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Taxonomic re-evaluation of the *Azolla* genus in Portugal

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Taxonomic re-evaluation of the *Azolla* genus in Portugal

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ABSTRACT - The Portuguese and the European Flora refer to the presence of two or three *Azolla* species in Portugal: *A. filiculoides* Lam., *A. caroliniana* Willd. and/or *Azolla mexicana* Presl., the latter included in the last edition of Flora Europaea. In the present work, the taxonomy of *Azolla* species is reviewed using the two most important characters that can distinguish between these two/three species: papillae in the dorsal leaf lobe and perine architecture of the megaspore apparatus. Other characteristics, such as the hyaline border cells of the dorsal leaf lobes and the number of glochidia septa in microsporangium massulae, are also used. All the *Azolla* specimens, collected from several locations in Portugal, were identified as *Azolla filiculoides* Lam. This identification disagrees with previous published reports on *Azolla* taxonomy in Portugal as well as with herbarium identification.

KEY WORDS - *Azolla filiculoides* Lam., perine surface, perine zonation, papillae, hyaline border, taxonomy

Azolla Lam. is an aquatic fern with deeply bilobed leaves. It has a very thin and hyaline ventral leaf lobe and an aerial and chlorophyllous dorsal lobe, which have an extracellular cavity. Populations of bacteria belonging to several genera, and a population of nitrogen-fixing cyanobacteria, *Anabaena azollae* live in this cavity (CARRAPIÇO, 1991; GRILLI CAIOLA & FORNI, 1999). This association is very important as a natural organic fertiliser for rice production in several countries (LUMPKIN & PLUCKNETT, 1980).

Research involving *Azolla* has often been hindered by confusion on the taxonomy of the genus. This genus is divided into two subgenera: *Azolla*, with two sections –

Azolla (*A. filiculoides* Lam., *A. rubra* R. Br., *A. caroliniana* Willd., *A. microphylla* Kaulf., *A. mexicana* Presl.) and *Rhizosperma* (*A. pinnata* R. Br.) - and subgenus *Tetrasporocarpia*, which only includes *A. nilotica* Decne (SAUNDERS & FOWLER, 1993). The taxonomy of the genus *Azolla* is traditionally based on vegetative characters such as plant dimensions, leaf characteristics (shape, papillae and hyaline border of dorsal leaves lobes), and on reproductive characters like the number of massulae and microsporangia (SVENSON, 1944). The number of glochidia septa in the microsporangium massulae has been used to distinguish between species. This is, however, a variable character, just like the above-mentioned

vegetative characters. PERKINS *et al.* (1985) studied the perine architecture of the megaspore of all extant *Azolla* species and found that this characteristic can be used as a distinctive character for interspecific *Azolla* identification.

According to several authors, in Portugal there are two or three *Azolla* species belonging to the section *Azolla*: *A. filiculoides* Lam. and *A. caroliniana* Willd. (ALMEIDA, 1986; FRANCO, 1971; LAWALRÉE, 1964; SAMPAIO, 1990; VASCONCELLOS, 1968) and/or *A. mexicana* Presl. (JERMY, 1993). In this paper, we revised the vegetative and reproductive characters in an attempt to re-evaluate and identify the *Azolla* specimens collected in several locations of Portugal.

MATERIALS AND METHODS

Fresh *Azolla* specimens with sporocarps were collected from six different sites in Portugal between 1992 and 1998 (Golegã, Nisa, Comporta, Pancas, Adema and

Guadiana River) (Table 1). A voucher with the specimen information (except for Adema, Pancas, Comporta and Nisa) was deposited in LISU. Additional plant material from the following Herbaria was examined: COI (Herbarium of Faculdade de Ciências e Tecnologia da Universidade de Coimbra), LISI (Herbarium of Instituto Superior de Agronomia), LISU (Herbarium of Faculdade de Ciências da Universidade de Lisboa) and PO (Herbarium of Faculdade de Ciências da Universidade do Porto) (Table 2).

For scanning electron microscopy (SEM), fresh sporophytes and sporocarps were fixed in a solution of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h at 4°C. After rinsing in the same buffer, the material was post-fixed with 2% osmium tetroxide aqueous solution for 1 h, following dehydration in a graded ethanol series, and finally dried with the CO₂ critical-point method in a Polaron E 3500. All the specimens were sputter-coated with gold in a Polaron E 5350, observed and photographed at 15-25 kV in JEOL

TABLE 1
Azolla specimens collected in the field between 1992 and 1998

Origin	Colector name	Date	Observations
Adema	F. Carrapiço	3/92	Channel
Guadiana river	F. Carrapiço	3/93	Bloom in 1993 near Mértola. High density of <i>Azolla</i> .
Golegã	G. Teixeira, A.L. Pereira	6/95, 3/96	Lagoon without direct connection to Tejo river. Some <i>Azolla</i> were rooted in the banks.
Pancas	G. Teixeira, A.L. Pereira	7/97, 4/98	From a marshy site near Tejo river. High density. Grown in Lisbon Botanical Garden.
Comporta	A.L. Pereira, G. Teixeira	8/97	Collected in rice fields. High density. Grown in Lisbon Botanical Garden.
Nisa	M. Bastos	7/97, 9/97, 3/98	Pool near Ribeira do Figueiró. Some <i>Azolla</i> were rooted in the banks. High density.

