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PHYLOGENETIC RELATIONSHIPS IN THE HETEROSPOROUS FERN GENUS *AZOLLA* (AZOLLACEAE) BASED ON DNA SEQUENCE DATA FROM THREE NONCODING REGIONS

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The taxonomic history of *Azolla* (Azollaceae) is long and complex. Previous studies have employed morphological, cytological, and molecular data in an attempt to circumscribe the seven extant species, but none has been completely successful. In this study, we employ DNA sequence data from three noncoding regions, two derived from the plastid genome (the *atp*B-*rbc*L and *trn*L-*trn*F regions) and a third from the internal transcribed spacers of the nuclear rRNA genes. Cladistic analyses of these data confirm the division of *Azolla* into two major clades, corresponding to the traditional classification of the genus into sections *Azolla* and *Rhizosperma*. Moreover, the monophyly of *A. pinnata* plus *A. nilotica* (sect. *Rhizosperma*) contradicts newer classifications of the family, in which these species were placed in different subgenera. In section *Azolla*, DNA sequence data support several past reports in suggesting that *A. rubra* and *A. filiculoides* are distinct species and that *A. caroliniana* is distinct from both *A. microphylla* and *A. mexicana*. However, distinct lineages representing *A. microphylla* and *A. mexicana* were not found, and these plants appear to represent a single evolutionary lineage.

Keywords: *Azolla*, heterosporous ferns, ITS, *atp*B-*rbc*L, *trn*L-*trn*F.

Introduction

The species of *Azolla* are all aquatic, heterosporous ferns distributed throughout the temperate and tropical regions of both the New and Old World (fig. 1). Classification of this genus varies widely (see fig. 2), but the most commonly accepted system is that of Svenson (1944), who recognized seven species in two sections (sects. *Azolla* and *Rhizosperma*, following Mettenius [1867]) on the basis of morphological discontinuities. Species native to the Old World represent members from both sections, including *A. pinnata* and *A. nilotica* from section *Rhizosperma*, and *A. rubra* from section *Azolla*. Native New World species include members from only section *Azolla* (viz., *A. filiculoides*, *A. mexicana*, *A. microphylla,* and *A. caroliniana*). Although *A. filiculoides* is native only in North and South America, the fossil record indicates that it was present in Europe before the last glaciation, and more recent introductions have led to its naturalization in southern Africa, eastern Asia, Europe, Australia, and parts of the southwest Pacific (Moore 1969). In the Neotropics and subtropics, there is a zone of overlap where all four native species co-occur that encompasses most of Central America and parts of northern South America (fig. 1).

Difficulties in the identification and consequently the classification of *Azolla* stem from the phenotypic similarity among all seven species, particularly when sterile. Most taxonomic systems focus primarily on reproductive characters of *Azolla*, but these structures are rarely present in nature and are often difficult to induce in culture. Cytology has proven taxonomically uninformative because all species exhibit $2n = 44$ except *A. nilotica*, with $2n = 52$ (Stergianou and Fowler 1989, 1990). The few vegetative characters that exhibit variation have proved too subtle to delineate species. The morphological similarity of *Azolla* species, together with their diminutive stature, have led to a long history of mistaken identifications, some of which have added to the taxonomic confusion. For example, Mettenius (1847), who published the first monograph of *Azolla,* carefully circumscribed each of the known species (fig. 2) but failed to use type materials in his descriptions and illustrations. Because the type specimen of *A. caroliniana* is sterile, Mettenius (1847) used fertile samples for his drawings of reproductive structures. Mettenius's (1847) illustrations of *A. caroliniana* were subsequently used by several other authors, including Strasburger (1873), who emerged as one of the leading authorities on *Azolla* taxonomy. Svenson (1944), however, believed that the samples used for these illustrations represented not *A. caroliniana* but *A. mexicana*, which may explain the taxonomic confusion that persists between these two species. Such examples abound in *Azolla* and are reflected in the complex synonymy of the genus (Evrard and Van Hove 2004) (table 1). Moreover, the confusion surrounding species-level taxonomy has been compounded by debates regarding generic classifications and the relationship of *Azolla* to other heterosporous ferns (Brown 1810; DuMortier 1829; Meyen 1836; Mettenius 1847; Baker 1887; Wettstein 1903; Saunders and Fowler 1993).

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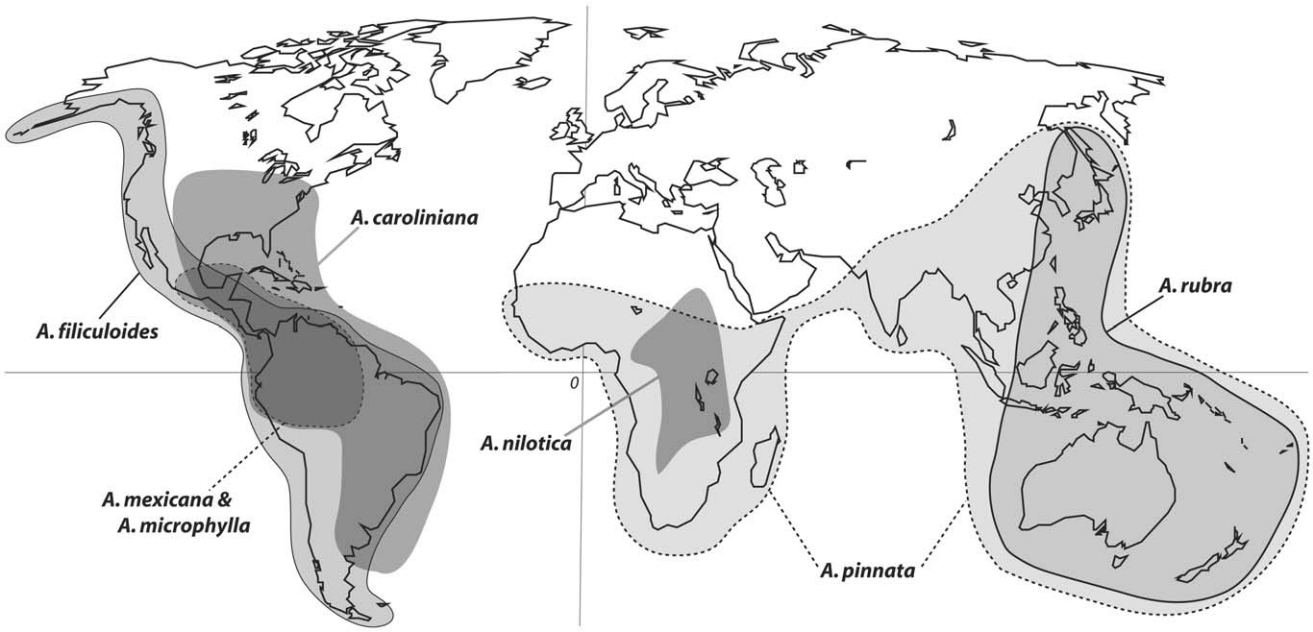

Fig. 1 Native ranges of the seven currently recognized species of *Azolla*. Note that *A. filiculoides* has been introduced into northern and central Europe, southern Africa, eastern Asia, Australia, and New Zealand; *A. caroliniana* has been introduced into western Europe (Moore 1969).

