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# COMPARATIVE MORPHOLOGY OF REPRODUCTIVE STRUCTURES IN HETEROSPOROUS WATER FERNS AND A REEVALUATION OF THE SPOROCARP

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Heterosporous water ferns (Marsileaceae and Salviniaceae) are the only extant group of plants to have evolved heterospory since the Paleozoic. These ferns possess unusual reproductive structures traditionally termed "sporocarps." Using an evolutionary framework, we critically examine the complex homology issues pertaining to these structures. Comparative morphological study reveals that all heterosporous ferns bear indusiate sori on a branched, nonlaminate structure that we refer to as the sorophore; this expanded definition highlights homology previously obscured by the use of different terms. By using a homology-based concept, we aim to discontinue the use of historically and functionally based morphological terminology. We recognize the sorophore envelope as a structure that surrounds the sorophore and sori. The sorophore envelope is present in Marsileaceae as a sclerenchymatous sporocarp wall and in *Azolla* as a parenchymatous layer, but it is absent in *Salvinia*. Both homology assessments and phylogenetic character-state reconstructions using the Cretaceous fossil *Hydropteris* are consistent with a single origin of the sorophore envelope in heterosporous ferns. Consequently, we restrict the term "sporocarp" to a sorophore envelope and all it contains. Traditional usage of "sporocarp" is misleading because it implies homology for nonhomologous structures, and structures historically called sporocarps in Salviniaceae are more appropriately referred to as sori.

*Keywords:* morphology, ferns, homology, Marsileaceae, Salviniaceae, sporocarp.

## Introduction

All known living and fossil heterosporous plant groups evolved in the Devonian or Carboniferous, except for the heterosporous water ferns, which are the only group to have evolved heterospory after the Paleozoic (Dilcher 2001). Heterosporous ferns evolved during the Mesozoic and diversified in the Late Cretaceous (Hall 1974; Collinson 1991; Kovach and Batten 1993; Pryer 1999; Lupia et al. 2000), at the same time as flowering plants. This fern group is the only extant lineage to exhibit extreme heterospory, that is, the combination of the plesiomorphic state of releasing sperm cells into the environment with the derived feature of forming a single viable megaspore per megasporangium (Schneider and Pryer 2002). This particular character combination is considered a critical step in the evolution of seedlike structures (Bateman and DiMichele 1994). All other extant heterosporous lineages either possess seeds (seed plants) or produce more than one megaspore per megasporangium (Isoetaceae and Selaginellaceae).

Heterosporous ferns are characterized not only by heterospory but also by the arrangement of spores into unusual reproductive structures. These structures, which are strikingly unique to each genus, do not show obvious similarities to the sporangia-bearing leaves of homosporous ferns, and consequently all have been termed "sporocarps," despite acknowledged morphological differences (Troll 1936, 1937; Tryon and Tryon 1982; Gifford and Foster 1989).

The heterosporous fern clade comprises the two families Marsileaceae and Salviniaceae, and this group is well nested within the homosporous leptosporangiate ferns (Hasebe et al. 1994, 1995; Rothwell and Stockey 1994; Pryer et al. 1995, 2001, 2004; Pryer 1999). Marsileaceae (~80 spp.) has three extant genera, *Marsilea* L., *Regnellidium* Lindm., and *Pilularia* L.; all are rooted semiaquatics and have creeping rhizomes with rather distinctive leaves (four part, two part, and filiform, for the genera, respectively) produced iteratively at nodes (Tryon and Tryon 1982; Johnson 1986; Kubitzki 1990). *Regnellidium* and *Pilularia* are sister taxa, and *Marsilea* is sister to that clade (Pryer 1999). Salviniaceae (~20 spp.) has two extant genera, *Salvinia* Seg. and *Azolla* Lam. (Copeland 1947; Tryon and Tryon 1982), although *Azolla* is sometimes segregated into Azollaceae (Kubitzki 1990). Both salviniaceous genera are wholly aquatic and float on the surface of freshwater ponds, lakes, and pools (Tryon and Tryon 1982; Kubitzki 1990). *Salvinia* has long branched rhizomes and, in a whorled arrangement at each node, two floating leaves plus a highly dissected submerged organ (Croxdale 1978, 1979, 1981; Lemon and Posluszny 1997); *Salvinia* lacks roots. *Azolla* has branched rhizomes with small bilobed leaves that overlap in an alternate arrangement on the upper surface and simple

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unbranched roots along the lower surface (Tryon and Tryon 1982; Kubitzki 1990; Saunders and Fowler 1993).

Marsileaceous ferns produce sori collectively encased in elliptical to globose structures (fig. 1*A*, 1*B*). These structures have thick, sclerenchymatous walls that protect against dryness and mechanical damage by herbivorous insects. These sclerified reproductive structures are thought to be modified leaflets (Johnson 1898*a*, 1898*b*, 1933*a*, 1933*b*; Johnson and Chrysler 1938; Puri and Garg 1953; Schmidt 1978) and are commonly referred to as "sporocarps" (Eames 1936; Bierhorst 1971; Tryon and Tryon 1982; Gifford and Foster 1989; Kubitzki 1990).

In *Salvinia*, the submerged organ is either sterile or fertile and branches dichotomously into multiple segments (fig. 1*C*). When fertile, one or two of the inner segments will each bear alternately arranged sori, typically called "sporocarps." In addition to bearing sori, the submerged organ may function as a stabilizer against drag and drifting and provide protection for the sori (Eames 1936; Kubitzki 1990). The exact nature of the submerged organ and its homology has been much disputed; typically it has been interpreted to be a submerged leaf (Pringsheim 1863; Campbell 1905; Croxdale 1978, 1981; Lemon and Posluszny 1997), but it also has been described as a branched shoot (Bonnet 1955; White and Turner 1995). More recently, it has been proposed to be part of a pinnately compound leaf, with two floating pinnae and three or more rootlike pinnae corresponding to the submerged organ (de la Solta 1999). Although at a glance the submerged organ appears rootlike by its dissected segments, it has rarely (Bischoff 1828) been labeled a root.

In *Azolla*, a submerged, dichotomously branching structure bears the sori; these are also termed "sporocarps" historically (fig. 1*D*). Sori occur in clusters, and each soral cluster is surrounded by a thin, parenchymatous tissue layer. This delicate layer has been termed an "involucre" (Konar and Kapoor 1972, 1974) or cowl/flange (Bower 1928) and has been identified by only some workers (fig. 1*D*; Strasburger 1873; Campbell 1893, 1905; Eames 1936; Calvert et al. 1983; Perkins and Peters 1993).

The reproductive structures of heterosporous ferns rarely have been critically compared to one another. This is probably because of the long-standing assumption that the two families were not closely related (Bower 1928; Eames 1936; Tryon and Tryon 1982) and because of the striking overall morphological differences among the spore-bearing structures. It has been stated previously that the sporocarps of Marsileaceae differ from the so-called sporocarps of *Azolla* and *Salvinia* (Troll 1936, 1937; Tryon and Tryon 1982; Gifford and Foster 1989). However, the reproductive structures of all heterosporous ferns have been termed "sporocarps" (Campbell 1893; Pfeiffer 1907; Eames 1936; Bierhorst 1971; Kubitzki 1990; Yamada and Kato 2002) and interpreted as homologous through phylogenetic character coding (see Rothwell and Stockey 1994; Pryer 1999). From the outset, we restrict the term "sporocarp" to the sclerenchymatous wall (and all it encloses) in Marsileaceae, and in Salviniaceae we investigate whether there are structures homologous to the marsileaceous sporocarp.