TABLE 2
Azolla specimens in Portuguese Herbaria used in this study

Herbaria	Taxa	Origin	Collector	Date
LISI-55224	<i>A. caroliniana</i>	Águas de Moura (rice fields)	J. Vasconcelos	8/55
LISU-P2784	<i>A. filiculoides</i>	Alcácer do Sal (rice fields)	L. Fernandes	5/21
LISU-P2788	<i>A. caroliniana</i>	Pinhal Novo (rice fields)	Palhinha	4/25
COI-9406	<i>A. filiculoides</i>	Geria, Coimbra (pool)	A. Fernandes	4/65
COI-22/38	<i>A. caroliniana</i>	Paúl da Arzila	Mota	5/32
PO-44133	<i>A. filiculoides</i>	Tabuaço, Adorigo (border of Douro river, quietness)	A. Duarte, A. Rozeira	9/45
PO-4977	<i>A. filiculoides</i>	Vendas Novas, Casa de Bragança (fountain)	A. Rozeira, J. Alte	9/45
PO-44132	<i>A. filiculoides</i>	Field in Coimbra	A. Cabral	11/20
PO-4441	<i>A. filiculoides</i>	S. Martinho do Porto (quiet stream)	A. Rozeira, J. Castro	7/44
PO-44134	<i>A. caroliniana</i>	Coimbra Botanical Garden	A. Cabral	11/20
PO-4214	<i>A. caroliniana</i>	Viscu, Dão river	J. Castro	8/44
PO-10220	<i>A. caroliniana</i>	Montemor-o-Velho, Barroca de Quinhentos	R. C. Teixeira	4/44

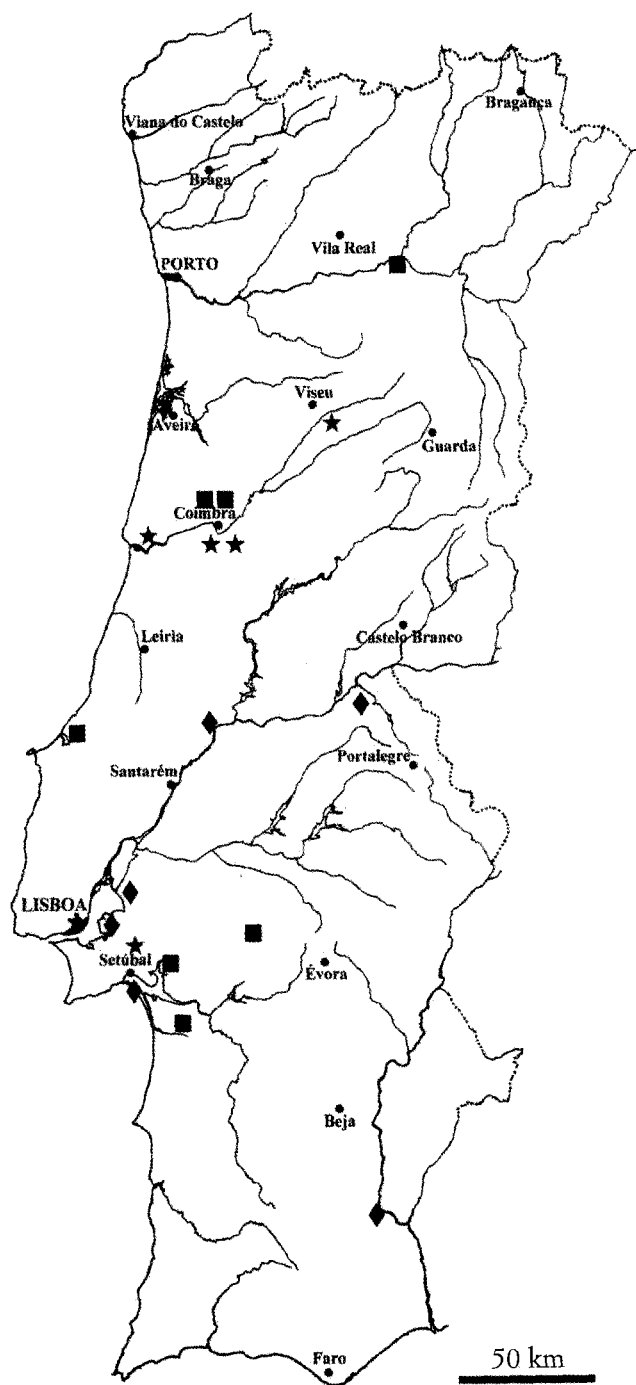


FIGURE 1 – Map of Portugal with the localisation of *Azolla filiculoides* (■) and *Azolla caroliniana* (★) from Herbaria. It also shows the location of field specimens (◆).

