NEW OBSERVATIONS AND SYNTHESIS OF PALEOGENE HETEROSPOROUS WATER FERNS

Margaret E. Collinson,[1,]* Selena Y. Smith,† Johanna H. A. van Konijnenburg-van Cittert,‡,§ David J. Batten,‖
Johan van der Burgh,‡ Judith Barke,‡ and Federica Marone#

*Department of Earth Sciences, Royal Holloway University of London, Egham, Surrey TW20 0EX, United Kingdom;
†Department of Earth and Environmental Sciences and Museum of Paleontology, University of Michigan, Ann
Arbor, Michigan 48109, U.S.A.; ‡Marine Palynology Group, Laboratory of Palaeobotany and Palynology,
Department of Earth Sciences, Utrecht University, Budapestlaan 4, 3584 CD Utrecht, The Netherlands;
§Naturalis Biodiversity Center, P.O. Box 9517, 2300 RA Leiden, The Netherlands; ‖School
of Earth, Atmospheric, and Environmental Sciences, University of Manchester,
Oxford Road, Manchester M13 9PL, United Kingdom, and Institute of
Geography and Earth Sciences, Aberystwyth University, Ceredigion
SY23 3DB, United Kingdom; and #Swiss Light Source, Paul
Scherrer Institute, CH-5232 Villigen, Switzerland

Premise of research.   Reproductive structures of modern genera of heterosporous water ferns (Marsileaceae and Salviniaceae) are widespread and abundant in plant mesofossil assemblages from the Paleogene. For Salviniaceae, whole fertile fossil plants give a good understanding of morphology. These fossils can be applied in paleoenvironmental analysis and to study water fern origin, evolution, and diversification.

Methodology.   New specimens were examined by SEM and TEM. Synchrotron x-ray tomographic microscopy (SRXTM) is evaluated as a nondestructive tool for investigating *Azolla* Lam. morphology.

Pivotal results.   *Azolla anglica* Martin and *Salvinia cobhamii* Martin (earliest Eocene, United Kingdom) are fully characterized using SEM and TEM. SRXTM enables digital rendering of the float system in *Azolla*, but individual floats are difficult to distinguish. Modern water fern genera characterize the Paleogene, but extinct sister taxa characterize the Cretaceous. Literature review documents that water ferns are intolerant of salinity over 5 psu.

Conclusions.   The oldest fully documented *Salvinia* Séguier sori and spores occur in earliest Eocene deposits at Cobham, United Kingdom, probably linked to warm climates. An unusual co-occurrence of *Salvinia* with *Azolla* is preserved at this site. The *Azolla* species differs from those present in the same region during other Eocene warm-climate intervals. SRXTM offers potential to retrieve taxonomically useful information on internal structures of *Azolla*. There is a major turnover in water ferns (dominantly extinct to almost entirely modern genera) across the Cretaceous-Paleogene transition. The utility of water ferns as indicator taxa is exemplified by recognition of freshwater ocean surfaces and widespread continental wetlands during the latest Early to earliest Middle Eocene in and around the Arctic and Nordic Seas.

Keywords: *Azolla*, Hydropteridales, megaspore, microspore, *Salvinia*, Salviniales, synchrotron radiation x-ray tomographic microscopy (SRXTM).

Introduction

Water ferns are a monophyletic group of heterosporous ferns comprising two extant families, Marsileaceae and Salviniaceae, placed in the order Hydropteridales (Rothwell and Stockey 1994; Yamada and Kato 2002) or Salviniales (Smith et al. 2006). In Salviniaceae, fertile whole-plant fossils are well known for *Azolla* Lam., with a couple of examples for *Salvinia* Séguier (reviewed in Collinson 1991, 2001, 2002; Hoffman and Stockey 1994; Collinson et al. 2002), and document plant morphology similar to that of the modern genera.

[1] Author for correspondence; e-mail: m.collinson@es.rhul.ac.uk.

The extinct genus *Hydropteris* Rothwell & Stockey (containing *Parazolla* Hall megaspores) is also based on fertile whole plants and may be basal within heterosporous ferns or an extinct sister taxon within Salviniaceae (Rothwell and Stockey 1994; Pryer 1999). *Regnellites nagashimae* Yamada & Kato (2002) included sporocarps (but no megaspores; see Batten et al. 2011a for discussion), but apart from this there are no fertile whole plants of fossil Marsileaceae, although there are sporocarps containing *Molaspora* Schemel (Batten et al. 2011a; described as *Regnellidium* Lindman in Lupia et al. 2000) and some examples of vegetative material attributed to Marsileaceae (Nagalingum 2007; Batten et al. 2011a). Salviniales have an excellent fossil record based on sori, megaspores, megaspore apparatuses,

microspore massulae, and microspores, often with organic connections between microspores or microspore massula and their respective megaspores/megaspore apparatuses (Collinson 1980, 1991, 2001, 2002; Vajda and McLoughlin 2005; Batten et al. 2011*a*, 2011*b*).

Water ferns include excellent paleoenvironmental indicator taxa (reviewed in Collinson 2002). The Paleogene record of modern genera of Marsileaceae is limited, lacking *Pilularia* L. and with only scattered Eurasian records of *Marsilea* L. and *Regnellidium* (Dorofeev 1981; Collinson 2001; Batten et al. 2011*a*). By contrast, fossil representatives of the modern genera of Salviniaceae were widespread in the Paleogene. They also showed considerable latitudinal range extension, far outside their modern distribution (Collinson 2001), including as far north as the central Arctic Ocean (Collinson et al. 2009*a*; Barke et al. 2012). This good fossil record supports inferences on water fern origin, evolution, and diversification.