Using an explicit phylogenetic framework (Rothwell and Stockey 1994; Pryer 1999), we examine and compare in detail the morphology of all heterosporous fern spore-bearing structures. We incorporate morphological data generated in the past 150 yr together with new data obtained using various microscopy techniques. We also include the sparse evidence available for the spore-bearing structures of fossil heterosporous ferns, in particular, the well-studied fossil *Hydropteris pinnata* Rothwell & Stockey (Rothwell and Stockey 1994). In doing so, we explore the importance of incorporating fossils into studies addressing the evolution of plant form. Our aim is to understand better the homology of the various components of the reproductive structures across extant heterosporous ferns.

## Material and Methods

### *Sampling*

Reproductive structures were collected from living plants and herbarium specimens from each of the five extant genera, for a total of 29 representative heterosporous fern species (table 1) that encompass the morphological diversity present in each of the genera. Fossils such as *Regnellidium upatoiensis* Lupia et al. (Lupia et al. 2000) and *Regnellites nagashimae* Yamada & M. Kato (Yamada and Kato 2002) possess sporocarp-like structures. These and other fossils exhibit a high similarity to extant taxa and therefore do not occupy critical positions in the phylogeny of heterosporous ferns. In contrast, the fossil *Hydropteris pinnata* is rather morphologically distinct from extant members of heterosporous ferns because it incorporates features from Marsileaceae, Salviniaceae, and homosporous ferns (Rothwell and Stockey 1994). Because of its unique combination of features and its critical position in heterosporous fern phylogeny, *H. pinnata* was included in our study.

### *Specimen Preparation*

Fresh specimens were fixed in 70% ethanol. Herbarium material was softened in water for one to several days and then fixed in 70% ethanol. Light microscopy observations were carried out with a dissecting microscope (Olympus SZX 12 and Leica MZ 12.5) and a compound microscope (Zeiss Axioskop and Zeiss Axioplan 2) utilizing brightfield optics. Images were recorded with a Sony DXC-970 1/2 RGB video system, a SPOT digital camera, or an AxioCam digital camera. For scanning electron microscopy (SEM), fixed fresh and herbarium material was further dehydrated in an ethanol series and dried using a Pelco 030 critical point dryer (100% $CO_2$/ethanol). Dried material was mounted on SEM stubs, sputter-coated with gold using a Denton Vacuum Desk II sputter-coater, and viewed with conventional SEM procedures using either an AMRAY 1810 or a Philips XL 30 ESEM TMP with an accelerating voltage between 7.0 and 15.0 kV. Scanning electron micrographs either were taken with Polaroid film (PIN55) and digitized using a flatbed scanner or were digitally captured. All images were edited using Adobe Photoshop 5.5.

### *Homology Assessment*

We assessed the homology of the various components of reproductive structures across extant heterosporous ferns. We used an evolutionary homology concept based on the criteria

