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STRUCTURE AND FUNCTION OF SPORES IN THE AQUATIC HETEROSPOROUS FERN FAMILY MARSILEACEAE

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Spores of the aquatic heterosporous fern family Marsileaceae differ markedly from spores of Salviniaceae, the only other family of heterosporous ferns and sister group to Marsileaceae, and from spores of all homosporous ferns. The marsileaceous outer spore wall (perine) is modified above the aperture into a structure, the acrolamella, and the perine and acrolamella are further modified into a remarkable gelatinous layer that envelops the spore. Observations with light and scanning electron microscopy indicate that the three living marsileaceous fern genera (*Marsilea*, *Pilularia*, and *Regnellidium*) each have distinctive spores, particularly with regard to the perine and acrolamella. Several spore characters support a division of *Marsilea* into two groups. Spore character evolution is discussed in the context of developmental and possible functional aspects. The gelatinous perine layer acts as a flexible, floating organ that envelops the spores only for a short time and appears to be an adaptation of marsileaceous ferns to amphibious habitats. The gelatinous nature of the perine layer is likely the result of acidic polysaccharide components in the spore wall that have hydrogel (swelling and shrinking) properties. Megaspores floating at the water/air interface form a concave meniscus, at the center of which is the gelatinous acrolamella that encloses a "sperm lake." This meniscus creates a vortex-like effect that serves as a trap for free-swimming sperm cells, propelling them into the sperm lake.

Keywords: acrolamella, exine, heterospory, hydrogel, *Marsilea*, Marsileaceae, perine, *Pilularia*, *Regnellidium*, reproductive biology, sperm lake, spore.

Introduction

Marsileaceae and Salviniaceae, two aquatic heterosporous fern families, have long held a special appeal for plant morphologists (Russow 1872; Campbell 1888; Sadebeck 1902; Shattuck 1910; Allsopp 1963; Bell 1996). They share many features in common with aquatic angiosperms, such as simplified vascular systems, polymorphic vegetative characters, and highly specialized reproductive systems. Although these features have contributed greatly to confounding our understanding of the relationships of these two families, recent phylogenetic studies robustly support them as a monophyletic group within the paraphyletic homosporous leptosporangiate ferns (Rothwell and Stockey 1994; Hasebe et al. 1995; Pryer et al. 1995; Schneider 1996; Stevenson and Loconte 1996; Kenrick and Crane 1997; Pryer 1999; Pryer et al. 2001). The Marsileaceae comprises three extant genera: *Marsilea* L. (ca. 70 species), *Pilularia* L. (ca. 5 species), and the monotypic *Regnellidium* Lindm. *Marsilea* is sister to the *Pilularia-Regnellidium* clade (Pryer 1999). The Salviniaceae includes only two extant genera, *Azolla* Lam. and *Salvinia* Séq. Both families differ in numerous morphological and ecological aspects; most notably, Salviniaceae are strictly floating aquatics, whereas Marsileaceae have an amphibious rooted habit. They share, however, several evolutionary innovations within the leptosporangiate ferns, including dioicy, heterosporangy, endospory, monomegaspory, and a highly modified perine.

Spore morphology of ferns, including heterosporous ferns, has been the subject of recent detailed studies that preferentially utilized scanning and transmission electron microscopy to augment significantly our knowledge of fern spore ultrastructure (Lugardon and Husson 1982; Tryon and Lugardon 1991). Despite these and other excellent studies (Tralau 1969; Ferrarini et al. 1986; Large and Braggins 1989, 1991; Stafford 1995), spore character variation within Marsileaceae has not been well documented, in part because important layers that can be viewed only with light microscopy, such as the gelatinous perine layer, were neglected. A gelatinous "spore envelope" has been reported in *Marsilea* (Shattuck 1910; Feller 1953; Machlis and Rawitscher-Kunkel 1967; Rice and Laetsch 1967; Pettitt 1971; Bilderback 1978; Pettitt 1979*a*, 1979*b*), *Pilularia* (Meunier 1888) and *Regnellidium* (Lindman 1904; Chrysler and Johnson 1939; Higinbotham 1941), but it has never been examined in detail or compared among genera. A second interesting but underexamined feature of marsileaceous spores is the modified perine above the aperture, which has been variously referred to either as an acrolamella, hilum, or papilla.

This article aims to provide a detailed description and comparison of marsileaceous spores. Thorough comparative studies of extant marsileaceous spores are necessary not only for interpreting similar structures observed in the rich fossil record

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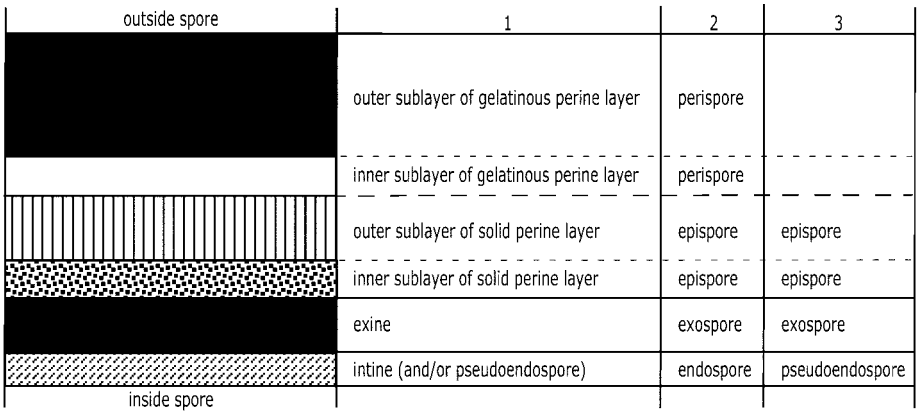

Fig. 1 Schematic cross section of Marsileaceae spore wall ultrastructure. Terminology used in this article (col. 1) is contrasted with corresponding terms used by Playford and Dettmann (1996; col. 2) and Tryon and Lugardon (1991; col. 3).

of heterosporous fern spores (Collinson 1991; Lupia et al. 2000) but also for addressing developmental, ecological, and evolutionary questions about these ferns and their close relatives. Among living heterosporous nonseed plants, the heterosporous ferns have the most sophisticated reproductive systems. These include characters such as monomegaspory—a feature otherwise restricted, among extant plants, to the seed plants (Bateman and DiMichele 1994)—that make these ferns particularly relevant to broader discussions on the evolution of heterosporic phenomena and the seed habit.

Material and Methods

Taxonomy

We follow Kramer (1990) for the classification of Marsileaceae. Our classification of *Marsilea* follows Johnson (1986) in large part and an ongoing phylogenetic study of *Marsilea* based on morphological and DNA sequence data (K. M. Pryer and H. Schneider, unpublished manuscript) that provides preliminary evidence for a basal division of the genus into two major clades. One clade (referred to herein as group I; table 1) unites *Marsilea mutica* Mett. with *Marsilea polycarpa* Hook. & Grev. and its relatives (section *Chlemys* D.M. Johnson). All other species of *Marsilea* are members of the sister clade (referred to herein as group II; table 1).

Plants, Culture, and Handling

Spores from an exhaustive list of marsileaceous ferns were examined and compared (table 1). Sporocarps were removed from herbarium vouchers and soaked in water until they released their spores. After their release, the spores were stored in water and observed nearly continuously for the first 4 h and at regular intervals thereafter for 2–4 d. If embryos eventually developed, observations were continued periodically on those megaspores with a young sporophyte attached. We mechanically opened a subset of sporocarps to better determine the time frame for the swelling of the gelatinous perine layer of the spore walls. Sporocarps and spores were maintained in small plastic petri dishes filled with tap water and were incubated in a growth chamber (Percival Scientific, CU-32L) with the following settings: day/night temperatures 28°–30°/22°C, humidity ca. 95%, light schedule 12 h light/12 h dark.

Light Microscopy

Whole and sectioned spores were examined with a Zeiss Axioskop light microscope with bright-field, dark-field, dif-

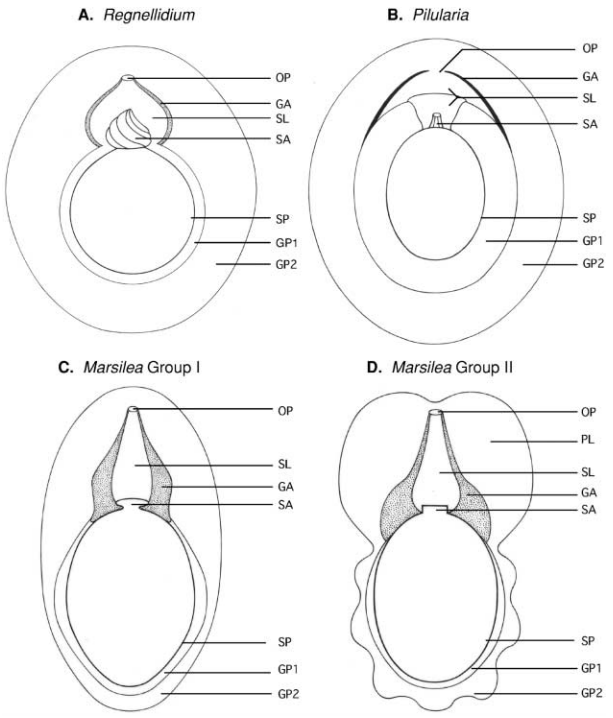

Fig. 2 Schematic longitudinal sections through fully hydrated Marsileaceae megaspores. *A*, *Regnellidium*. *B*, *Pilularia*. *C*, *Marsilea* group I. *D*, *Marsilea* group II. *GA* = gelatinous acrolamella layer; *GP*1 = inner sublayer of the gelatinous perine layer; *GP*2 = outer sublayer of the gelatinous perine layer; *OP* = opening of the sperm lake; *PL* = proximal lobes of the outer sublayer of the gelatinous perine layer (this character is unique to *Marsilea* group II). *SA* = solid acrolamella layer; *SL* = sperm lake; *SP* = solid perine layer (surrounds the exine and other internal spore regions not illustrated here).

Table 1

Marsileaceae Taxa Examined and Vouchers

Species	Country source and voucher information (herbarium accession numbers)
Regnellidium Lindm.:	
**Regnellidium diphyllum* Lindm.	Brazil: *Bloom s.n.* (F 1709990), *Durta s.n.* (F 1433068), *Rau s.n.* (US 1593512)
Pilularia L.:	
**Pilularia americana* A. Braun	USA: *Hill 8654* (F 1866831), *Pryer et al. 954* (DUKE)
**Pilularia globulifera* L.	France: *Chevallier s.n.* (F 802279)
Pilularia minuta Durieu	France: *Neyra 820* (F 799940)
Pilularia novae-zelandiae Kirk	New Zealand: *Kirk s.n.* (F 35940)
Marsilea L. (group I):	
**Marsilea crotophora* D.M. Johnson	Bolivia: *Arroyo 36* (F 2185246), *Rolleri 9* (US 2849627); Nicaragua: *Baker 7781* (US 398726)
**Marsilea deflexa* A. Braun	Costa Rica: *Jimenez 348* (F 1607254), *Williams & Williams 24936* (F 1623778); Venezuela: *Pittier 10153* (US 1120869)
**Marsilea mutica* Mett.	Australia: *Boorman s.n.* (F 197668), *Constable NSW P8312* (US 241634)
Marsilea polycarpa Hook. & Grev.	Bolivia: *Beck 5518* (F 1896684); Colombia: *Elias 11374* (F 880833); Costa Rica: *Gomez 12557* (F 2151327); Panama: *Dressler 6004* (US 3140953), *Standely 30765* (US 16200); West Indies: *Curtis 220* (F 165133)
Marsilea L. (group II):	
Marsilea aegyptica Willd.	Egypt: *Schweinfurth s.n.* (US 816363); Yemen: *Wood 2948* (F 1870462)
**Marsilea ancylopoda* A. Braun	Ecuador: *Asplund 15265* (US 2652614); Mexico: *Lott and Sanders 3987* (F 2120246)
Marsilea angustifolia R. Br.	Australia: *Chinnock P899* (US 2952878)
**Marsilea azorica* Launert & Paiva	United Kingdom: cultivated in Chelsea Physic Garden, London (original collection from Azores) (no voucher)
**Marsilea botryocarpa* F. Ballard	Kenya: *Faden 72/101* (US 2690545), *Luke TPR 783* (US 3215831), *Evans and Maikweki 62* (US 2650940)
Marsilea burchelii A. Braun	South Africa: *Son 18042* (F 653429), *Schelpe 4552* (F 2423789)
Marsilea capensis A. Braun	South Africa: *Schweickerdt 9346* (US 2414736)
Marsilea coromandelina Willd.	Ceylon: *Faden 77/154* (F 2014306); Kenya: *Faden and Smeenk 72/76* (US 2690542)
**Marsilea drummondii* A. Braun	Australia: *Chinnock P1067* (F 17934307); Switzerland: *Schneller s.n.* (cultivated, original collection from Australia) (Z); USA: *Hoshizaki 577* (cultivated, original collection from Australia) (UC 1730195)
**Marsilea ephippiocarpa* Alston	South Africa: *Son 18026* (F 653428)
Marsilea exarata A. Braun	Australia: *Winkworth P7525* (US 2414194)
**Marsilea fadenia* Launert	Kenya: *Evans and Maikweri 55* (US 3183268)
Marsilea farinosa Launert	Kenya: *Faden 70/902* (US 2650941)
**Marsilea gibba* A. Braun	Kenya: *Evans 10* (US 2690525), *Faden 69/1293* (US 26114135), *Faden 74/576* (F 1794168), *Faden 87/33* (US 2991993)
Marsilea hirsuta R. Br.	Germany: *Braun s.n.* (cultivated, original collection from Australia) (F 893225)
**Marsilea macrocarpa* C. Presl	Kenya: *Agnew 10750* (US 3113520), *Faden 77/295* (F 206935); USA: *Hoshizaki 236* (cultivated, original collection from Tanzania) (F 2195891)
Marsilea macropoda Engelm. ex A. Braun	USA: *Landry 7895* (US 2951819), *Webster and Wilbur 3057* (US 2139942)
**Marsilea minuta* L.	Australia: *McKee 9048* (US 2416383); Japan: *Saiki 2270* (F 2096977); Kenya: *Evans and Glover 64* (US 2690526); Malaysia: *Molesworth 4784* (F 1592394); Pakistan: *Rodin 5438* (F 1448313); Philippines: *Merrill 194* (F 132301); USA: *Hoshizaki 900* (cultivated, original source not known) (UC)
Marsilea mollis Robinson & Fernald	Mexico: *Hartman 604* (F 49637), *Pringle 2434* (F 10499)
**Marsilea nashii* Underw.	West Indies: *Correll 46631* (F 1769479), *Correll 49945* (F 1866099)
Marsilea oligospora Goodding	USA: *Howell 36949* (US 2631045)
**Marsilea quadrifolia* L.	France: *Le Brun s.n.* (F 802284); USA: *Fosberg 44302* (US 2692394)
Marsilea strigosa Willd.	Germany: *Braun 755* (cultivated, original collection from France) (US 828812)
**Marsilea vestita* Hook. & Grev.	USA: *Howell 47460* (US 783150), *Palmer 13465* (F 741964), *Hoshizaki 238* (F 2195893)
**Marsilea villosa* Kaulf.	USA: *Degener 9049* (US 1657099), *Fosberg 9204* (F 944653)