In an attempt to clarify the classification and phylogeny of *Azolla*, data from morphology (Svenson 1944), megasporocarp ultrastructure (Perkins et al. 1985), cytology (Nayak and Singh 1989), and molecular characters (Zimmerman et al. 1989) have been used. However, a coherent picture of species circumscriptions and relationships has not emerged. Currently, most authors accept seven species placed in two sections, differentiated by the number of megaspore floats (Moore 1969; but see Saunders and Fowler 1993). *Azolla* section *Rhizosperma* is characterized by nine floats on its megaspore and includes *A. pinnata* and *A. nilotica*. Section *Azolla*, with three floats, includes all remaining species (fig. 2). The major taxonomic questions that persist include (1) the distinction between *A. rubra* and *A. filiculoides* (Zimmerman et al. 1989); (2) the status of *A. caroliniana*, *A. microphylla*, and *A. mexicana* as distinct species (Mettenius 1867; Dunham and Fowler 1987; Zimmerman et al. 1989); and (3) the placement of *A. nilotica* together with *A. pinnata* in section *Rhizosperma* (as opposed to the alternative placement of *A. nilotica* in a monotypic subgenus, as proposed by Saunders and Fowler [1993]).

We employ DNA sequence data to clarify phylogenetic relationships among the seven species of *Azolla* and to shed light on problems that may be the source of the historical taxonomic difficulties. No previous molecular studies of *Azolla* have employed either cladistic analyses or DNA sequence data. As was done for other recent studies of interspecific relationships in ferns (Hoot and Taylor 2001; Van den Heede et al. 2003), we chose noncoding sequences for use in our study because they hold the greatest potential for exhibiting interspecific variation. Our study incorporates data from three noncoding regions (each roughly 1 kb in length), including the *trn*L-*trn*F region (the *trn*L intron and *trn*L-*trn*F intergenic spacer [IGS]) and the *atp*B-*rbc*L IGS of the chloroplast, as well as the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. This sampling provides data from two separate genomes (nuclear and chloroplast) and three types of noncoding sequences (introns, transcribed spacers, and nontranscribed spacers). Sequence data also provide a complement to earlier phenetic studies based on RAPD and RFLP data (Chen et al. 2003; Van Coppenolle et al. 1995), but without some of the potential difficulties associated with those techniques (most notably the difficulty in distinguishing the genomic source of mutations when using total DNA extractions, a significant concern in plants containing endosymbionts; Chen et al. 2003).

Material and Methods

At least two accessions, representing different geographic source locations, were sampled from each of the seven currently recognized species of *Azolla*, with the exception of *A. nilotica*, for which only a single accession was available. Plants were obtained from cultures maintained by G. A. Peters on nitrogen-free liquid medium (IRRI medium; Peters et al. 1980) under a 16L : 8D regime and a photon flux density of 150–180 μmol/m^2/s (provided by fluorescent and incandescent bulbs). Original field sources of these plants are detailed in table 2, together with voucher specimen information. Before DNA extraction, living cultures were propagated clonally from a single frond. In addition, three accessions of endophyte-free plants, representing both sections *Azolla* and *Rhizosperma*, were generated by an apical cutting procedure (G. A. Peters, personal communication). A commercially available species of *Salvinia* (Carolina Biological Supply) was sampled for outgroup comparison based on evidence from

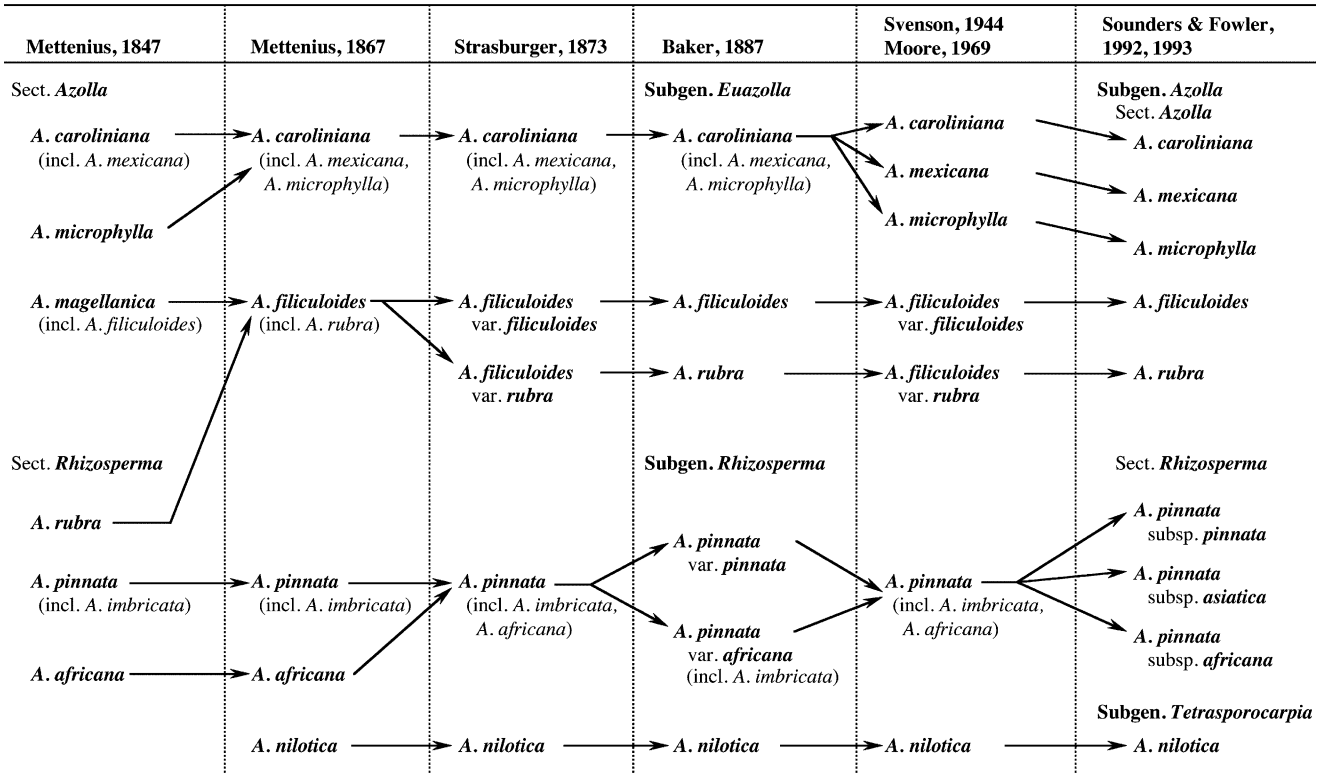

Fig. 2 Synopsis of major taxonomic treatments of *Azolla*.

characters of sporocarp and embryo development (Campbell 1893) and molecular data (Pryer 1999), which indicate that *Salvinia* is the closest extant relative to *Azolla*.

