	0.09	0.17	
	c				0.33	
	d			0.09		
	e	0.17				
	f	0.83				1.00
XDH	a		0.83		0.66	
	b			0.10		
	c		0.17	0.20		
	d	1.00		0.60	0.17	
	e			0.10		
	f				0.17	0.67
	g					0.33
AAT-3	a	0.75			0.33	
	b	0.25				
	c		0.29			
	d		0.71	1.00	0.67	1.00
TPI-1	a		1.00		1.00	1.00
	b	1.00		1.00		
TPI-2	a			1.00	1.00	
	b	1.00	1.00			0.33
	c					0.67
TPI-3	a	1.00				
	b					0.67
	c		1.00	1.00	1.00	
	d					0.33

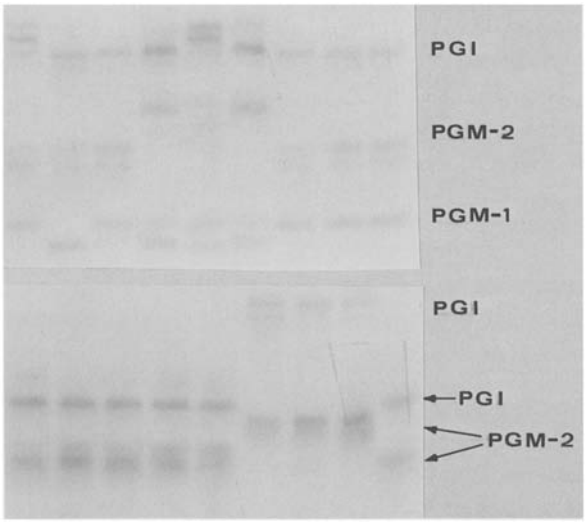

Fig. 1. A) PGI/PGM zymogram comparing accessions of *A. microphylla* with *A. filiculoides* (1001), *A. caroliniana* (3001), and questionable *A. rubra* (6003) (located in the three center lanes). B) PGI/PGM zymogram comparing questionable *A. rubra* accessions with *A. rubra* from Australia-New Zealand (2nd–4th lanes from the right).

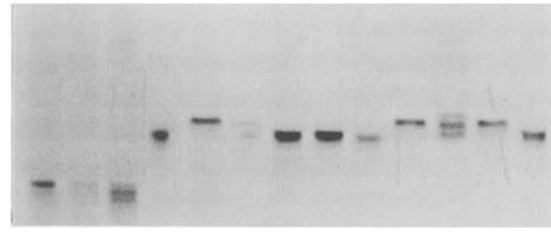

Fig. 2. IDH zymogram comparing accessions of *A. filiculoides* and questionable *A. rubra* (first three lanes from the left) with accessions from the three other species.

Classification

A proposed phenogram (Fig. 4) was composed from the initial cumulative cluster analysis by grouping all apparently conspecific accessions. The inherent difficulties in species separation are illustrated in the principle component analysis (PCA). Three species – *A. microphylla, A. caroliniana,* and *A. mexicana* – clustered closely and were not easily defined (Fig. 5). The combination of principal components I and III showed better phenetic separation than components I and II, and they represented only 18.6% of the total variation of the correlation matrix.

A. filiculoides was the most easily discernible of the five species by its zymograms. *A. filiculoides* differed from *A. rubra,* the other distinctive species, through its enzymes and by its leaf trichomes which are more prominent relative to the other epidermal cells according to the trichome key. However, some accessions of *A. filiculoides* appeared to have trichomes intermediate in cell size (e.g., no. 1010; not pictured).

A. rubra, sometimes classified as a variety of *A. filiculoides* (Svenson, 1944; Seto & Nasu, 1975), was partitioned into two subgroups. The subgroup of accessions from Japan (also once named *A. japonica* Fr. & Sav.; Tuzimura et al., 1957) was almost identical enzymatically to *A. filiculoides* and should be thus labelled. The subgroup of *A. rubra* from Australia-New Zealand was phenetically unique (Fig. 5). *A. filiculoides* and *A. rubra* (Aus-NZ) differed in allelic comparisons only slightly less to each other than to the other species (Table 3).

The leaf trichome structures of these accessions confirmed this evaluation (Fig. 3b–d). We suggest that the *Azolla* collected in Japan was *A. filiculoides* and may have been introduced into that country. Sporophytic similarity between the Japanese strains and New World *A. filiculoides* has been previously noted (Moore, 1969), although one study reported discrepancies between the two groups (Lin, 1980). On the other hand, the development of distinctive flora in the geographically isolated Australian continent is a known phenomemon. This would ostensibly include *A. rubra,* and the indigenous *A. pinnata.* The lack of true *A. rubra* accessions from Japan in our collections does not rule out the possibility of the existence of this species in that country.