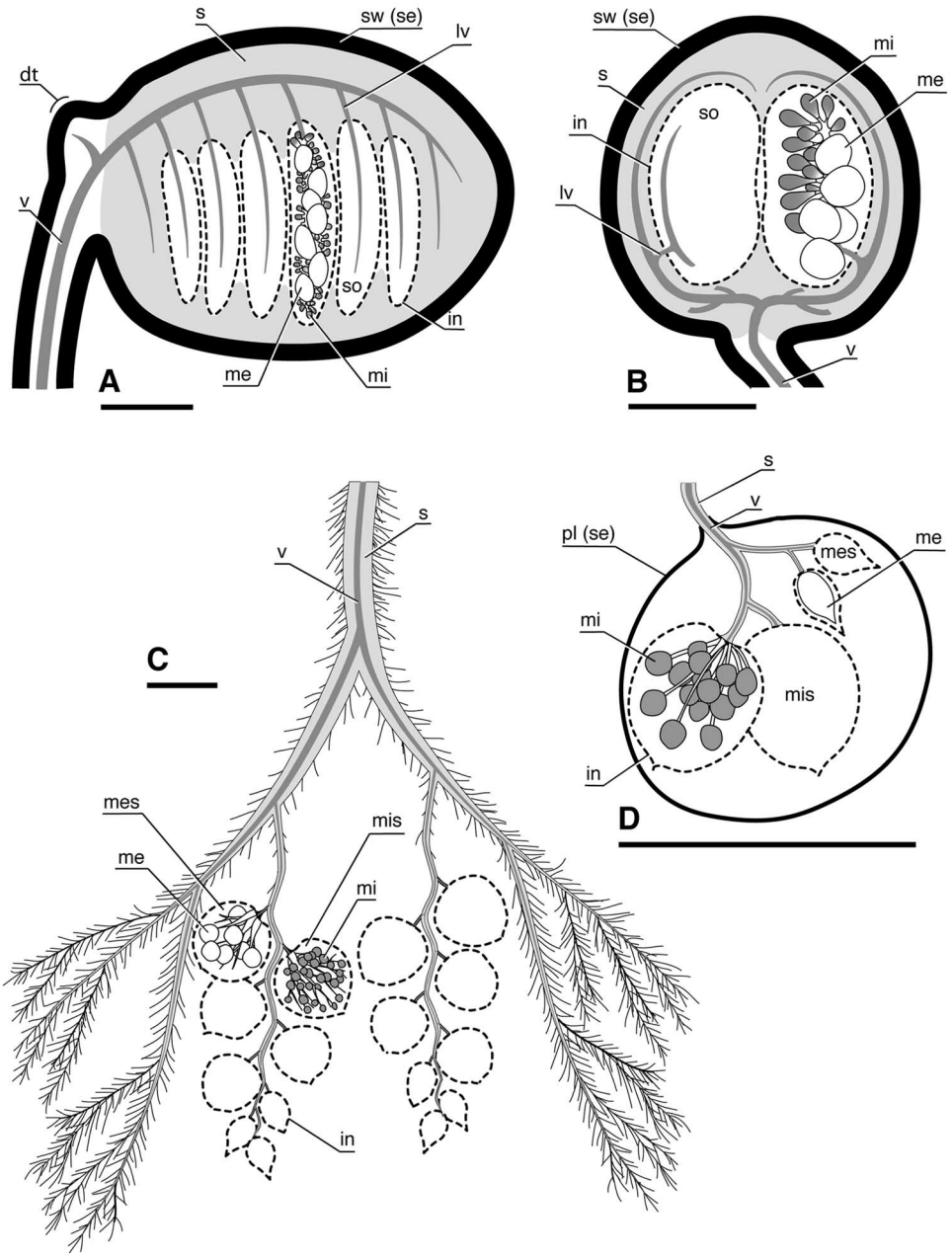



**Fig. 1** Schematic drawings of spore-bearing structures of Marsileaceae (*A*, *B*) and Salviniaceae (*C*, *D*). *A*, *Marsilea*: longitudinal section of an elliptical sporocarp. The sporocarp wall (*sw*) (also termed the sorophore envelope [*se*]) surrounds the sorophore (*s*), main (*v*) and lateral (*lv*) veins, and sori (*so*). Each sorus is delimited by an indusium (*in*). One sorus is detailed to show the arrangement of megasporangia (*me*) and microsporangia (*mi*); all sori enclose a similar arrangement of sporangia, and all are heterosporangiate. *B*, *Pilularia*: longitudinal section of a globose sporocarp. The sporocarp wall (*sw*) (also termed the sorophore envelope [*se*]) surrounds the sorophore (*s*), main (*v*) and lateral (*lv*) veins, and sori (*so*). Each sorus is delimited by an indusium (*in*). One sorus is detailed to show the arrangement of megasporangia (*me*) and microsporangia (*mi*); all sori enclose a similar arrangement of sporangia, and all are heterosporangiate. *C*, *Salvinia*: longitudinal section of a submerged organ. The submerged organ, also termed the sorophore (*s*), bears alternately arranged sori. The sori are indusiate (*in*) and homosporangiate; that is, they either are megasporangiate (*mes*) and bear only megasporangia (*me*) or are microsporangiate (*mis*) and contain only microsporangia (*mi*). All fertile segments arise at the second dichotomy of the submerged organ, but species can differ in the arrangement of sori on the segment; *Salvinia minima* is shown here. *D*, *Azolla*: longitudinal section of a reproductive structure. The parenchymatous layer (*pl*), also termed the sorophore envelope (*se*), encloses the sorophore (*s*) and sori. The sorophore bears two megasporangiate sori (*mes*) and two microsporangiate sori (*mis*). One megasporangiate sorus is detailed to show the single megasporangium (*me*), and a microsporangiate sorus shows the multiple microsporangia (*mi*). Each sorus is delimited by an indusium (*in*). Only *Azolla nilotica* bears four sori per reproductive structure; all other species have two. One reproductive structure can include any combination of megasporangiate and/or microsporangiate sori. Scale bars = 1 mm. *A*, *C*, *D* based on personal observations; *B* after Meunier (1888).

**Table 1**

**Heterosporous Fern Taxa Examined and Vouchers (Herbarium and Accession Numbers When Available)**

| Species | Source and vouchers |
|---|---|
| Marsileaceae: | |
| *Marsilea azorica* Launert & Paiva | United Kingdom: cultivated Chelsea Physic Gardens, London (original from Azores), no voucher |
| *Marsilea berhautii* Tardieu | Burkina Faso: *Martin 500* (SENCK) |
| *Marsilea botryocarpa* Ballard | Kenya: *Evans and Maikweki 62* (US 2650940) |
| *Marsilea crotophora* D. M. Johnson | Bolivia: *Rolleri 9* (US 2849627) |
| *Marsilea deflexa* A. Braun | Costa Rica: *Jiminez 348* (F 1607254) |
| *Marsilea drummondii* A. Braun | Switzerland: *Schneller s.n.* cultivated Zurich Botanic Garden (original from Australia) (Z) |
| *Marsilea ephippiocarpa* Alston | South Africa: *Son 18026* (F 653428) |
| *Marsilea fadeniana* Launert | Kenya: *Evans and Maikweri 55* (US 3183268) |
| *Marsilea minuta* L. | Kenya: *Evans and Glover 64* (US 2690526) |
| *Marsilea mutica* Mett. | Australia: *Constable NSW P8312* (US 241634); U.S.A.: *Nagalingum s.n.* cultivated Duke University Greenhouse (original from Lilypons Water Gardens Nursery) (DUKE) |
| *Marsilea polycarpa* Hook. & Grev. | Bolivia: *Beck 5518* (F 1896684) |
| *Marsilea quadrifolia* L. | U.S.A.: *Fosberg 44302* (US 2692394) |
| *Marsilea vestita* Hook. & Grev. | U.S.A.: *Palmer 13465* (F 741964) |
| *Pilularia americana* A. Braun | U.S.A.: *Pryer et al. 954* (DUKE), *Hill 8654* (F 186631) |
| *Pilularia globulifera* L. | France: *Chevallier s.n.* (F 802279); Germany: *Schneider s.n.* cultivated Göttingen Botanic Garden (original from near Düsseldorf) (GOET) |
| *Regnellidium diphyllum* Lindm. | Brazil: *Bloom s.n.* (F 1709990), *Rau s.n.* (US 1593512) |
| Salviniaceae: | |
| *Azolla caroliniana* Willd. | U.S.A.: *Duncan 19969* (F 1581435) |
| *Azolla filiculoides* Lam. | France: *Saino 1809* (F 802585) |
| *Azolla microphylla* Kaulf. | Guatemala: *Steyermark 31827* (F 1050147) |
| *Azolla nilotica* Dcne. ex Mett. | Zambia: *Parris and Croxall 10157* (F 1954760); Tanzania: *Goetze* (F 820344) |
| *Salvinia auriculata* Aubl. | Belize: *Davidse 32892* (F 199340); Bolivia: *Ritter et al.* (NHA 78697); Costa Rica: *Robles 1313* (F 2054048); El Salvador: *Fassett 28662* (F 1512754) |
| *Salvinia biloba* Raddi | Brazil: *Schneller s.n.* (Z) |
| *Salvinia hastata* Desv. | Madagascar: *Appert 6203* (Z), *Perrier 7128* (Z) |
| *Salvinia herzogii* de la Sota | Brazil: *Schneller s.n.* (Z) |
| *Salvinia minima* Baker | U.S.A.: *Pryer et al. s.n.* (unvouchered); Brazil: *Schneller 9* (Z) |
| *Salvinia natans* (L.) All. | Japan: *Tagawa 7174* (F 1483842) |
| *Salvinia nymphellula* Desv. | Nigeria: *Adams s.n.* (Z), *Thorold 2003* (BM) |
| *Salvinia oblongifolia* Mart. | Brazil: *Glaziou 16650* (F 63698); *Pereira 9745* (F 1629587); U.S.A.: *Nagalingum s.n.* cultivated Duke University Greenhouse, donated by T. Lemieux, University of Colorado, Boulder (origin unknown) (DUKE) |
| *Salvinia sprucei* Kuhn in Mart. | Venezuela: *Johnson 789* (MICH) |

of similarity, conjunction (i.e., position), and congruence (Patterson 1982).

### *Phylogenetic Hypotheses and Character Reconstruction*

There is a single phylogenetic hypothesis for relationships of extant heterosporous ferns: Salviniaceae (*Azolla*, *Salvinia*) is sister to Marsileaceae (*Marsilea,* [*Regnellidium*, *Pilularia*]) (Rothwell and Stockey 1994; Pryer 1999). However, there are two phylogenetic hypotheses regarding the position of the fossil *Hydropteris*: one proposed by Rothwell and Stockey (1994) and Pryer (1999) and another proposed by Pryer (1999). We investigated character evolution by plotting morphological characters onto the phylogeny of extant taxa alone and the two hypotheses incorporating the fossil *Hydropteris* (Rothwell and Stockey 1994; Pryer 1999). To determine the plesiomorphic condition for heterosporous ferns, we added outgroups based on a previous study showing that the sister group of heterosporous ferns is tree ferns plus polypod ferns and that Schizaeaeceae is sister to all of these (Pryer et al. 2004). The characters were reconstructed using both ACCTRAN and DELTRAN optimizations in MacClade, version 4.06 (Maddison and Maddison 2003). Character states for *H. pinnata* were interpreted from the original publication of Rothwell and Stockey (1994), and character states for all other taxa were derived from personal observations. All characters were treated as equally weighted and unordered.

## Results and Discussion

### *Fertile Morphology of Marsileaceae*

Of the Marsileaceae representatives we sampled, the reproductive structures were similar in overall morphology and anatomy. Therefore, the structures described here apply to all three genera, unless otherwise noted.