This article has three aims: (1) to describe new data on important UK Paleocene-Eocene thermal maximum (PETM) water ferns, (2) to evaluate the usefulness of synchrotron radiation x-ray tomographic microscopy (SRXTM) for the study of water fern megaspores, and (3) to use new literature to test our existing hypothesis (Collinson 1991) of major turnover in water ferns at the Cretaceous-Paleogene transition. New data on water ferns from the PETM Cobham Lignite, United Kingdom, are important in the context of the then prevailing warm and humid climates, the early fossil record of *Salvinia* megaspores and microspore massulae, and comparison with other European and Nordic Sea species including the UK Thanetian *Azolla teschiana* Florschütz emend. Batten & Collinson (Batten and Collinson 2001) and the five species now recorded from the Arctic and Nordic Seas (Barke et al. 2012). However, *Azolla anglica* Martin and *Salvinia cobhamii* Martin were originally documented using LM, while the taxa with which the species need to be compared are documented by LM, SEM, and TEM. Therefore, new material of *A. anglica* and *S. cobhamii* from the type locality and bed is reinvestigated here with these methods and compared with other species of similar age or geographic distribution.

In addition, new approaches have become available in recent years, and here we explore the suitability of SRXTM for nondestructive analysis of *Azolla* morphology and for obtaining three-dimensional information on internal structure normally hidden from view. Multifloated species of *Azolla* are often totally covered by filosum (Sweet and Hills 1976; Collinson et al. 2009*a*, 2010). The filosum obscures the underlying floats, the number and arrangement of which are important systematic characters (Batten and Collinson 2001; Collinson et al. 2009*a*, 2010). X-ray methodologies offer the potential to render digitally the floats and hence determine their number and arrangement. In addition, digital sections can be obtained nondestructively and have the potential to reveal details otherwise available only after time-consuming and destructive embedding and sectioning. However, *Azolla* megaspores are commonly less than 500 μm in length, the filosum elements are narrow (0.3–2.0 μm in diameter) and of a similar size to the smaller vacuoles of the floats (fig. 3*I*), and the filosum zones between the floats are often only a total of 2–3 μm thick (fig. 3*I*). All these factors make x-ray approaches challenging. *Azolla jutlandica* Collinson et al. was

previously studied using micro-CT, which showed low resolution, and although areas of pseudovacuolate structure were visualized, it was not possible to recognize the float boundaries (Collinson et al. 2010). Therefore, the same specimen of *A. jutlandica* has now been studied by SRXTM along with specimens of three other species.

Finally, much new literature is available on megaspore assemblages. This can be used to test the previous suggestion (Collinson 1991) of turnover at the Cretaceous-Paleogene transition, from dominantly extinct to dominantly extant water fern genera.

Material and Methods

Cobham Lignite PETM Water Ferns

Water ferns from the Cobham Lignite were first described by Martin (1976*b*). However, their stratigraphic position, within the bed or with respect to the PETM, was not then known. Recent work on the Cobham Lignite Bed (Collinson et al. 2009*b*) has shown that the water ferns occur in the blocky lignite at the very base of the PETM, i.e., in the earliest Eocene. Their occurrence is coincident with major environmental changes, including increased rainfall, loss of the pre-PETM episodic fire regime, and a shift from fern-dominated to mixed palm, conifer, and dicot-dominated vegetation (Collinson et al. 2009*b*). Mesofossils were studied in the >125-μm fraction from the same 5-g sediment samples as prepared for palynology (Collinson et al. 2009*b*). Preparation for SEM and TEM follows that described by Collinson (1991) and used by Batten and Collinson (2001) and Collinson et al. (2009*a*, 2010). Instrumentation is that used by Collinson et al. (2009*a*, 2010). Specimens are housed in the Natural History Museum, London (NHMUK); specimen numbers begin with "V."