JSM 5200Lv and JEOL JSM T220 scanning electron microscopes.

For light microscopy, dried and fresh material was observed without staining and photographed using

Leitz Wetzlar Dialux and Nikon Labophot light microscopes.

RESULTS

Azolla is well adapted to the Portuguese climate, ranging from north to south in some international rivers such as Douro, Tejo and Guadiana but also in others national rivers, lagoons and small pools (Figure 1). Probably because of environmental conditions (especially the warmer climate) it is more frequent in the centre and south of Portugal.

Many vegetative and reproductive characters were observed, but in this study, we only used the characters described in table 3, which indicate similarities and differences between the *Azolla* specimens.

The sporophyte of *Azolla* is planar and/or erect depending on environmental conditions and plant density. This character, in nature, is highly variable and should not be used as a character to distinguish between *A. filiculoides*, *A. caroliniana* and *A. mexicana*. As for the shape of the sporophyte, it is always polygonal.

The number of hyaline border cells and papillae have been considered as diagnostic characters to identify *Azolla* species of the section *Azolla*. However, all the specimens collected in the field showed a high variability in the hyaline border, which consisted of two (Figure 2) to four (Figure 3) cell layers. The Herbarium specimens of *A. caroliniana* have two to three cell layers (Figures 4, 5) and those of *A. filiculoides* range from two to five cell layers (Figures 6, 7). The papillae of dorsal lobes from *Azolla* collected in the field are unicellular, *i.e.*, with only one cell above the epidermal cell (Figure 8). The specimen from Pancas, identified as *A. caroliniana*, also has unicellular papillae (Figure 9). Both *A. filiculoides* (Figure 10) and *A. caroliniana* (Figure 11) from Herbaria also showed unicellular papillae.

The presence or absence of septa in the anchor-shape glochidia has been used to distinguish between *A. filiculoides*, *A. caroliniana* and *A. mexicana*. In the specimens collected in the field, the number of septa is highly variable, varying between none (Figure 12) to one (Figure 13), two or three (Figure 14) even within the same massula. Herbarium specimens of *A. caroliniana* have none or one (Figure 15) septum, whereas *A. filiculoides*, also from Herbaria, has one (Figure 16) or two (Figure 17) septa in the apex of the glochidia. These observations demonstrate the high variability of this character, that depends on environmental conditions. It is therefore evident that some vegetative characters and the number of glochidium septa are not useful for identification.

Species recognition in the genus *Azolla* should be based on differences in the structure of the megaspore apparatus. The megaspore apparatus of field-collected *Azolla* always shows the same perine architecture, including the one from Pancas identified as *A. caroliniana*. Some herbarium specimens sporulated and were identified as *A. caroliniana* and *A. filiculoides*. In all of them, the perine surface has distinct raised hexagonal markings tied by the ends and covered with few filaments (Figures 18, 20). In longitudinal section, the perine that surrounds the megaspore consists of three layers: the innermost exine, an intermediate endoperine and the outermost exoperine. The endoperine in the raised areas is highly alveolated, but in the depressions becomes finely alveolated. In contrast, the exoperine is granular but thicker and denser in the depression areas than on top of raised areas (Figure 19). The perine surface of *A. caroliniana* from Herbaria also showed the same raised hexagonal markings (Figures 21, 23) and a highly alveolated endoperine (Figure 22), as observed also in *A. filiculoides*.