No appreciable phenetic distance was found between *A. microphylla* and the *A. mexicana-A. caroliniana* group, and some accessions overlapped (Fig. 5). Most isolates from these species sorted into two separate, adjacent entities. The relative similarity among these species is apparent from their allelic data summarized in Table 3. This closeness also corroborates the fact that Svenson's

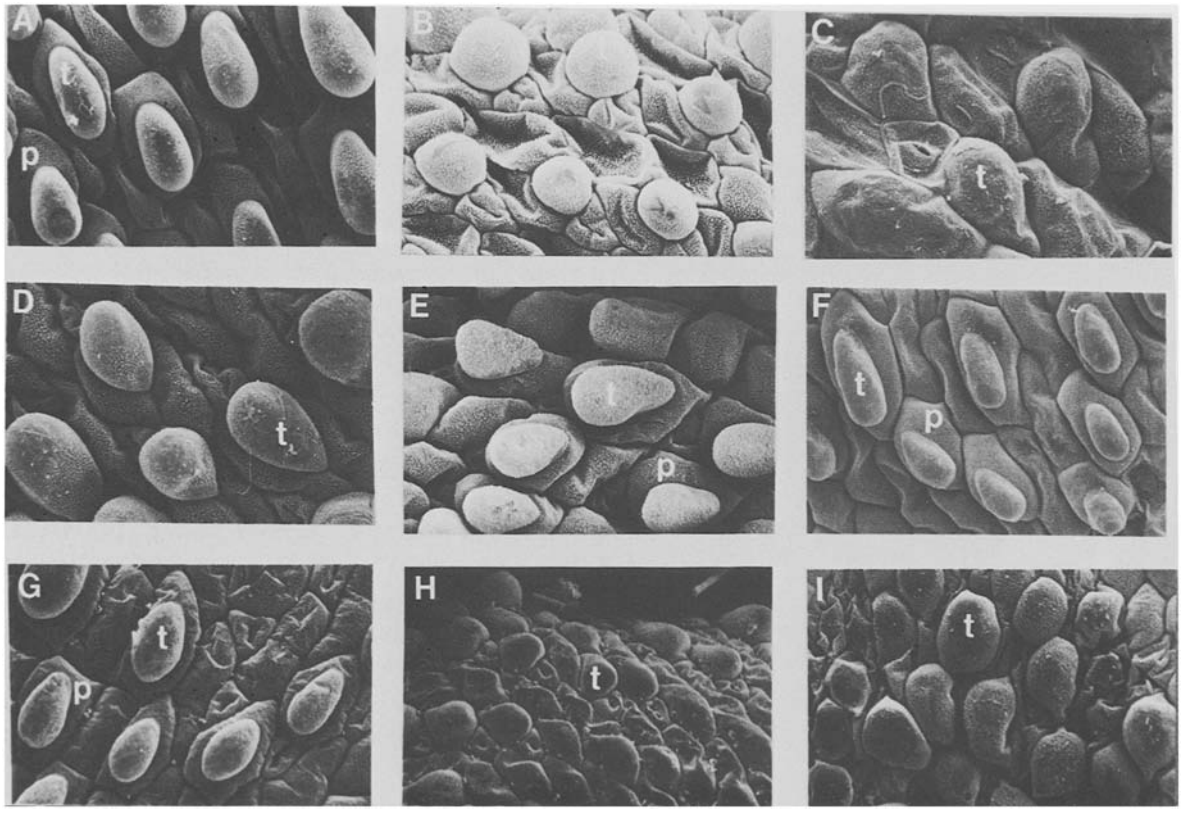

Fig. 3. Leaf trichomes of the five species of section Azolla: A) questionable *A. filiculoides* (1023), B) *A. filiculoides* (1005), C) *A. rubra* (6502), D) questionable *A. rubra* (6003), E) *A. mexicana* (2002), F) *A. caroliniana* (ADUL 45), G) *A. microphylla* (4001), H) *A. caroliniana* (3001), and I) *A. filiculoides* (1026). t = apical trichome cell, p = pedicel cell.

(1944) modern botanical definitions of *A. microphylla, A. caroliniana,* and *A. mexicana* are just a newer interpretation of the amalgamated *A. caroliniana* created by Mettenius (1867) from several morphologically similar taxa.

Leaf trichomes of these three species resembled each other. All of our accessions exhibited immature growth morphology under laboratory maintenance conditions, so the number of trichome cells in any of these species did not exceed two cells. The *A. mexicana-A. caroliniana* group, according to the trichome key, should be distinguished by a broad pedicel cell. This diagnostic character was only partially effective in that those accessions with broad pedicel cells were always members of *A. mexicana* or *A. caroliniana* and never *A. microphylla.* However, apical cells were often similar in size among the three species.