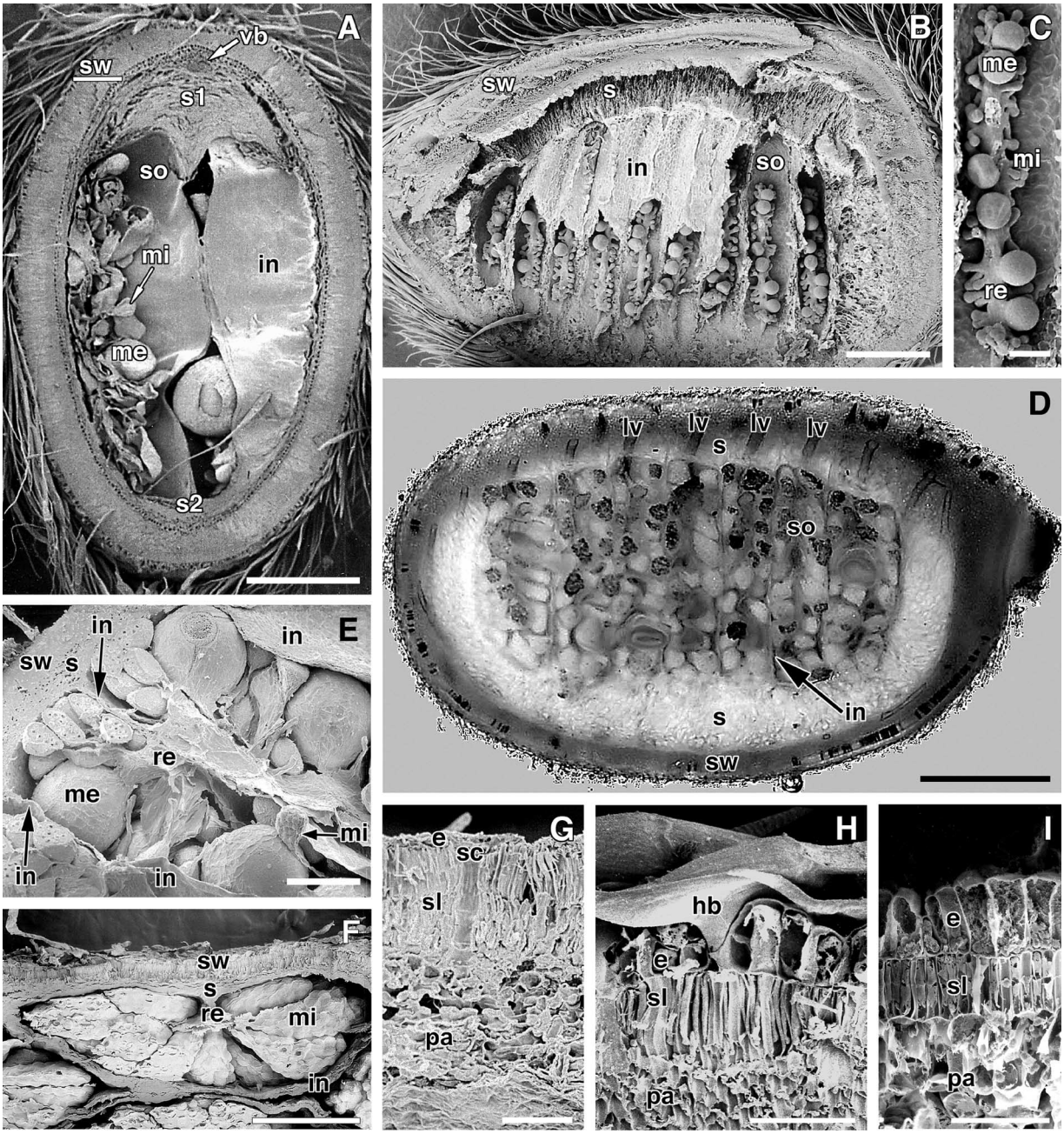



**Fig. 2** Fertile morphology of Marsileaceae. *A*, *Marsilea drummondii* sporocarp: scanning electron micrograph (SEM) of a longitudinal radial section. Long, unbranched hairs surround the sporocarp. The sporocarp is delimited by a sporocarp wall (*sw*). The sorophore is visible as a ring with dorsal (*s1*) and ventral (*s2*) segments; it is greater in size dorsally and is absent along the lateral wall. In the dorsal region of the sorophore is the vascular bundle (*vb*); the vascular bundle lies adjacent to the parenchymatous layer of the sporocarp wall. The sorus (*so*) on the left contains megasporangia (*me*) and microsporangia (*mi*). The sorus on the right has an indusium (*in*) that is partially broken, exposing one large megasporangium and three smaller microsporangia near the base. *B*, *Marsilea drummondii* sporocarp: SEM of a transverse longitudinal section. Long, unbranched hairs surround the sporocarp. The sporocarp wall (*sw*) is underlain by the sorophore (*s*). Each sorus (*so*) is delimited by an indusium (*in*). *C*, *Marsilea drummondii* sorus: close-up of *B* showing detail within a single sorus. The sorus contains an elongated receptacle (*re*) bearing megasporangia (*me*) on its crest and microsporangia (*mi*) on its lateral margins. *D*, *Marsilea mutica* sporocarp: light micrograph of a transverse longitudinal section, stained with safranin. The sporocarp wall (*sw*) lies adjacent to the sorophore (*s*), and in the dorsal region of the sorophore are lateral veins (*lv*). Most lateral veins lead to a sorus (*so*). The sori are delimited by an indusium (*in*) and contain megasporangia and microsporangia. *E*, *Regnellidium diphyllum* sporocarp: SEM of a transverse section. The sporocarp wall (*sw*) is underlain by the sorophore (*s*). The limit of one sorus is defined by its indusium (*in*; arrows) and contains megasporangia (*me*), microsporangia (*mi*), and a receptacle (*re*). *F*, *Pilularia globulifera* sporocarp: SEM of a transverse section. The sporocarp wall (*sw*) is underlain by the sorophore (*s*). One complete sorus is visible; it comprises an indusium (*in*) and a receptacle (*re*) that bears elongate microsporangia (*mi*). Although present in this sorus, the megasporangia are

In mature sporocarps, the wall is rigid and thickened (*sw* in fig. 1*A*, 1*B*; fig. 2*A*, 2*B*, 2*D*–2*F*) and is composed of epidermal, sclerenchymatous, and parenchymatous zones (*e*, *sl*, and *pa*, respectively, in fig. 2*G*–2*I*). Respectively, these layers are equivalent to the terms "epidermis," "hypodermis," and "transitional zone" used by Johnson and Chrysler (1938) and Bilderback (1978*a*). The epidermis is one cell layer thick and incorporates hairs and stomata. The sclerenchymatous zone is composed of one or two layers of regularly arranged, oblong, sclerified cells and confers rigidity to the sporocarp wall. The parenchymatous zone comprises several layers of loosely organized, circular to elliptic, parenchymatous cells.

In *Marsilea*, the sorophore lies adjacent to the parenchymatous zone of the sporocarp wall (*s* in fig. 1*A*; fig. 2*A*, 2*B*, 2*D*), forming an asymmetric ring that is larger dorsally. However, the sorophore is absent along the lateral sporocarp walls and between sori. In *Pilularia* and *Regnellidium*, the sorophore is also absent between the sori, and in contrast to *Marsilea*, the sorophore is relatively thinner and lies throughout the sporocarp adjacent to the parenchymatous zone (*s* in fig. 1*B*; fig. 2*E*, 2*F*). The sorophore is composed of closely packed, parenchymatous cells with large polysaccharide-filled vacuoles (Bilderback 1978*b*). When the polysaccharides are hydrated, the cells that make up the sorophore undergo a massive expansion in size; this swelling forces the sporocarp to open.