DNA was extracted using the DNeasy extraction kit (Qiagen), following the manufacturer's instructions. Target sequences were PCR-amplified in 50-μL reactions containing the following components: 5.0 μL of 10$\times$ reaction buffer, 2.0 μL of 25-mM $MgCl_2$, 4.0 μL of 10-mM dNTPs, 2.5 μL each of forward and reverse primers (5 μM), 0.75 units of Qiagen *Taq*, 4.0 μL of unquantified total DNA, and sterile water to the final volume. The PCR cycling program for ITS included 40 cycles at 94°C for 30 s, at 48°C for 1 min, and at 68°C for 1 min. For the two chloroplast regions, PCR parameters were set to 35 cycles at 94°C for 1 min, at 49°C for 1 min, and at 72°C for 2 min. Negative controls were performed for each round of PCR to test for contamination. Primers for all PCR reactions are provided in table 3.

After purification using the QIAquick PCR cleanup kit (Qiagen) and visual quantification using agarose gel electrophoresis, PCR amplicons were sequenced directly. Cycle sequencing reactions were carried out on both (complementary) strands using primers (see table 3) and the BigDye Terminator reaction kit (PE Applied Biosystems), according to the manufacturer's instructions. Samples were purified using the DyeEx spin kit (Qiagen) and separated electrophoretically on an ABI Prism 377 DNA sequencer (PE Applied Biosystems). Resulting sequences were assembled and edited using Sequencher (ver. 4.1, GeneCodes). Length mutations were rare, facilitating visual alignment.

Each of the three molecular data sets was analyzed separately using PAUP* 4.0 (Swofford 2002) with both maximum parsimony (MP) and maximum likelihood (ML) methods. Equally weighted parsimony analyses employed branch-and-bound searches (with ACCTRAN optimization and MULPARS in effect), treating gaps as missing data. To estimate support of individual clades, bootstrap (BS) analyses (Felsenstein 1985) were completed using PAUP*. Each BS analysis employed 1000 replicates using branch-and-bound searches. ML searches employed the GTR + Γ + I model, determining the base frequencies empirically and estimating both the substitution model and the gamma distribution. MacClade 4 (Maddison and Maddison 2000) and/or PAUP* were used to assess homoplasy, transition to transversion ratios, nucleotide composition, and pairwise distances (estimated using the Kimura three-parameter algorithm). The three sequence regions were first analyzed separately. Before they were combined into a single analysis, a partition homogeneity test (Farris et al. 1995) was performed to assess congruence.

Because of the symbiotic association between *Azolla* and its cyanobacterium, there was concern that some sequences may have been derived from the cyanobiont rather than from the fern. Although our method of DNA extraction is not expected to disrupt the prokaryotic capsule (thereby leaving the cyanobacterial cells intact), we employed two tests to ensure that our sequences were derived only from the ferns. First, we sequenced three cyanobiont-free accessions from section *Azolla* (*A. microphylla, A. mexicana,* and *A. caroliniana)* for comparison with samples derived from plants growing in

Table 1

The Seven Currently Recognized Species of *Azolla* along with Their Synonyms and/or Other Names Associated with These Species in Early Classifications

Currently recognized species	Synonyms or other associated names
A. caroliniana Willd.	*A. densa* Desv.
	A. mexicana Presl
	A. mexicana Schlect.
	A. portoricensis Spreng.
	A. bonariensis Bertol.
	A. cristata Kaulf.
	A. microphylla Kaulf.
	Salvinia azolla Raddi
A. filiculoides Lam.	*A. magellanica* Willd.
	A. arbuscula Desv.
	A. rubra R. Br.
	A. squamosa Molina
	A. bonariensis Bertol.
A. microphylla Kaulf.	*Salvinia azolla* Raddi
A. mexicana Presl	*A. caroliniana sensu* Mett. non Willd.
	A. densa Desv.
A. rubra R. Br.	*A. filiculoides* Lam.
A. pinnata R. Br.	*Salvinia imbricata* Roxb.
	Rhizosperma pinnata (R. Br.) Salomon
	A. imbricata Desv.
	A. guineensis Schum.
	A. decomposita Zoll.
	A. japonica Franch. et Sav.
	A. imbricata (Roxb. ex Griff.) Nak.
A. nilotica Decne. ex Mett.	none

Note. Data are from Saunders and Fowler (1993) and Evrard and Van Hove (2004).

association with the cyanobiont. Sequences derived from symbiotic and endophyte-free accessions were identical or nearly identical for each species tested. As a second test, we compared sequences derived from endosymbiont-containing plants against all sequences in the GenBank database. In all cases, putative fern sequences most closely matched those of other land plants (both bryophytes and vascular plants) and not those of prokaryotic sequences.

Results

atp*B*-rbc*L IGS*. The aligned sequence length of the entire intergenic spacer region between *atp*B and *rbc*L (plus flanking regions of the genes) was 1133 bp, ranging in length from 1083 to 1096 bp among the 13 accessions (and all seven species) sampled from *Azolla*. In *Salvinia*, the *atp*B-*rbc*L IGS region was 1063 bp long. Alignment of ingroup and outgroup sequences was straightforward, requiring the insertion of 31 gaps. Of these, six gaps were needed exclusively to align the ingroup to *Salvinia*. The data set included 255 variable characters, of which 97 were potentially informative. Nucleotide composition was A = 29.5%, C = 20.7%, G = 21.5%, T = 28.4%, and the transition to transversion ratio (ts : tv) was 1.18 : 1. Pairwise distances ranged from identity (among most samples of *A. microphylla* and *A. mexicana*) to 16.2% (between *Salvinia* and *A. pinnata*).