Note. Spores from all taxa were studied with light microscopy. Taxa preceded by an asterisk were also examined with scanning electron microscopy.

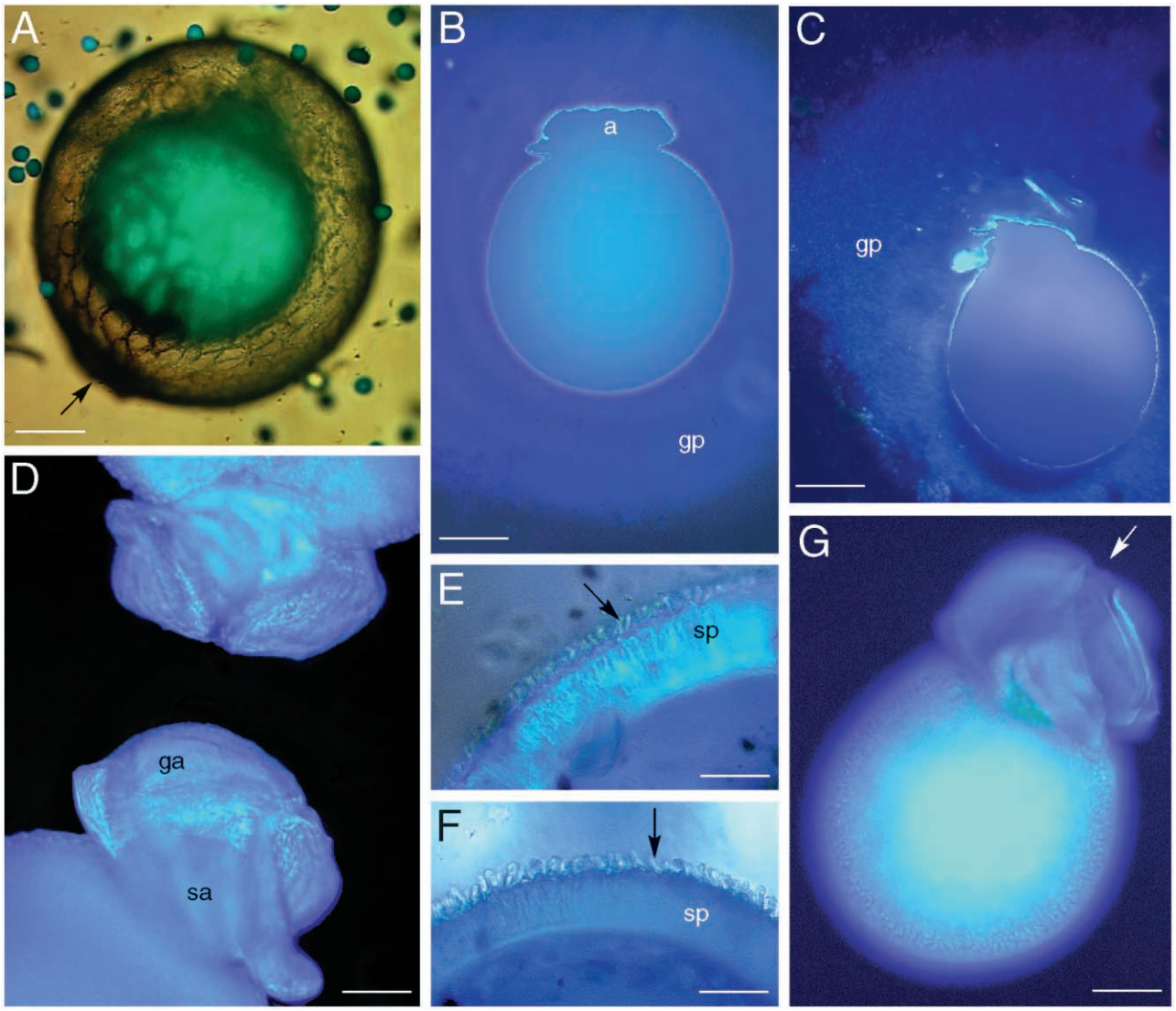

Fig. 3 Megaspores of *Regnellidium diphyllum* (*Bloom s.n.*, F). *A*, Megaspore inside megasporangium with acrolamella (appears as indistinct bulge through translucent megasporangium wall) oriented in the opposite direction of the attachment point (arrow) of the megasporangium stalk, which was removed in this view. Note the free-floating microspores visible in the vicinity of the hydrated megasporangium. Scale bar = 120 μm. *B*, Fully hydrated megaspore with acrolamella (*a*) surrounded by gelatinous perine (*gp*) layer, which has a low optical contrast. Scale bar = 110 μm. *C*, Hydrated megaspore that has dried. The gelatinous perine (*gp*) layer is deposited as a thin layer on the slide surface. Scale bar = 120 μm. *D*, Acrolamellae of two megaspores, each showing an inner part that is solid (*sa*) enclosed by an outer part that is gelatinous (*ga*) and that mirrors the twisted lobes of the solid part. Scale bar = 41 μm. *E*, *F*, Cross section of the megaspore wall showing the solid perine (*sp*) layer with an alveolate sublayer that grades outward into baculae (arrow). Scale bars = 27 μm and 32 μm, respectively. *G*, Whole megaspore 6–7 h after release from megasporangium, at which time the gelatinous perine layer has disintegrated. The gelatinous part of the acrolamella that completely encloses the solid part remains intact. Note the twisted lobes of the solid part of the acrolamella and the small opening (arrow) at top of acrolamella that leads to a breach between the solid and gelatinous parts; the breach is occupied by the sperm lake. Scale bar = 90 μm. Epifluorescence optics used for *B–D*, *G*; epifluorescence combined with bright-field optics used for *A*, *E*, *F*.

ferential interference contrast (DIC), and epifluorescence optics (fluorescence filter sets CZ 902 and CZ 905). Samples of whole spores were observed in water, absolute ethanol, or glycerin and were stained with the following dyes: alcian blue for acidic polysaccharides (Pearse 1968), aniline black for proteins (O'Brien and McCully 1981), aniline blue for callose (O'Brien and McCully 1981), calcofluor white for carboxylated polysaccharides (Hughes and McCully 1975), iodine-potassium iodide (Lugol's reagent) with acids or zinc-chloride for cellulose (O'Brien and McCully 1981), periodic acid–Schiff's reaction (PAS) for insoluble polysaccharides (Jensen 1962), ruthenium red (Jensen 1962) for polysaccharides with acidic groups (Amarasinghe 1990), safranin for polysaccharides (O'Brien and McCully 1981), and Sudan IV for lipids (O'Brien and McCully 1981).

We sectioned spores in water or glycerin with a freezing

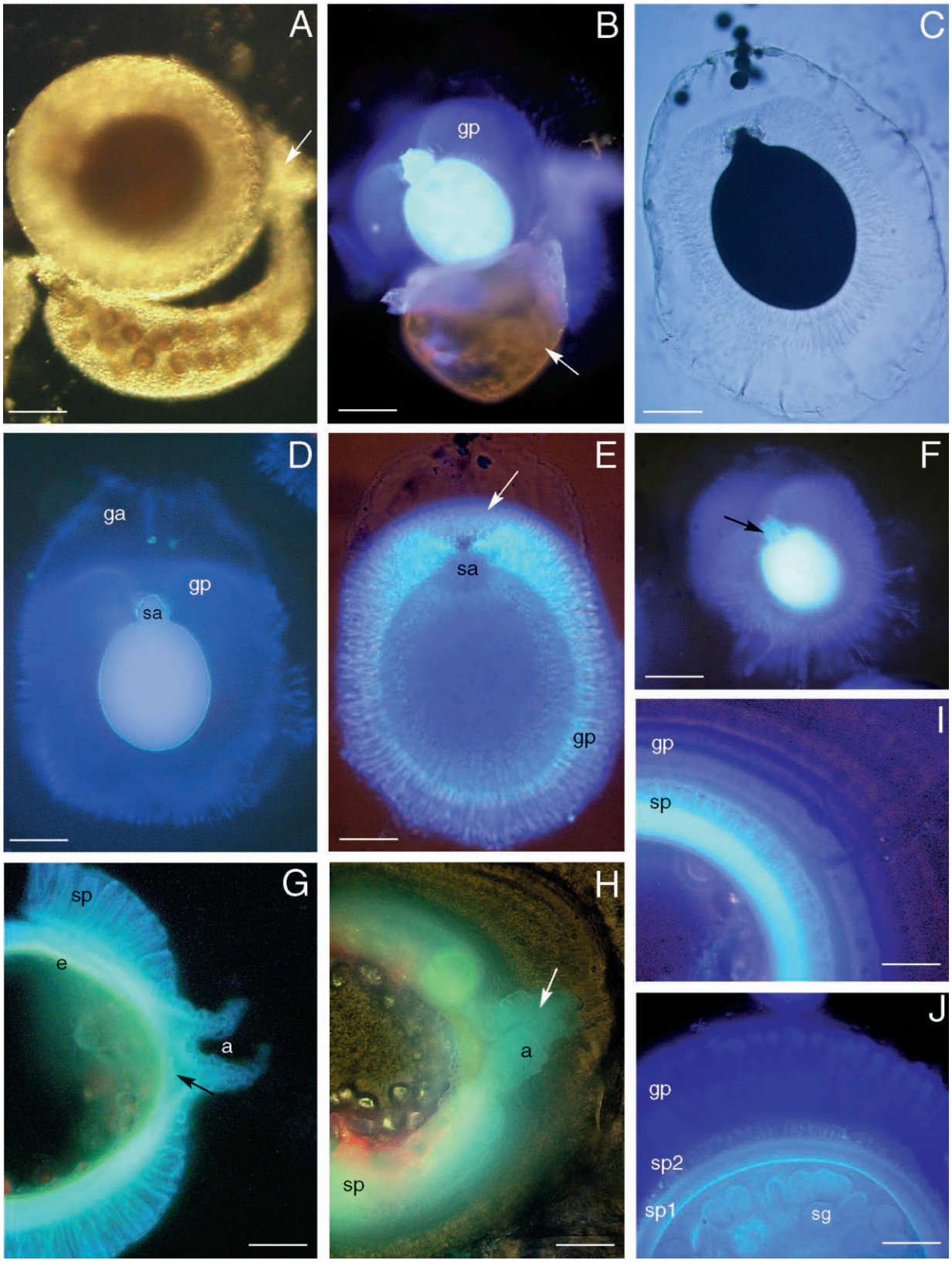

Fig. 4

microtome (Leica SM2000R with a Physitemp BFS series freezing stage). Sections were 0.1–0.2 mm thick. They were transferred to and observed in water, absolute alcohol, or glycerin and stained with the same dyes as those used for whole spores.