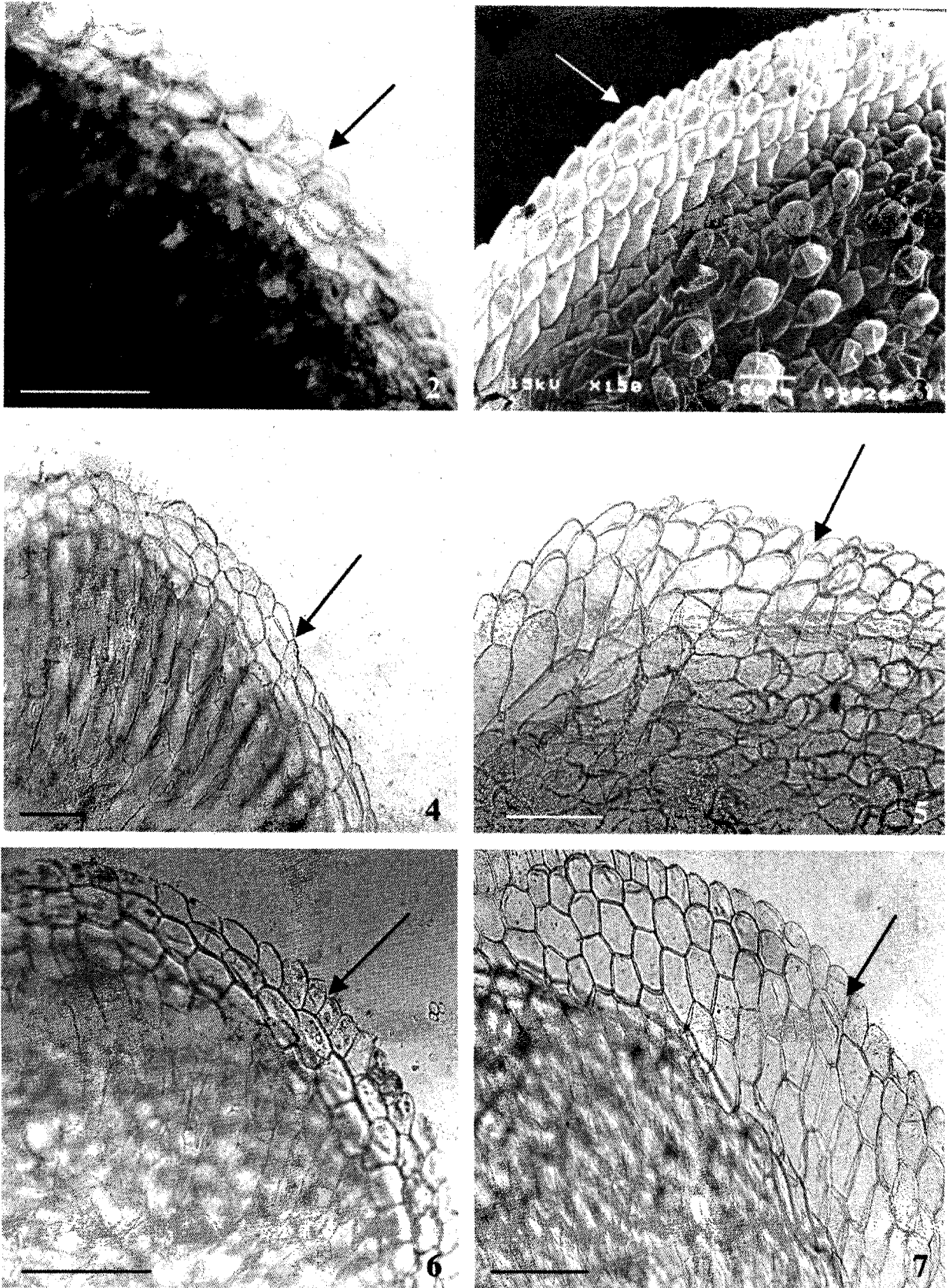
DISCUSSION

The vegetative characters used to distinguish between *A. caroliniana*, *A. filiculoides* and *A. mexicana* were plant and lobe dimensions, shape and degree of imbrication of leaves around the stem, leaf colour, and number of hyaline border cells. Based on our observations, these characters were highly variable. The sporophyte of *Azolla* frequently had a vertical growth and, in this case, the leaves were less imbricate around the stem, which may lead to incorrect identification. The other two characters – hyaline border and papillae of dorsal leaf lobes – seemed to have more credibility for taxonomic purposes, but only when the number of hyaline border and papillae cells was specified. Only ALMEIDA (1986) in Flora Ibérica indicates the number of cell layers of the hyaline border, since others Flora still use the words “membranous margins”, “thick” and “thin” hyaline border. The number of papilla cells of dorsal lobes is also a problematic issue. Some authors (ALCAREZ, 1995; FRANCO, 1971; LAWALRÉE, 1964; SAMPAIO, 1990) do not report the number of papilla cells, while others do