Phylogenetic analysis yielded a single most parsimonious (MP) tree (fig. 3*a*), which was 305 steps long and had a consistency index (CI) of 0.841 (excluding uninformative characters) and a retention index (RI) of 0.877. Four major clades are apparent in this single tree. The clade labeled "MIC-MEX" (fig. 3*a*) contains all accessions of *A. microphylla* and *A. mexicana*. The "CAR" clade contains only accessions of *A. caroliniana*. The "FIL-RUB" clade comprises the accessions of both *A. filiculoides* and *A. rubra*, and the "PIN-NIL" clade contains *A. pinnata* and *A. nilotica*. Each of the four major clades had strong BS support, with values ranging from 88% to 100%. The ML tree (fig. 3*b*) was identical in topology to the MP tree.

trn*L*-trn*F region*. Sequences from the *trn*L-*trn*F region were derived from 24 accessions (representing all seven species of *Azolla* and one sequence from *Salvinia*) and included data from the *trn*L intron and the IGS between *trn*L and *trn*F. Sequence length ranged from 847 to 863 bp in *Azolla* and was 751 bp in *Salvinia*. The alignment required the addition of 56 indels (16 of which were needed to align the ingroup to *Salvinia*), resulting in a data set of 989 characters. Of these, 277 characters were variable and 147 were potentially informative. Nucleotide composition was A = 32.4%, C = 18.8%, G = 21.1%, T = 27.7%, and the ts : tv was 1.24 : 1. Pairwise distances ranged from identity (within species and within the MIC-MEX group) to 22.9% (between *Salvinia* and *A. pinnata*).

Phylogenetic analysis yielded three MP trees, each 346 steps (CI = 0.913, excluding uninformative characters; RI = 0.963). The analysis resolved all four major clades identified in the *atp*B-*rbc*L IGS tree. A strict consensus of the three shortest trees reveals polytomies among the samples of *A. pinnata*, among the samples of *A. caroliniana*, and within the MIC-MEX clade (fig. 4*a*). The BS analysis supported all four major clades, with values ranging from 97% to 100%. The ML tree (fig. 4*b*) was identical in topology to the strict consensus of the MP trees.

Internal transcribed spacer region. The ITS sequences represented the entire length of ITS1, ITS2, the intervening 5.8S coding region, and flanking regions of the 18S and 26S genes. Across 26 accessions (representing all seven species of *Azolla*), the length of the ITS region varied from 808 to 858 bp. Alignment required the insertion of 24 gaps, resulting in total length of 874 bp. An ITS sequence was also derived from one sample of *Salvinia* for use as an outgroup, but this sequence could not be reliably aligned to ingroup sequences. Therefore, we rooted the ITS trees at the node between the *A. nilotica* + *A. pinnata* clade and the clade comprising the remaining species of *Azolla*, on the basis of results from the chloroplast data sets. Of the 874 aligned characters, 304 were variable and 238 were potentially parsimony informative. Nucleotide composition was A = 20.8%, C = 27.5%, G = 28.9%, T = 22.8%, and the ts : tv was 1.39 : 1. Pairwise distances ranged from identity (between several accessions within the MIC-MEX group) to 27.7% (between *A. rubra* and *A. pinnata*).

Phylogenetic analysis yielded 174 MP trees (fig. 5*a*). Each of these trees was 413 steps long, with a CI of 0.888 (excluding uninformative characters) and an RI of 0.952. These trees resolved the same four major clades identified in both the

Table 2

Accessions Used in This Study, with Voucher Numbers and Original Field Source Localities

Species	Voucher	Original field source of culture and corresponding IRRI number (if applicable)	ITS	*trn*L-*trn*F	*atp*B-*rbc*L
Azolla caroliniana	*Reid 65*	Ohio, USA; CA3001	DQ066475	DQ066501	n/a
A. caroliniana[a]	*Reid 66*	Endophyte-free culture of *Reid 65*	DQ066476	DQ066500	DQ066464
A. caroliniana	*Reid 67*	Madison, Wisconsin USA; CA3002	DQ066474	DQ066502	DQ066465
A. caroliniana	*Reid 86*	Santo Domingo, Philippines; PI0001	DQ066473	n/a	n/a
A. filiculoides	*Reid 68*	Brazil	DQ066494	DQ066498	DQ066456
A. filiculoides	*Reid 92*	Hangzhou, China; FI1534	DQ066495	DQ066499	DQ066457
A. mexicana	*Reid 71*	Sutter County, California, USA	DQ066484	DQ066508	DQ066461
A. mexicana	*Reid 72*	Butte County, California, USA; ME2001	DQ066478	DQ066519	n/a
A. mexicana[a]	*Reid 73*	Endophyte-free culture of *Reid 72*	DQ066477	DQ066514	DQ066462
A. mexicana	*Reid 74*	Leticia, Columbia IRRI No. ME2016	DQ066485	n/a	n/a
A. mexicana	*Reid 69*	Maui, Hawaii, USA; FI1539	DQ066479	DQ066513	DQ066463
A. mexicana	*Reid 76*	California, USA	DQ066480	DQ066518	n/a
A. microphylla	*Reid 77*	Chaco, Paraguay	DQ066481	DQ066509	n/a
A. microphylla[a]	*Reid 78*	Endophyte-free culture of *Reid 77*	n/a	DQ066510	n/a
A. microphylla	*Reid 79*	Logo Cañada, Paraguay; MI4075	DQ066486	DQ066511	DQ066460
A. microphylla	*Reid 80*	Santa Cruz Island, Ecuador; MI4021	DQ066482	DQ066515	DQ066459
A. microphylla	*Reid 81*	Paraguay; MI4001	DQ066472	DQ066516	n/a
A. microphylla	*Reid 91*	Ifugao, Philippines; PI0004	DQ066471	DQ066512	n/a
A. microphylla	*Reid 75*	Guayana; ME2003	DQ066483	n/a	n/a
A. microphylla	*Reid 70*	Sutter County, California, USA	DQ066487	n/a	n/a
A.a microphylla	*Reid 88*	Sacramento County, California, USA	DQ066488	DQ066517	DQ066466
A. nilotica	*Reid 82*	Kosti, Sudan; NI5001	DQ066470	DQ066507	DQ066468
A. pinnata	*Reid 83*	Shandong, China; PI0022	DQ066489	DQ066506	n/a
A. pinnata	*Reid 84*	Bumbong Lima, Malaysia; PI0002	DQ066490	DQ066504	DQ066467
A. pinnata	*Reid 87*	Uttar Preadesh, India	DQ066491	DQ066505	n/a
A. rubra	*Reid 89*	North Island, New Zealand; RU6501	DQ066493	DQ066497	n/a
A. rubra	*Reid 90*	Victoria, Australia; RU6502	DQ066492	DQ066496	DQ066458
Salvinia sp.	*Reid 93*	Burlington, North Carolina, USA	n/a	DQ066503	DQ066469

Note. All vouchers were deposited at Virginia Commonwealth University. IRRI = International Rice Research Institute, Manila, Philippines. GenBank accession numbers are supplied for all sequences; n/a indicates sequence not obtained for that sample.

[a] DNA samples derived from endophyte-free cultures.

*atp*B-*rbc*L and *trn*L-*trn*F trees, and each of these clades was again strongly supported (BS values ranging from 91% to 100%). The ML analysis (fig. 5*b*) yielded the same basic topology, apart from minor differences in the degree of resolution.