Light micrographs were taken with a Sony DXC-970 1/2 RGB video system. Using the highest resolution, we transferred the video system signal directly to a PowerMac 7500 outfitted with an AG-7 frame-grabber. Images were manipulated with Adobe Photoshop 5.0.

Scanning Electron Microscopy

Spores were prepared for scanning electron microscopy with three different methods. The first method involved an acidic treatment to remove the gelatinous perine layer, whereby hydrated spores were transferred to glacial acetic acid (2–10 min) before they were treated with concentrated sulfuric acid (1–3 min). They were then transferred back to glacial acetic acid and slowly dehydrated with an alcohol/water dehydration series (50%, 75%, 90%, 95%, 100% EtOH). The second method involved hydrating spores in water for 4–6 h and then transferring them to 100% EtOH. This permitted the gelatinous perine layer to be distinguished from the solid perine layer in cross section. The third method involved mechanically removing spores from the dry sporocarps and observing them without any further treatment. Spores prepared by the first two methods were critical-point dried with a Balzers critical point dryer 030 (100% EtOH, CO_2). Fractured wall sections were obtained with a razor blade or fine-point forceps. Dry spores were affixed to stubs with double-sided tape, coated with a thin gold film (2–4 min) in a sputter coater (Denton Vacuum Desk II), and viewed with an AMRAY 1810 scanning electron microscope at 10 kV and 60 μA. Scanning electron micrographs were recorded on Polaroid film (PIN 55).

Spore Terminology

Most descriptions of fern spores (Tryon and Lugardon 1991; Lellinger and Taylor 1996) differ in terminology from descriptions of pollen and other spores (Blackmore 1990; Playford and Dettmann 1996). We follow the terminology compiled in Punt et al. (1994; a second edition is available on-line at http://www.bio.uu.nl/~palaeo/glossary/glos-int.htm).

Although most descriptions of marsileaceous spores agree as to the presence of three spore wall layers, they differ in the terminology applied to these layers (Feller 1953; Boterberg 1956; Pettitt 1966, 1971, 1979*a*, 1979*b*; Southworth and Myles 1984; Tryon and Lugardon 1991). Figure 1 provides a comparison of terminology used in recent literature (Tryon and Lugardon 1991; Playford and Dettmann 1996). For a comparison of older terms, see Southworth and Myles (1984). Stafford (1995) differs from all other authors in that he defines the entire acid-resistant spore wall as "exine" and uses "sexine" to refer to the outermost exine ("perine" in this study) and "nexine" for the innermost exine ("exine" in this study).

Transmission electron microscope studies by Tryon and Lugardon (1991) indicated the presence of additional inner spore wall layers—the pseudoendospore and intine—in marsileaceous spores that are formed during germination. It was not possible for us to distinguish these two layers with scanning electron or light microscopy.

The middle spore wall layer, or exine, is homologous to the exospore of homosporous ferns (*sensu* Lugardon and Husson 1982; Tryon and Lugardon 1991). The terminology associated with the outer spore wall of marsileaceous ferns is particularly confusing with various terms having been applied previously, such as exospore, perine, perispore, or epispore. Although the term perispore is usually associated with the outer spore wall of homosporous ferns by Lugardon and Husson (1982) and Tryon and Lugardon (1991), these authors introduced the term "epispore" for the outer spore wall of heterosporous ferns to highlight ultrastructural differences between heterosporous and homosporous ferns. Using this new term, however, disregards the strong likelihood that the outer spore walls of both heterosporous and homosporous ferns are homologous and that the epispore is simply a highly modified perine ("perispore" of Lugardon).

An often-ignored layer of marsileaceous spores is the gelatinous mass surrounding hydrated spores (fig. 2). Although the

Fig. 4 Megaspores of *Pilularia americana* (*Pryer et al. 954*, DUKE). *A*, Falcate microsporangium containing numerous microspores and ellipsoidal megasporangium (appears almost spheroidal in this view) containing a single megaspore with an acrolamella (appears as indistinct bulge through translucent megasporangium wall) that is oriented in the opposite direction of the attachment point of the megasporangium stalk (arrow). Scale bar = 145 μm. *B*, Megaspore initiating its release from the megasporangium (arrow) via the swelling of the gelatinous perine (*gp*) layer. Scale bar = 140 μm. *C*, Megaspore surrounded by the fully expanded gelatinous perine layer with a fibrillose inner sublayer visible. Scale bar = 110 μm. *D*, Megaspore surrounded by the gelatinous perine (*gp*) layer. The acrolamella has an inner part that is solid (*sa*) and an outer part that is gelatinous (*ga*) and ephemeral. Scale bar = 140 μm. *E*, Megaspore enclosed by gelatinous perine (*gp*) layer with only the fibrillose inner sublayer still visible. The inner solid part of the acrolamella (*sa*) is surrounded by a funnel-shaped sperm lake (arrow) soon to be exposed by the disintegration of the gelatinous part of the acrolamella. Scale bar = 90 μm. *F*, Megaspore, not fully hydrated, showing the fibrillose inner sublayer of the gelatinous part of the perine and the straight lobes (arrow) of the solid part of the acrolamella. Scale bar = 210 μm. *G*, Longitudinal section of megaspore at acrolamella (*a*). The exine (*e*) is yellow. The solid part of the perine (*sp*) is yellowish green and grades outward to blue with a thick alveolate sublayer that becomes reduced at the acrolamella (*a*). Note the small chamber (arrow) between the exine and the perine at the base of the acrolamella lobes. Scale bar = 10 μm. *H*, Longitudinal section of a megaspore at acrolamella (*a*). The solid part of the perine (*sp*) is yellowish green. Starch grains are visible in spore lumen, and the acrolamella (*a*) has a central channel (arrow). Scale bar = 34 μm. *I*, Cross section of the megaspore wall showing the alveolate sublayer of the solid perine (*sp*) layer and the concentric nature of the gelatinous perine (*gp*) layer. Scale bar = 14 μm. *J*, Cross section of the megaspore wall showing starch grains (*sg*) in the spore lumen and the uniform inner sublayer (*sp1*) and the alveolate outer sublayer (*sp2*) of the solid perine layer, both of which are surrounded by the gelatinous perine (*gp*) layer. Scale bar = 18 μm. Dark-field optics used for *A*; epifluorescence optics used for *B*, *D*–*G*, *I*, *J*; epifluorescence combined with bright-field optics used for *H*; epifluorescence combined with DIC optics used for *C*.

gelatinous mass has been recognized as an integral part of the spore wall by some authors (Meunier 1888), many authors viewed it as ancillary and restricted their descriptions of the spore wall to the solid layers (Chrysler and Johnson 1939) or to the acetolysis-resistant layers (Pettitt 1979*b*), suggesting that the authors considered the presence of sporopollenin to be the defining character of a spore wall. In homosporous ferns, the perine is formed by material provided by the degenerating tapetum (Tryon and Lugardon 1991; van Uffelen 1991, 1993, 1996; Parkinson 1995*a*, 1995*b*, 1996; Parkinson and Pacini 1995; Oldenhof and Willemse 1999), and this seems also to be true in heterosporous ferns (Tryon and Lugardon 1991; Parkinson and Pacini 1995). Although detailed studies are not available, observations of early spore development (Meunier 1888; Chrysler and Johnson 1939; Higinbotham 1941) suggest that the solid and gelatinous layers of the perine likely undergo similar developmental processes. Location, developmental similarities, and phylogenetic arguments support the homology of the perine of homosporous ferns with both the solid and gelatinous layers of the outer spore wall of marsileaceous ferns.

A variety of terms have been used to describe the strong modification of the perine that covers the exine aperture of marsileaceous spores, i.e., acrolamella, gula, hilum, and papilla (Collinson 1991; Stafford 1995). We follow Tschudy (1966) and Hall (1975) in preferring "acrolamella."

Spore "Behavior" Observations

A time series was recorded for specific "behavioral" and developmental events for spores of *Pilularia americana* from the moment of spore release from the sporocarp, through "germination" and gametophyte development and until fertilization, or the apparent initiation of sporophyte development. Times and events reported here are based on the average of 10 independent observation periods. For each observation period, a fresh sporocarp was opened mechanically, placed in a small glass vial filled with tap water (4 cm deep), and incubated at 25°C with a 12-h photoperiod (Mahlberg and Baldwin 1975; Mahlberg and Yarus 1977). We also made similar observations for several species of all three genera using the recorded times of *P. americana* for comparison.

Character Evolution

A phylogeny of heterosporous ferns based on Pryer (1999) and an ongoing phylogenetic study of *Marsilea* (K. M. Pryer and H. Schneider, unpublished manuscript) provided the simplified topology that we used to interpret spore character evolution in Marsileaceae. We mapped character state changes onto this tree using Acctran and Deltran optimization with MacClade 3.05 (Maddison and Maddison 1992).

Results

Mega- and Microsporangium Morphology

Marsileaceae has two types of sporangia: megasporangia, which contain a single megaspore (monomegasporangy), and microsporangia, which contain up to 64 microspores. Both types of sporangia develop within a single sorus. Sporangia are of the leptosporangiate type (i.e., single-celled sporangium wall), but neither an annulus nor a stomium is differentiated. In *Regnellidium*, both the mega- and microsporangia (fig. 3*A*; fig. 7*A*, 7*B*) are differentiated into a capsule and a long stalk. A clearly differentiated stalk is absent (figs. 4*A*, 7*C*) in *Pilularia*. In *Marsilea*, the capsules and stalks (figs. 5*A*, 6*A*; fig. 7*D*, 7*E*) are differentiated but the stalk is very short. The megasporangium capsule essentially captures the shape of its single resident megaspore. *Regnellidium* (fig. 3*A*) has a spheroidal capsule, while *Pilularia* and *Marsilea* have ellipsoidal to obovoidal capsules (figs. 4*A*, 5*A*, 6*A*). The three genera are distinct from one another in the shapes of their microsporangia. The microsporangium capsule is spheroidal in *Regnellidium* (fig. 7*A*, 7*B*), falcate in *Pilularia* (fig. 7*C*), and ellipsoidal to obovoidal in *Marsilea* (fig. 7*D*, 7*E*).

Marsilea differs in its orientation of the megaspore inside the megasporangium from *Pilularia*, *Regnellidium*, and other heterosporous ferns. The spore aperture is oriented toward the sporangial stalk in *Marsilea* (figs. 5*A*, 6*A*), whereas the aperture is oriented in the opposite direction (toward the sporangium apex) in *Regnellidium* and *Pilularia* (figs. 3*A*, 4*A*) and in all other heterosporous ferns (H. Schneider and K. M. Pryer, personal observation).

Megaspore Morphology

The megaspore outline is either prolate, as in *Marsilea* (fig. 2*C*, 2*D*; figs. 5*B*, 6*E*) and *Pilularia* (figs. 2*B*, 4*C*), or spheroidal, as in *Regnellidium* (figs. 2*A*, 3*B*). Megaspores of *Pilularia globulifera* (fig. 8*C*) differ from all other species of *Pilularia* in having an obovoidal shape and an equatorial furrow subproximal to the aperture (also figs. 29 and 33 in Lupia et al. 2000). Observations from light microscopy indicate that the outermost layer of the spore wall of *Marsilea*, *Pilularia*, and *Regnellidium* is modified into a remarkable gelatinous envelope. An additional notable characteristic of marsileaceous megaspores is that the perine is modified above the exine aperture into an acrolamella (fig. 2).

