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ORIGINAL ARTICLE

Morphological and genetic diversity of the family Azollaceae inferred from vegetative characters and RAPD markers

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Abstract Family Azollaceae has seven species with a controversial taxonomy. The identification of species without reproductive structures relies on vegetative characters but some are variable, leading to misinterpretations. The molecular methods may be helpful, but until now, they did not provide a conclusive *Azolla* taxonomy. Therefore, we studied the family Azollaceae at vegetative and molecular levels. Analysis of vegetative, random amplified polymorphic DNA (RAPD) and combined data showed a comparable grouping of the *Azolla* species in two main clusters: cluster I, referred to as section *Rhizosperma* (*A. pinnata* and *A. nilotica*) and cluster II, referred to as section *Azolla* (*A. filiculoides*, *A. microphylla*, *A. caroliniana* and *A. mexicana*), with the exception of *A. rubra*, which clustered differently depending on the method. All the *Azolla* species were distinguished by the 13 polymorphic vegetative characters, the 211 RAPD markers or the combined data, with the latest showing the highest discrimination. The Shannon Index diversity was greater with RAPD (2.276) than with vegetative characters (0.054), highlighting the higher discriminating power of the molecular data. The partitioning of diversity was, as expected, high among species for all the types of data and low within species, with the lowest diversity obtained for morphological data. Both data sets (vegetative and RAPD) allowed the distinction of all the species and their clustering into sections *Rhizosperma* and *Azolla*, suggesting this as the most correct for this family. The dendrogram from the combined data was the most accurate, highlighting the benefit of integrating different types of data to study the family Azollaceae.

Keywords *Azolla* · Azollaceae · RAPD markers · Relatedness · Taxonomy · Vegetative characters

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Introduction

The seven species of *Azolla* belonging to the family Azollaceae are small heterosporic free-floating aquatic pteridophytes with tropical and temperate worldwide distribution. The deeply bilobed leaves (dorsal and ventral lobes) cover the entire rhizome, and the cavities of the dorsal lobe harbour a colony of a filamentous heterocystous cyanobacterium *Anabaena azollae* and several genera of bacteria. The *Azolla* species can be used as biofertiliser in

rice culture, animal feed and wastewater phytoremediation (Lumpkin and Plucknett 1980; Wagner 1997; Carrapiço et al. 2000).

The taxonomy of the family Azollaceae is highly controversial and several classifications have been proposed over the years (see Dunhan and Fowler 1987; Reid et al. 2006 for a synopsis). The *Azolla* classification has been mainly based on vegetative characters, but the high variability of some of those characters, such as the habit and leaf colour, can lead to misinterpretations. The reproductive characters are more reliable, but sporophytes with megasporocarps and microsporocarps are scarce. The shape and septum number of glochidia that surround the massulae microsporocarps are important taxonomic characters (Svenson 1944; Saunders and Fowler 1993). However, the septum number was variable within some species and should be used with precaution (Godfrey et al. 1961; Pereira et al. 2000, 2001). On the other hand, the surface and stratification of the megasporocarp perine may be regarded as a fingerprint for each *Azolla* species (Perkins et al. 1985) since it is an evolutionary highly conserved character. An additional problem in the *Azolla* taxonomy is the existence of sporulated specimen types. The most problematic is *A. caroliniana* because the type specimen is not sporulated and their status as a species is uncertain; so, the sporulated collected *A. caroliniana* specimens cannot be compared with the species-type.

Presently there are two classifications for family Azollaceae based on vegetative and reproductive characters: (1) two sections: *Rhizosperma* (*A. nilotica* Decne. ex Mett., *A. pinnata* R.Br. var. *pinnata* R.Br. and var. *imbricata* (Roxb.) Bonap.) and *Azolla* (*A. caroliniana* Willd., *A. mexicana* Presl., *A. microphylla* Kaulf., *A. filiculoides* Lam., *A. rubra* R.Br.) (Svenson 1944; Lumpkin and Plucknett 1980; Tan et al. 1986), which is mostly accepted; and (2) two subgenera: *Tetrasporocarpia* (only with *A. nilotica*) and *Azolla* divided in the sections *Azolla* (*A. caroliniana*, *A. microphylla*, *A. mexicana*, *A. filiculoides*, *A. rubra*) and *Rhizosperma* (*A. pinnata* subsp. *africana* (Desv.) R.M.K. Saunders & K. Fowler, subsp. *asiatica* R.M.K. Saunders & K. Fowler and subsp. *pinnata*) (Saunders and Fowler 1992, 1993), with a small number of citations in the literature. Isozymes, restriction fragment length amplification (RFLP) and hybridization analyses of the section *Azolla* allowed the distinction of two groups, *A. filiculoides* + *A. rubra* (FI-RU) and *A. caroliniana* + *A. mexicana* + *A. microphylla* (CA-ME-MI) evaluated as one species (Zimmerman et al. 1989, 1991a, b). A previous random amplified polymorphic DNA (RAPD) analysis further confirmed the section *Azolla* observed with the isozymes and the section *Rhizosperma* containing *A. nilotica* and *A. pinnata* (van Coppenolle et al. 1993). On the other hand, *A. nilotica* has a chromosome number $2n = 52$ while the remaining species have $2n = 44$, pointing to a higher phylogenetic relatedness between New World species, *A. rubra* and *A. pinnata* (Stergianou and Fowler 1990) and thus supporting the classification in two subgenera proposed by (Sauders and Fowler 1993). More recently, the use of DNA sequences of noncoding plastid regions showed the division of the extant species in the sections *Azolla* and *Rhizosperma* (Reid et al. 2006; Metzgar et al. 2007), which was in disagreement with the two subgenera proposal. Saunders and Fowler (1992) based on 38 vegetative and reproductive characters indicate for *A. pinnata* three subspecies correlate with their geographical origin: subsp. *africana* from Africa, subsp. *asiatica* from Asia and subsp. *pinnata* from Australia. In addition, Evrard and van Hove (2004) proposed a new classification for the New World species with only two species, *A. filiculoides* and *A. cristata*, based on three morphological characters. Therefore, the family Azollaceae, even after morphological and molecular research, is still under discussion.

Taking into account the controversy in the classification of the family Azollaceae, the aim of this study was to analyse the diversity and relatedness of the *Azolla* species using specimens representative of diverse origins from all around the world applying both vegetative characters and RAPD markers.

Materials and methods

Plant material

The 53 *Azolla* accessions from several locations around the world used in this study were obtained from the International Rice Research Institute (IRRI) germplasm collection and the Portuguese *A. filiculoides* specimen from the Botanical Garden of Lisbon University (BGLU) (Table 1).

Vegetative characters

The sporophytes of all the *Azolla* accessions were hydrated (Evrard and van Hove 2004) before evaluation of the 16 vegetative characters (Table 2). Observations were made with a binocular stereomicroscope (Olympus, UK) and a light microscope (Olympus BX60) coupled to a Leica DP50 camera (Leica Microsystems, Germany) for image acquisition.