In *Marsilea*, the main vein (*v* in fig. 1*A*) of the sporocarp dichotomizes into two branches: one enters the dorsal tooth (*dt* in fig. 1*A*), and the other extends along the dorsal axis (figs. 1*A*, 2*A*) and produces lateral veins (*lv* in figs. 1*A*, 2*D*). Except for one or two distal and proximal lateral veins, each lateral vein bears a single sorus (figs. 1*A*, 2*D*). In *Pilularia*, the main vein (*v* in fig. 1*B*) dichotomizes into two branches at the base of the sporocarp (fig. 1*B*). Each branch dichotomizes twice to produce three subbranches. One subbranch produces a lateral vein (*lv* in fig. 1*B*) that bears a sorus (*so* in fig. 1*B*); this sorus is encircled by the two other vein subbranches (not fully visible in the plane shown in fig. 1*B*).

The sori (*so*) are longitudinally aligned in the sporocarp and are borne by the lateral veins of the sorophore (fig. 1*A*, 1*B*; fig. 2*A*, 2*B*, 2*D*). Each sorus contains both mega- and microsporangia (=heterosporangiate sorus) and is delimited by an indusium that is one cell layer thick (*in* in fig. 2*A*, 2*B*, 2*D*–2*F*). The indusium surrounds an elongate, unbranched receptacle (*re* in fig. 2*C*, 2*E*, 2*F*) and all of the mega- and microsporangia that are attached to it. In *Marsilea* and *Pilularia,* the receptacle is attached to the indusium wall closest to the sporocarp wall (fig. 2*A*–2*C*, 2*F*), whereas in *Regnellidium* it is located on the indusium cross-wall, that is, on a wall adjacent to the next sorus (fig. 2*E*). In the *Marsilea* sorus, the crest of the receptacle bears megasporangia (*me* in fig. 2*C*), whereas the lateral margins produce microsporangia (*mi* in fig. 2*C*). In *Pilularia* and *Regnellidium* sori, microsporangia are produced distally to the megasporangia (fig. 1*B*). All marsileaceous megasporangia (*me*) are elliptical to globose, several times larger than microsporangia, and bear a single megaspore (fig. 1*A*, 1*B*; fig. 2*C*, 2*E*). Marsileaceous microsporangia bear 16–64 microspores (Tryon and Lugardon 1991) but morphologically differ by genus. They are ovate with a poorly developed stalk in *Marsilea* (*mi* in fig. 2*C*), globose with a well-developed stalk in *Regnellidium* (*mi* in fig. 2*E*), and elongate-falcate with a strongly reduced stalk in *Pilularia* (*mi* in fig. 2*F*).

### *Fertile Morphology of Salviniaceae*

The fertile structures of *Salvinia* and *Azolla* differ substantially from one another. They therefore are described separately.

***Salvinia.*** All parts of the submerged organ are densely hirsute (fig. 1C; fig. 3*A*, 3*B*, 3*D*). The main vein (*v*) and its resulting branches and subbranches divide dichotomously with the segments. In sterile and fertile *Salvinia*, the main axis (also called the pedicel) of the submerged organ undergoes one dichotomous division to produce two major secondary segments (fig. 1C; fig. 3*A*, 3*B*). The secondary segments usually dichotomize further into subsequent segments, ultimately producing more than 15 divisions (fig. 1C; fig. 3*A*, 3*B*). In fertile *Salvinia*, one or two segments are each transformed into a fertile segment (figs. 1*C*, 3*A*). At the second dichotomy, on one or both sides of the submerged organ is a fertile segment. The fertile segments are typically shorter than the surrounding sterile segments and at maturity bear four to 20 sori (*so* in fig. 1C; fig. 3*A*, 3*B*). Sori are spherical and homosporangiate, that is, consisting of either several megasporangia or multiple microsporangia (figs. 1*C*, 3*G*). The megasporangiate and microsporangiate sori are individually delimited by an indusium consisting of two parenchymatous cell layers (*in* in fig. 3*E*, 3*F*). The sporangia are borne on a receptacle that is attached at the base of the sorus (*re* in fig. 3*C*). At maturity, megasporangiate sori are more or less the same size as microsporangiate sori (*mes* and *mis* in figs. 1*C*, 3*G*), although megasporangia (with one megaspore) are roughly twice the size of microsporangia (with 64 microspores; *me* and *mi* in fig. 3*G*).

***Azolla.*** In *Azolla*, the reproductive structures are also submerged. The sori are borne on a short, dichotomizing nonlaminate structure (fig. 1*D*). This structure divides once into two branches for most species of *Azolla*, or twice into four branches for *Azolla nilotica*, and terminal on every branch is a sorus (fig. 1*D*).

The sori are delimited by a parenchymatous indusium (*in* in fig. 4*E*, 4*F*) and are homosporangiate with either one megasporangium (bearing one megaspore; figs. 1*D*, 4*E*) or multiple microsporangia (each bearing 64 microspores; figs.

**Fig. 2** (*Continued*) not visible at this plane. *G–I*, Marsileaceae sporocarp walls: SEMs of longitudinal sections of *Regnellidium diphyllum* (*G*), *Marsilea drummondii* (*H*), and *Pilularia americana* (*I*). In all taxa, the sporocarp wall is composed of epidermal (*e*), sclerenchymatous (*sl*), and parenchymatous (*pa*) zones. In *Regnellidium*, a stomatal chamber (*sc*) is visible perforating the sporocarp wall, and in *Marsilea*, a hair base (*hb*) occurs at the epidermal zone; in all taxa, stomates and, when present, hairs occur at the epidermal zone. Note that in *Pilularia*, the sporocarp wall is immature, and the cell walls, in particular those of the sclerenchymatous zone, are not yet thickened. Scale bars = 1 mm (*A*, *B*, *D*), 200 μm (*C*), 500 μm (*E*, *F*), 100 μm (*G–I*). *A–C*, *H*, *Marsilea drummondii* Schneller s.n. (Z); *D*, *Marsilea mutica* Nagalingum s.n. (DUKE); *E*, *G*, *Regnellidium diphyllum* Bloom s.n. (F); *F*, *Pilularia globulifera* Chevallier s.n. (F); *I*, *Pilularia americana* Hill 8654 (F).