Hydrated megaspores are surrounded by a gelatinous perine layer (fig. 3*B*, 3*C*; fig. 4*C*; fig. 5*B*, 5*C*; fig. 6*C*). This layer swells rapidly when in contact with water, but in dehydrated spores, it is a very thin sheath that tightly envelops the solid perine layer (fig. 8*B*, 8*D*, 8*F*). The gelatinous perine layer reaches its maximum size about 2–4 h after the spore's release in water and begins to disintegrate after 6 h. Only a thin gelatinous residue remains after 20 h in water. The gelatinous perine layer appears to be divided into two sublayers. In *Regnellidium*, the outer gelatinous sublayer is spheroidal, but the inner one mirrors the shape of the solid perine layer, including the solid acrolamella (fig. 2*A*). In *Pilularia*, the outer gelatinous sublayer (figs. 2*B*, 4*C*) is ellipsoidal, but the inner sublayer is modified around the solid acrolamella to form a funnel-shaped region termed the "sperm lake" (figs. 2*B*, 4*D*–4*F*; Machlis and Rawitscher-Kunkel 1967). In *Marsilea* group I taxa (table 1), the outer gelatinous sublayer is obovoidal (fig. 2*C*; fig. 5*B*, 5*C*), but the inner sublayer forms a bell-shaped structure that surrounds the solid acrolamella (fig. 2*C*; fig. 5*D*, 5*H*). In *Marsilea* group II taxa (table 1), the inner gelatinous sublayer is identical to that observed in *Marsilea* group I taxa, whereas the outer sublayer has numerous folds at the distal end of the spore (corresponding to the "basal envelope" of Machlis and

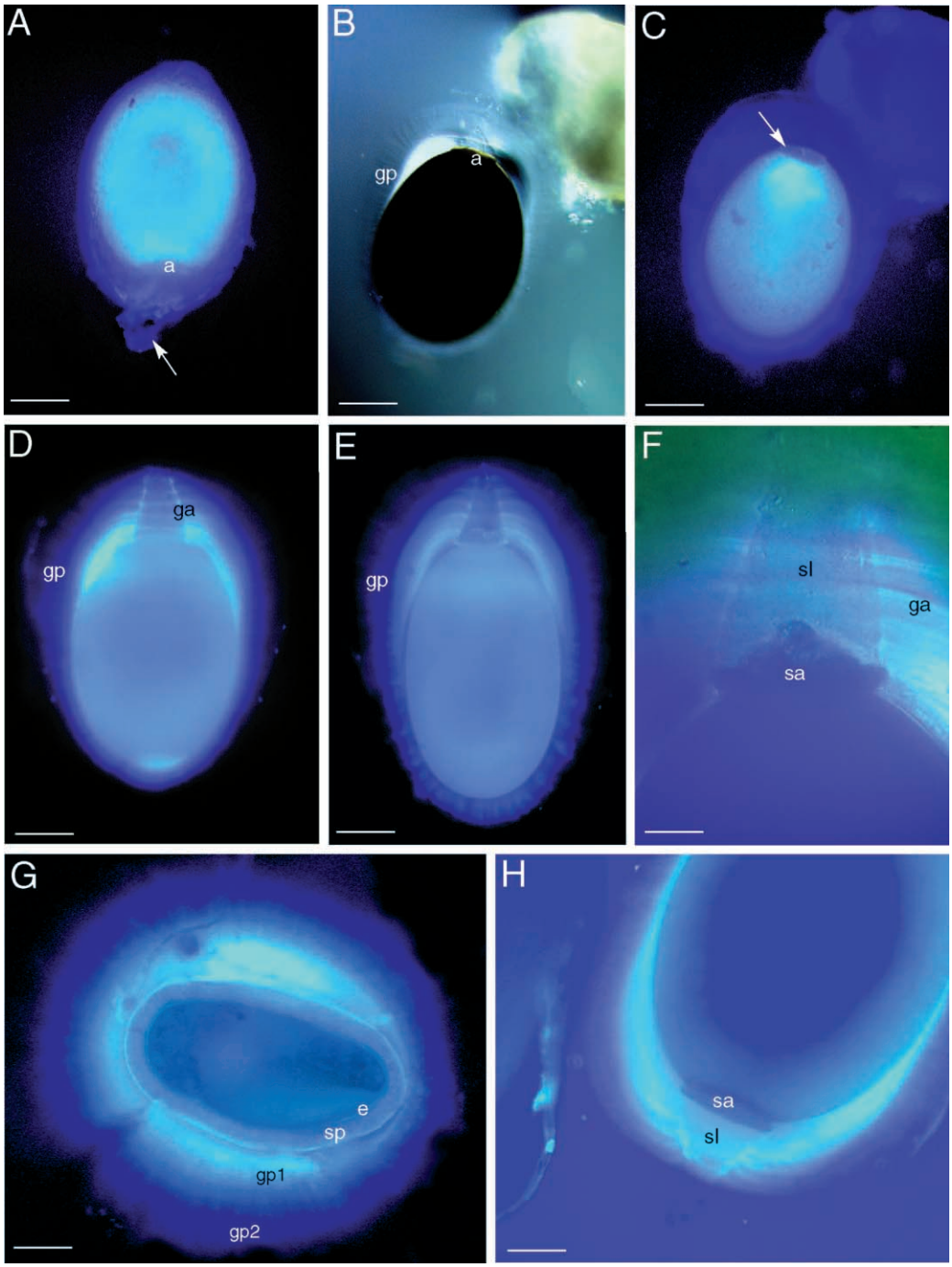

Fig. 5 Megaspores of *Marsilea mutica* (representative of *Marsilea* group I) (*Boormann s.n.*, F). *A*, Megaspore inside megasporangium, with acrolamella (*a*) oriented toward megasporangium stalk (arrow). Scale bar = 150 μm. *B*, Newly released megaspore (ca. 10 min after release from megasporangium), showing the gelatinous perine layer that has swelled (especially evident close to the acrolamella [*a*]). Note the fibrillose nature of the inner part of the gelatinous perine (*gp*) layer. Scale bar = 150 μm. *C*, Same megaspore as in *B* but viewed with epifluorescence optics to highlight the disk-shaped solid part of the acrolamella (arrow). Scale bar = 150 μm. *D*, Megaspore 1 h after release. Along the polar axis, the gelatinous perine (*gp*) layer is slightly asymmetrical; the conical "bell" within the gelatinous part of the acrolamella (*ga*) is not fully expanded and shows layered rings. Scale bar = 120 μm. *E*, Megaspore 4 h after release. The gelatinous perine (*gp*) layer is somewhat more expanded toward the acrolamella but is beginning to disintegrate. Scale bar = 100 μm. *F*, Close-up of *E* showing the disk-shaped solid part of the acrolamella (*sa*) and the conical bell within the gelatinous part of the acrolamella (*ga*) that encloses the sperm lake (*sl*). Note the layered ringlike folds in the gelatinous part of the acrolamella. Scale bar = 57 μm. *G*, Tangential section through the megaspore showing the exine (*e*), the solid perine (*sp*) layer, and the inner (*gp1*) and outer (*gp2*) sublayers of the gelatinous perine layer. Scale bar = 80 μm. *H*, Same spore as in *E* but with the focus directed toward the edge of the disk-shaped solid part of the acrolamella (*sa*). The conical bell within the gelatinous part of the acrolamella surrounds the sperm lake (*sl*). Scale bar = 70 μm. DIC optics used in *B*; epifluorescence optics used in *A*, *C*–*E*, *G*, *H*; epifluorescence optics combined with bright-field optics used in *F*.

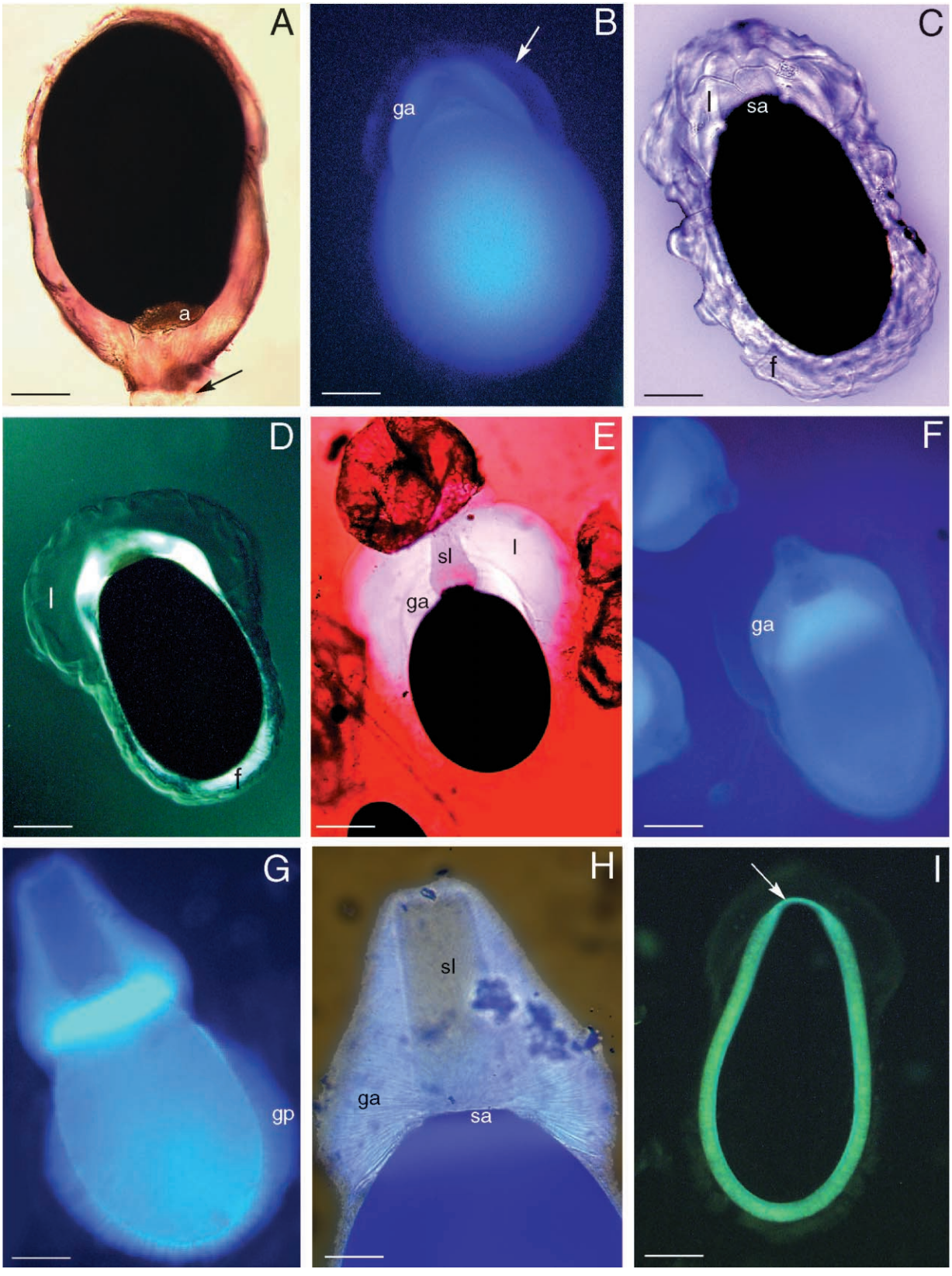

Fig. 6

Rawitscher-Kunkel 1967) and is distinctly lobed at the proximal end (corresponding to the "papillar envelope" of Machlis and Rawitscher-Kunkel 1967) that surrounds the acrolamella (figs. 2*D*, fig. 6*C*–6*E*).

Acetolysis or similar acid treatments destroy the gelatinous layer, thus allowing the surface sculpture of the outer sublayer of the solid perine to be clearly observed with scanning electron microscopy (fig. 8*A*, 8*C*, 8*E*). The surface sculpture is reticulate in *Marsilea* (fig. 8*A*) and coarsely baculate in *Regnellidium* (fig. 8*E*). Spores of all species of *Pilularia* are reticulate, except for *P. globulifera*, where the distal pole region is undulate and the proximal pole region is reticulate and a subproximal furrow forms a border between both types of surface sculpture (fig. 8*C*). Untreated spores (no acetolysis) observed with scanning electron microscopy have an undulate surface sculpture resulting from the dehydrated gelatinous mass forming a sheath that surrounds the spore (data not shown, but see Tryon and Lugardon 1991, figs. 221.7, 221.8).

Megaspore Wall Ultrastructure

In all three marsileaceous fern genera, the exine is two-layered (the "blechnoid type" *sensu* Tryon and Lugardon 1991), and the exine surface sculpture is psilate to slightly rugulate. Relative to the exine, the perine is exceptionally thick and is divided into an inner solid layer and an outer gelatinous layer (figs. 1, 2).

In dehydrated spores, the gelatinous perine layer is thin in comparison to the solid perine layer (fig. 8*B*, 8*D*, 8*F*), and no notable details are detectable with light or scanning electron microscopy. In hydrated spores, the gelatinous perine layer is much thicker than the solid perine layer (figs. 4*J*, 5*G*). This gelatinous layer appears to be divided into two sublayers in hydrated spores (figs. 1, 2, 4*C*). The inner sublayer often is fibrillose (fig. 4*C*, 4*E*, 4*F*; fig. 5*B*), which is especially visible in *Pilularia*.