Genomic DNA extraction

All the 53 *Azolla* specimens were surface disinfected with aqueous sodium hypochlorite (1:10), washed in distilled water and stored at −70°C. Genomic DNA was extracted from approximately 4 g of *Azolla* sporophyte according to

Table 1 List of *Azolla* accessions used in the study

Accession[a]	Species	Origin and harvest year	Source[c]
PI1	*A. pinnata* var. *imbricata*	Philippines, Santo Domingo, Albay, 1975	IRRI
PI5		Thailand, Bangkok, 1977	T Lumpkin
PI13		Nepal, Lalitpur, 1978	DA Nepal
PI18		Vietnam, Hanoi, 1979	DA Vietnam
PI19		Vietnam, Hanoi, 1979	DA Vietnam
PI32		China, Jianci, 1980	FAAS
PI39		Australia, Griffith (NSW), Murranbridge, 1980	IRRI
PI72		Indonesia, Java, from Becking, 1984	T Lumpkin
PI79[b]		Japan, Chisato Mie, 1984	T Lumpkin
PI527		Germany, Munich, 1987	–
FI1001	*A. filiculoides*	East Germany (ex-GDR), 1979	IB China
FI1008		USA, Cranmore Road, Sutter Co., California, 1981	D. Rains
FI1010		Peru, PUFFI, Lima, 1982	CIAT
FI1034		China, from megaspore of FI 301, *Azolla* Center, 1986	Lin Chang
FI1042		Brazil, Parana, 1987	I. Watanabe
FI1052		France, North of Lyon, 1989	P Roger
FI1090		Japan, Tanabe-cho, 1992	S Kitoh
FI1091		Japan, Tanabe-cho, *Anabaena* free from FI 1090, 1992	S Kitoh
FI1501		Belgium, Harchies, 1987	A Lawalree
FI1505		South Africa, Verwoerd dam, 1987	D Toerien
FI1507		Colombia, Zipaquira, 1987	Y Lopez
FI1518		Sweden, Lund Botanical Garden, 1987	–
FI1522		Switzerland, Zurich Botanical Garden, 1987	–
FI1530[b]		Ireland, Dublin, 1987	J Akeroyd
FIPort		Botanical Garden, Lisbon University, 2001	AL Pereira
ME2001[b]	*A. mexicana*	USA, Graylodge, California, 1978	D Rains
ME2008		Colombia, Cali, from Dr. Seko, 1982	CIAT
ME2011		Japan, Osaka, 1984	T Lumpkin
ME2026		Brazil, Solimoes river, Pacencia Island, Iranduba, Amazonas (BLCC 18), 1984	T Lumpkin
CA3001[b]	*A. caroliniana*	USA, Ohio, 1978	D Rains
CA3002		USA, Madison, Wisconsin, 1981	D Rains
CA3017		Brazil, Rio Grande Sul, 1987	I Watanabe
CA3502		Egypt, Moshtohor University, 1987	C Myttenaere
CA3507		Surinam, Boxel, 1987	H Lardinois
CA3513		Zimbabwe, Causeway Botanical Garden, 1987	T Muller
CA3524		Netherlands, 1987	E Ohoto
CA3525		Rwanda, Cyili Rice Research Centre, 1987	C Van Hove
CA3537		Philippines, Banawe, IRRI station, 1988	C Van Hove
MI4018[b]	*A. microphylla*	Paraguay, 1981	D Rains
MI4021		Equator, Santa Cruz Island, Galapagos, 1982	T Lumpkin
MI4025		Philippines (MI 4018 megaspore, *Anabaena* free), 1985	Lin Chang
MI4028		Philippines, hybrid (MI4018xFI1001) with *Anabaena*, 1985	Do Van Cat
MI4054		Brazil, Baia, 1987	I Watanabe
MI4510		Philippines, Los Baños, IRRI, 1987	C Van Hove
NI5001	*A. nilotica*	Sudan, Kosti, 1982	T Lumpkin
NI5002[b]		Sudan, Kosti, 1989	T Lumpkin
NI5501		Burundi, Bujumbura, 1987	J Bouharmont
RU6502[b]	*A. rubra*	Australia, Victoria (37.40 S–144.48 E), 1987	–

Table 1 continued

Accession[a]	Species	Origin and harvest year	Source[c]
PP7001[b]	*A. pinnata* var. *pinnata*	Australia, Kakadu Northern Park, Northern Territory, 1982	Yatazawa
PP7506		Sierra Leone, 1982	C Dixon
PP7509		Nigeria, Moor plantation, 1987	C Van Hove
PP7511		Guinea-Bissau, Contuboel, 1987	H Diara
PP7512		Zaire, Kisantu, 1987	B Bruyneel

[a] The accession numbers were listed according to the IRRI code number except for the Portuguese specimen (FIPort)

[b] Species used for the screening of the 120 RAPD primers

[c] *IRRI* International Rice Research Institute, *DA Nepal* Department of Agriculture, Nepal, *DA Vietnam* Department of Agriculture, Vietnam, *FAAS* Fujian Academy of Agricultural Sciences, *China, IB* China-Institute of Botany, Academia Sinica, Beijing, China, *CIAT* International Centre for Tropical Agriculture, Colombia

Table 2 Vegetative characters applied to all *Azolla* accessions

Character	Description
1	Sporophyte shape: 0, polygonal; 1, deltoid (triangular)
2	Polygonal branching pattern: 0, isotomous opposite (dichotomous); 1, anisotomous opposite
3	Deltoid branching pattern: 0, elongate alternate; 1, sub-pinnate alternate
4	Rhizome indumentum: 0, glabrous; 1, pubescent
5	Rhizome papillae: 0, unicellular; 1, bi- or multicellular
6[a]	Root arrangement: 0, solitary; 1, fascicles
7	Dorsal lobe apex shape: 0, sub-round; 1, round
8	Apex dorsal lobe angle: 0, acute; 1, obtuse
9	Dorsal lobe shape: 0, elliptical; 1, obovate
10[a]	Dorsal lobe border shape: 0, entire; 1, crenate
11	Hyaline border symmetry: 0, asymmetrical; 1, symmetrical
12	Number of cells of the hyaline border: 0, 2 to 6 layers; 1, 3 to 4 layers
13	Dorsal lobe papillae: 0, unicellular; 1, bicellular
14	Dorsal lobe stomata: 0, annular without middle longitudinal ridge; 1, annular with middle longitudinal ridge
15[a]	Dorsal leaf lobe stomata type: 0, anomocytic; 1, non-anomocytic
16	Ventral lobe stomata: 0, absent; 1, present

[a] Monomorphic vegetative characters not used for the cluster analysis and Shannon diversity

van Coppenolle et al. (1993) with modifications. Specifically, each sample was ground to fine powder in liquid nitrogen and DNA extracted with preheated extraction buffer (33.3 mM Tris–HCl pH 8.0, 1.03 M NaCl, 6.67 mM EDTA, 1.6% CTAB) incubated at 60°C for 30 min, followed by two extractions with chloroform:isoamyl alcohol (24:1) centrifuged at 5,000*g* for 10 min. The DNA was precipitated with isopropanol at −20°C for 2 h and the pellet obtained was dissolved in TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). RNA was removed by digestion with RNAse A for 1 h at 37°C followed by protein digestion with proteinase K for 30 min at 37°C. The impurities were removed with chloroform. Total DNA was precipitated with 3 M sodium acetate pH 5.2 and 100% ethanol overnight at −20°C. The precipitate was washed with 10 mM ammonium acetate in 76% ethanol and the dried pellet dissolved in TE buffer was stored at 4°C. The DNA quality and quantity were evaluated by spectrophotometry in a GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech, UK) and in 1% agarose gel electrophoresis running in 1× TAE buffer.