SRXTM of Azolla Megaspore Apparatuses

Four specimens of *Azolla* megaspores were analyzed with SRXTM imaging during two sessions at the TOMCAT beamline (Stampanoni et al. 2006) of the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland). The x-rays transmitted by the samples were converted into visible light by a 20-μm-thick Ce-doped LAG scintillator screen (Crytur, Turnov, Czech Republic). A microscope objective of ×20 was used to magnify projection data that were then digitized by a high-resolution CCD camera (PCO2000; PCO, Kelheim, Germany), resulting in a final pixel size of 0.35 μm. The energy was set to 10 keV, and the exposure time per projection was 1 s or 115 ms. For each scan, a total of 1501 or 1601 (2048 × 2048 pixels) projections were acquired over 180°. Tomographic reconstructions were performed on a 60-node Linux PC cluster with a highly optimized routine based on the Fourier transform method and a gridding procedure (Marone and Stampanoni 2012). Three-dimensional data sets were visualized using Avizo (ver. 5, 6; Visualization Sciences Group, http://www.vsg3D.com). Images of digital transverse, longitudinal, and oblique sections were captured. Two types of three-dimensional visualizations were used: (1) volume renderings to visualize the whole specimen and (2) manual image segmentation to visu-
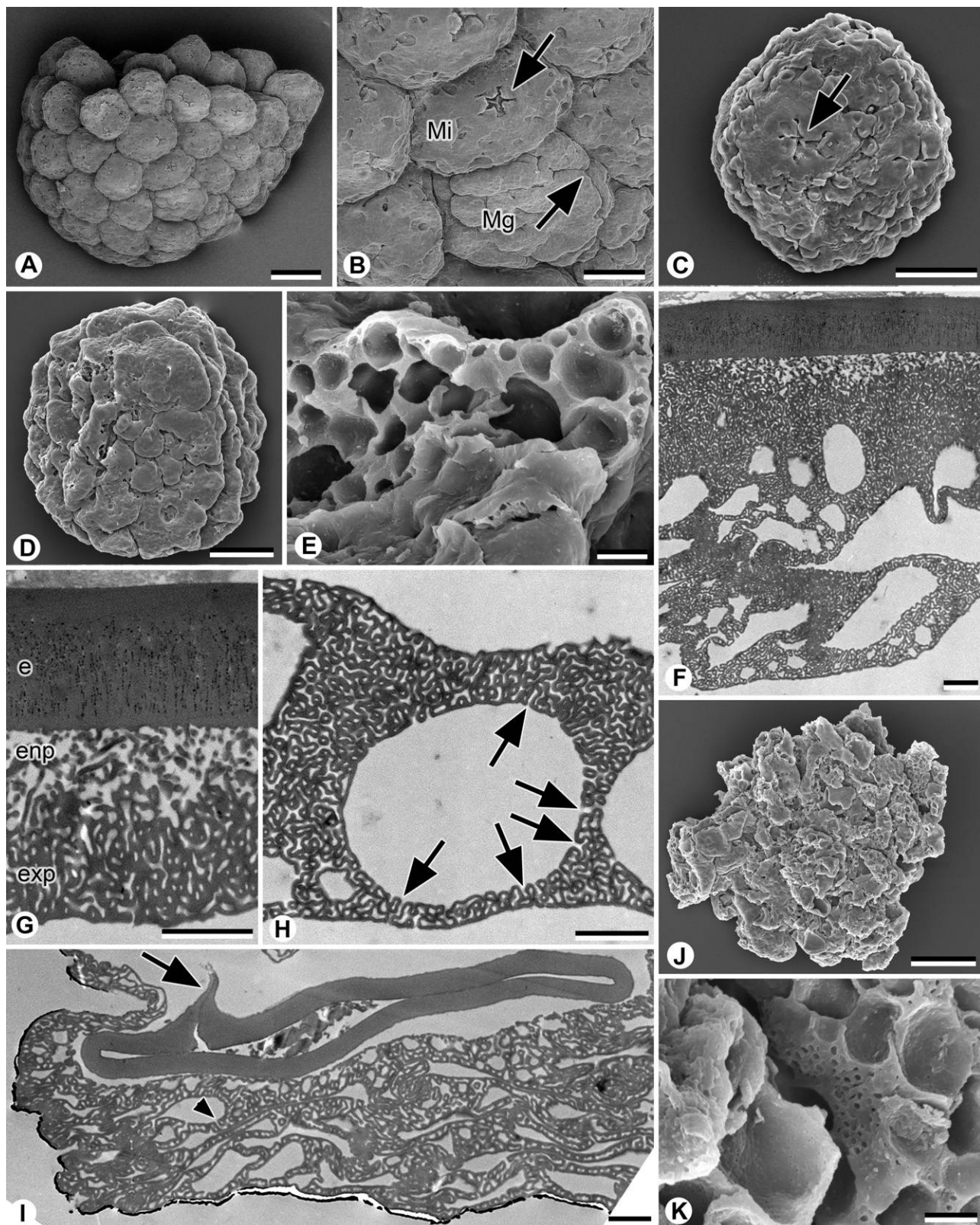
Fig. 1 SEM (*A–E, J, K*) and TEM (*F–I*) of *Salvinia cobhamii* and probable coprolite containing *S. cobhamii* from the early PETM, Cobham, United Kingdom. *A*, Bisporangiate sorus; V68456(1). *B*, Detail of *A* showing megaspore (Mg) with trilete mark (arrow) and stellate opening (arrow) on microspore massula (Mi). *C*, Dispersed microspore massula with stellate opening (arrow); V68457(1)$1 now in TEM block. *D*, Dispersed megaspore; V68457(2). *E*, Fracture through pseudovacuolate megaspore exoperine; V68457(3)$1 now in TEM block. *F*, Section

alize only the floats. Snapshots of volume renderings in Avizo were captured in that program. Image contrast adjustments were performed using Adobe Photoshop CS4.

Results

Cobham Lignite PETM Water Ferns

New observations of Salvinia cobhamii. Martin (1976*b*) did not report sori in his material. The newly recognized sori are bisporangiate, containing both megaspores and microspore massulae (fig. 1*A*, 1*B*). Up to 45 former sporangial contents are visible on one surface of the compressed sori (*n* = 3), suggesting more than 100 sporangia per sorus. Sori contain many more microspore massulae than megaspores. No sorus wall or sporangial remnants were seen by SEM or TEM. Both sori (fig. 1*A*, 1*B*) and dispersed microspore massulae (fig. 1*C*) and megaspores (fig. 1*D*) are present in the samples.

In SEM and TEM, the megaspore wall is irregularly pseudovacuolate with variable sizes and distribution of vacuoles (fig. 1*E*, 1*F*). In TEM, the exine (fig. 1*G*, e) shows a faint radial arrangement of minute pores but is otherwise solid. The endoperine (fig. 1*G*, enp) consists of narrow, hairlike filaments in various orientations (appearing circular or elongate in section). These filaments curl and anastomose to form the complex intertwined meshwork of the exoperine (fig. 1*G*, exp). In most (but not all) cases, the exoperine vacuoles have discontinuous indented boundaries (fig. 1*H*, arrows).