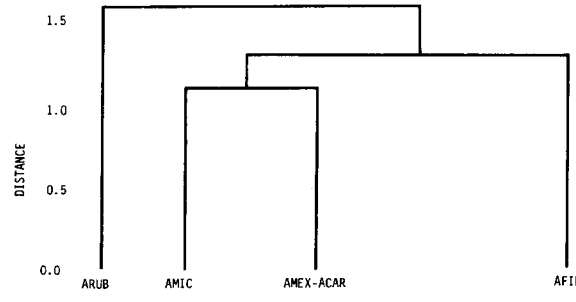

Fig. 4. Phenogram of *Azolla* species, section *Azolla,* as derived from cluster and PCA analyses. Cophenetic correlation coefficient = 0.901. (I) AFIL = *A. filiculoides,* (II) ARUB = *A. rubra,* (IIIa) AMIC = *A. microphylla,* (IIIb) AMEX-ACAR = *A. mexicana-A. caroliniana.*

Our determination that *A. caroliniana* is unrelated to *A. filiculoides* conflicts with conclusions from a study of megaspore type descriptions by Dunham & Fowler (1987), based predominantly on herbarium specimens. They stated that *A. caroliniana* was synonymous with *A. filiculoides*. Tan et al. (1986) suggested that *A. caroliniana* could be made synonymous with either *A. filiculoides* because of its aseptate glochidia (Svenson, 1944) or *A. microphylla* because of the filamentose surface of the megasporoderm (Perkins et al., 1985). The classification of *A. caroliniana* is further confused by the lack of naturally sporulating strains for additional documentation. Tan et al. induced sporulation in a few accessions from IRRI's 3000 series (designated *A. caroliniana*), which are primarily Brazilian isolates, and identified them by megasporocarp structures to be *A. microphylla* or *A. mexicana*. They and Dunham & Fowler both proposed that *A. caroliniana* be eliminated as a species.

If we chose to follow this rationale together with our results, *A. mexicana* and *A. caroliniana* are noted in Table 3 to have more loci with identical alleles (27%) than do any other pair of species, and have a low percentage of loci with no shared alleles (20%). A reinterpretation of the results illustrated in Figs. 4 and 5 would then suggest the following four clusters or suspected species: (I) *A. filiculoides*, (II) *A. rubra*, (IIIa), *A. microphylla*-like, and (IIIb) *A. mexicana*-like. The initial and final categorizations of the examined accessions (our cluster IIIb represents *A. mexicana-A. caroliniana*) are given in Table 4.

While our results strongly indicate little affinity between *A. filiculoides* and the controversial *A. caroliniana*, and present new evidence for *A. rubra* as a separate species while possibly combining *A. mexicana* and *A. caroliniana*, more research is required. Excluding certain reproductive features, no one classification scheme is accepted universally. A combination of biochemical, anatomical, and physiological information is evidently necessary for accurate classification with current technology.

For example, the response to phosphorus starvation may be useful to delineate accessions or species (unpublished results). Tolerance to stressful high temperatures is often another useful parameter for discrimination. In a separate series of experiments conducted at IRRI (IRRI, 1987, and unpublished results), screening of accessions from the 1000 series by stepwise temperature elevation (up to 33° C) revealed that neotropical accessions (1013–1027) of the 1000 series were tolerant to high temperatures while others were not. Since valid *A. filiculoides* and *A. rubra* are sensitive to high temperatures (Lumpkin & Plucknett, 1982), this finding correlates well with the results from our zymograms and leaf trichome morphology which demonstrated that these strains belong to other species.

In conclusion, this report does not address the question of the relative effects of human cultural practices vs. natural evolutionary causes on differences among *Azolla* taxa, but does assist in compartmentalizing groups of *Azolla*. *A. filiculoides*, *A. rubra*, and *A. microphylla* can be recognized via their enzymes. This biochemical assessment intends to serve as a 'working taxonomy' for those who utilize this fern as a field-grown biofertilizer in rice paddies. Our continuing work on DNA and direct genetic variation should further clarify the taxonomic situation of *Azolla*.