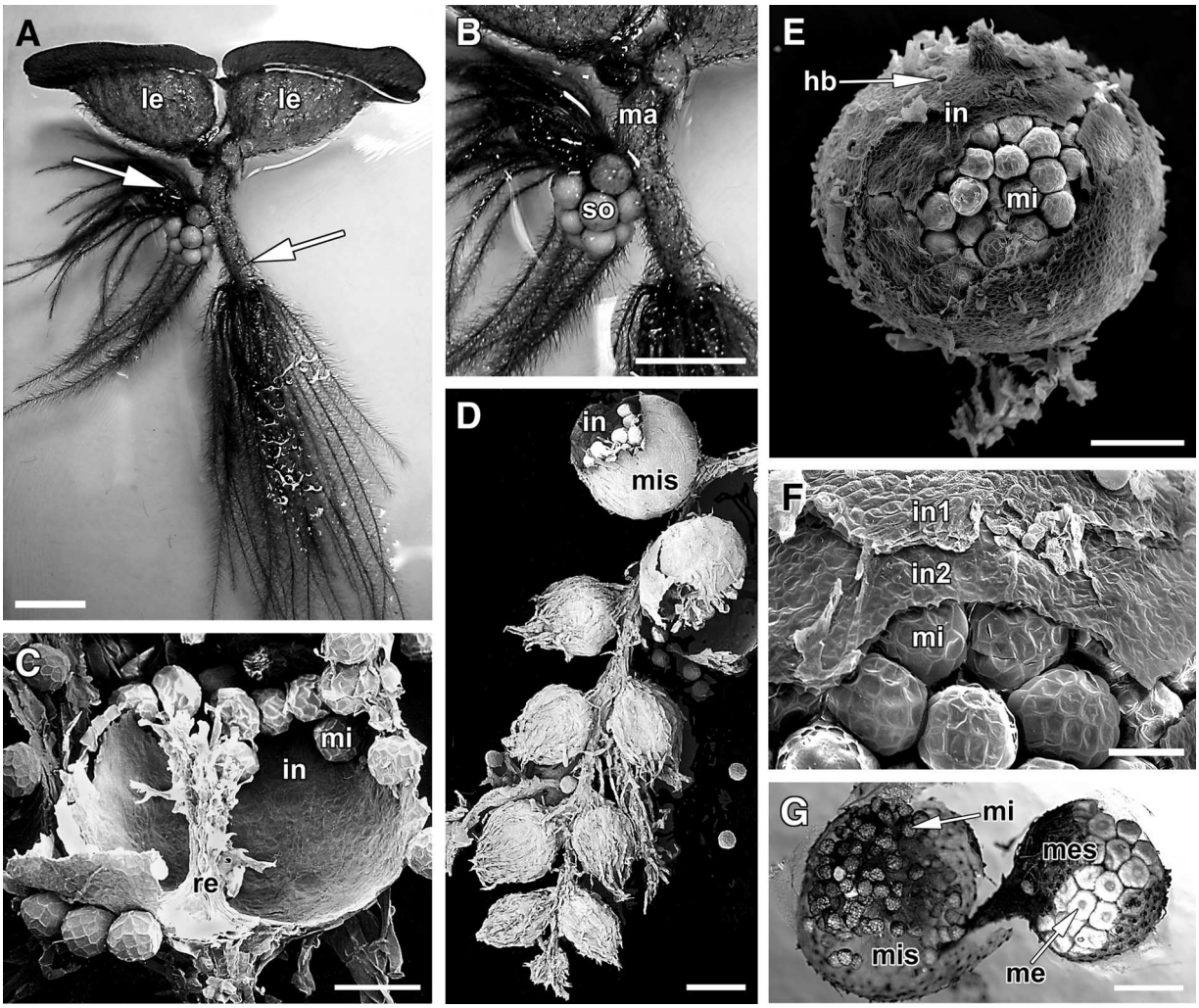



**Fig. 3** Fertile morphology of *Salvinia*, Salviniaceae. *A*, *Salvinia oblongifolia* fertile node: photograph of node comprising two floating leaves (*le*) and a submerged organ (or sorophore). The main axis of the submerged organ divides dichotomously into two secondary segments (arrows), one of which bears an immature fertile segment. *B*, *Salvinia oblongifolia* submerged organ: enlarged view of *A*. The main axis (*ma*) bears a segment with an immature fertile axis that has multiple sori (*so*). *C*, *Salvinia auriculata* sorus: scanning electron micrograph (SEM) of a microsporangiate sorus. The indusium (*in*) is broken to show the receptacle (*re*) and detached microsporangia (*mi*). *D*, *Salvinia minima* fertile branch: SEM of one sorophore segment bearing alternately arranged microsporangiate sori (*mis*); the indusium (*in*) of one sorus is broken to show the enclosed microsporangia. *E*, *Salvinia oblongifolia* sorus: SEM of a whole microsporangiate sorus. The hairs have been removed (*hb*), and the indusium (*in*) is broken to show the microsporangia (*mi*). *F*, *Salvinia oblongifolia* sorus: enlarged view of *E*. The two-layered indusium is composed of outer (*in1*) and inner (*in2*) layers that surround the microsporangia (*mi*). *G*, *Salvinia auriculata* sori: light micrograph of two homosporangiate sori. The microsporangiate sorus (*mis*) bears microsporangia (*mi*), and the megasporangiate sorus (*mes*) has megasporangia (*me*). Scale bars = 1 mm (*A*, *B*, *D*, *G*), 200 $\mu$m (*C*), 500 $\mu$m (*E*, *H*). *A*–*C*, *E*, *F*, *Salvinia oblongifolia* Nagalingum s.n. (DUKE); *D*, *Salvinia minima* Schneller 9 (Z); *G*, *Salvinia auriculata* Ritter et al. (NHA).

1*D*, 4*F*) that are borne on a receptacle. Because of the difference in number of sporangia per sorus, microsporangiate sori (*mis* in figs. 1*c*, 4*c*) are more globose and considerably larger at maturity (at least twice the size) than the ovoid megasporangiate sori (*mes* in figs. 1*C*, 4*C*).

A parenchymatous layer (*pl* in figs. 1*D*, 4*A*–4*D*) encases both the dichotomizing structure and the immature sori; the combination of the branching structure, sori, and parenchymatous layer comprises the entire reproductive unit. The delicate parenchymatous layer is composed of a single layer of cells, lacks vascular tissue, and breaks as the sori enlarge; hence, it is often difficult to detect when the sori are at full size, and this layer has rarely been described. The pair of sori (in the case of most *Azolla*) or two pairs (for *A. nilotica*) within the parenchymatous layer can be any combination of micro- or megasporangiate sori (e.g., fig. 4*B*, four microsporangiate sori; fig. 4*C*, two microsporangiate sori and two megasporangiate sori).

### *Assessments of Homology*

By examining extensive morphological information on the reproductive structures from all representatives of all extant