Microspore massulae contain multiple microspores close to the surface (fig. 1*I*). Five microspores are seen in a single plane of section by TEM; a total of 24–42 were reported by Martin (1976*b*) based on LM observations. All are enclosed within a common perine. As in the megaspores, the perine is irregularly pseudovacuolate (fig. 1*I*) and comparable to the megaspore exoperine (fig. 1*F*–1*H*); it also has some discontinuous vacuole boundaries (fig. 1*I*, arrowhead). Martin (1976*b*) reported individual perines per microspore based on LM observations, but this is not supported by TEM. Microspore massulae open to the exterior by small stellate, five-partite openings (fig. 1*B*, 1*C*, arrows) beneath which are the trilete laesurae of the microspores (fig. 1*I*). The microspore exine is amorphous with a smooth surface (fig. 1*I*).

New observations of Azolla anglica. The *A. anglica* megaspore apparatus (fig. 2*A*) has limited suprafilosum such that the exoperine is always partially visible, especially near the distal pole (fig. 2*B*), but often also visible elsewhere (fig. 2*C*, 2*D*). Infrafilosum, arising from the exoperine, is infrequent but consistently present (figs. 2*B*, 2*C*, 3*D*). The exoperine surface is reticulate-foveolate (fig. 2*C*) with large foveolate lumina surrounded by broad, rounded muri sometimes elevated into rounded papillae of the same diameter as, or smaller than, the muri (fig. 2*B*, 2*C*). In SEM (fig. 2*E*) and TEM (fig. 3*B*–3*D*) sections, the exoperine is composed of large, broad, solid clavate masses supported on short, narrower pillars. The endoperine has an open structure, and its surface is ornamented with small rugulae or papillae. The SEM and TEM data entirely vindicate the megaspore wall interpretation by Martin (1976*b*, text fig. 1a) based on LM observations (Martin 1976*b*, pl. 27, fig. 3), although Martin (1976*b*) did not report the presence of infrafilosum arising from exoperine that has been observed here (figs. 2*B*, 2*C*, 3*D*). The exoperine becomes strongly modified at the proximal pole (fig. 3*F*, lower wall layer from right to left) and gives rise to suprafilosum (fig. 3*E*, 3*G*, arrows) that enmeshes the floats (fig. 3*G*, 3*I*). There is a clear central columella formed of endoperine and modified exoperine (fig. 3*G*, c) and possibly a diminutive collar (fig. 3*G*). Floats are clearly pseudovacuolate (fig. 3*G*, 3*I*), and at least five are visible in a single longitudinal section (parts of which are shown in fig. 3*F*, 3*G*), although the tiering is obscured by compression. Martin (1976*b*) reported up to 24 floats in three tiers, and the new observations by TEM are entirely consistent with this. A remnant of sporangial wall ("cap") one cell thick is present at the proximal pole of the megaspore apparatus (fig. 3*H*, arrow).

Microspore massulae occur in connection with the megaspore apparatus in many specimens (figs. 2*A*, 2*F*–2*H*, 3*A*), a connection mediated by the grapnel tips of the glochidia becoming entangled in megaspore filosum (fig. 2*G*). In TEM section, the massula is pseudovacuolate, and the microspores have a solid exine with a smooth surface (fig. 3*A*). Massula surfaces and glochidia stalks lack hairs and may be slightly wrinkled, sometimes foveolate (fig. 2*F*–2*H*). Glochidia are short, have a wide basal attachment and a narrow basal stalk, and widen in the upper stalk to a distal dilation with a constriction beneath the grapnel-shaped tip. Glochidia fluke tips are always recurved. Martin (1976*b*, text fig. 1b) shows narrow and twisted lower glochidia stalks widening abruptly to the upper stalk, a reconstruction not supported by SEM data (fig. 2*F*–2*H*).

Evidence for paleoenvironment from associated mesofossils. A single fragment of a probable coprolite (fig. 3*J*) composed of *Salvinia* megaspore wall fragments (fig. 3*K*) suggests possible arthropod feeding on *Salvinia* in the local environment. Mesofossils associated with *Azolla* and *Salvinia* at Cobham include *Minerisporites* Potonié megaspores (fig. 4*A*), produced by isoetalean plants likely to have been aquatics or marginal aquatics (Batten and Collinson 2001). There are several morphologies of seed cuticle (fig. 4*B*, 4*C*) that might include a poorly preserved example of *Typha* L. (fig. 4*B*). An anther