Table 3. Proportion of loci among *Azolla* species which share no alleles (above the diagonal) or share all alleles (below the diagonal). The number of unique alleles is given in the last column

Designated species	*filiculoides*	*caroliniana*	*microphylla*	*mexicana*	*rubra*	Unique alleles
filiculoides	–	0.87	0.67	0.80	0.60	11
caroliniana	0.13	–	0.33	0.20	0.73	7
microphylla	0.07	0.07	–	0.13	0.73	18
mexicana	0.00	0.27	0.13	–	0.67	10
rubra	0.07	0.07	0.07	0.07	–	9

Table 4. Classification of *Azolla* accessions

Accession code	Preliminary species designation	Origin	Cluster
1001[a]	*A. filiculoides*	Germany (DDR)	I
1005		Germany (FDR, Hamburg)	I
1006		Germany (FDR, Hamburg)	I
1010		Peru (Lima)	I
1013		Brazil (Parana)	IIIb
1014		Colombia (CIAT)	(?)
1015		Japan (Osaka)	IIIb
1016		Peru (Lima)	I
1017		Colombia (Monteria)	IIIa(?)
1021		Colombia (Leticia)	IIIa/b
1023		Colombia (Leticia)	IIIa/b
1025		Colombia (Cartagena)	IIIa(?)
1026		USA (Florida)	(?)
1027		Colombia (CIAT)	(?)
Pasco		USA (Washington)	I
SWD		Sweden (collection)	I
2001[a]	*A. mexicana*	USA (California)	IIIb
2002		Guyana	IIIb
2003		Guyana	IIIb
2004		Guyana	IIIb
2007		USA (collection)	IIIb
60		USA (California)	IIIa(?)
3001[a]	*A. caroliniana*	USA (collection)	(?)
3006		Brazil (Amazonas)	IIIb
3007		Brazil (Amazonas)	IIIb
3008		Brazil (Amazonas)	IIIb
3009		Brazil (Para)	IIIb
3010		Brazil (Amazonas)	IIIb
3011		Brazil (Amazonas)	IIIb
3012		Brazil (Rio Grande do Sul)	IIIa/b
3014		Brazil (Amazonas)	IIIb
3015		Brazil (Rio Grande do Sul)	IIIa
3503		Brazil (Para)	IIIb
ADUL43		Brazil (Para; equiv. to 3503)	IIIa/b
ADUL45		Brazil (Amazon; equiv. to 3505)	IIIb
CNPAF57		Brazil (Sta. Cta; equiv. to 3016)	(?)
UYCC2		Uruguay (Treinta-y-tres)	IIIb
USCC1		USA (collection)	IIIb
4001	*A. microphylla*	Paraguay	IIIa
4003		Paraguay	IIIa
4009		Paraguay	IIIa
4014		Paraguay	IIIa
4018[a]		Paraguay	IIIa
4021		Equador (Santa Cruz Is.)	IIIa
4022		Philippines (origin 4018)	IIIa
4024		Equador (Galapagos Is.)	IIIa
4032		Philippines (China collection)	IIIa
4033		Philippines (*filiculoides* symbiont; origin 4032)	IIIa
4035		Philippines	IIIa

Table 4. Continued

Accession code	Preliminary species designation	Origin	Cluster
6003	*A. rubra*	Japan (Kyoto)	I
6005		Japan (Kyoto)	I
6006		Japan (Osaka)	I
6007		Japan (Furuoka)	I
6008		Japan (Matsue)	I
6501[a]		New Zealand	II
6502		Australia (Victoria)	II
6503		New Zealand	II

[a] Accessions identified and used as 'typical' members of their respective species.

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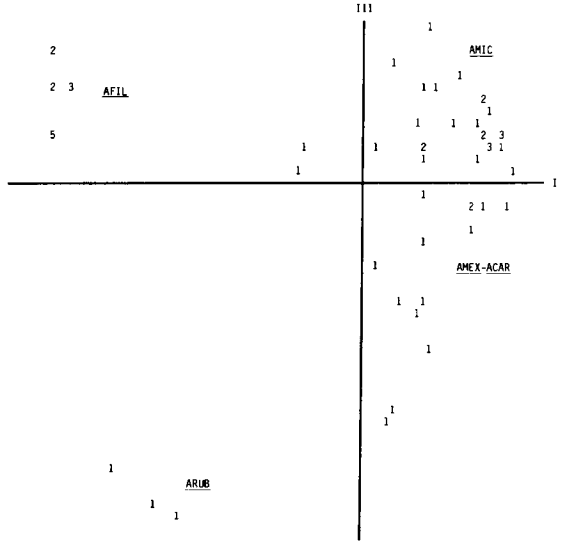

Fig. 5. Projection of the 57 populations of *Azolla* onto the first and third principal components. (I) AFIL = *A. filiculoides,* (II) ARUB = *A. rubra,* (IIIa) AMIC = *A. microphylla,* (IIIb) AMEX-ACAR = *A. mexicana-A. caroliniana.*

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