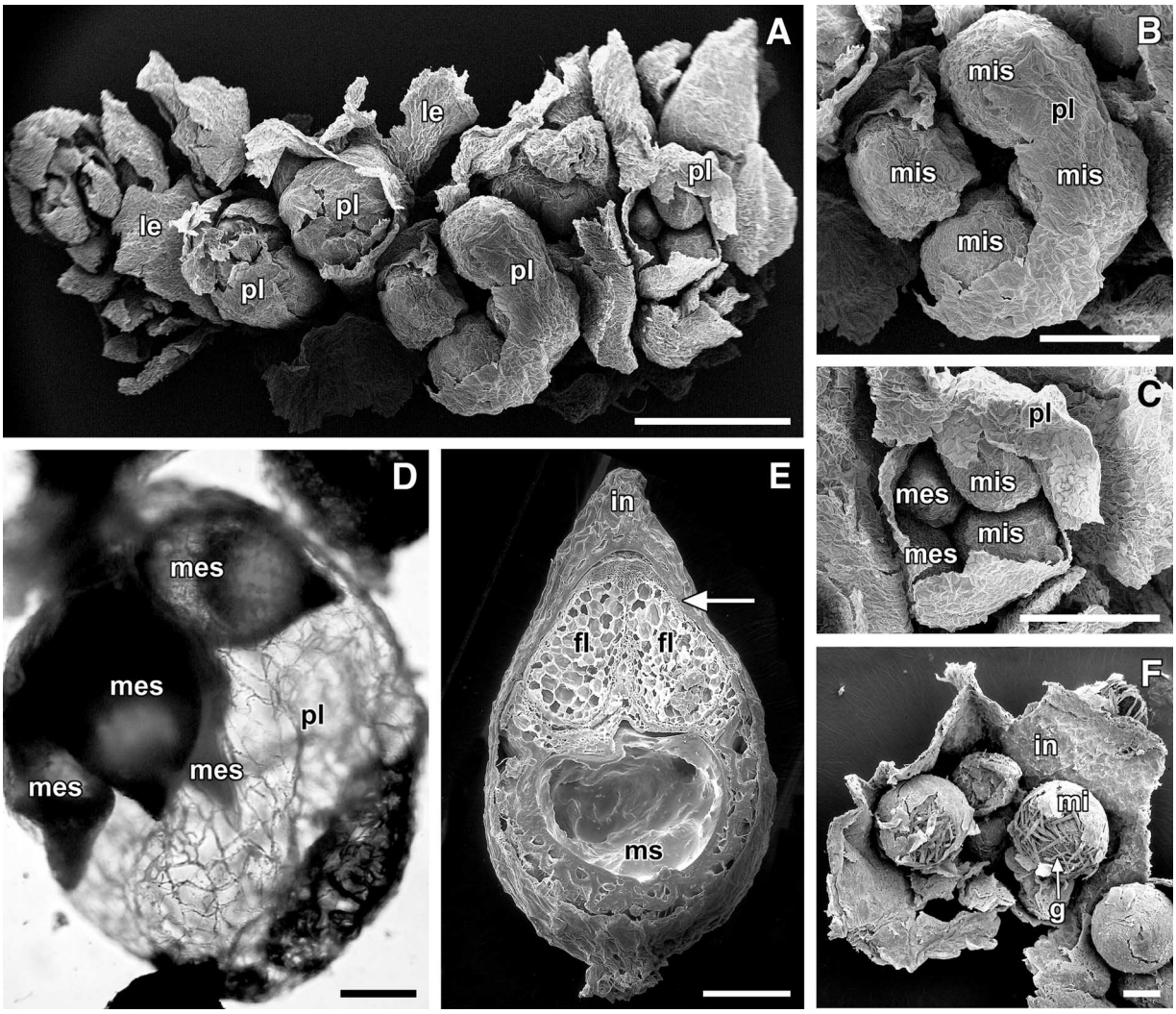
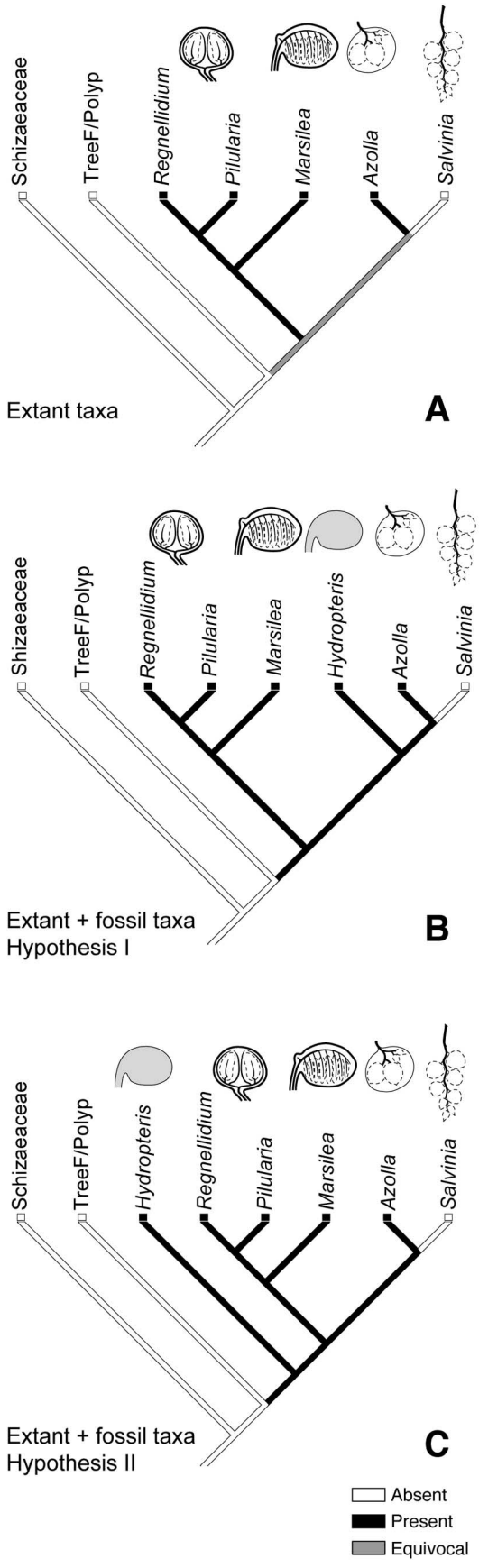



**Fig. 4** Fertile morphology of *Azolla,* Salviniaceae. *A*, *Azolla nilotica* fertile plant: scanning electron micrograph (SEM) of leaves (*le*) and reproductive units or structures that are each delimited by a parenchymatous layer (*pl*). In some clusters, the expanding sori have ruptured the parenchymatous layer; however, in one cluster (far right), the parenchymatous layer was broken deliberately to expose the enclosed sori. *B*, *C*, *Azolla nilotica* reproductive units: enlarged views of *A* each showing four sori surrounded by a parenchymatous layer (*pl*). One reproductive unit contains four microsporangiate sori (*mis*) (*B*); the other has two microsporangiate sori (*mis*) and two megasporangiate sori (*mes*) (*C*). *D*, *Azolla nilotica* reproductive units: light micrograph of the parenchymatous layer (*pl*) and four megasporangiate sori (*mes*). *E*, *Azolla filiculoides* megasporangiate sorus: SEM of a longitudinal section. The indusium (*in*) surrounds the megasporangium wall (arrow); within the sporangium is a single mature megaspore (*ms*) and its attached floats (*fl*). *F*, *Azolla filiculoides* sorus: SEM of a microsporangiate sorus. The indusium (*in*) is opened to reveal microsporangia (*mi*); the microsporangium wall is broken, showing the elongate glochidia (*g*) of a massula, that is, the vacuolate meshwork in which microspores are embedded. Scale bars = 1 mm (*A*), 500 μm (*B–D*), 100 μm (*E*, *F*). *A–C*, *Azolla nilotica* Goetze s.n. (F); *D*, *Azolla nilotica* Parris and Croxall 10157 (F); *E*, *F*, *Azolla filiculoides* Saino 1809 (F).

genera of heterosporous water ferns, we can compare these structures within a phylogenetic framework. The homology of the structures is interpreted using an evolutionary homology concept based on three criteria: similarity, conjunction (i.e., position), and congruence (Patterson 1982). Congruence is assessed using our current understanding of the relationships of extant heterosporous ferns where the clade comprising *Regnellidium-Pilularia* and *Marsilea* (Marsileaceae) is sister to *Azolla* and *Salvinia* (Salviniaceae; Pryer 1999). Assessment of the homology of the various components of the reproductive structures (sporocarps) in these ferns has been hampered by their morphological diversity and complexity. However, the complexity can be broken down into three major components: sorus (sori), a sorus-bearing structure hereafter referred to as the sorophore, and a structure surrounding the sorophore and attached sori, which we term the "sorophore envelope."

In homosporous ferns, each sorus is composed of sporangia that are attached to a receptacle, and the sorus is sometimes protected by a parenchymatous indusium. In all heterosporous ferns, the sporangia are also borne by a receptacle and surrounded by a thin, parenchymatous tissue referred to as the indusium. Collectively, these are homologous to what are




called sori in homosporous ferns. This homology is supported by phylogenetic congruence, conjunction, and structural similarity (i.e., composed of similar cells and tissues).