RAPD amplification

A pre-screening with six RAPD primer kits (OPA to OPF, Operon Technologies, Alameda, CA) was performed using one specimen of each species (highlighted with asterisk in Table 1). The screening of the 53 *Azolla* accessions was made with the selected 17 RAPD primers. All the samples were PCR amplified in a 25-µl reaction mixture containing 20 ng of template DNA, 1× *Taq* polymerase buffer, 2.0 mM $MgCl_2$, 200 µM dNTPs, 1 µM primer, 1 U/µl *Taq* DNA polymerase (GibCo-BRL, UK) and bidistilled sterilized water. The PCR amplifications were performed in a Biometra UnoThermoblock thermocycler (Biometra, Germany) with an initial denaturing step at 94°C for 3 min,

Table 3 Vegetative characters contributing for clusters and subclusters distinction

Cluster I	Cluster II
Deltoid sporophyte	Polygonal sporophyte
Pubescent rhizome	Glabrous rhizome
Sub-round dorsal lobe apex	Round dorsal lobe apex
Acute angle of the dorsal lobe	Obtuse angle of the dorsal lobe
Asymmetrical hyaline borders	Symmetrical hyaline borders
3–4 layers of cells on the hyaline border	2–6 layers of cells on the hyaline border
Subcluster Ia	Subcluster Ib
Sub-pinnate alternate deltoid branching pattern	Elongate alternate deltoid branching pattern
Absence of stomata on the ventral lobe	Presence of stomata on the ventral lobe
Subcluster IIa	Subcluster IIb
Anisotomous opposite polygonal branching pattern (except *A. rubra*)	Isotomous opposite polygonal branching pattern
Unicellular dorsal lobe papillae (except *A. microphylla*)	Bicellular dorsal lobe papillae
Annular stomata with middle longitudinal ridge of the dorsal lobe (variable character depending of specimen/species)	Annular stomata without middle longitudinal ridge of dorsal lobe (except three specimens of *A. caroliniana*)
Absence of ventral lobe stomata (except Portuguese *A. filiculoides*)	Presence of ventral lobe stomata (except *A. mexicana*)

followed by 40 cycles of denaturation at 94°C for 3 min, annealing at 36°C for 1 min and extension at 72°C for 2 min, without a final extension step. At least two PCR amplifications were performed for each sample with the RAPD primers to evaluate the reproducibility of the bands. A negative control (without DNA) was included in each set of reactions. The amplification products were separated by 2% agarose gel electrophoresis running in 1× TAE and stained with ethidium bromide. GeneRuler™ DNA Ladder Mix (Fermentas, Germany) was used as molecular size marker. The gels were observed at UV light with a BioRad Gel Doc 2000 (Hercules, CA) and photographed with Quantity One software (version 4.0.1).

Data analysis

The vegetative characters and the amplification profiles of the selected primers were evaluated in a 0/1 binary system. Only distinct, reproducible, well-resolved fragments were scored as RAPD markers. For cluster analysis and genetic diversity, only the polymorphic markers were used (Semagn et al. 2000). Pairwise similarity was estimated by the Jaccard coefficient (S_J), an algorithm that considers individuals similar only when they possess a common band (Sneath and Sokal 1973), and the similarity matrices were used to construct dendrograms for the vegetative, RAPD and vegetative + RAPD characters. The cluster analyses were performed by Sequential Agglomerative Hierarchical Nested (SAHN) method based on Unweighted Pair-Group Method Arithmetic Average (UPGMA), where *Azolla* specimens were grouped according to their similarity. The clustering congruence was estimated by the Mantel test with 1,000 permutations (Rohlf 2000). The numerical analysis had been made with NTSYS-pc Exeter Software version 2.1 (Setauket, USA).

The Shannon Index (H), used to estimate the morphological, genetic and combined data diversity, was defined as $H = -\Sigma p_i \log_2 p_i$ where p_i is the frequency of the presence/absence of a marker in each population (each species/variety is considered as a population). The average diversity over all populations was calculated as $H_{pop} = 1/n\Sigma H$ where n is the number of populations. The species diversity was calculated as $H_s = -\Sigma p_s \log_2 p_s$ where p_s is the frequency of presence/absence of a marker in the whole sample. The partitioning of diversity for each marker within populations (H_{pop}/H_s) and the component between populations $[(H_s - H_{pop})/H_s]$ was calculated (Bussell 1999; Jorge et al. 2003).

Results

Morphological and genetic markers

From the 16 vegetative characters analysed in all the 53 *Azolla* accessions, 13 characters were polymorphic and therefore further used for the cluster and Shannon diversity analysis. Six of the 13 polymorphic vegetative data (sporophyte shape, rhizome indumentum, dorsal lobe apex shape, apex dorsal lobe angle, hyaline border symmetry and number of cells of the hyaline border) allowed the distinction of the sections *Azolla* and *Rhizosperma*. In addition, two polymorphic vegetative data allowed the distinction of the *Azolla* species *A. pinnata* and *A. nilotica* (Table 3).

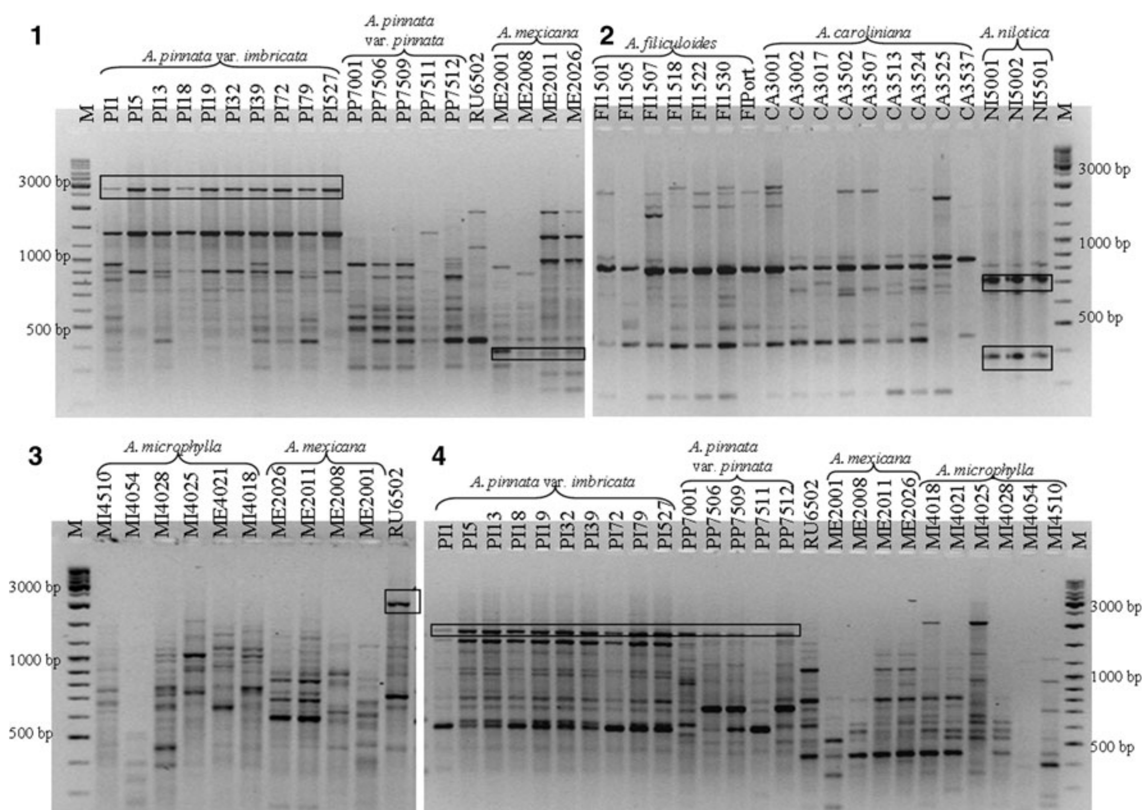
For the RAPD markers, the pre-screening of 120 RAPD primers in eight *Azolla* specimens revealed that 68 primers did not generate any amplification product, two were not reproducible, 33 showed faint, irregular and/or poor distinct bands, and only 17 primers had profiles with reproducible, high intensity and sharp bands. Using the PCR optimized conditions and these 17 selected primers, all the *Azolla* accessions were scrutinized. The screening of the 53 *Azolla* accessions with the 17 RAPD primers generated 254 bands ranging from 220 to 3,000 bp of which 211 fragments (83%) were polymorphic. The number of polymorphic bands ranged from 6 to 21, with an average of 12.5 bands per primer and 60–100% of polymorphism per primer. The integration of the polymorphic data obtained for 13 vegetative characters and 211 RAPD fragments into a single matrix yielded a total for 224 markers.