through megaspore wall showing exine (top of image), endoperine, and pseudovacuolate exoperine; V68457(3)$1 TEM block and grids. G, Detail of exine (e) showing radial rows of small pores, endoperine (enp) with sectioned filaments, and exoperine (exp) with anastomosing filaments forming a meshwork; V68457(3)$1 TEM block and grids. H, Detail of anastomosing and twisting filaments of exoperine and pseudovacuole with discontinuous indented (arrows) boundary; V68457(3)$1 TEM block and grids. I, Part of microspore massula showing pseudovacuolate common perine, in places with discontinuous boundaries to pseudovacuoles (arrowhead) and microspore with solid amorphous exine with section through trilete laesura beneath massula opening (arrow); thin black outermost layer is gold coating from SEM study; V68457(1)$1 TEM block and grids. J, Probable coprolite fragment consisting of multiple plant fragments; V68455(1). K, Detail of J showing typical morphology of fragments having the pseudovacuolate structure characteristic of *Salvinia* megaspore wall (cf. E). Scale bars: A, 250 µm; B–D, J, 100 µm; E, 10 µm; F–I, 2 µm; K, 5 µm.
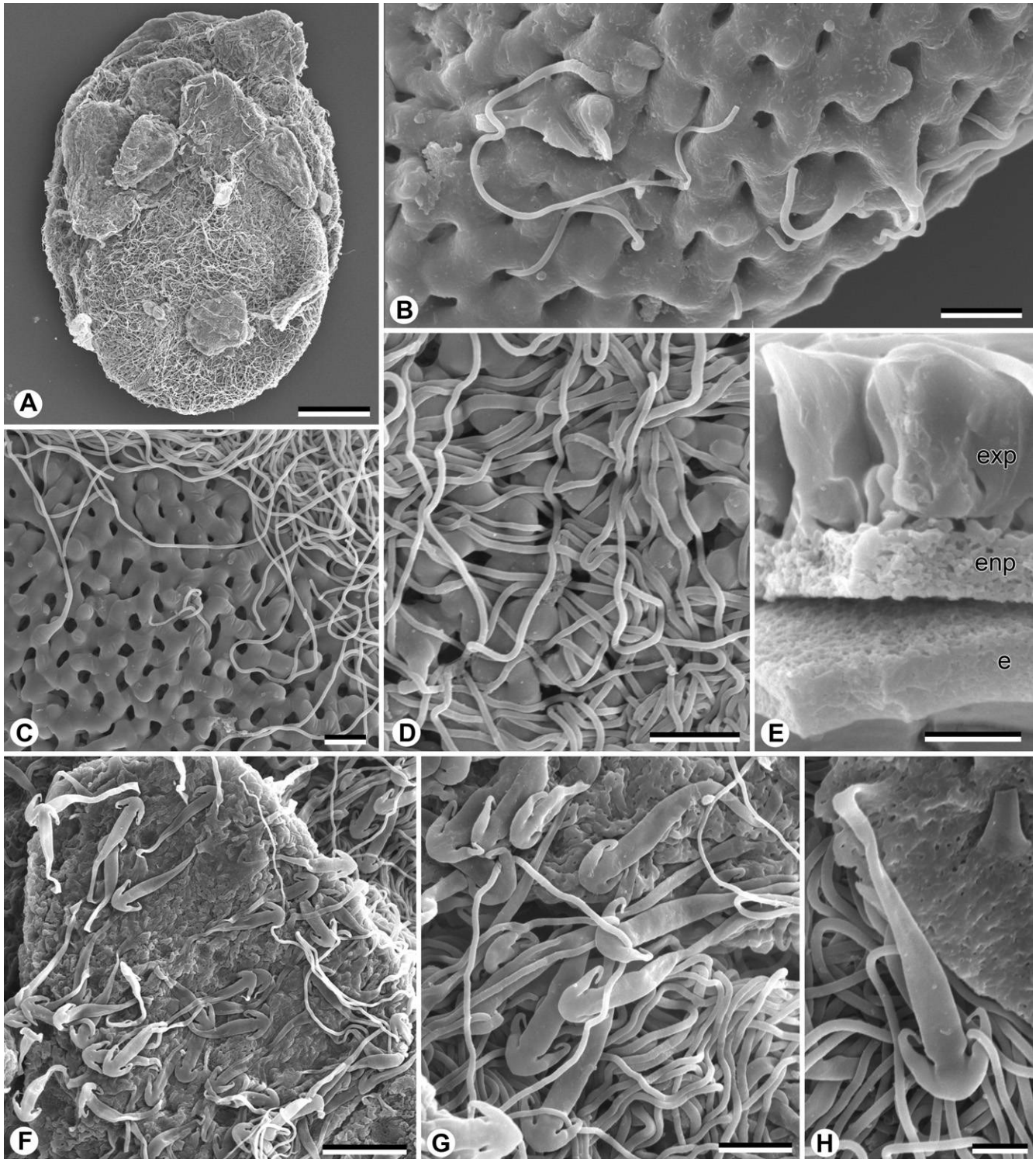
Fig. 2 SEM of *Azolla anglica* from the early PETM, Cobham, United Kingdom. *A,* Megaspore apparatus with numerous attached microspore massulae; V68455(2). *B,* Detail of distal part of another megaspore showing filosum arising from exoperine (infrafilosum) but no cover of suprafilosum; V68455(3)$1 now in TEM block. *C,* Megaspore exoperine; note reticulate pattern with large lumina to the reticulum and rounded muri sometimes topped with rounded papillae, partial covering of suprafilosum, and scattered infrafilosum arising from surface; V68455(4). *D,* Megaspore surface covered with suprafilosum but not totally obscured; V68455(5). *E,* Fracture through megaspore wall showing exine (e), porous endoperine (enp), and large, solid, clavate masses of exoperine (exp) supported by short pillars; V68455(4). *F,* Microspore massula attached to *A,* with short glochidia and wrinkled massula surface lacking hairs. *G,* Detail of glochidia from *A;* note grapnel tips entwined in suprafilosum elements. *H,* Single glochidium showing lack of hairs on stalk or massula surface, wide stalk base, narrower lower stalk, and wider

of a pollen type similar to *Sparganiaceaepollenites* Thiergart (but differing, e.g., in having an annulus around the pore), and likely to have been produced by Sparganiaceaeae or Typhaceae, was illustrated by SEM and TEM in Smith et al. (2010). This pollen is common in the samples and has been found on surfaces of the *Azolla* megaspore apparatuses, suggesting a close proximity of the pollen-producing plants and the *Azolla* depositional setting. All of these associated fossils characterize a wetland paleoenvironment.