In homosporous ferns, the sori are usually attached to the abaxial leaf surface or leaf margin, whereas the sori of heterosporous ferns are terminal on branched, nonlaminate structures called sorophores. All sorophores extend from the base of, or attach directly to, a petiole. The position of the sorophores of heterosporous ferns suggests that they are homologous. The sorophores show similarities in the presence of vascular tissue, attachment of indusiate sori, and dichotomous branching. In most cases, the sorophore branching is isodichotomous, but in *Marsilea* the branching appears to be the result of strong anisodichotomous branching. The sorophores also display remarkable structural differences. In Marsileaceae, the cortex of the sorophore is differentiated into a gelatinous tissue, and in Salviniaceae the cortex is nongelatinous and sometimes becomes aerenchymatous (e.g., *Salvinia cucullata* Roxb. ex Bory). Further differences among sorophores lie in the number of divisions, as indicated by vascular dichotomies. The number of dichotomies usually corresponds to the relative size of the sorophore. This is most obvious in Marsileaceae, in which the small sorophores of *Pilularia* show only a few orders of branching (about four), whereas more dichotomies contribute to the larger sorophores of *Regnellidium* and *Marsilea*. Similarly in Salviniaceae, the sorophore of *Azolla* has one or two orders of branching, whereas the *Salvinia* sorophore has multiple orders of division (>8) that form the sterile and fertile segments of the highly dissected submerged organ. However, under an alternative interpretation, the submerged organ of *Salvinia* represents several less divided sorophores (similar to those of *Azolla* but either sterile or fertile) that are repeated metamers and merged into a single structure. Despite a few structural differences, the fulfillment of the positional and similarity criteria supports the homology of the sorophore in all heterosporous ferns. This is further supported by the congruence criterion: the phylogeny for these taxa indicates that the sorophore is a synapomorphy for the heterosporous fern lineage.

The third component of the heterosporous fern reproductive structure is the sorophore envelope. It is present as a thick sporocarp wall in Marsileaceae and as a thin parenchymatous layer in *Azolla*. A sorophore envelope is absent in *Salvinia*. The envelope completely surrounds the sorophore and sori in Marsileaceae and *Azolla*, and thus its position suggests it is homologous across these taxa. When present, the sorophore envelope shows striking variation in its structure—it

**Fig. 5** Evolution of the sorophore envelope in heterosporous ferns. The presence or absence of the sorophore envelope is plotted on three tree topologies. *A*, Extant taxa alone; *B*, hypothesis 1 with *Hydropteris* as sister to Salviniaceae; *C*, hypothesis 2 with *Hydropteris* as sister to all extant heterosporous. The outgroups of heterosporous ferns are tree ferns plus polypod ferns (TreeF/Polyp) and Schizaeaceae. The schematic illustrations represent simplified versions of the reproductive structures in each genus. Note that the sporocarps of *Regnellidium* and *Pilularia* are essentially identical in structure and are shown by one schematic, and the reproductive structure of *Hydropteris* lacks detail because its internal organization is not known.

is multilayered and partly sclerenchymatous in Marsileaceae, and in *Azolla*, it comprises a single parenchymatous layer. Although these structural differences do not strongly support homology according to the similarity criterion, such variation may have arisen by modification after the evolution of the sorophore envelope. The third criterion, phylogenetic congruence, is equivocal when using only extant heterosporous ferns (fig. 5*A*) because there are two equally parsimonious explanations for the evolution of the sorophore envelope: it either evolved twice (once in Marsileaceae and once in *Azolla*) or it arose once in heterosporous ferns and was lost altogether in *Salvinia*. Thus, the homology of the sorophore envelope is supported by two of three criteria, and according to one criterion it is ambiguous. Fossil taxa with new or intermediate character states have the potential to resolve this ambiguity or at least to provide added insight into the evolution of these structures.

### *Incorporation of Fossils*

The fossil *Hydropteris pinnata* occupies a critical position in the phylogeny of heterosporous ferns, and it incorporates features from Marsileaceae, Salviniaceae, and homosporous ferns (Rothwell and Stockey 1994). The reproductive structure of this fossil fern is similar to the marsileaceous sporocarp in position and shape. It is delimited by a wall-like structure that may have been parenchymatous or sclerenchymatous, but it was noted that it appeared "fleshy and possibly photosynthetic" (Rothwell and Stockey 1994, p. 488). Based on its original description, the *Hydropteris* wall corresponds to the sorophore envelope in our morphological character analysis.

Several studies have investigated the phylogenetic position of this fossil within heterosporous ferns. Rothwell and Stockey (1994) first suggested that *Hydropteris* was sister to Salviniaceae (hypothesis 1; fig. 5*B*). Pryer (1999) later proposed two hypotheses (1 and 2) in which *Hydropteris* was sister either to Salviniaceae (hypothesis 1; fig. 5*B*) or to all extant heterosporous ferns (hypothesis 2; fig. 5*C*). Assuming that the sorophore envelopes of all heterosporous ferns are homologous (by the positional and similarity criteria), we attempted to reconstruct the evolution of the sorophore envelope on the two available hypotheses incorporating *Hydropteris* (fig. 5*B*, 5*C*).

According to hypotheses 1 and 2, the sorophore envelope arose once in the heterosporous ferns (fig. 5*B*, 5*C*). It has been retained in all extant taxa except for *Salvinia*, where its absence is interpreted as a loss. The plesiomorphic morphology of the sorophore envelope is unclear—it may have been parenchymatous as in *Azolla* or sclerenchymatous as in Marsileaceae, or it may have a unique morphological state not present in any known taxon.

### *Ecological Implications*

It is possible that the loss and delicate nature of the sorophore envelope in Salviniaceae may be related to the wholly aquatic habitats in which these ferns are found and that the sporocarps of Marsileaceae are an adaptation to amphibious environments. The ephemeral, amphibious nature of the habitats of marsileaceous ferns provides a small window of opportunity for reproduction, and the sporocarp may serve to facilitate and ensure spore survival as well as the simultaneous release of megaspores and microspores. This function would not be required for Salviniaceae because water for reproduction is always readily available, resulting in a loss of or a delicate sorophore envelope. Alternatively, the sclerenchymatous sorophore envelope of *Marsilea* may serve for spore protection during digestion by water birds (endozoochory has been documented in *Marsilea* [Malone and Proctor 1965], but it has not been studied in *Pilularia* and *Regnellidium*). Endozoochory has not been suggested or documented for *Salvinia* (or *Azolla*); consequently, the sorophore envelope is not needed in these plants, leading to its delicate nature or loss.

### *Concluding Remarks and Future Directions*

Based on three criteria (position, structure, and phylogenetic congruence), in heterosporous ferns we recognize the sorophore as a sorus-bearing structure and the sorophore envelope as a structure surrounding the sorophore and attached sori. All heterosporous ferns possess a sorophore (gelatinous in Marsileaceae and nongelatinous in Salviniaceae), and the sorophore envelope of Marsileaceae (sporocarp wall) is homologous with that of *Azolla* (parenchymatous layer). The term "sporocarp" is defined here as a sorophore and sori surrounded by a sorophore envelope. Under this definition, sporocarps occur in Marsileaceae and *Azolla* but not in *Salvinia* because a sorophore envelope is absent.

Further work is required to understand the development and origin of the sorophore envelope. Previous workers have recognized the foliar origin of the marsileaceous sporocarp wall (Johnson 1898*a*, 1898*b*, 1933*a*, 1933*b*; Eames 1936; Johnson and Chrysler 1938; Puri and Garg 1953; Schmidt 1978), and others have interpreted the parenchymatous layer of *Azolla* as a modified leaf lobe (Bower 1928; Konar and Kapoor 1972, 1974). However, modern comparative developmental studies are needed to confirm further the homology of the sorophore envelope of Marsileaceae and *Azolla*.

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