Combined molecular analyses. Both visual inspection and the results of the partition homogeneity test ($p = 0.10$) indicated that the separate data sets are not incongruent and are therefore combinable. Because sampling overlap across individual data sets was imperfect, two strategies were used in

Table 3

Primers Used for PCR and Sequencing

Sequence and primer	Location	3′-sequence-5′	Reference
*rbc*L-*atp*B:			
*rbc*L 49 (R)	External	CAC CAG CTT TGA ATC CAA CAC TTG C	Pryer 1999
*atp*B 166 (F)[a]	External	CTC GGC GAT AYG GAG CCR AAA GRT C	This article
ITS:			
ITS5 (F)	External	GGA AGT AAA AGT CGT AAC AAG G	White et al. 1990
CA26A (R)	External	TTT CTT TTC CTC CGC T	Wen et al. 1998
ITS2 (F)	Internal	GCA TCG ATG AAG AAC GTA GC	Modification of White et al. 1990
ITS2 (R)	Internal	GCT ACG TTC TTC ATC GAT GC	Modification of White et al. 1990
N5.8S (R)	Internal	TGC GTT CAA AGA CTC GAT	Wen et al. 1998
*trn*L-*trn*F:			
c (F)	External	GGA AAT CGG TAG ACG CTA CG	Taberlet et al. 1991
f (R)	External	ATT TGA ACT GGT GAC ACG AG	Taberlet et al. 1991

Note. External primers were used for both PCR amplification and sequencing; internal primers were used only for sequencing where needed. (F) and (R) denote forward and reverse primers, respectively.

[a] *atp*B 166 (F) is the complement of the unpublished primer *atp*B 166R (K. M. Pryer, personal communication).

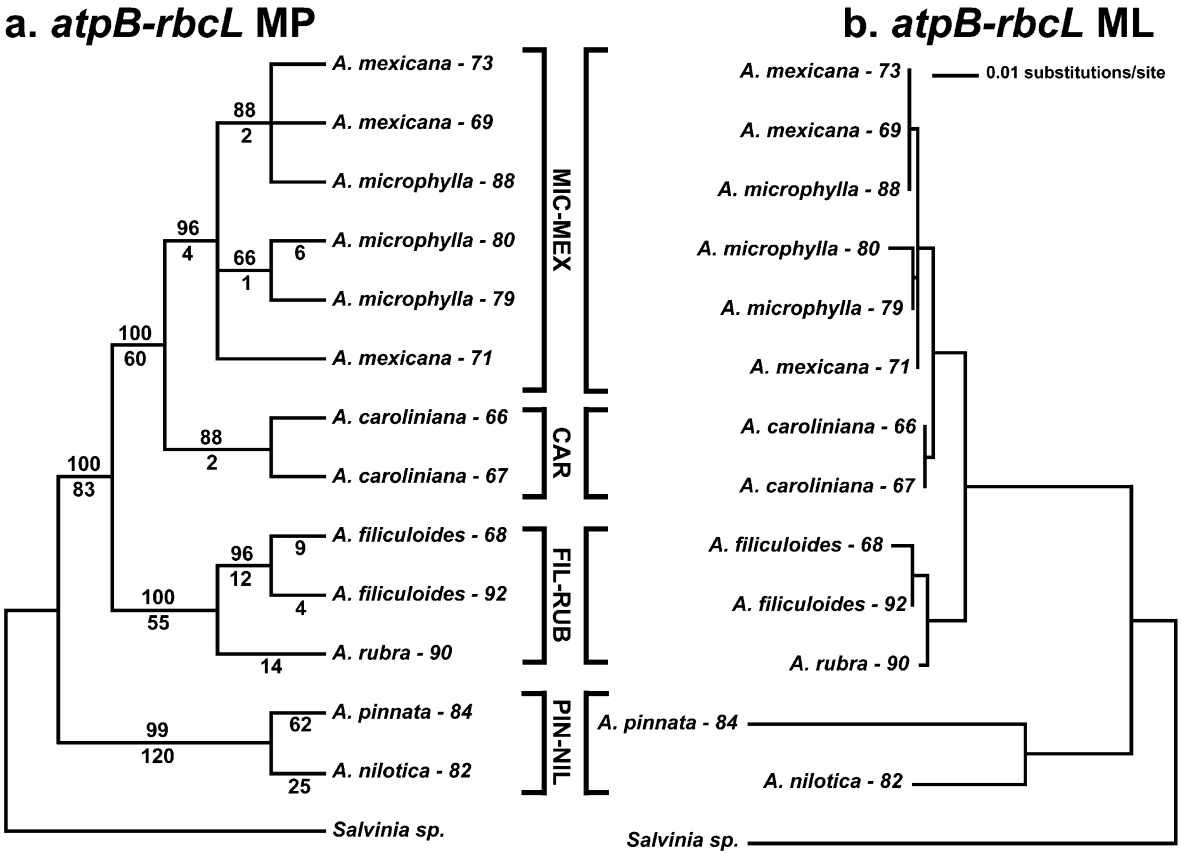

Fig. 3 Phylogenetic trees based on intergenic spacer sequences of the *atp*B-*rbc*L region of cpDNA from 14 samples (representing all seven species of *Azolla* plus one *Salvinia*). *a*, Single tree resulting from maximum parsimony (MP) analysis; tree length = 305 steps; consistency index = 0.841 (excluding uninformative characters); retention index = 0.877. Numbers above branches are bootstrap percentages; those below are branch lengths (0 branch lengths not shown). *b*, Maximum likelihood (ML) tree based on the same data set.

combining data sets. In one approach, only sequences from ITS and *trn*L-*trn*F were combined, for a total of 23 taxa, to take advantage of the heavier overlap in sampling represented by these data sets. This resulted in a total length of 1863 bp, 576 variable characters, and 383 potentially parsimony-informative characters. This analysis yielded 31 MP trees (many more trees than *trn*L-*trn*F alone, but many fewer than ITS alone), which were 749 steps long (CI = 0.897, excluding uninformative characters; RI = 0.953; fig. 6*a*). In a second approach, a combined analysis was based on the 14 samples common to all three data sets, resulting in 2996 bp, 826 variable characters, and 337 potentially parsimony-informative characters. This analysis yielded a single MP tree, which was 1037 steps long (CI = 0.853, excluding uninformative characters; RI = 0.889; fig. 6*b*). In both of the combined analyses, *Salvinia* was used as the outgroup, with all ITS characters scored as missing data (unrooted trees resulting from analyses where *Salvinia* was omitted produced identical topologies; trees not shown). Because the two combined trees are essentially identical in topology, only the ITS + *trn*L-*trn*F trees (with their greater taxon sampling) will be considered hereafter. The strict consensus (fig. 6*a*) is fully resolved except for relationships in the MIC-MEX clade. When these topologies were compared with topologies resulting from the separate analyses, the same four major clades were again resolved (MIC-MEX, CAR, FIL-RUB, and PIN-NIL), with BS support ranging from 99% to 100%.