The analysis with the 17 primers also showed the occurrence of some species-specific bands in the genus *Azolla*: 2,520 bp for *A. pinnata* var. *imbricata* and 360 bp for *A. mexicana* with the primer A9 (Fig. 1); 380 and 700 bp with the primer A10 (Fig. 2), 1,700 bp with the primer C04, 910 bp with the primer E01 and 880 bp with the primer E02 for *A. nilotica*; and 420 bp with the primer A11, 1,000 bp with the primer C10 and 2,300 bp with the primer D18 (Fig. 3) for *A. rubra*. In addition, the amplicons 1,000 bp with the primer A10, 780 bp with the primer A16, 800 bp with the primer C07 and 1,800 bp with the primer C11 (Fig. 4) enabled the genetic distinction of both *A. pinnata* varieties from the other *Azolla* species.

Morphological and genetic relatedness

The goodness-of-fit between the cophenetic and similarity matrixes was assessed by the Mantel test for vegetative characters (0.97), RAPD markers (0.95) and combined data (0.96). These high values of the dendrogram branching indicate a very good congruence. Additionally, the dendrograms obtained for morphologic and molecular data were compared with the Mantel statistical test with a good value of congruence (0.68).

The dendrograms obtained using the cluster analysis for the three data sets (vegetative characters, RAPD markers and combined data) generated two main clusters matching the sections *Rhizosperma* (cluster I) and *Azolla* (cluster II), but differing in the species grouping mostly in the section *Azolla* (Figs. 5, 6, 7). The exception was *A. rubra*, which



Figs. 1–4 Amplification profiles with some primers showing *Azolla* species-specific bands (highlighted in *boxes*). **1** Primer A9 showing a specific band of 2,520 bp for *A. pinnata* var. *imbricata* and 360 bp for *A. mexicana*. **2** Primer A10 showing two specific bands of 700 and 380 bp for *A. nilotica*. **3** Primer D18 showing a specific band of 2,300 bp for *A. rubra*. **4** Primer C11 showing a specific band of 1,800 bp for *A. pinnata* var. *imbricata* and *A. pinnata* var. *pinnata*. M-1 Kb plus DNA ladder RU6502-*A. rubra*

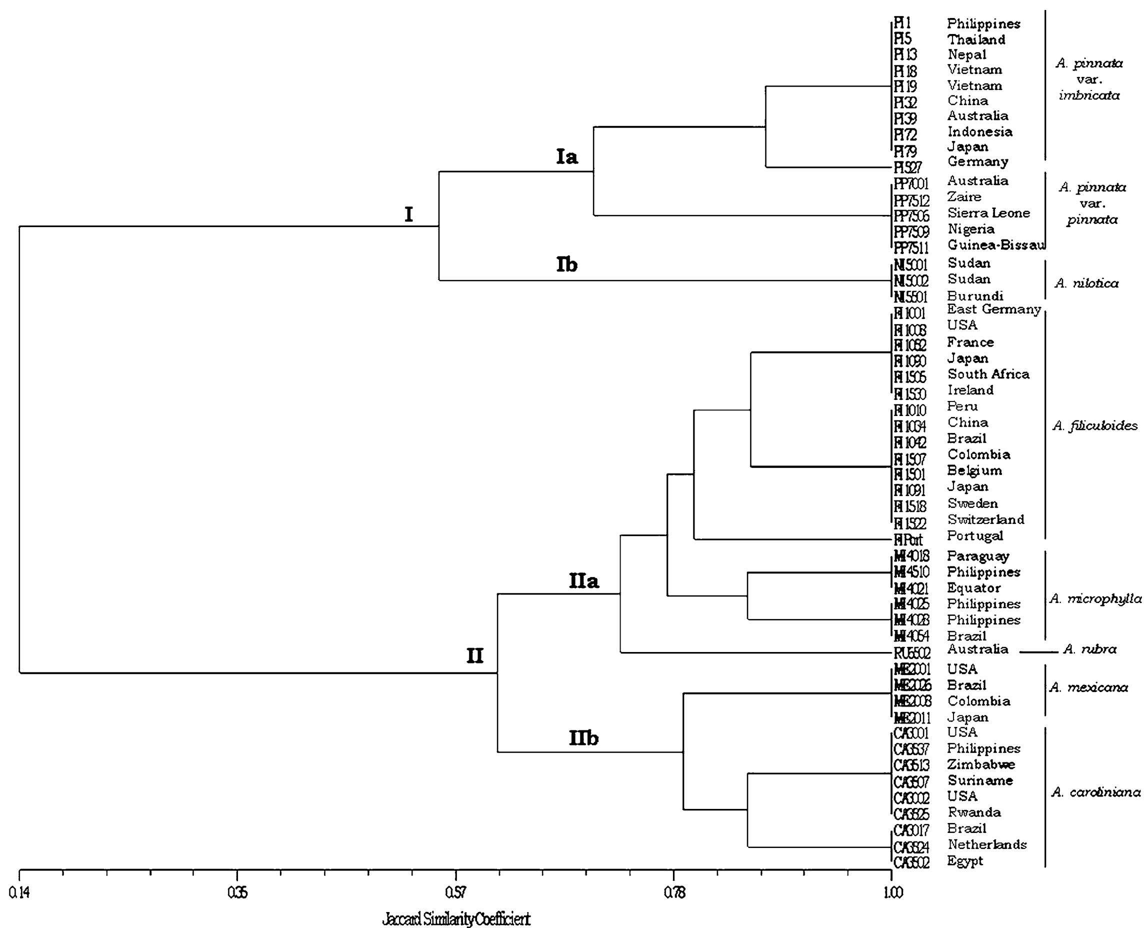


Fig. 5 Dendrogram derived from the 53 *Azolla* accessions and 13 polymorphic vegetative characters using the Jaccard similarity coefficient and UPGMA for the cluster analysis

grouped with section *Rhizosperma* when the RAPD data were analysed (Fig. 6) and associates with section *Azolla* when the morphological (Fig. 5) and combined (Fig. 7) data were analysed. The clusters of *Azolla* species were compared in Fig. 8. Also the Jaccard similarity coefficient at which cluster I and II separate was similar for the three dendrograms (0.14, 0.17 and 0.16 with vegetative characters, molecular markers and combined data, respectively), further supporting their status as two distinct groups.

The two main clusters obtained with the three types of data showed essentially the same *Azolla* species grouping: (a) *A. pinnata* and *A. nilotica* (cluster I); (b) *A. filiculoides*, *A. caroliniana*, *A. mexicana*, *A. microphylla* and *A. rubra* (cluster II). *A. rubra* was the exception, since it locates in cluster II with vegetative and combined data and in cluster I with the molecular data. The similarity coefficients at which clusters I and II form subclusters were higher with vegetative data ($S_J = 0.55$ and $S_J = 0.61$, respectively) and lower with the RAPD data ($S_J = 0.18$ and $S_J = 0.19$, respectively), which is in agreement with a higher discriminating power of the molecular data. Combined data, as expected, showed intermediate similarity coefficients ($S_J = 0.20$ and $S_J = 0.25$, respectively).

The *Azolla* species of the section *Rhizosperma* (cluster I) formed two subclusters with morphological (Fig. 5) and combined (Fig. 7) data, and a third subcluster with RAPD data (Fig. 6): (a) subcluster Ia with *A. pinnata* var. *imbricata* and var. *pinnata* ($S_J = 0.70$, morphological; $S_J = 0.45$, combined data; and $S_J = 0.38$, RAPD); (b) subcluster Ib with *A. nilotica* ($S_J = 1.0$, morphological; $S_J = 0.74$, combined data; and $S_J = 0.71$, RAPD); (c) subcluster Ic, for RAPD data, containing *A. rubra*. Distinction of the section *Rhizosperma* species belonging to the subclusters *A. pinnata* and *A. nilotica* can be made with two polymorphic vegetative characters (Table 3). Further, two vegetative characters can distinct the two varieties of *A. pinnata*: var. *imbricata* has bicellular rhizome papillae and obovate dorsal lobe shape and var. *pinnata* has unicellular rhizome papillae and elliptical dorsal lobe shape.