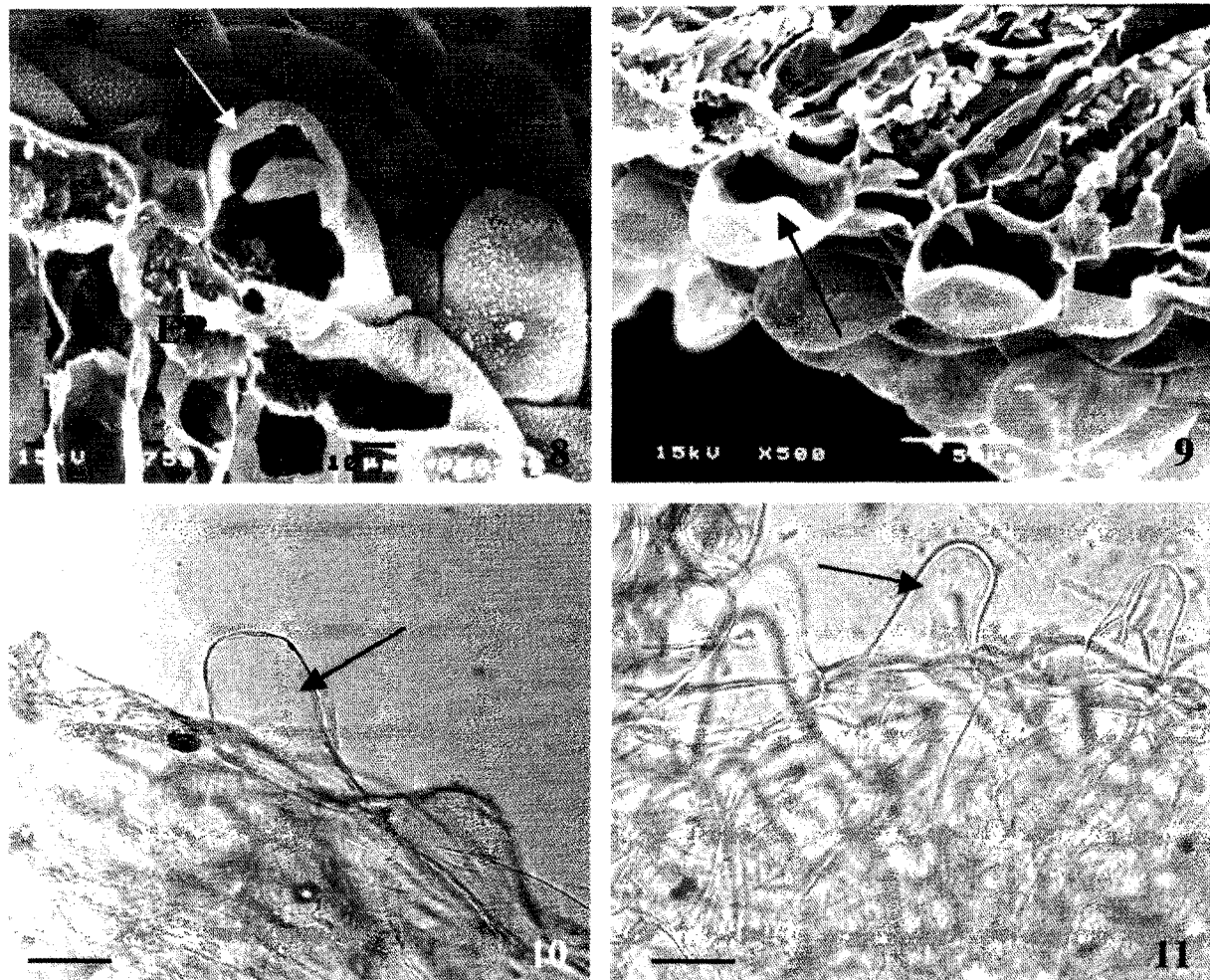
TABLE 3
Key characters used in the identification of *Azolla* field and herbarium specimens

	Character										
	1	2	3	4	5	6	7	8	9	10	11
Adema	0	0,1	1,2	0	0,1,2	0	0	0	0	1	0
Guadiana river	0	0,1	1,2	0	0,1,2	0	0	0	0	1	0
Golegã	0	0,1	1,2	0	0,1,2	0	0	0	0	1	0
Pancas	0	0,1	1,2	0	0,1,2	0	0	0	0	1	0
Comporta	0	0,1	1,2	0	0,1,2	0	0	0	0	1	0
Nisa	0	0,1	1,2	0	0,1,2	0	0	0	0	1	0
LISI-55224	0	?	1,2	0	0,1,2	0	0	0	/	/	/
LISU-P2784	0	?	1,2	0	0,1,2	0	0	0	/	/	/
LISU-P2788	0	?	1,2	0	0,1,2	0	0	0	/	/	/
COI-9406	0	?	1,2	0	0,1,2	0	0	0	/	/	/
COI-22/38	0	?	1,2	0	0,1,2	0	0	0	/	/	/
PO-44133	0	?	1,2	0	0,1,2	0	0	0	0	1	?
PO-4977	0	?	1,2	0	/	/	/	/	/	/	/
PO-44132	0	?	1,2	0	0,1,2	0	0	0	/	/	/
PO-4441	0	?	1,2	0	/	/	/	/	/	/	/
PO-44134	0	?	1,2	0	/	/	/	/	/	/	/
PO-4214	0	?	1,2	0	/	/	/	/	/	/	/
PO-10220	0	?	1,2	0	/	0	0	0	0	1	0

(1) Shape of sporophyte: 0 = polygonal, 1 = pinnate. (2) Habit at maturity: 0 = erect, 1 = planar. (3) Number of cells in hyaline border (dorsal lobe): 0 = one, 1 = two, 2 = three or more. (4) Papillae of dorsal lobes: 0 = unicellular, 1 = bicellular. (5) Number of septa in glochidia: 0 = none, 1 = one, 2 = two or more. (6) Perine surface: 0 = visible with few filaments, 1 = covered with filaments. (7) Perine surface: 0 = hexagonal excrescence, 1 = unpitted. (8) Hexagonal excrescence: 0 = tied by the ends, 1 = not tied by the ends. (9) Endoperine: 0 = highly alveolated in raised areas, 1 = scarcely alveolated in the raised areas. (10) Endoperine: 0 = thin between depressions, 1 = thick between depressions. (11) Exoperine: 0 = granular and dense in depression areas, 1 = regular. ? = uncertain. / = not observed



FIGURES 2 to 7 – Number of hyaline border cells of dorsal leaf lobes of *Azolla* specimens showing the variability of this character. Figures 2 and 3. *Azolla* collected in the field with two- and four-cell layers (arrows). Figures 4 and 5. *Azolla caroliniana* from Herbaria with two- and three-cell layers (arrows). Figures 6 and 7. *Azolla filiculoides* of Herbaria with two- to four-cell layers (arrows). Scale bar = 100 μ m.