Discussion

Sequence comparisons. This study represents the first attempt to use DNA sequence data in a phylogenetic study of interspecific relationships in *Azolla*. Evolutionary rates among the three markers were compared by estimating genetic distances from five identical pairs of taxa for each marker. Rates for *trn*L-*trn*F and *atp*B-*rbc*L were very similar, with *trn*L-*trn*F evolving ca. 1.3 times faster than *atp*B-*rbc*L IGS. The ITS region evolved 2.1 times faster than *atp*B-*rbc*L and 1.6 times faster than *trn*L-*trn*F. All three markers resolved relationships among the species of *Azolla* except *A. microphylla* and *A. mexicana* (see below), suggesting that they may have broad utility at the interspecific level among ferns.

Phylogenetic relationships. All of the molecular analyses rooted with *Salvinia* yielded the same four major clades and indicate identical relationships among these four clades. In

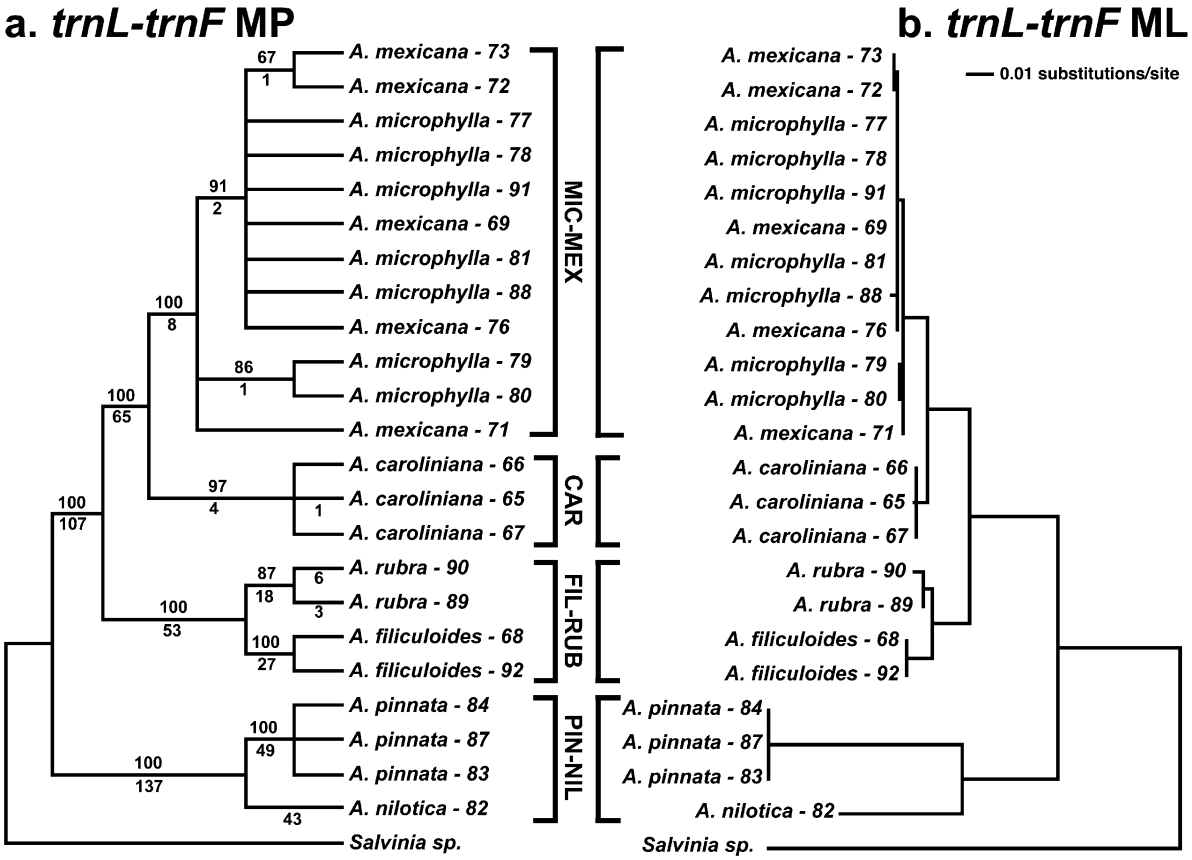

Fig. 4 Phylogenetic trees based on sequences from the *trn*L-*trn*F region of cpDNA from 24 samples (representing all seven species of *Azolla* plus one *Salvinia*). *a*, Strict consensus of the three shortest trees resulting from maximum parsimony (MP) analysis; tree length = 346 steps; consistency index = 0.913 (excluding uninformative characters); retention index = 0.963. Numbers above branches are bootstrap percentages; those below are branch lengths (0 branch lengths not shown). *b*, Maximum likelihood (ML) tree based on the same data set.

all trees, the MIC-MEX clade is sister to the CAR clade, the MIC-MEX + CAR clade is sister to the FIL-RUB clade, and this larger clade (MIC-MEX + CAR + FIL-RUB) is sister to the PIN-NIL clade. In addition, each of the four major clades has very strong BS support across all trees; poor support was found only among the accessions within the MIC-MEX group. Therefore, none of the three molecular markers, separately or in combination, were able to resolve species-specific subclades among the samples of *A. microphylla* and *A. mexicana*.

The resolution and placement of the PIN-NIL clade indicates that *A. pinnata* and *A. nilotica* share a single common ancestor that diverged early in the diversification of *Azolla*. This finding agrees with most taxonomic treatments, which have placed these two species in section *Rhizosperma*. Although these two species exhibit geographic overlap in Africa (fig. 1), they can be readily distinguished on the basis of vegetative morphology alone. This is the result in large part of several distinctive features unique to *A. nilotica*, including its branching pattern and stem vasculature, as well as the production of sporocarp tetrads. This species also differs from *A. pinnata* (and all other species of *Azolla*) in its chromosome number. For these reasons, Saunders and Fowler (1993) recommended placing *A. nilotica* in its own subgenus, *Tetrasporocarpia*. The results from our study, however, indicate that recognition of this new subgenus would render subgenus *Azolla* paraphyletic. Despite the many morphological and cytological differences separating *A. nilotica* and *A. pinnata*, they do share a number of distinctive morphological characters, such as nine megaspore floats, pubescent stems, and unbarbed massula trichomes. Their close relationship is also supported by 123 synapomorphies in the three-sequence combined analysis (fig. 6*b*), confirming the traditional placement of both species in section *Rhizosperma*. Thus, our results indicate that recognition of subgenus *Tetrasporocarpia* is both unnecessary and phylogenetically undesirable.