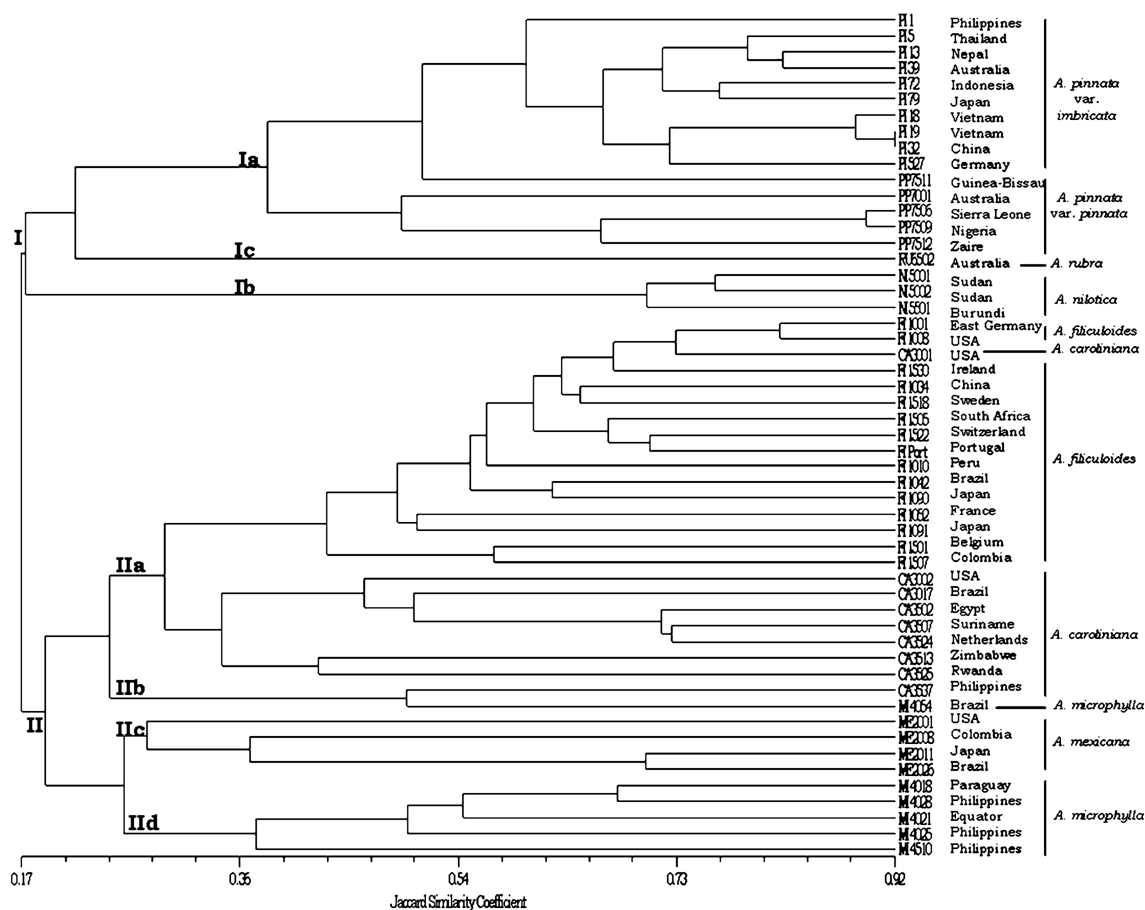


Fig. 6 Dendrogram derived from the 53 *Azolla* accessions and 211 polymorphic RAPD markers using the Jaccard similarity coefficient and UPGMA for the cluster analysis

The subclustering of section *Azolla* (cluster II) was more dissimilar between the different types of data. With morphological data (Fig. 5) cluster II is divided into subcluster IIa with *A. filiculoides*, *A. microphylla* and *A. rubra* (FI-MI-RU, $S_J = 0.72$) and subcluster IIb with *A. mexicana* and *A. caroliniana* (ME-CA, $S_J = 0.79$). Distinction of the section *Azolla* species belonging to these subclusters can be made with four polymorphic vegetative characters (Table 3). Molecular data allowed the division of cluster II into four subclusters (Fig. 6): *A. filiculoides* and *A. caroliniana* ($S_J = 0.30$, subcluster IIa); specimens CA3537 and MI4054 ($S_J = 0.50$, subcluster IIb); *A. mexicana* ($S_J = 0.29$, subcluster IIc); and *A. microphylla* ($S_J = 0.38$, subcluster IId). For the combined data, cluster II was divided into five subclusters (Fig. 7): *A. mexicana* ($S_J = 0.36$, subcluster IIa); *A. microphylla* ($S_J = 0.45$, subcluster IIb); *A. filiculoides* and specimens CA3001 and MI4054 ($S_J = 0.48$, subcluster IIc); *A. caroliniana* ($S_J = 0.37$, subcluster IId); and *A. rubra* (subcluster IIe), matching almost perfectly to the five species in the section *Azolla*.

Morphological and genetic diversity

The Shannon Index of diversity (H_0) for the seven species and two varieties was low (up to 0.054) for the 13 vegetative characters, intermediate (up to 1.307) for the 224 markers of the combined data and high (up to 2.276) for the 211 RAPD markers (Table 4). The specimens of *A. pinnata* var. *pinnata*, *A. nilotica* and *A. mexicana* showed no morphological diversity, indicating complete homogeny of the vegetative characters.

The Shannon Index for each species (H_{pop}) and total specimens (H_s) were used for partitioning within (H_{pop}/H_s) and between [$(H_s - H_{pop})/H_s$] the *Azolla* species for each data set (Table 5). The within-species variability obtained with the vegetative characters was very low (4%), but it had higher values with the molecular markers (32%). This means that the specimens belonging to a given species showed little vegetative diversity and higher genetic diversity. As for the diversity between the eight *Azolla* species, the vegetative characters gave the highest diversity index (96%) and the molecular data