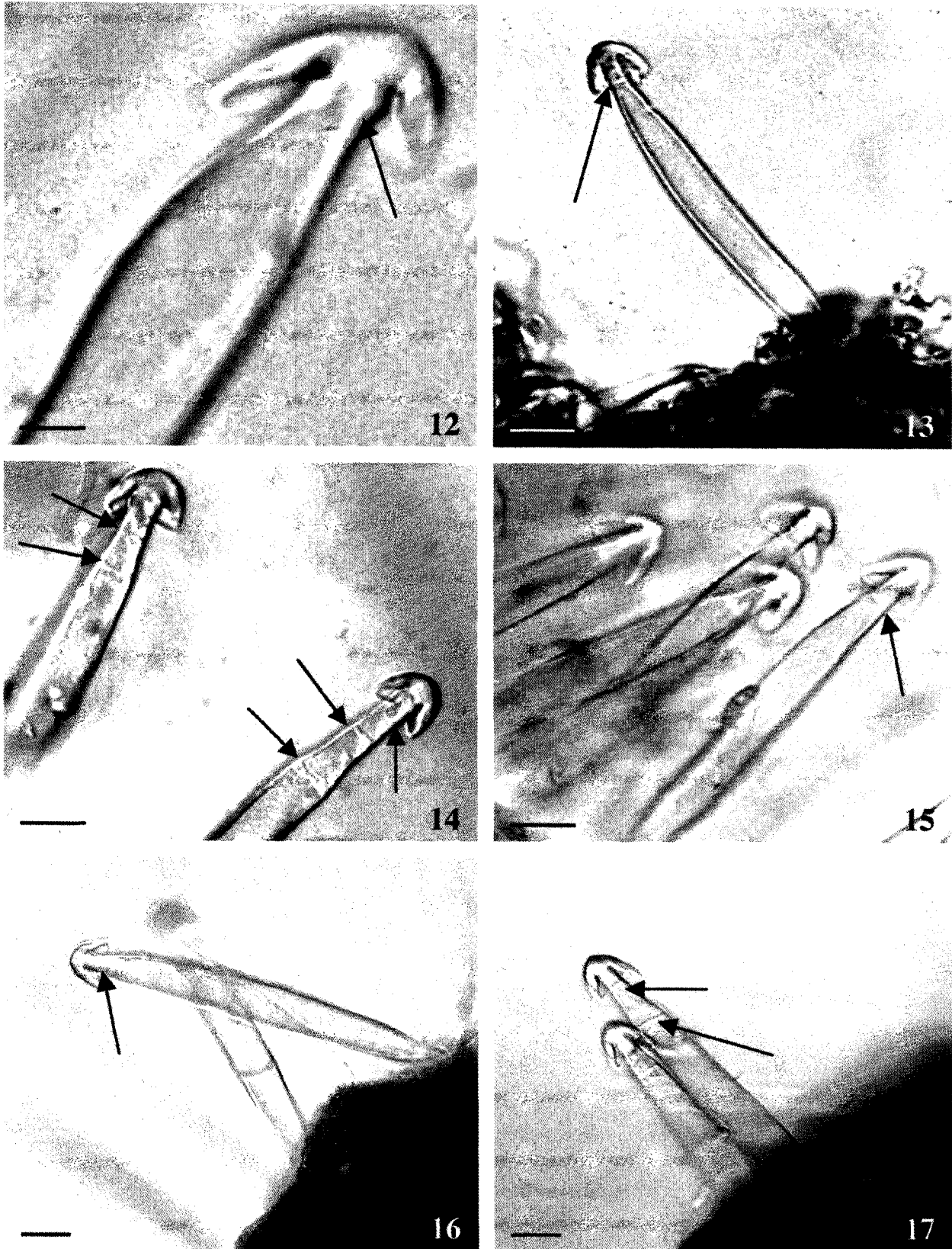
FIGURES 8 to 11 - Papillae of dorsal leaf lobes above the epidermal cells of *Azolla*. *Azolla filiculoides* collected in the field (figure 8) and from Herbaria (figure 10) with unicellular papillae (arrow). *Azolla caroliniana* collected in the field (figure 9) and from Herbaria (figure 11) also with unicellular papillae (arrow). Scale bar = 20 μ m.