The clade comprising all species of section *Azolla* is supported by 124 synapomorphies (based on the three-sequence combined analysis; fig. 6*b*). Within this large clade, there is a patristic distance of 83 characters separating the FIL-RUB clade from the rest of section *Azolla*. The monophyly of *A. filiculoides* and *A. rubra* is supported by many morphological synapomorphies, including unicellular leaf trichomes, glochidial septation, and megasporocarps with raised hexagonal markings. The two species can, however, be readily distinguished using characters of stem vasculature, perispore

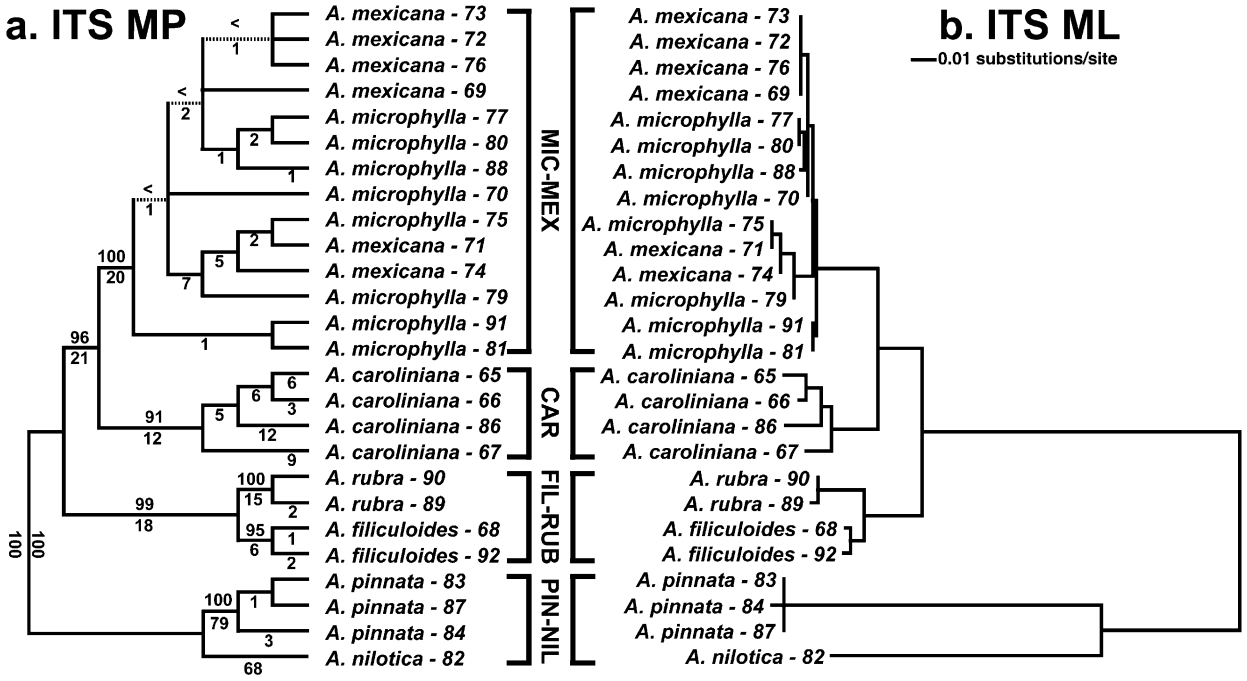

Fig. 5 Phylogenetic trees based on nuclear ITS sequence data from 26 samples of *Azolla* (representing all seven species). *a*, One of the 174 shortest trees resulting from the maximum parsimony (MP) analysis (the strict tree can be derived by collapsing the dashed branches); tree length = 413 steps; consistency index = 0.888 (excluding uninformative characters); retention index = 0.952. Numbers above branches are bootstrap percentages; those below are branch lengths (0 branch lengths not shown). *b*, Maximum likelihood (ML) tree based on the same data set.

structure, and the presence/absence of filosum on the megaspore collar. Molecular data also clearly distinguish these two species, with a patristic distance of 42 characters separating *A. filiculoides* from *A. rubra*. Therefore, in contradiction to the many treatments that have united these two taxa under a single species (Mettenius 1867; Strasburger 1873; Svenson 1944; Moore 1969) (fig. 2), both molecular and morphological characters support the current recognition of *A. rubra* as a species distinct from *A. filiculoides*.

Among the other clades of section *Azolla*, the CAR clade (comprising only samples identified as *A. caroliniana sensu* Svenson [1944]) is well supported (BS = 100%; fig. 6*b*) and is separated from the MIC-MEX clade by a patristic distance of 53 changes (fig. 6*b*). Earlier treatments (Mettenius 1847; Strasburger 1873; Baker 1887) questioned the morphological distinctiveness of *A. caroliniana* from *A. mexicana* and *A. microphylla*, but these studies were based solely on the sterile type specimen of *A. caroliniana*. Moreover, phenetic similarities based on molecular data collected from the cyanobacterial symbionts of *Azolla* have led some researchers to speculate that *A. caroliniana, A. mexicana,* and *A. microphylla* may represent a single species (Franche and Cohen-Bazire 1987; Plazinski et al. 1988; Van Coppenolle et al. 1995). By contrast, fertile specimens identified as *A. caroliniana sensu* Svenson (1944) are always morphologically distinct from all other species, and our DNA results confirm Svenson's circumscription. The source of the confusion appears to be discrepancy between the nomenclatural type of *A. caroliniana* and the morphological species concept that was developed later by Svenson (1944) and subsequent workers (Moore 1969; Saunders and Fowler 1992, 1993). In these more recent classification systems, definitive species determinations require reproductive structures, but the type specimen of *A. caroliniana* is sterile. Thus, despite the morphological distinctiveness of fertile specimens identified as *A. caroliniana,* some researchers have suggested that its sterile type may in fact represent an individual currently referable to *A. filiculoides*, *A. microphylla*, or an interspecific hybrid (Mettenius 1867; Dunham and Fowler 1987). While there appears to be ample morphological and molecular evidence to support the circumscription of the species commonly called "*A. caroliniana*," this lineage may require a nomenclatural change pending the satisfactory identification of the current type.

The MIC-MEX group is a well-supported clade (BS = 100%, branch length = 33; fig. 6*b*), but there is no evidence indicating the monophyly of either species. Rather, samples of *A. microphylla* and *A. mexicana* are interdigitated in the MIC-MEX clade in all trees (figs. 3–6), whether based on the rapidly evolving ITS sequences, the more conservative chloroplast sequences, or their combinations. This indicates that the lack of resolution results neither from excessive noise (character saturation) nor from insufficient variation. Moreover, pairwise distances among the MIC-MEX samples (and branch lengths within the MIC-MEX clade) are more similar to values within other conspecific clades (e.g., among samples within the CAR or PIN clades) than to values between interspecific clades (e.g., between FIL and RUB or PIN and NIL).