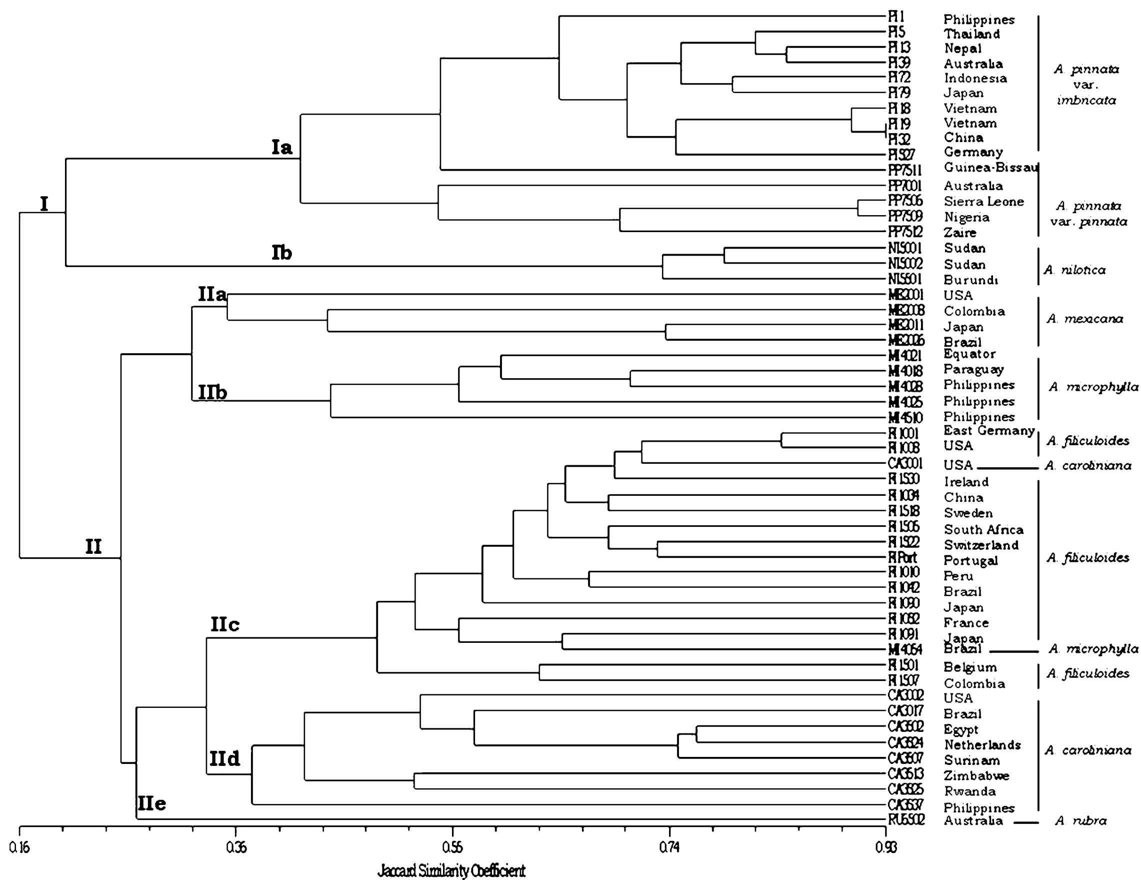


Fig. 7 Dendrogram derived from the 53 *Azolla* accessions using the 13 vegetative characters and 211 RAPD markers combined in a single matrix data. The Jaccard similarity coefficient and UPGMA were used for cluster analysis

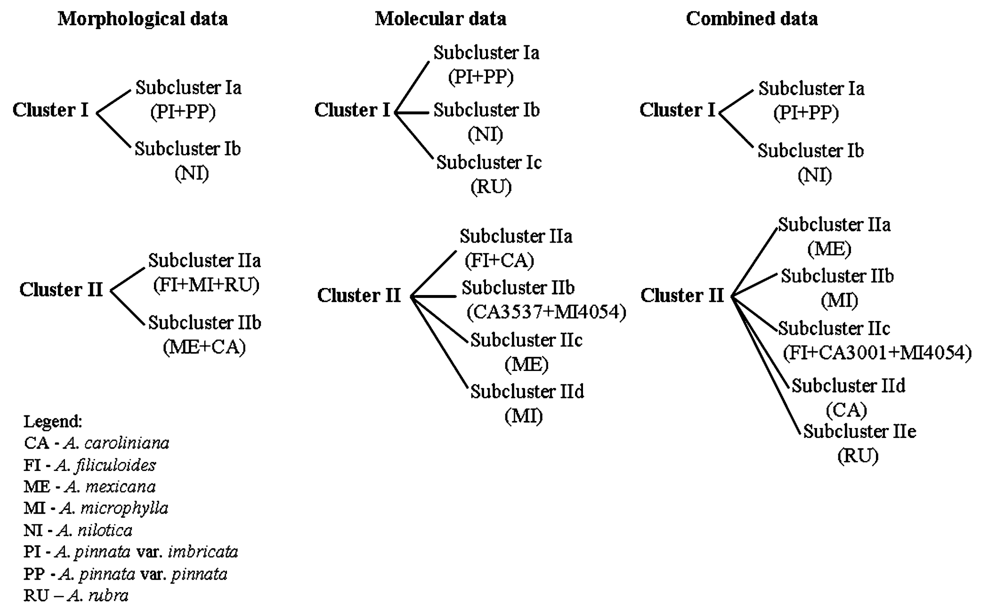


Fig. 8 Scheme of the *Azolla* species clustering using the morphological, molecular or combined data

the lowest (68%). The partition was in accordance with the cluster analysis since the dendrograms point towards the homogeneity of the vegetative characters with most of the specimens within a species with 100% Jaccard similarity coefficient. However, with the RAPD markers and combined data, the dendrogram showed several branches

Table 4 Shannon index (H) estimation per vegetative character and primer and mean Shannon index (H_0) within the eight populations (seven species and two varities) of the Family Azollaceae

	Populations (species and/or varities)[a]							
	PP	PI	NI	FI	CA	RU	ME	MI
Vegetative characters								
H	0.000	0.000–0.332	0.000	0.000–0.442	0.000–0.528	0.000	0.000	0.000–0.500
H_0	0.000	0.026	0.000	0.054	0.041	0.000	0.000	0.038
RAPD markers								
H	0.000–3.022	0.000–3.066	0.000–1.975	0.272–3.952	0.352–4.125	0.000	0.811–3.811	0.528–3.378
H_0	1.509	1.090	0.618	1.952	2.276	0.000	1.735	1.950
Vegetative characters + RAPD markers								
H	0.000–3.022	0.000–3.066	0.000–1.975	0.000–3.952	0.000–4.125	0.000	0.000–3.811	0.000–3.378
H_0	0.855	0.629	0.350	1.130	1.307	0.000	0.983	1.121

[a] *PP-A. pinnata* var. *pinnata*, *PI-A. pinnata* var. *imbricata*, *NI-A. nilotica*, *FI-A. filiculoides*, *CA-A. caroliniana*, *RU-A. rubra*, *ME-A. mexicana*, *MI-A. microphylla*

Table 5 Partitioning of the overall vegetative and genetic diversity within and between the eight *Azolla* groups

	H_s[a]	H_{pop}[b]	H_{pop}/H_s[c]	$(H_s - H_{pop})/H_s$[d]
Vegetative characters				
Range	0.200–0.547	0.000–0.042	0.000–0.352	0.895–1.000
Mean	0.457	0.020	0.040	0.960
RAPD markers				
Range	1.793–6.880	0.450–2.240	0.224–0.411	0.589–0.776
Mean	4.352	1.391	0.320	0.680
Vegetative characters + RAPD markers				
Range	0.200–6.880	0.025–2.240	0.125–0.411	0.589–0.875
Mean	2.664	0.820	0.250	0.750

[a] H_s-total diversity of the 53 *Azolla* accessions considered together

[b] H_{pop}-average diversity overall the populations (i.e., the seven species and two varieties)

[c] H_{pop}/H_s-proportion of diversity within populations

[d] $(H_s - H_{pop})/H_s$-proportion of diversity between populations

separating from each other at a wide range of similarity coefficients.

Discussion

The vegetative characters are essential for the *Azolla* taxonomy since the identification of *Azolla* species is still based on those characters. However, the correct identification of unfertile specimens can be difficult because of differences in some vegetative characters that depend on environmental factors and/or population density such as the leaf imbrication, planar or vertical growth, leaf colour, sporophyte dimensions and others. Therefore, the morphological characters, although essential, should be used carefully for taxonomical purposes. In the last years, genetic analysis has started to be applied to improve the taxonomical ranking of the *Azolla* species and to increase the amount of data available for phylogenetic purposes. The molecular profiles can also allow the identification of unique markers assigned to a single species that may be used as a molecular signature. Nevertheless, in the *Azolla* taxonomy, no one ever performed an analysis combining morphological and molecular markers. The current research uses, for the first time, morphological and molecular data in a relatedness analysis, further complemented with a diversity analysis to assist in the clarification of the *Azolla* taxonomy following an integrative approach.