Two other small mesofossils are present in this association and are therefore documented here for completeness. The unidentified objects (fig. 4D, 4E) may be arthropod fragments, and *Burtonella emarginata* Chandler (Chandler 1962) of the Capparadaceae (fig. 4F–4J) is represented by a single seed. *Burtonella emarginata* is previously known from the early Eocene Dorset Pipe Clays at Lake, United Kingdom (Chandler 1962), and the Eocene of the former USSR (Budantsev 2005), so the Cobham *Burtonella* is the oldest record of the species. The seed is campylotropous (fig. 4F), with a single sclerotic outer testa and a thin inner cuticular tegmen (fig. 4F, 4G). The surface of the sclerotesta is ornamented with discontinuous ridges and covered with a thin (partially decomposed?) outer cuticle (fig. 4H). The sclerotesta is one cell thick (fig. 4J), and cell surfaces are micropapillate (fig. 4I). The tegmen has a fine surface ornament of striae, sometimes straight and sometimes swirling in a "fingerprint" pattern. The environment in which the extinct *Burtonella* parent plant lived is not known.

SRXTM of *Azolla* Megaspores

Results are variable (fig. 5). Some specimens are so strongly compressed that, apart from distinguishing the distal position of the internal spore (slit at base of fig. 5A), no other information is visible (fig. 5A). In other specimens, the spore wall and surrounding filosum are visualized as well as patches of pseudovacuolate floats (fig. 5B–5D). In one case the microspores in an attached microspore massula are clearly seen (fig. 5D, arrow). In the specimen of *Azolla jutlandica*, visualized by volume rendering in Avizo (fig. 5F), the cellular structure of the proximal cap (top of image) is seen, as is the filosum covering, visible over the distal part of the megaspore apparatus. The infilling of some parts of the pseudovacuolate floats by pyrite (white in images), which prevented TEM sectioning of the float zone (Collinson et al. 2010), is well visualized in longitudinal section (fig. 5E), and the extent of pyrite presence is clear in transparent volume rendering (fig. 5G). The pseudovacuolate float structure is visible in all planes of section (fig. 5E, 5I, 5K), and an attempt has been made to digitally render the floats (fig. 5H, 5J, 5L). The transparent volume rendering of the megaspore apparatus with the rendered floats (fig. 5H) shows that floats have been separated from the filosum covering that envelops them. Longitudinal sections show two long floats on either side of the midline, with one in the plane of section (fig. 5I),

which is consistent with the interpretation of six large floats in one tier in this species by Collinson et al. (2010). The transverse section of the rendered floats is less successful in demonstrating float number but is consistent with the presence of six floats (fig. 5L).

Discussion

Cobham Lignite PETM Water Ferns

Salvinia cobhamii significance. *Salvinia cobhamii* is the oldest Paleogene *Salvinia* from the Northern Hemisphere. It is also the oldest *Salvinia* documented by LM, SEM, and TEM from reproductive structures. A few records of material assigned to *Salvinia* are known from the Late Cretaceous and Paleocene. Weber (1973) described fertile plants with sporocarps but lacking spores from the Olmos Formation, Late Campanian to Early Maastrichtian (Estrada-Ruiz et al. 2010), of Mexico. Wing et al. (2009, supplementary information, p. 69) illustrated foliage assigned to *Salvinia* from the late Paleocene Cerrejon Formation of Colombia. However, it is difficult to assess the generic attribution fully because there are no records of spores with the Colombian Paleocene *Salvinia* foliage. Nambudiri and Chitaley (1991) described sporocarps, megaspores, and vegetative parts named *Salvinitis* Nambudiri and Chitaley, of which some features resemble *Salvinia*, from the ?latest Cretaceous or earliest Paleogene Deccan Intertrappean Series of India. In addition, Paradkar and Barlinge (1979) described *Salvinia intertrappea* Mahabale based on putative megaspores, microspore massulae, and vegetative parts also occurring in the Deccan Intertrappean cherts. This material was studied by cellulose acetate peel sections and not by SEM or TEM, so it is difficult to compare fully with *Salvinia*.

Salvinia cobhamii occurs at the base of the PETM in the earliest Eocene. General ultrastructural organization of the megaspore wall and microspore massula is the same as that of other species studied by TEM (Kempf 1971; Vanhoorne 1992; Van Bergen et al. 1993; Batten and Collinson 2001; Collinson et al. 2002, fig. 8). However, one aspect of megaspore exoperine ultrastructure (discontinuous or continuous linings to the vacuoles) was considered of potential taxonomic significance at the infrageneric level by Collinson et al. (2002). In this respect, surprisingly, *S. cobhamii* (fig. 1H) is similar to the Neogene *Salvinia intermedia* complex sensu Collinson et al. 2002 (section *Salvinia*) in having mainly discontinuous vacuole linings (although the exoperine of the *S. intermedia* complex has a much more open meshwork of intertwined filaments overall). *Salvinia cobhamii* also has some small foveolae in the outer surface of the exoperine (fig. 1D), a feature typifying members of the *S. intermedia* complex but lacking in *Salvinia cerebrata* Dorofeev ex Nikitin (section Cerebrata). However, in external megaspore morphology, *S. cobhamii* is quite unlike the *S. intermedia* complex; instead, it is similar to Neogene *S. cerebrata* (see