In all marsileaceous ferns, the solid perine layer is composed of two sublayers (figs. 1, 4*J*). In cross section, the inner sublayer appears to be smooth to granular (depending on magnification), and the outer sublayer is alveolate (fig. 8*B*, 8*D*, 8*F*; figs. 25, 30, 34, 38 in Lupia et al. 2000). The alveolate outer sublayer is always considerably thicker than the inner sublayer, with the exception of *Pilularia americana*, where the two sublayers are more or less comparable in thickness (figs. 4*J*, 8*D*; fig. 30 in Lupia et al. 2000). Unfortunately, ultrastructural data are not available for the two southern hemispheric species of *Pilularia* (*P. novae-hollandiae* and *P. novae-zelandiae*). Differences observed in the solid perine surface sculpture can be attributed directly to the organization of the alveolate outer sublayer of the solid perine layer. For example, in *Regnellidium* the alveolate outer sublayer grades outward into fused papillae resulting in a baculate surface sculpture (fig. 8*E*; also fig. 25 in Lupia et al. 2000), whereas the alveolate outer sublayer grades outward into an open reticulum in *Marsilea* (fig. 8*A*; also fig. 38 in Lupia et al. 2000).

Megaspore Acrolamella

Marsilea group I and group II taxa, *Regnellidium*, and *Pilularia* each have a characteristic acrolamella (figs. 2, 3*G*; fig. 4*D*, 4*F*; fig. 5*D*, 5*F*, 5*H*; fig. 6*E*, 6*H*). The acrolamella is positioned above the nonprominent aperture of the exine and is extended along the polar axis of the spore. A small chamber between the exine and perine at the base of the acrolamella lobes was observed in all taxa (figs. 4*G*, 8*F*). As with the perine, the acrolamella is differentiated into an inner solid layer and an outer gelatinous layer. A breach between these two layers is consistently observed, resulting in a space that leads to the outside through a small opening (figs. 2, 3*G*). We refer to this as the "sperm lake" following Machlis and Rawitscher-Kunkel (1967).

In *Regnellidium*, the acrolamella has a prominent solid layer with six to seven twisted, leaflike folds (fig. 2*A*; fig. 3*D*, 3*G*; fig. 8*E*). The gelatinous acrolamella layer mirrors the solid layer in shape but is somewhat wider (fig. 3*D*, 3*G*). A breach between the solid and gelatinous layers leads to an opening at the proximal end (figs. 2*A*, 3*G*). The solid layer of the acrolamella has an alveolate ultrastructure similar to that observed in the solid perine layer, but it is considerably thinner (fig. 8*F*; also fig. 26 in Lupia et al. 2000). The solid acrolamella layer has a psilate surface sculpture (fig. 8*E*, 8*F*; also fig. 26 in Lupia et al. 2000).

Fig. 6 Megaspores of *Marsilea azorica* (*A–C*, *G*, *H*) (*Schneider and Pryer 99/4*, F), *Marsilea vestita* (*D*, *E*, *I*) (*Palmer 13465*, F), and *Marsilea villosa* (*F*) *Degener 9049*, US), representatives of *Marsilea* group II. *A*, Megaspore inside megasporangium. The acrolamella (*a*) is oriented toward the megasporangium stalk (arrow). Scale bar = 90 μm. *B*, Megaspore 10 min after release. The gelatinous part of the acrolamella (*ga*) is undergoing expansion. Two sublayers are visible, though the outer sublayer is faint (arrow). Scale bar = 100 μm. *C*, Megaspore 1 h after release showing the disk-shaped solid part of the acrolamella (*sa*) and the gelatinous perine layer, which is fully hydrated with folds (*f*) at the distal end of the spore and lobes (*l*) at the proximal end that surround the acrolamella. Folds and lobes correspond to the "basal envelope" and the "papillar envelope," respectively, of Machlis and Rawitscher-Kunkel 1967. Scale bar = 100 μm. *D*, Megaspore 1 h after release showing fully hydrated lobes (*l*) and folds (*f*) of the gelatinous perine layer. Scale bar = 110 μm. *E*, Megaspore in Sudan red IV stain 3 h after release. The conical "bell" of the gelatinous part of the acrolamella (*ga*) is fully hydrated and encloses the sperm lake (*sl*). The lobes (*l*) of the gelatinous perine layer at the proximal end that surround the acrolamella are fully hydrated and still visible, but the folds that were previously at the distal end of the spore have completely disintegrated and are no longer visible. Scale bar = 140 μm. *F*, Megaspore 5 h after release. The conical bell of the gelatinous part of the acrolamella (*ga*) is visible, but the lobes of the gelatinous perine layer that were previously at the proximal end of the spore have mostly disintegrated and are only faintly visible. Scale bar = 110 μm. *G*, Megaspore 7 h after release. The distal end of the spore is covered only with the resistant inner sublayer of the gelatinous perine (*gp*) layer. Scale bar = 95 μm. *H*, Megaspore 6 h after release showing the fully hydrated conical bell of the gelatinous part of the acrolamella (*ga*), which encloses the sperm lake (*sl*) above the flat disk-shaped solid part of the acrolamella (*sa*). Scale bar = 55 μm. *I*, Longitudinal section of megaspore showing only the solid perine layer, which becomes reduced toward the acrolamella (arrow). Scale bar = 80 μm. Bright-field optics used for *E*; DIC optics used for *C* and *D*; epifluorescence optics used for *B*, *F*, *G*, *I*; epifluorescence optics combined with bright-field optics used for *A*, *H*.

Pilularia also has a prominent solid layer of the acrolamella, but with six to seven straight, leaflike folds (figs. 2*B*, 4*F*, 8*C*; also figs. 29 and 33 in Lupia et al. 2000). The gelatinous acrolamella layer forms a dome that sits above a funnel-shaped sperm lake that is partly formed by an indentation of the inner sublayer of the gelatinous perine layer (fig. 2*B*; fig. 4*D*, 4*E*). The solid acrolamella layer is centrally located in the funnel-shaped sperm lake. The solid layer of the acrolamella has a uniform (not alveolate) ultrastructure (fig. 8*D*), and its surface sculpture is psilate (fig. 8C; also figs. 29 and 33 in Lupia et al. 2000).

The solid acrolamella layer is extremely reduced in *Marsilea*. It forms a disk shape in both *Marsilea* group I and group II taxa (figs. 2*D*, 6*H*) but is slightly raised from the spore body in *Marsilea* group I taxa (fig. 2*C*; fig. 5*F*, 5*H*). The gelatinous acrolamella layer has a striking bell shape in both groups of *Marsilea* that completely encloses the sperm lake except for a small opening at the apex (fig. 2*C*, 2*D*; fig. 5*D*, 5*E*, 5*H*; fig. 6*E*–6*H*). A unique feature of the gelatinous acrolamella layer of *Marsilea* is the fibrillose to ringlike folds observed in the basal region of the acrolamella (fig. 5*D*–5*F*, 5*H*; fig. 6*H*). The solid acrolamella layer is thin and undifferentiated (figs. 6*I*, 8*B*), and its surface sculpture is more or less psilate (fig. 8*A*; also fig. 37 in Lupia et al. 2000).

Microspore Morphology

Microspores are spheroidal in *Marsilea*, *Regnellidium*, and *Pilularia* (fig. 7*I*–7*K*). When freshly released, microspores from a single sporangium are united in a common gelatinous mass that envelops all microspores (fig. 7*F*). The common gelatinous mass disintegrates 2–4 h after release, and the spores are independent (fig. 7*G*). Each individual spore is surrounded by its own gelatinous perine layer, which appears to be divided into two sublayers (fig. 7*G*, 7*K*). The gelatinous perine layer uniformly surrounds the microspore in *Marsilea* and *Regnellidium*; it is, however, somewhat asymmetric in its distribution around the microspore in *Pilularia*, with a small region appearing almost uncovered (arrow in fig. 7*J*). Both sublayers are retained until the male gametophyte is fully developed. The gelatinous sublayers disappear shortly before the release of the sperm cells, which occurs ca. 6–8 h after the spores are first released from the microsporangium.

The trilete microspores have a solid perine layer, with *Pilularia* and *Regnellidium* both having a rugulate surface sculpture and *Marsilea* distinguished by a surface that is baculate (figs. 27, 31, 35, and 39 in Lupia et al. 2000). The surface sculpture is only visible after the gelatinous perine layer is removed by acetolysis.

An acrolamella-like structure was rarely observed for microspores of *Marsilea*. In *Regnellidium*, a solid acrolamella layer (fig. 7*I*), reminiscent of that present in the megaspores, was sometimes seen. Such a structure was not observed for *Pilularia* in this study. The use of transmission electron microscopy could confirm the status of the acrolamella in these microspores.

Microspore Wall Ultrastructure

The ultrastructure of the marsileaceous microspore wall is similar to that of the megaspore wall in that it has an exine and a perine (fig. 7*H*).

Chemical Composition of the Perine

We used routine histological methods to determine the chemical composition of the perine (table 2). Sporopollenin is present in both the exine and the solid perine layers of the spore wall. Its presence can be inferred from the resistance of the layers to acids and acetolysis and from a yellow (exine) to yellowish green, grading outward to blue (perine) autofluorescence emission. Positive results observed with the periodic acid–Schiff's (PAS) reaction and other stains (table 2) lead us to conclude that polysaccharides are a major component of the solid perine layer and that they nearly exclusively make up the gelatinous perine layer. Nothing is known about the nature of these polysaccharides. The inner sublayer of the gelatinous perine layer shows a weak positive reaction to stains for lipids and cellulose; however, reactions for proteins and callose were negative. In contrast with the solid perine layer, the gelatinous perine layer expands when in contact with water, and most of this layer is soluble. Remarkably, spores that were fixed in 100% alcohol and later placed in water still were able to show this expansion reaction of the gelatinous perine layer. Maintaining the spores in solutions with a low water content decreased the speed of the expansion process but did not stop it. The expansion of the gelatinous perine layer was only prevented when the spores were placed in completely water-free fluids such as glycerin. Therefore, the expansion of the gelatinous perine layer is not dependent on the action of enzymes (which would be rendered inactive in 100% alcohol).

Behavior of Spores in Water

The entire reproductive biology of marsileaceous ferns occurs in water, including the release of spores from the sporocarp, the dispersal of the spores to the air/water interface where fertilization occurs, and finally the sinking of the young developing embryo to the water/soil interface (fig. 9*A*). Spores of all three genera were examined for their behavior in water. The timing and speed of the reproductive process is rapid but highly variable and is easily influenced by such factors as temperature and available water supply. Therefore, only a representative scenario is described here, but the sequence of events during the reproductive process is constant for all taxa (fig. 9*B*). When dry sporocarps were introduced to the vials, they would sink to the bottom. Usually, after a few hours (<12 h), they would open, and sporangia would begin to release their spores over a period of 4–5 h. In general, differences in access to water appeared to influence the different times of spore release. Because of the presence of a sorophore, the sporangia remain attached to the sporocarp in most species of *Marsilea*; however, the sporangia in *Pilularia* and *Regnellidium* become detached and float independently. The delicate sporangia, which lack an annulus or a fixed opening point (figs. 3*A*, 4*A*, 5*A*, 6*A*, 7*A*–7*E*), tear apart soon after their release because of the rapid swelling of the gelatinous perine layer of the spores (fig. 4*B*). This gelatinous layer of the released micro-