Sections versus subgenera

Currently, two simultaneous classifications are used for ranking the seven species of *Azolla* both based on morphological characters: one with two subgenera (Saunders and Fowler 1993) and another with two sections (Svenson 1944; Lumpkin and Plucknett 1980; Tan et al. 1986). In the

present study, the 53 *Azolla* accessions analysed by UPGMA always grouped in two distinct main clusters, using morphological, molecular or combined data: cluster I containing *A. pinnata* and *A. nilotica* and cluster II with *A. filiculoides*, *A. caroliniana*, *A. mexicana* and *A. microphylla* mainly corresponding to sections *Rhizosperma* and *Azolla* respectively. Thus, both types of data, morphological and molecular, support the most accepted two sections classification *Azolla* and *Rhizosperma* (Lumpkin and Plucknett 1980; Tan et al. 1986) and declining the two subgenera classification, *Tetrasporocarpia* and *Azolla*, proposed by Saunders and Fowler (1993), which highlights the importance of the morphological characters and molecular markers to the taxonomy of the family Azollaceae. The clustering of *A. nilotica* in a different subgenera was supported by Stergianou and Fowler (1990) based on different chromosome number. The two subgenera proposed by Saunders and Fowler (1993) were based on morphological characters common to the *Azolla* species (synapomorphies) and do not find those common characters for *Azolla* species of section *Rhizosperma*. In the present research we found two distinct (autapomorphic) vegetative characters that allow distinguishing *A. pinnata* from *A. nilotica* (Table 3), and 6 synapomorphic characters (deltoid sporophyte shape, presence of rhizome indumentums, subround dorsal lobe apex shape, acute apex dorsal lobe angle, asymmetrical hyaline border, 2–6 layers of cells of the hyaline border) common to *A. pinnata* and *A. nilotica*.

The two sections obtained in the present study using morphological, molecular and combined data, respectively, were supported by: (1) the low Jaccard similarity coefficients at which the two sections separate; (2) the high values of goodness-of-fit of all the dendrograms; (3) the low within species variability of 4, 32 and 25%, respectively; (4) the high between species variability of 96, 68 and 75%, respectively. This indicates homogeneity of the morphological characters and a reasonable genetic variability inside a given species and heterogeneity of the vegetative characters and molecular markers among the *Azolla* species, allowing the discrimination of all the species and the two varieties, and their grouping in two sections. The same taxonomic two-section ranking has been obtained by other authors, using either morphological data (Svenson 1944; Lumpkin and Plucknett, 1980; Tan et al. 1986) or molecular data (van Coppenolle et al. 1993; Reid et al. 2006, Metzgar et al. 2007). In our study, the use of both types of data has further proven to be advantageous since: (1) the six vegetative characters identified to discriminate the sections *Rhizosperma* and *Azolla* (Table 3) may be used to ascertain field harvest specimens; (2) the amplification profiles of eight of the selected primers contain species-specific bands that can be used to identify *Azolla* specimens when the classification by vegetative characters is insufficient. Altogether, the 13 polymorphic vegetative characters used in this study provided a good discriminative power for the *Azolla* species and varieties, but the RAPD markers were more informative establishing in more detail the relationships between the specimens of the different *Azolla* species.

Azolla clustering: morphological and molecular data

The species corresponding to section *Rhizosperma* (cluster I) grouped in two and three subclusters using morphological and molecular data, respectively. For *A. pinnata* (PP and PI), the morphological data gave low diversity ($H_0 = 0.000$ and 0.026 for PP and PI, respectively) and lesser dendrogram branching (higher homogeneity) than the molecular data with higher genetic variability as shown by the higher Shannon Index ($H_0 = 1.509$ and 1.090 for PP and PI, respectively) and high dendrogram branching. In the present study, the two varieties of *A. pinnata* (var. *pinnata* and *imbricata*) formed two distinct groups that were grouped in one subcluster. Some of the vegetative data assessed disagreed from previous studies. It is the case of the unicellular rhizome papillae observed in *A. pinnata* var. *pinnata* and bicellular in var. *imbricata* as opposed to the bicellular papillae for *A. pinnata* observed by Saunders and Fowler (1992, 1993) or the bicellular dorsal lobe papillae for both *A. pinnata* varieties instead of the unicellular papillae observed by Teixeira (1999). In the *A. pinnata* group, there is some misunderstanding about the taxonomic ranking in two varieties or three subspecies. Saunders and Fowler (1992), who analysed specimens from Africa, Asia and Australia with 38 morphological characters, proposed the three subspecies correlating with their origin. Other studies, using RAPD and plastid genome loci, just discriminate the *A. pinnata* varieties and not the subspecies (van Coppenolle et al. 1993; Reid et al. 2006; Metzgar et al. 2007). In the present research, using both vegetative characters and molecular markers, it was not possible to make any correlation between the harvest location (Africa, Asia or Australia) and an *A. pinnata* ecotype. In addition, two vegetative characters were observed that can distinct the two varieties: *A. pinnata* var. *imbricata* has bicellular rhizome papillae and obovate dorsal lobe shape, and *A. pinnata* var. *pinnata* has unicellular rhizome papillae and elliptical dorsal lobe shape, supporting the classification in two varieties. Further, in the present study, we obtained one variety-specific band for *A. pinnata* var. *imbricata* and four species-specific amplicons for *A. pinnata*; these fragments may be used to identify field-harvested specimens when the morphological characters fail in their identification. The grouping of the specimen PP7511 (*A. pinnata* var. *pinnata*) with the specimens of var. *imbricata*, when using RAPD markers,

suggests that this specimen could be a genetic intermediate or hybrid between both varieties of *A. pinnata*. van Coppenolle et al. (1993) identified PP7512 as a possible intermediate specimen of both A. *pinnata* varieties, but in the present investigation, the specimen PP7512 grouped with the var. *pinnata* specimens.

The *A. nilotica* specimens group in subcluster Ib with morphological and with molecular data. Those *A. nilotica* specimens are morphologically distinct from the other *Azolla* including the *A. pinnata* in some specific characters (tetrads of sporocarps, root disposition, etc.) (Saunders and Fowler 1992, 1993; Perkins et al. 1985; Tan et al. 1986) and chromosome number (Stergianou and Fowler, 1990). The molecular analysis using RAPDs (van Coppenolle et al. 1993) or plastid genome loci (Reid et al. 2006, Metzgar et al. 2007) confirmed the uniqueness of *A. nilotica*. Our study further supported the genetic and morphological distinction of *A. nilotica* from *A. pinnata*. Of special interest were the five species-specific amplicons identified in this research, which can be used as genetic markers.

The section *Azolla* continues to be the most controversial in the family Azollaceae mostly due to different interpretations of the morphological characters. In the present study, it was possible to distinguish two and four subclusters within the section *Azolla* (cluster II) by using morphological and molecular data respectively. The taxonomy of the New World species (*A. filiculoides*, *A. mexicana*, *A. caroliniana* and *A. microphylla*) was revised by Evrard and van Hove (2004), who, considering three morphological characters (leaf papillae number, perine surface and number of glochidia septae), rearranged the *Azolla* cluster in only two species, *A. filiculoides* (grouping *A. filiculoides*, *A. caroliniana* Willd. and *A. microphylla* Kaulf.) and *A. cristata* (including *A. caroliniana* auct. non-Willd., *A. microphylla* auct. non-Kaulf. and *A. mexicana* Presl.). However, the character "number of glochidia septa" varies among specimens of *A. caroliniana* (Godfrey et al. 1961) and *A. filiculoides* (Teixeira 1999; Pereira et al. 2000, 2001), and thus it is not a good taxonomic character. The classification proposed by Evrard and van Hove (2004) disagrees with the distinction of all the New World species (*A. caroliniana*, *A. filiculoides*, *A. microphylla* and *A. mexicana*) made by Perkins et al. (1985) who considered the perine architecture and stratification to be species-specific.