upper stalk with distal dilation and constriction beneath grapnel tip, flukes long and recurved, angled upward; V68455(4). Scale bars: A, 100 μm; B–D, G, 10 μm; E, H, 5 μm; F, 25 μm.
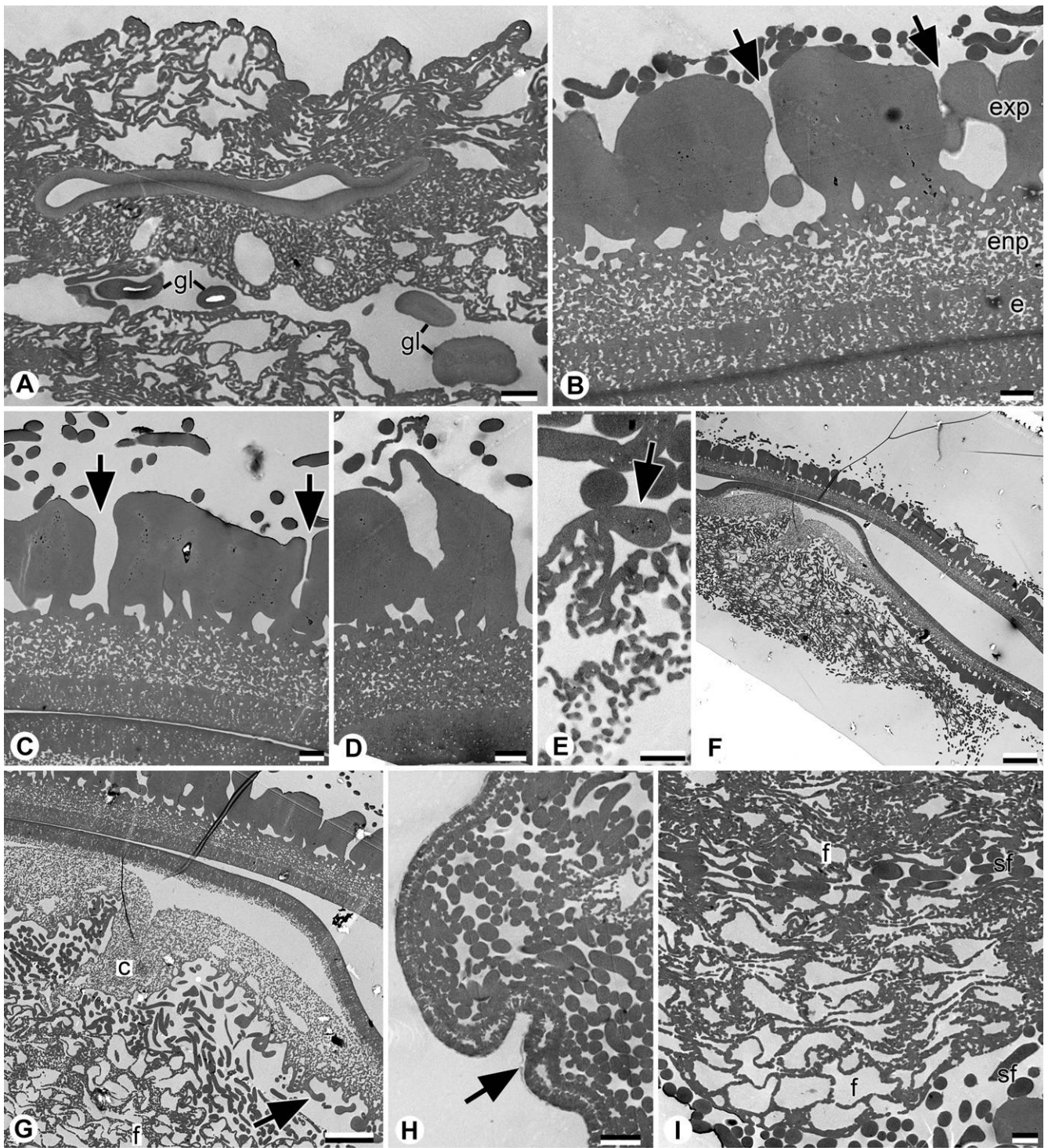
Fig. 3 TEM sections of *Azolla anglica* from the early PETM, Cobham, United Kingdom. *A*, Parts of two microspore massulae that were attached to a megaspore apparatus, showing pseudovacuolate structure and amorphous microspore exine and some sectioned glochidia (gl; white centers to two glochidia sections are artifacts of poor resin penetration); V68462 TEM block and grids. *B–D*, Megaspore wall showing exine (e) with somewhat radially aligned small spaces, porous endoperine (enp) with rugulate to papillate surface, and exoperine (exp) formed of large, broad, solid clavate masses supported on short, narrower pillars, outer surface partly covered by suprafilosum; *B*, *C*, Spaces between clavae (arrows) represent lumina of reticulum seen at surface (as in fig. 2C) and variation in size of clavae. *D*, Origin of infrafilosum hair from exoperine surface. *B*, *D*, V68462 TEM block and grids; *C*, V68455(3)\$1 TEM block and grids. *E*, Detail of origin of suprafilosum element (arrow) from the strongly modified exoperine near the proximal pole among the floats; V68462 TEM block and grids. *F*, Portion of compressed specimen in longitudinal section with gradual modification (right to left in image) of exoperine in the proximal part of the spore underlying the floats; a diminutive
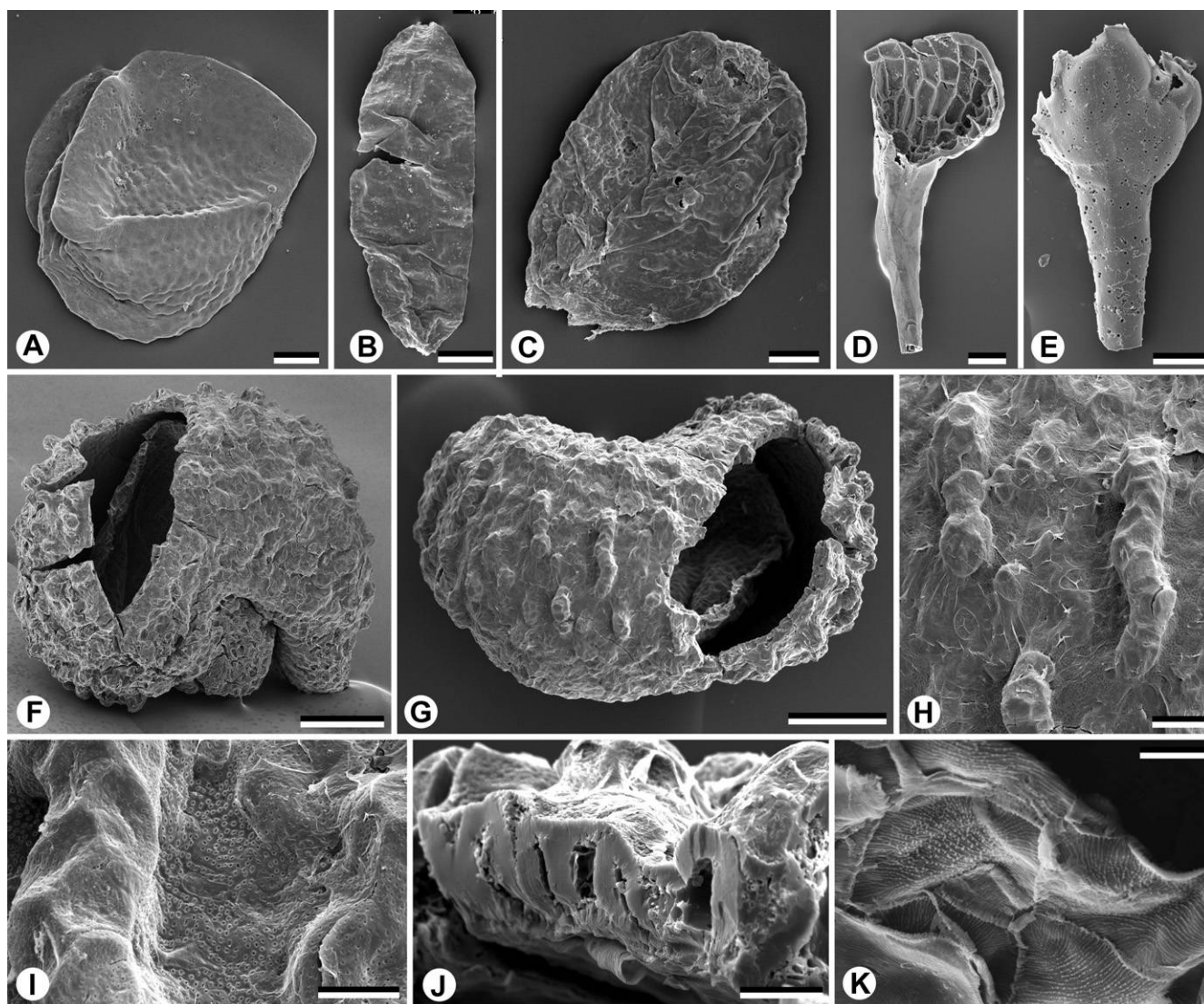
Fig. 4 SEM of various mesofossils from the early PETM, Cobham, United Kingdom. *A, Minerisporites* (isoetalean) megaspore; V68458(1). *B, C,* Seed cuticles. *B,* Possible *Typha* seed; V68459(1). *C,* V68458(2). *D, E,* Unidentified possible arthropod fragments, two specimens showing opposite sides; *D,* V68460(1); *E,* V68461(1). *F–K,* Seed of *Burtonella emarginata* (Capparidaceae); V68461(2). *F, G,* Seed side (*F*) and top (*G*) views; note campylotropus morphology, surface ridges, and inner cuticular tegmen. *H,* Detail of ridged surface with thin, discontinuous outer cuticle. *I,* Detail of ridged surface showing cells with micropapillate surface. *J,* Broken edge of sclerotesta showing single cell thickness and thick-walled cells. *K,* Detail of cuticular tegmen; note striate surface. Scale bars: *A–E,* 100 μm; *F, G,* 250 μm; *H,* 50 μm; *I, J,* 25 μm; *K,* 10 μm.