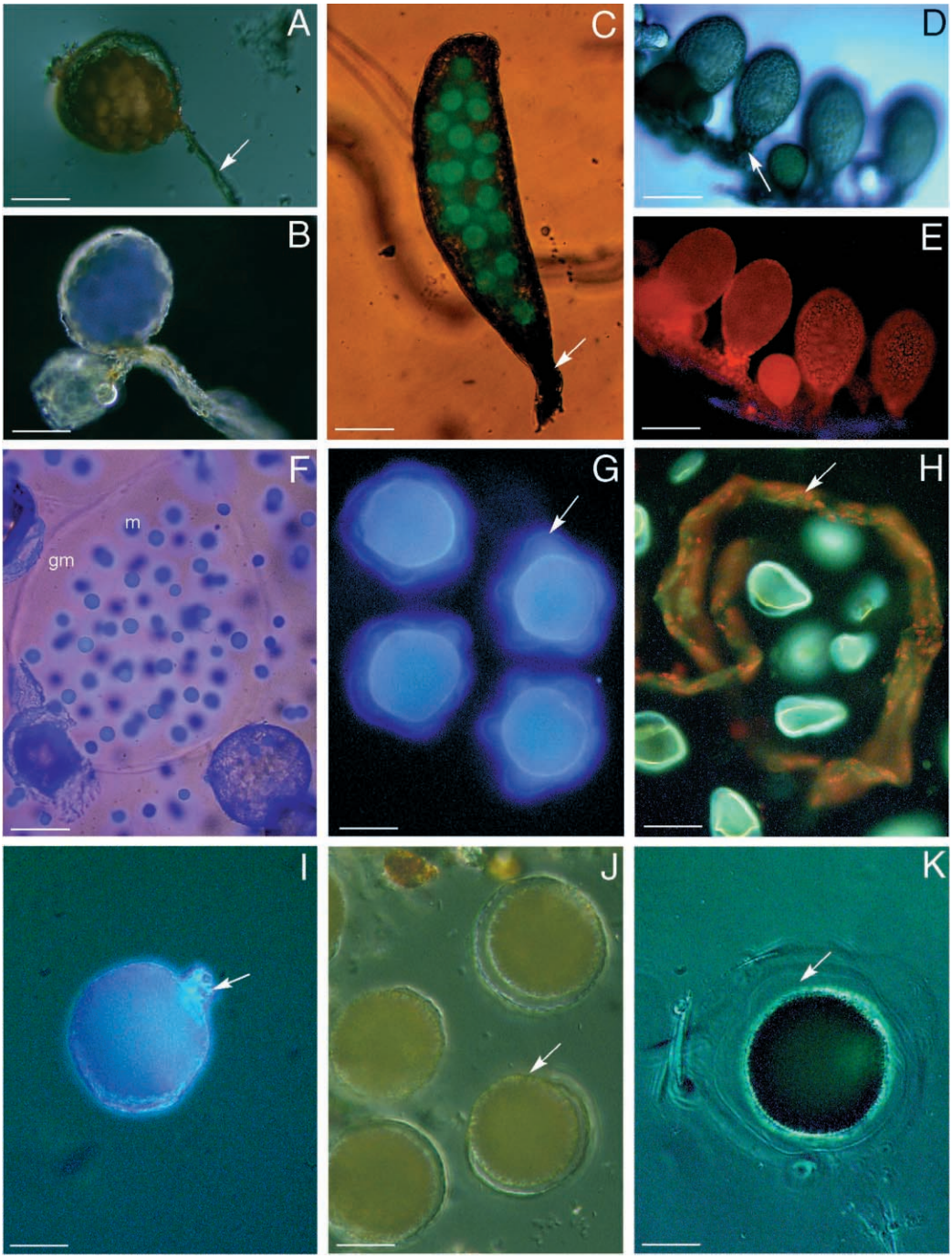

Fig. 7 Microsporangia and microspores of marsileaceous ferns. *A*, *B*, Microsporangia of *Regnellidium diphyllum* (*Rau s.n.*, US), each showing a spherical capsule containing numerous microspores and a thin long stalk (arrow). Scale bars = 162 μm. *C*, Microsporangium of *Pilularia americana* (*Hill 8654*, F), showing a falcate capsule and the lack of a clearly differentiated stalk (arrow). Scale bar = 165 μm. *D*, *E*, Microsporangia of *Marsilea vestita* (*Howell 47460*, US) at various stages of development, showing ellipsoidal capsules subtended by short stalks (arrow). Scale bars = 180 μm. *F*, *G*, Microspores of *Regnellidium diphyllum* (*Durta s.n.*, F). *F*, When released from the microsporangium, the microspores (*m*) are embedded in a unified gelatinous mass (*gm*). Scale bar = 162 μm. *G*, Microspores 2 h after release have become separated. Each microspore retains one to two gelatinous sublayers, the inner one irregularly lobed (arrow). Scale bar = 33 μm. *H*, Cross section through the microsporangium of *Marsilea vestita* (*Palmer 13465*, US), the sporangium wall (arrow) with starch grains (red), and the sectioned spores showing the exine (yellow) and perine (blue green). Scale bar = 65 μm. *I*, Microspore of *R. diphyllum* (*Durta s.n.*, F) showing a view of the acrolamella (arrow). Scale bar = 19 μm. *J*, Microspores of *Pilularia americana* (*Pryer et al. 954*, DUKE) surrounded by an asymmetrical gelatinous layer (arrow). Scale bar = 26 μm. *K*, Microspore of *M. vestita* (*Palmer 13465*, US) showing an inner gelatinous sublayer (arrow) surrounded by a faint outer sublayer. Scale bar = 22 μm. Bright-field optics used for *A*; dark-field optics used for *B*; DIC optics used for *D*, *J*, *K*; epifluorescence optics were used for *E*, *G*, *H*; epifluorescence optics combined with bright-field optics used for *C*, *F*; epifluorescence optics combined with DIC optics used for *I*.

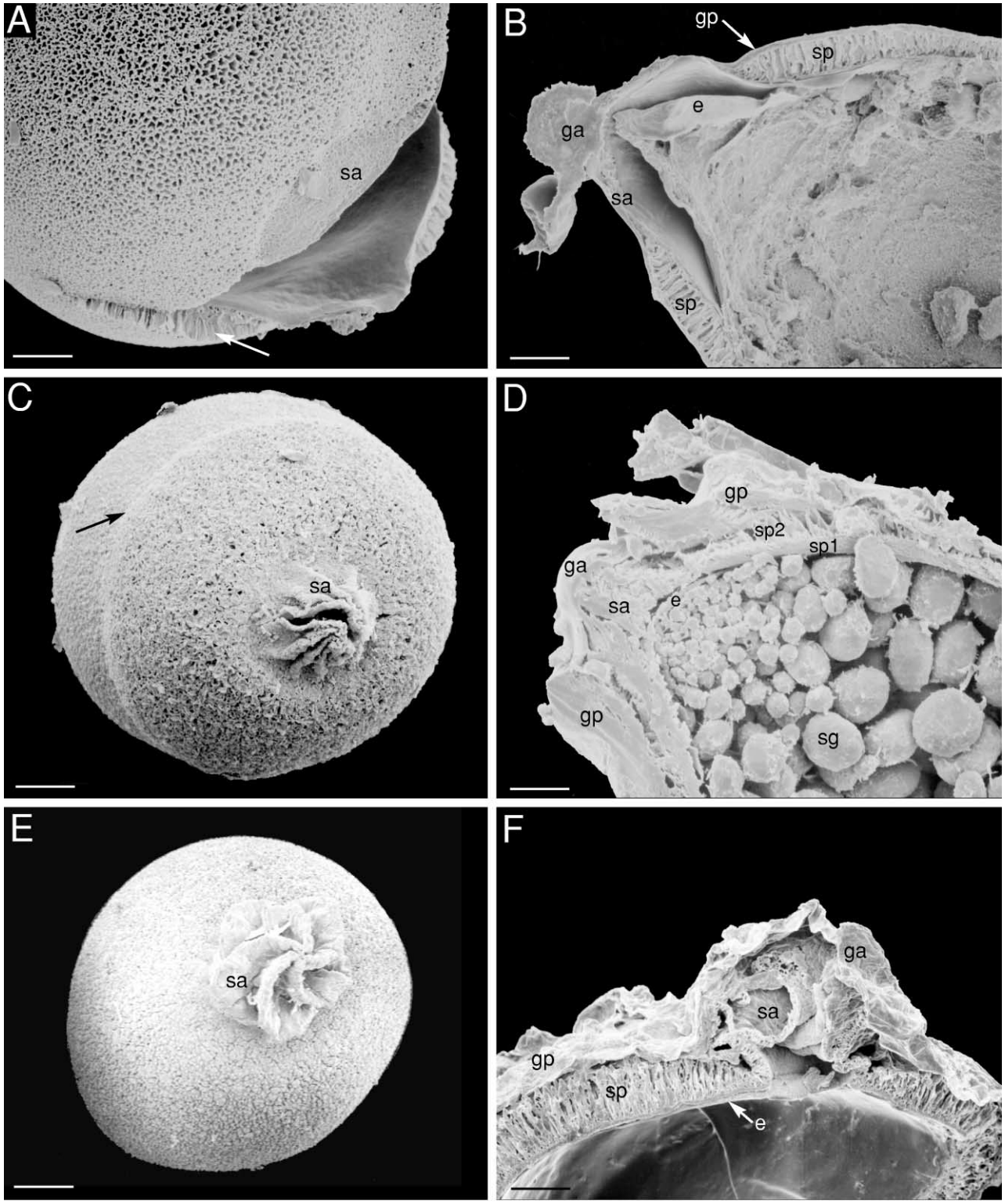

Fig. 8 Scanning electron microscope (SEM) images of megaspores of marsileaceous ferns. *A*, Megaspore of *Marsilea drummondii* (*Schneller s.n.*, Z). The solid perine layer shows a reticulate surface sculpture. The flat disk-shaped solid part of the acrolamella (*sa*) has a more or less psilate surface. The alveolate outer sublayer (arrow) of the solid perine layer is visible via a break in the megaspore wall. Scale bar = 58 μm. *B*, Longitudinal section of a megaspore of *Marsilea vestita* (*Palmer 13465*, US) showing the gelatinous part of the perine/acrolamella (*gp*/*ga*) forming a thin layer enveloping the solid part of the perine/acrolamella (*sp*/*sa*). The solid perine (*sp*) layer shows an alveolate sublayer that is

and megaspores continues to expand (figs. 4*B*, 5*B*) as they drift toward the air/water interface. Newly released microspores initially float in groups that comprise all of the spores from a single microsporangium (fig. 7*F*), but they become separated from one another after 1 h (fig. 7*G*). Both micro- and megaspores promptly rise to the air/water interface and can stay there for at least 10 h (fig. 9).

The gelatinous perine layer of the megaspores is fully expanded 2–3 h after they are released from the sporangia (fig. 4*C*), and the sperm lake region is fully developed after 6–8 h (fig. 4*E*), when the outer sublayer of the gelatinous perine layer begins to disintegrate. Megaspores float close to the water surface for several hours (<14 h) after their release from the sporangia. During this time, the acrolamella is oriented toward the surface, and the polar spore axis is oriented at an angle of ca. 20°–60° to the water surface. The acrolamella was never observed to lie parallel to the water surface or downward. After ca. 12 h, the bulk of the gelatinous perine layer had disintegrated, and the spores had begun to sink slowly. After 24 h, most megaspores had settled at the bottom of the vials. In contrast, the microspores did not sink, perhaps because by this time they had expended most of their mass by the release of the sperm cells (fig. 9).

Most male gametophytes were mature 6–8 h after the time of spore release, and free-swimming sperm cells were usually observed after 8–10 h (fig. 9). We did not determine the active life span of sperm cells. Female gametophytes develop somewhat more slowly than male gametophytes, and most have appeared 8–10 h after the time of spore release. Free-swimming sperm cells were observed in the sperm lake soon after the release of sperm cells, often before the female gametophyte was fully developed. The developing female gametophyte expels some material (perhaps cytoplasm from the archegonium neck cells) into the sperm lake shortly before the archegonium neck is visible (ca. 10 h after spore release). Each megagametophyte develops a single archegonium. Young embryos were observed as early as 2 d after the time of spore release, and the gelatinous acrolamella layer envelops the embryo (fig. 9). The female gametophyte forms rhizoids only after successful fertilization has occurred, and they develop primarily on the side of the gametophyte that is oriented toward the substrate. The orientation of the rhizoids appears to be influenced by the light source. Rhizoids break through the gelatinous acrolamella layer, which remains in place until it is destroyed by the first expanding leaf and root (fig. 9*A*).

Discussion

Preparation Methods

Since the advent of scanning and transmission electron microscopy, most studies of fern spores have relied strictly on these two approaches (e.g., Tryon and Lugardon 1991). Spore studies that combine electron and light microscopy are now rare by comparison (Large and Braggins 1991). Relatively few fern spore studies use acetolytic preparation methods (Erdtman 1960; Erdtman and Sorsa 1971; Stafford 1995), which are used regularly in investigations of pollen. Although standardized methods present tremendous advantages for comparative studies across various groups, they are not necessarily adequate for describing structural diversity. In extreme cases, acetolysis can destroy structures of biological importance (Hesse and Waha 1989; van der Ham et al. 1998). Studies that combine various approaches are better able to characterize structures because they utilize a broader set of information tools and can identify artifacts through comparison. Researchers often ignore the value of light microscopy in spore studies, and thus they ignore information that could be obtained from a combination of optical and staining techniques.