(GUINOCHET & VILMORIN, 1973; JERMY, 1993; VALDÉS *et al.*, 1987; VASCONCELLOS, 1968). *A. caroliniana* has bicellular papillae and thin or 1-celled hyaline border cells, while *A. filiculoides* has unicellular papillae and thick or 2-4 celled hyaline border cells (ALMEIDA, 1986). All the specimens of *Azolla* observed had at least two-celled hyaline borders and unicellular papillae on the dorsal lobes, which may indicate that the fern in Portugal is *A. filiculoides*. PIETERSE *et al.* (1977) studied *Azolla* specimens from Netherlands and distinguished *A. filiculoides* from other samples that had papillae on dorsal leaf lobes, since in the former they are unicellular. Recently, Veys *et al.* (2000) proposed the pore of the leaf cavity as another vegetative character to distinguish between sections *Azolla* and *Rhizosperma*. According to these authors, the shape of the pore, the number of teat-

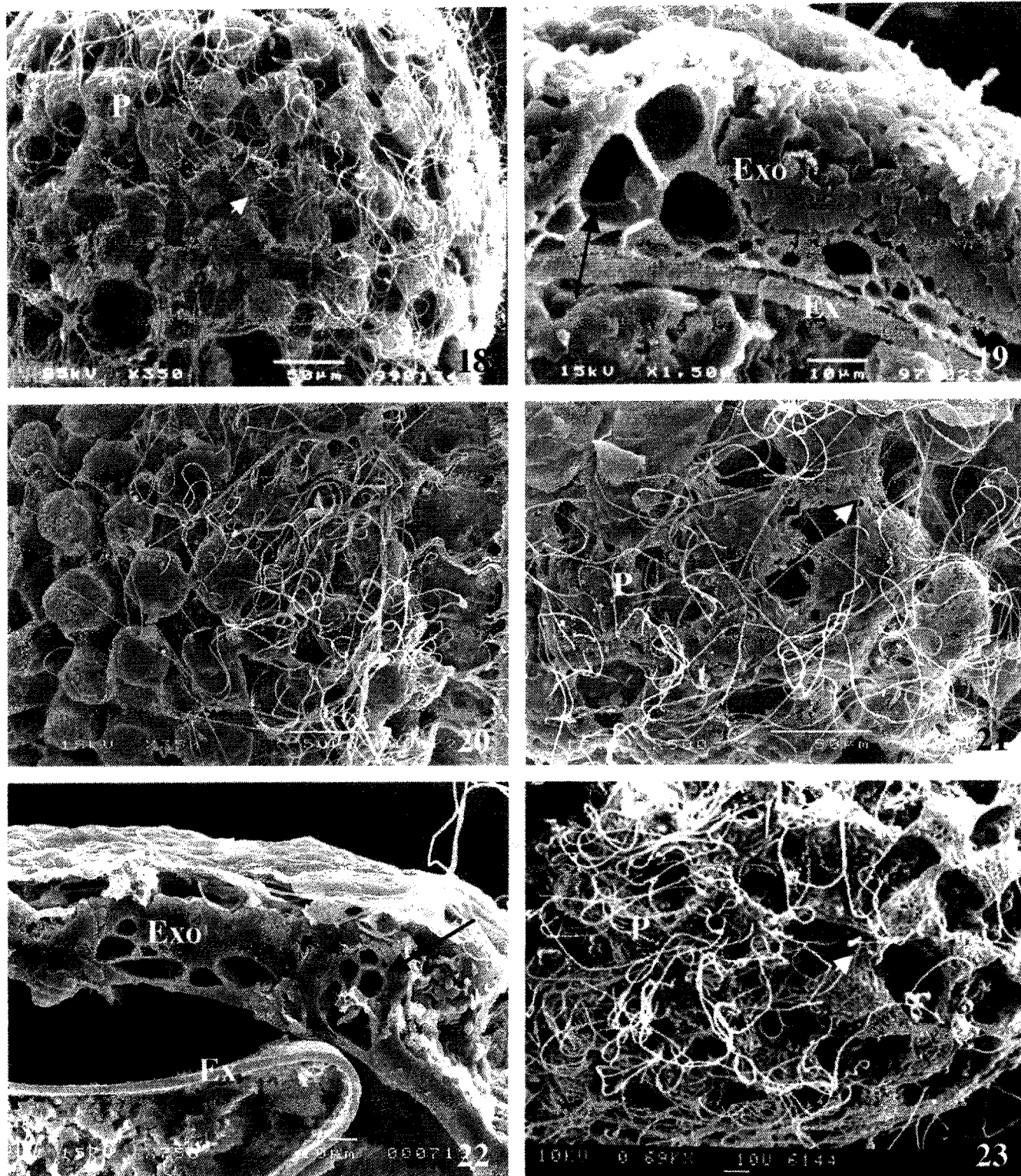
cell tiers, and the protrusion of the pore margin are similar in specimens of section *Azolla*.