In the present research, two clusters were formed using morphological characters: (1) subcluster IIa with *A. filiculoides*, *A. rubra* and *A. microphylla* and (2) subcluster IIb with *A. caroliniana* and *A. mexicana*, both subclusters with high similarity coefficients and low diversity Shannon Index. However, some of the scrutinized vegetative characters showed divergences from data reported by other authors, such as the presence of a longitudinal ridge in the stomata in *A. filiculoides* specimens in opposition with stomata without middle longitudinal ridge (Teixeira 1999) or no stomata in the ventral lobe of *A. filiculoides* (except for the Portuguese specimen), while Teixeira (1999) detected stomata in the ventral lobe in the same species. Therefore, once more, conclusions based on morphological characters, especially vegetative ones, should always be made cautiously.

The four subclusters (*A. filiculoides* + *A. caroliniana*, CA3537 + MI4054, *A. mexicana* and *A. microphylla*) obtained with the molecular markers differed from the groups *A. filiculoides*, *A. rubra* and the cluster *A. caroliniana* + *A. mexicana* + *A. microphylla* (CA-ME-MI) previously obtained with RAPDs (van Coppenolle et al. 1993), isozymes and RFLP (Zimmerman et al. 1989, 1991a, b). The clade ME-MI has been considered as one species supported by good bootstrapping values (Reid et al. 2006) and due to their recent divergence event that did not allowed their separation as independent species (Metzgar et al. 2007). In our study, although *A. mexicana* and *A. microphylla* appear to be closely related, morphological and molecular data support these as independent species. *A. caroliniana* is a source of intense debate since some authors say that this is a valid species and others say it is not, mostly because of the non-sporulated specimen type. Our results show that *A. caroliniana* could be an independent and valid taxon because of the grouping of the specimens in one subcluster using either morphological or molecular data. In addition, Reid et al. (2006) and Metzgar et al. (2007) demonstrated the monophyly of *A. caroliniana* as a valid taxon using plastid genome loci, in contradiction to the isozymes and RFLP studies of Zimmerman et al. (1989, 1991a, b) or the RAPDs of van Coppenolle et al. (1993). The specimen CA3001 was an outlier, grouping with *A. filiculoides* when analysed by molecular markers, but clustering in the correct group when using morphological characters. The genetic similarity of CA3001 with *A. filiculoides* was also detected by Zimmerman et al. (1989) who verified that this specimen had an isozyme profile similar to the *A. filiculoides* specimen FI1026. Cluster analysis based only on molecular data also created an unexpected subcluster IIb with the specimens CA3537 (*A. caroliniana*) and MI4054 (*A. microphylla*). For these two specimens, as well as for CA3001, extended molecular analysis should be performed to clarify their grouping.

A. rubra is another species under debate. Some authors considered it as an *A. filiculoides* variety (Fowler and Stennett-Willson 1978), while others, based on isozymes, RFLPs, RAPDs and plastid genome loci, considered it as an independent species, although close to *A. filiculoides* (Zimmerman et al. 1989, 1991a, b; van Coppenolle et al. 1993; Reid et al. 2006; Metzgar et al. 2007). In our survey,

the clustering using the RAPD fragments rendered an unexpected subcluster of *A. rubra* in the section *Rhizosperma*, while with the morphological data it grouped in section *Azolla*. This result suggests a closer genetic relatedness of *A. rubra* with the species of section *Rhizosperma*. Interestingly, these represent the three species native to the Old World (*A. rubra*, *A. pinnata* and *A. nilotica*), with *A. rubra* and *A. pinnata* exhibiting some geographic overlap, and therefore these results may point to a common origin. However,these results should be confirmed using more accessions of *A. rubra*. In addition, three species-specific *A. rubra* amplicons were identified that can be used to ascertain field-harvested specimens difficult to distinguish by morphological characters.

Azolla clustering: combined data

The two dendrograms constructed with the vegetative or molecular data showed some differences in the clustering (Fig. 8), especially on the section *Azolla*. Comparing morphological and molecular dendrograms with the Mantel test resulted in a good goodness-of-fit of 0.68, which indicates a positive relation between both dendrograms. So, it was decided to analyse all the data (morphology and molecular) in a unique matrix with equal weight for all the characters. Covering both types of data gave important, valid complementary information for *Azolla* taxonomy with increased discriminating power. The combined data provided an intermediate within and between species variability as compared to genetic and morphological data alone.

The species of the section *Rhizosperma* (cluster I) were grouped in two subclusters, one with *A. pinnata* composed by the two varieties and the other subcluster with *A. nilotica*. Once again, as with morphological and molecular data alone, the three subspecies of *A. pinnata* proposed by Saunders and Fowler (1992) were not revealed in the present study when combining morphology with molecular information. Probably, the morphological adaptations to a particular environment cause small variations in the phenotype, which however were not sufficient to modify the *A. pinnata* clustering. Again, the specimen PP7511 clustered with var. *imbricata* instead of var. *pinnata,* pointing to an intermediate specimen between both varieties and revealing a higher strength of the molecular markers over the morphological characters.

For the section *Azolla* (cluster II), the combined clustering was similar to the one obtained only with RAPDs and again it disagreed from the clusters obtained by Evrard and van Hove (2004), van Coppenolle et al. (1993), and Zimmerman et al. (1989, 1991a, b), which defined the cluster CA-ME-MI as a single species. Based on the present data, such classification does not seem to be correct since all the species of the section *Azolla* were discriminated. The variability detected in the dendrogram of the combined data was predominantly determined by the molecular results, but in some cases, the morphological characters were crucial to elucidate the taxonomical ranking of species such as *A. rubra*. Thus, the use of a combined matrix to construct a combined dendrogram is recommended, since it allows much better species discrimination and more precise relatedness within and between species. The clustering of *A. rubra* was corrected with the use of the combined data where it clustered with section *Azolla* as expected, but not with *A. filiculoides* as in previous molecular studies. *A. caroliniana* clustered in a single subcluster, emerging as a valid taxon.

Only two specimens, CA3001 and MI4054, appeared as outliers, grouping with *A. filiculoides* specimens despite their morphological differences. We therefore suggest that their revision as *A. filiculoides* should be considered, although more information using other molecular tools should be first gathered.

Conclusions

The taxonomy of the family Azollaceae and species ranking is still a continuous process that changes depending on the data analysed. The different interpretations and mistakes about morphological characters might be solved by joining a wide group of researchers in a common effort to homogenise strategies and criteria, and integrating observations of Azolla-type specimens, Herbarium and field-collected material, thus reducing inconsistencies and misinterpretations. Molecular tools definitely contribute to a better and more precise ranking of *Azolla* species. The present study highlighted the high diversity between species, independently of using morphological or molecular data. The diversity within species was very low when using the vegetative characters and intermediate with the RAPD markers pointing to a low diversity at the morphological level (conservation of the vegetative characters) and a higher diversity at the genetic level. The clustering analysis with the different types of data (morphological, RAPD and combined) supported the two-section ranking, the *A. pinnata* and *A. nilotica* in section *Rhizosperma* and five distinct species on section *Azolla Azolla* (*A. mexicana*, *A. microphylla*, *A. caroliniana*, *A. filiculoides* and *A. rubra*). However, further studies are needed for *A. rubra* to clarify its grouping since the only specimen included in this research grouped in section *Rhizosperma* when the RAPD data were analysed and in section *Azolla* when considering the vegetative and combined data. The species-specific bands obtained by RAPD analysis can be used in field-harvest specimens for species identification. In addition,

the three subspecies suggested for *A. pinnata* do not seem to be supported by our morphological or molecular data, which only revealed two varieties (var. *imbricata* and var. *pinnata*). In this study, for the first time, morphological and molecular data were simultaneously analysed in a representative sample of the family Azollaceae. Moreover, a significant contribution towards the analysis of the diversity and taxonomy of the *Azolla* species was achieved using a novel integrative approach.

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