Because of the presence of a gelatinous perine layer that has a low optical contrast and that also reacts rapidly to changes in water availability, a thorough description of marsileaceous spores is only possible to achieve by using a combination of methods. Observation of these spores is particularly troublesome because many microscopic techniques require fixation and/or dehydration treatments that modify (sometimes drastically) their morphology/anatomy (see also discussion in O'Brien and McCully 1981; Platt et al. 1997). While this risk can be ignored for many biological structures, its potential for misrepresentation of form is important to recognize. In Marsileaceae, dry spores are enveloped by a thin sheath, whereas hydrated spores reveal a thick gelatinous mass that surrounds them. The low optical contrast, plasticity, and transformations that this gelatinous mass undergoes over time are but some of the problems encountered in attempting to make consistent and reproducible observations. Acetolytic treatments allow one to observe the surface of the solid perine layer, but they eradicate the gelatinous perine layer. A careful combination of microscopic approaches and techniques permits a thorough reconstruction of the biological structures under study.

absent in the solid part of the acrolamella (*sa*). Note the breach between the exine (*e*) and solid part of the acrolamella (*sa*) that forms a small chamber. Scale bar = 41 μm. *C*, Megaspore of *Pilularia globulifera* (*Chevallier s.n.*, F) showing an irregularly reticulate surface sculpture of the solid perine layer at the proximal end, an equatorial furrow (arrow), and relatively straight lobes of the solid part of the acrolamella (*sa*). Scale bar = 88 μm. *D*, Longitudinal section of a megaspore of *Pilularia americana* (*Pryer et al. 954*, DUKE) showing the gelatinous perine (*gp*) layer enveloping the solid perine layer, which is composed of an outer alveolate sublayer (*sp2*) and an inner uniform sublayer (*sp1*). Both the solid (*sa*) and gelatinous (*ga*) parts of the acrolamella are visible. Note the breach between the exine (*e*) and solid part of the acrolamella (*sa*) and the starch grains (*sg*) present in the spore lumen. Scale bar = 32 μm. *E*, Megaspore of *Regnellidium diphyllum* (*Bloom s.n.*, F) showing the baculate surface sculpture of the solid perine layer and the twisted lobes of the solid part of the acrolamella. Scale bar = 87 μm. *F*, Longitudinal section of a megaspore of *Regnellidium diphyllum* (*Rau s.n.*, US) showing the gelatinous part of the perine/acrolamella (*gp*/*ga*) enveloping the solid part of the perine/acrolamella (*sp*/*sa*), which has a clearly visible alveolate sublayer. Note the breach between the exine (*e*) and the solid part of the acrolamella (*sa*) that forms a small chamber. Scale bar = 49 μm. Megaspores prepared using acidic treatment to remove gelatinous part of perine (*A*, *C*, *E*); sections of megaspores fixed in 100% EtOH after 5 h of hydration in water (*B*, *D*, *F*).

Table 2

Chemical Composition of Marsileaceous Fern Spore Exine and Perine Layers

Histological treatment → positive reaction suggests presence of chemical component that is indicated	Exine	Solid perine layer	Gelatinous perine layer
Resistant to acids/acetolysis → sporopollenin	+	+	−
Autofluorescence (450–600 nm)[a]	Yellow	Yellowish green grading outward to blue	Blue
Alcian blue → polysaccharides	(+)	+	(+)
Aniline blue → callose	−	−	−
Aniline black → proteins	−	(+)	−
Calcofluor white → polysaccharides	−	+	+
Lugol's reagent with zinc chloride/acids → cellulose	(+)	+	(+)
Periodic acid–Schiff's (PAS) → polysaccharides	(+)	+	(+)
Ruthenium red → polysaccharides	−	+	(+)
Safranin → polysaccharides	(+)	+	(+)
Sudan IV → lipids	+	(+)	(+)

Note. + = positive reaction; (+) = weak positive reaction; − = negative reaction.

[a] Yellow autofluorescence emission indicates the presence of sporopollenin; blue autofluorescence emission indicates the presence of polysaccharides.

Spore Character Evolution

The morphological and ultrastructural characters of Marsileaceae megaspores and the distribution of several representative characters on a phylogeny of heterosporous ferns are summarized in table 3 and figure 10, respectively. Spores of Marsileaceae differ in many features from spores of Salviniaceae and homosporous ferns, but they do share a plain, blechnoid (two-layered) exine and a trilete aperture with Salviniaceae and closely related dennstaedtioid and cyatheoid homosporous ferns. The megaspore exine profile is spheroidal in *Azolla*, *Salvinia*, and *Regnellidium* and prolate in *Marsilea* and *Pilularia*. The microspores of all five genera have a spheroidal exine profile. The closely related dennstaedtioid and cyatheoid homosporous ferns have a spore exine profile in which the polar axis and the equatorial diameter are approximately equal, which suggests that a spheroidal exine profile is the plesiomorphic character state for heterosporous ferns (fig. 10*A*). The ancestral character state for the Marsileaceae, however, is ambiguous (fig. 10*A*).

Both the Salviniaceae and Marsileaceae have a modified perine, but the modifications are very different. In Marsileaceae, the perine is divided into an outer gelatinous layer and an inner solid layer, whereas Salviniaceae have a vacuolate perine (Tryon and Lugardon 1991). Closely related homosporous ferns have varied perine ultrastructures, but they are never vacuolate or gelatinous, making it impossible, so far, to determine the ancestral character states. The presence of a gelatinous perine is correlated with the presence of starch grains as the main reserve unit in the spores of the amphibious Marsileaceae, whereas lipids are found in spores of homosporous ferns and the floating aquatic Salviniaceae (fig. 10*B*). The megaspores in both Marsileaceae and Salviniaceae have a modified perine above the exine aperture and the acrolamella of marsileaceous megaspores is most likely homologous to similar structures observed in salviniaceous megaspores (H. Schneider and K. M. Pryer, unpublished results).

A large solid acrolamella layer is found in the *Pilularia-Regnellidium* clade (fig. 10*C*), whereas the solid acrolamella layer is reduced to a small disk in *Marsilea*. In contrast, a bell-shaped gelatinous acrolamella layer is a synapomorphy of *Marsilea*, as is the orientation of the acrolamella in the megasporangium (figs. 5, 6, 10*C*). Group I and group II taxa of *Marsilea* can be distinguished by the structure of their outer gelatinous layer, which is continuous in *Marsilea* group I and in *Pilularia* and *Regnellidium* but discontinuous and differentiated into folds at the distal end and lobes at the proximal end of the spore in *Marsilea* group II (fig. 2). *Pilularia* differs from all other genera in the structure of its gelatinous acrolamella that forms a funnel-shaped sperm lake.

Developmental Aspects

Only certain details of the development of marsileaceous spores have been studied critically with electron microscopy (Pettitt 1971, 1979*a*, 1979*b*; Bell 1985, 1996). These studies could be expanded by examining more closely the important steps in the development of the tapetum. Meunier (1888) and Chrysler and Johnson (1939) suggested there were similarities in the development of heterosporous spores and spores of homosporous, leptosporangiate ferns, but very few ferns have been studied well enough to permit such a comparison (van Uffelen 1991, 1993, 1996; Parkinson 1995*a*, 1995*b*, 1996; Parkinson and Pacini 1995; Oldenhof and Willemse 1999). A comparison of the structure and function of the tapetum in pteridophytes and seed plants (Parkinson and Pacini 1995; Parkinson 1996) indicates that there are significant differences not only between pteridophytes and angiosperms (as might be expected) but also among ferns (unexpected). The limited data that are available appear to support a common developmental scheme in leptosporangiate ferns, but the length of the process may be reduced in Marsileaceae and Salviniaceae (Parkinson and Pacini 1995). If this is true, the gelatinous perine layer that is present in Marsileaceae spores could be explained as a result of the early release of spores, i.e., before the complete development of the perine (a heterochronic shift in the timing of spore maturity). This is, of course, only a hypothesis, which needs to be tested with thorough developmental studies that are conducted within a robust phylogenetic framework. It is relevant to note, however, that heterochronic changes have

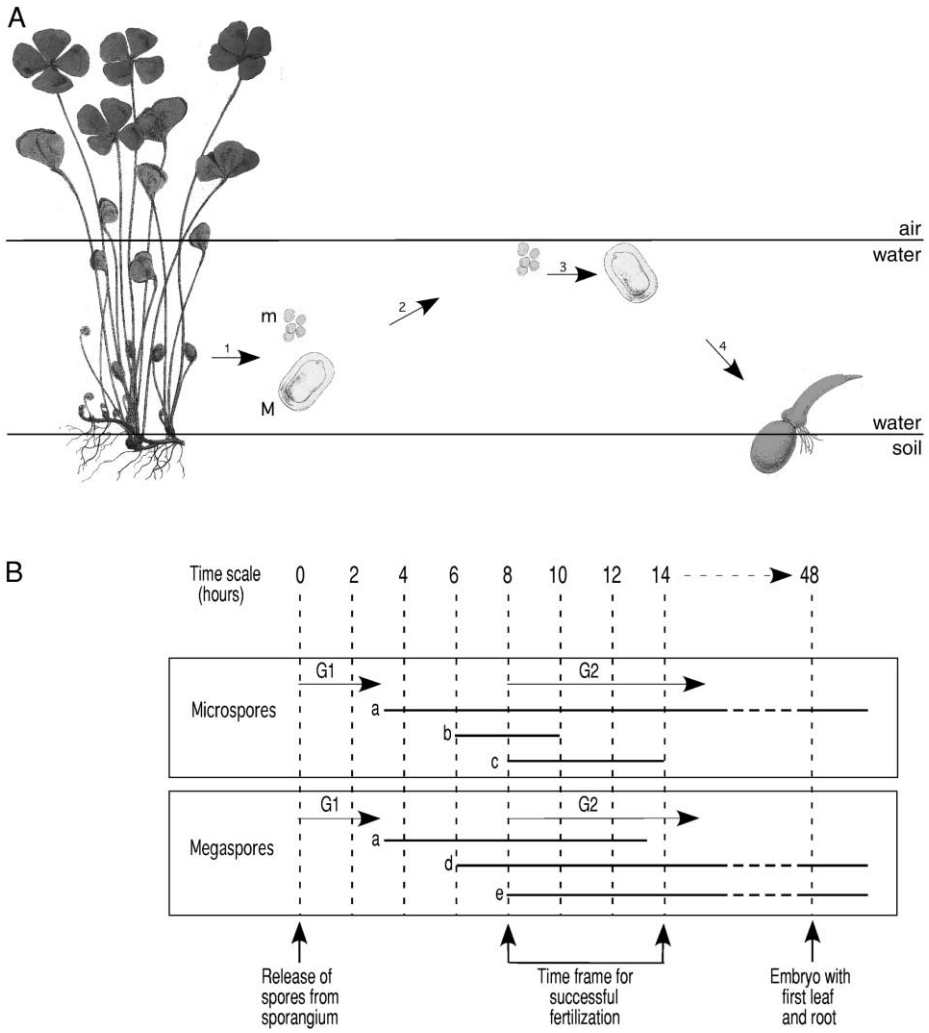

Fig. 9 Representative sketches illustrating the aquatic reproductive biology of Marsileaceae (not drawn to scale). *A*, Release of spores from the sporangium (*1*), the dispersal of spores to the air/water interface (*2*) where fertilization occurs (*3*), and finally, the sinking of the young developing embryo into the water/soil interface (*4*); *m* = microspores; *M* = megaspore. *B*, Sexual reproduction time points and intervals starting with the time of spore release from the sporangium (time 0 h) and ending with embryo formation (time 48 h); *a* = spores floating at water surface; *b* = mature male gametophyte; *c* = free-swimming sperm cells; *d* = fully developed sperm lake; *e* = mature female gametophyte; *G1* = gelatinous perine layer expanding; *G2* = gelatinous perine layer disintegrating. Ca. 8–14 h after the time of spore release from the sporangium is the estimated time frame for successful fertilization, which leads to visible embryos ca. 48 h later. Plant and spore illustrations modified from Foster and Gifford (1989) and Hooker (1862).

been implicated in the evolution of heterosporous reproduction, especially in seed plants (DiMichele et al. 1989; Friedman and Carmichael 1998).

Despite impressive progress, our understanding of spore and pollen development is still incomplete, especially with regard to the regulation of spore wall formation (Barnes and Blackmore 1986; Dickinson and Sheldon 1986; Owens et al. 1990; van Uffelen 1996; Oldenhof and Willemse 1999). Marsileaceous spores present an exceptional opportunity to explore the development of spore walls. The main advantages are the large size of the megaspores, the easy cultivation of these ferns, and their rapid development. In good conditions, an individual of *Pilularia americana* grown from spores can begin to form sporocarps within 4–5 wk. Each sporocarp is fully developed after another 2–3 wk (H. Schneider and K. M. Pryer, unpublished data).