These findings indicate that *A. microphylla* and *A. mexicana* represent not two distinct species but rather a single evolutionary lineage, and a number of studies lend support to this

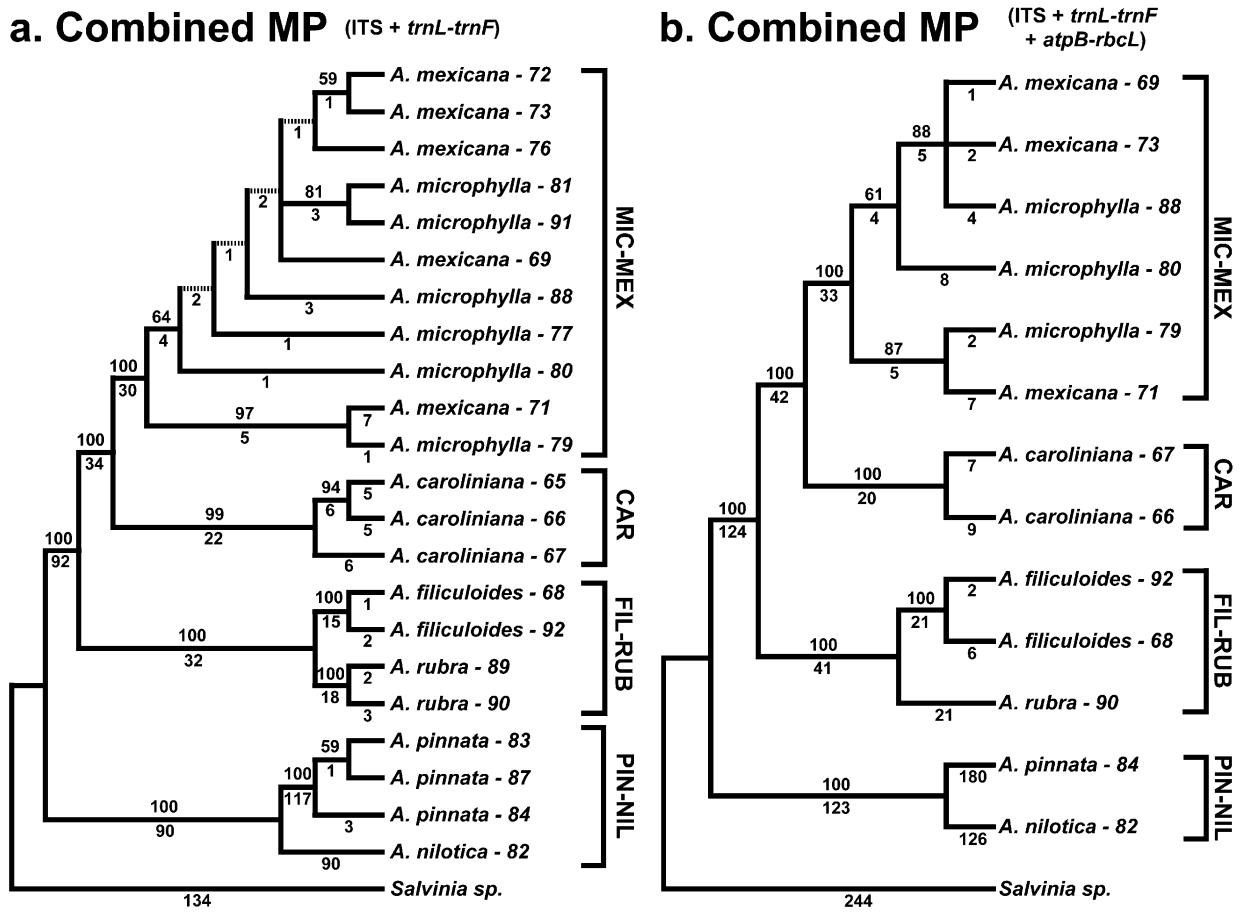

Fig. 6 Phylogenetic trees based on maximum parsimony (MP) analyses of combined molecular data sets. Numbers above branches are bootstrap percentages; those below are branch lengths (0 branch lengths not shown). *a*, One of the 31 trees resulting from the analysis of 23 samples for which both ITS and *trn*L-*trn*F sequences were available (the strict tree can be derived by collapsing the dashed branches); tree length = 749 steps; consistency index (CI) = 0.897 (excluding uninformative characters); retention index (RI) = 0.955. *b*, Single tree resulting from the analysis of data from 14 samples for which all three data sets were available (ITS, *atp*B-*rbc*L, and *trn*L-*trn*F); tree length = 1037; CI = 0.853 (excluding uninformative characters); RI = 0.889.

conclusion. For example, experiments by Stergianou and Fowler (1990) reported a high frequency of aneuploid offspring resulting from all interspecific crosses in *Azolla* but not from crosses within species. Crosses between *A. microphylla* and *A. mexicana* generally yielded normal offspring, indicating conspecific status. They did report several aneuploid offspring resulting from a few crosses between *A. microphylla* and *A. mexicana*, but these cultures may not have been maintained in a purely vegetative state, allowing for the possibility of self-fertilization, which is frequently associated with aneuploidy (Brochmann and Hapnes 2001; Ma and Tarumoto 2002). In another study, Chen et al. (2003) used RAPD data to test relationships in *Azolla*. Estimates of genetic similarity among the samples from *A. microphylla* and *A. mexicana* were so high that the authors concluded these samples did not represent two species.

Taxonomic considerations. The phylogenetic trees presented herein provide clear, well-resolved, and well-supported relationships among the species of *Azolla*. Despite differences in interpretations, our results also agree largely with the results of earlier phenetic analyses in *Azolla* based on RAPD and RFLP data. In addition, they appear to resolve many of the long-standing problems that have plagued *Azolla* taxonomy. On the basis of our results, we make the following taxonomic recommendations. (1) Section *Azolla* ("*A. caroliniana*," *A. microphylla* + *A. mexicana*, *A. filiculoides*, *A. rubra*) and section *Rhizosperma* (*A. nilotica*, *A. pinnata*) are strongly supported monophyletic groups that should be maintained taxonomically; we see no reason to recognize the monotypic subgenus *Tetrasporocarpia*. (2) The typification of *A. caroliniana* must be carefully scrutinized. Our evidence clearly indicates that there is a clade corresponding to the morphological definition of *A. caroliniana*, but the type specimen of this species, which is sterile, may be conspecific with *A. filiculoides* (or some other species). If so, the name "*A. caroliniana*" should be conserved under a better lectotype (or neotype), or a new name should be provided to this lineage. (3) *Azolla mexicana* should be treated as a synonym under *A. microphylla* (which has taxonomic priority). Therefore, "*Azolla microphylla*" should be applied to all individuals represented by the MIC-MEX lineage. (4) The taxonomy of the remaining species of *Azolla* can be maintained unaltered.

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