The septation of glochidia that surround the microsporangium massulae is a reproductive character that may differentiate the species of section *Azolla*. *A. filiculoides* is traditionally described as non-septated or rarely septated with one or two septa, whereas *A. caroliniana* and *A. mexicana* have glochidia with one or two septa (ALMEIDA, 1986). According to GODFREY *et al.* (1961), this overlap in the number of septa is due to morphological variations within a given species, and may lead to contradictory identifications of *Azolla* specimens belonging to the same species. Our *Azolla* specimens had zero to three septa, indicating the high variability of this reproductive character.

PERKINS *et al.* (1985) studied all extant *Azolla* species



FIGURES 12 to 17 – Number of septa in the apex of glochidia present in the massulae of *Azolla* specimens, showing the variability of this character. *Azolla filiculoides* collected in the field with none (figure 12), one (figure 13), two and three (figure 14) septa (arrows). *Azolla caroliniana* from Herbaria with none or one (figure 15) septum (arrow). *Azolla filiculoides* from Herbaria with none (figure 16), one or two (figure 17) septa (arrows) even in the same massula. Scale bar = 10 μm.



FIGURES 18 to 23 - Perine surface and cross-section of megaspore apparatus from *Azolla filiculoides* collected in the field (figures 18, 19), *Azolla filiculoides* from Herbaria (figure 20) and *Azolla caroliniana* from Herbaria (figures 21, 22, 23). All the megaspore apparatus show a perine surface (P) with raised hexagonal markings tied by the ends (arrowhead) and few filaments. In longitudinal section the megaspore shows a 3-layered perine: an exine (ex), an alveolated endoperine (arrows) in the raised areas and a granular exoperine (exo).

with regard to the perine architecture, showing that this character is the most reliable for *Azolla* identification because it is different and unique for each *Azolla* species. This is also a useful character for studying the megaspore apparatus of fossil *Azolla* (ZHOU, 1983). SVENSON (1944) studied all the *Azolla* species and already indicated the perine surface as a distinctive character within *Azolla* species, particularly the hexagonal markings of *A. filiculoides*. DI FULVIO (1961) also studied the megaspore apparatus of *A. caroliniana*, *A. filiculoides* and *A. mexicana* and found differences in these three species, especially in the perine zonation.

In cross-section, the megaspore apparatus has three distinctive layers: the exine in close contact with the megaspore, the endoperine, and the outermost exoperine. *A. caroliniana* has a regular perine surface, unpitted and densely covered with filaments making it almost impossible to distinguish other structures. The cross-sections showed a finely granular endoperine that becomes slightly alveolated towards the exoperine. In *A. mexicana* the perine surface is pitted and almost without filaments (PERKINS *et al.*, 1985), and in cross-section the endoperine is granular whereas the exoperine is thick (DI FULVIO, 1961). *A. filiculoides* has a perine surface with raised hexagonal markings, tied by the ends and surmounted by few filaments. In cross-section the raised zones are perceptible with an alveolated endoperine that alternates with depressions, whose endoperine is thin and slightly granular (FOWLER & STENNETT-WILLSON, 1978; PERKINS *et al.*, 1985).

The new edition of *Flora Europaea* states the existence of *A. mexicana* Presl., and not *A. caroliniana* Willd., in Europe (including Portugal) based on papillae and number of glochidium septa (JERMY, 1993). However, LUMPKIN (1999), in the online edition of *Flora of North America*, uses the perine architecture to distinguish *Azolla* species and does not mention the glochidium septa as a distinctive character. The *Azolla* specimens collected in several sites in Portugal has a perine surface with hexagonal raised markings, clearly visible since they have few filaments. In cross-section, the endoperine is alveolated in the raised zone and less alveolated in the depression zones; the exoperine is finely granular and thick. Therefore, our observations match the ones made by FOWLER & STENNETT-WILLSON (1978) and PERKINS *et al.* (1985) for *A. filiculoides*. All the herbarium material already investigated, including the material identified as *A. caroliniana*, is in fact *A. filiculoides* (TEIXEIRA, 1999; PEREIRA *et al.*, 1998).

The taxonomy of the genus *Azolla*, particularly the species belonging to section *Azolla*, is difficult and controversial since the vegetative and some reproductive

characters used to identify these species are highly variable, depending on the collection site and of the environmental conditions.

Our observations on perine architecture and papillae of dorsal leaf lobes point towards the existence of only one species in Portugal, *A. filiculoides*, with a very distinct perine surface from that of the other *Azolla* species. With regard to the existence of two species in Portugal, we offer two explanations: either *A. caroliniana* never existed in our country and the herbarium specimens were incorrectly identified or, if it existed, it probably overlapped with *A. filiculoides*. There is at present no field or lab evidence for the presence of *A. mexicana*, and all the data strongly suggest that only *A. filiculoides* Lam. is present in Portugal.

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