Functional Aspects

The spores of heterosporous ferns differ from homosporous ferns in two functional aspects: they are dispersed in water rather than air, and the gametophytes are always enclosed within the spores (endospory). Water-dispersed spores need to reckon with similar obstacles as air-dispersed spores, namely, their release into the water/air flow, transport in the flow, and landing on and adhering to a substrate (Crane 1986; Punt 1986; Tryon 1990). Unlike air-dispersed spores, heterosporous fern spores do not need to be resistant to dehydration because

Table 3

Comparison of Morphological and Ultrastructural Megaspore Characters in Marsileaceae

	Marsilea group I	*Marsilea* group II	*Pilularia*	*Regnellidium*
Megaspore shape	Prolate	Prolate	Prolate	Spheroidal
Exine sculpture	Psilate	Psilate	Psilate	Psilate
Solid perine:				
Number of sublayers	2	2	2	2
Inner sublayer[a]	Granular[b]	Granular[b]	Granular[b]	Granular[b]
Outer sublayer[a]	Alveolate	Alveolate	Alveolate	Alveolate
Sculpture	Reticulate	Reticulate	Reticulate[c]	Baculate
Gelatinous perine:				
Number of sublayers	2	2	2	2
Outer sublayer	Weakly asymmetrical	Strongly asymmetrical[d]	Symmetrical	Symmetrical
Solid acrolamella shape	Disk[e]	Disk[e]	Straight conical	Twisted conical
Gelatinous acrolamella shape	Bell	Bell	Distal conical, proximal funnel	Twisted conical

[a] Outer sublayer of solid perine thicker than inner sublayer, except in *Pilularia americana*, where they have roughly the same thickness.
[b] Finely granular, appearing uniform with light microscopy.
[c] Except in *Pilularia globulifera*, which is reticulate/undulate.
[d] Proximal part differentiated into cone-shaped lobes.
[e] In *Marsilea* group I, the disk-shaped acrolamella is slightly raised from the spore body, whereas in *Marsilea* group II, it sits directly on the spore body.

they remain in water. Ordinarily, spores are the units for long-distance dispersal and dormancy; however, in marsileaceous ferns these are the primary functions of the sporocarps (Johnson 1985, 1986) because the spores, once released into water, germinate quickly and are susceptible to dryness, if exposed. The dissemination of sporocarps (spore packets) rather than individual spores has likely had a profound impact on the dispersal biology of these ferns, though population-level studies have not been carried out to verify this. One example of how these sporocarp dispersal units impact reproductive biology and maximize reproductive success is by releasing both micro- and megaspores at the same time, resulting in the synchronization of the development of the short-living micro- and megagametophytes. Effectively, these heterosporous ferns release the equivalent of sperm and egg, a high-risk reproductive strategy that keeps them confined to aquatic and amphibious habitats (DiMichele et al. 1989; Bateman and DiMichele 1994; DiMichele and Bateman 1996).

The functional aspects of the gelatinous perine layer of marsileaceous spores are of particular interest to us here. Marsileaceae are amphibious ferns, and these gelatinous layers may represent a special adaptation to reproduction in this environment. Spores are mostly released close to the substrate because either the sporocarps are attached to the base of the petiole or they are actually embedded in the substrate (e.g., *Marsilea ancylopoda*). Newly released spores are discharged into the water flow, assisted in their exit from the sporangia by the rapid increase of the gelatinous perine layer, which serves to reduce the spore density by increasing the spore volume. Similar polysaccharide-rich gelatinous envelopes with analogous functions are known in various phytoplanktonic organisms (discussions in Reynolds 1984; Vogel 1994). Fully hydrated marsileaceous spores are not necessarily provided with positive buoyancy, but if any positive lift (e.g., provided by convection streams) transports them to the water/air interface, they can stay there for hours because of the gelatinous mass, which makes them "slow sinkers." The eventual disintegration of the gelatinous perine layer (after ca. 10 h; fig. 9) results in a drastic reduction in spore volume and a simultaneous but minimal decrease in spore mass. The final result is an increase in the spore density, which assists the spore in sinking to the substrate.

A recent study of xylem pit membranes (Brown 2001; Zwieniecki et al. 2001) demonstrated that their constituent acidic polysaccharides (pectins) with hydrogel properties are involved in the regulation of hydraulic resistance in plants. We believe that the gelatinous perine layer that we describe here is also an example of acidic polysaccharides with hydrogel properties. Acidic polysaccharides are major components in the perine of homosporous and heterosporous ferns (Tryon and Lugardon 1991; Parkinson and Pacini 1995), and the swelling of the polysaccharide matrix when it comes in contact with water contributes to the general expansion of fern spores in water (Pacini 1990). However, a gelatinous perine layer is only known in marsileaceous ferns. A further unique feature is the temporally restricted stability of this gelatinous layer. Cell wall pectins or hydrogels are known for their gel-like properties and their swelling and shrinking behavior (Brett and Waldron 1996). This is also observed for the gelatinous perine layer of marsileaceous spores if water is only accessible for short periods followed by dry phases. If the gelatinous layer is exposed to water for an extended period, it slowly disintegrates. Currently, it is unknown if ions would play a similar role in mediating the shrinking and swelling behavior of the gelatinous layer as shown for hydrogels in the xylem (Zwieniecki et al. 2001).

The gelatinous perine layer does provide the spores with a mechanism to float at the water/air interface for an extended period. This may have various advantages. Water/wind currents can drift floating spores across the water surface and may thereby mix spores of different individuals, thus increasing the potential for outbreeding. The reduction of the "search area"

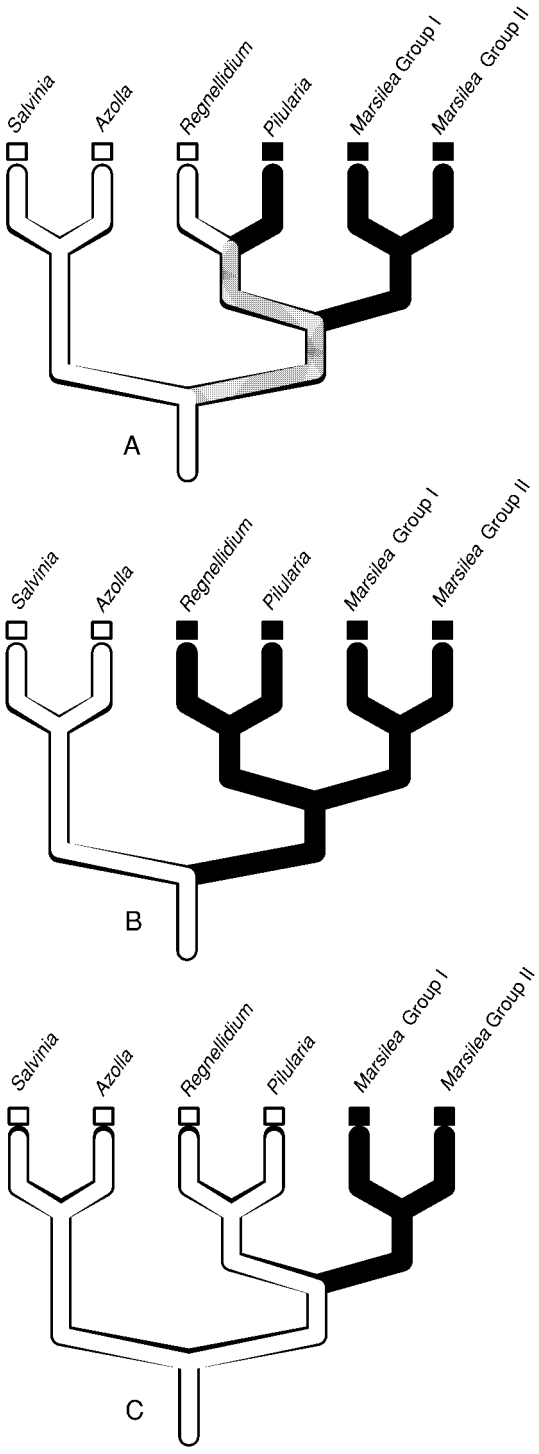

Fig. 10 Evolution of representative spore character states in heterosporous ferns. *A*, Two characters with identical character state distributions. Megaspore exine profile: prolate (black), spheroidal (white), equivocal (gray). Microsporangium capsule shape: longer than wide (black), as long as wide (white). *B*, Three characters with identical character state distributions: gelatinous perine layer; starch grains as main reserve units; amphibious habit: present (black), absent (white). *C*, Two characters with identical character state distributions. Bell-shaped gelatinous acrolamella layer: present (black), absent (white). Orientation of megaspore in megasporangium: acrolamella oriented toward megasporangium stalk (black), acrolamella oriented in the opposite direction of the attachment point of the megasporangium stalk (white).

in which the swimming sperm cells need to locate the megagametophtyes from a three-dimensional space to a two-dimensional plane at the water surface may increase the likelihood of successful fertilization (Haig and Westoby 1988). Although the water surface offers advantages for the fertilization process, it is not ideal for the developing sporophyte, which needs to be rooted. Increasing the spore density (as described above) by the disintegration of the gelatinous perine layer allows the spores to sink to the water/soil interface. The final obstacle to the young developing sporophyte is the requirement to adhere to the substrate. Adherence may be promoted at first by the sticky nature of the polysaccharide remains of the gelatinous perine layer, then by the fast-developing rhizoids, and finally by the first root.

The function of the acrolamella, which covers the spore aperture of marsileaceous spores, is also of special interest. Fern spore apertures operate by a harmomegathic mechanism and function mainly as "gates" for the transfer of water and to release the developing gametophyte (e.g., Blackmore and Barnes 1986; Lugardon 1986). At the aperture point, a small chamber is formed between the exine and perine at the base of the acrolamella lobes (figs. 4*G*, 8*F*). In megaspores, this chamber may function to protect the early growth stages of the developing female gametophyte, which eventually breaks through the solid acrolamella layer and exposes the single archegonium to the free space (sperm lake) between the solid and gelatinous acrolamella layers (fig. 5*F*). The sperm lake has a small opening to the surrounding environment, which allows the sperm cells to gain entry. Free-swimming sperm cells are found in the sperm lake shortly after their release from the male gametophytes. The cells swim there until the archegonium is fully developed and ready for fertilization. The sperm lake may not only provide protection for the sperm cells but may also function primarily as a "trap." The trap results from the large size of the megaspore, which results in a concave meniscus being formed at the water/air interface. The resulting vortex-like effect would force sperm cells in the local vicinity to be caught up in the vortex and to be propelled into the sperm lake. Hemsley et al. (1999) described a somewhat similar syndrome for heterosporous plants with dispersed spores and referred to it as a "hydrochory/meniscus encounter" and cited similarities with hydrophilous pollination observed in some aquatic flowering plants, such as *Vallisneria* (Cox 1988). Likely additional functions of the gelatinous acrolamella are protection of the embryo until the first leaf and root are developed and protection of the embryo against dehydration, mechanical stress, pathogens, and perhaps harmful amounts of light. It is unknown whether the different acrolamella shapes for each of the three genera correlate with ecological parameters unique to each of their environments because there is an absence of detailed ecological description.

Observations by Hemsley et al. (1999) of fossilized Marsi-

leaceae megaspores found with microspores in tight association led to the conclusion that the gelatinous perine layer of the megaspores likely has a "velcro-like retention" capability to sample or collect microspores or otherwise attract them. Based on our observations of the reproductive process in all three living genera, we can state that the fully hydrated outer gelatinous perine layer of the megaspores does not have a sticky quality and that when both spore types are maintained in hydrated conditions they never are seen to bind to one another. We can offer this explanation for why mega- and microspores are found in close association in fossil preparations: megaspores that have undergone hydration and that are then exposed to air become slightly sticky.

Oligosaccharides created by the disintegration of the gelatinous perine layer may transmit signals between the micro- and megaspores' respective endosporic gametophytes. Oligosaccharides have been reported to be signal transducers in plants (Ryan and Farmer 1991), but they are not known to function as sperm cell attractants or as antheridiogens—gibberellin-like pheromones released by gametophytes and involved in sex determination and reproduction in homosporous ferns (Chiou and Farrar 1997; Banks 1999). Antheridiogens are not likely to be reported from heterosporous ferns because the sex of the gametophyte is determined before spore release. The colonization of aquatic habitats has strongly altered the morphology and reproductive biology of heterosporous ferns, and this may include communication between gametophytes.

Although we have provided here some adaptive explanations for why in the evolution of these heterosporous ferns such characters as the gelatinous perine and acrolamella may have been advantageous to the reproduction of plants with free spores in amphibious habitats, these explanations need to be tested with rigorous experimental and field observations.

Conclusions

Our results provide the first attempt to examine the evolution of spore characters in marsileaceous ferns by using a phylogenetic, comparative, and functional approach. Marsileaceae has highly specialized spores with many unique features that can provide insight into the modification of morphological structures involved in reproductive biology that are necessary for the transition from terrestrial to aquatic habitats. A critical examination of the living taxa is also important for the interpretation of the fossil spore record of heterosporous ferns, which shows an enormous range of forms with possible relationships to Marsileaceae and/or Salviniaceae (Collinson 1991; Rothwell and Stockey 1994; Lupia et al. 2000).

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