Collinson et al. 2002) in being more or less equiaxial and in having a flattened apex and a surface ornamented by rounded ridges or folds and tubercles (fig. 1*D*). Details of surface ornament distinguish species in this section, and *S. cerebrata* has narrow, elongate branching ridges and very few tubercles (Collinson et al. 2002), which differentiates it from *S. cobhamii.*

Collinson et al. (2002, p. 271) reported preliminary TEM observations on other *Salvinia* from Paleocene-Eocene transi-

tional strata in the United Kingdom. These are from Felpham and St. Pancras and are also earliest Eocene in age (slightly younger than those from Cobham; Collinson et al. 2003). These megaspores and microspore massulae have continuous vacuole linings (Van Bergen et al. 1993), as do *Salvinia* microspore massulae from Paleocene-Eocene transitional strata in the Netherlands (Batten and Collinson 2001). Therefore, earliest Eocene *S. cobhamii* represents the first occurrence of

collar may be present; V68455(3)$1 TEM block and grids. *G,* Detail of *F* showing the columella (c) part of a pseudovacuolate float (f) and suprafilosum with obvious origin of some elements from modified exoperine (arrow). *H,* Detail of "cap," single cell layer (anticlinal wall at arrow), overlying suprafilosum; V68462 TEM block and grids. *I,* Detail of parts of two adjacent, closely packed pseudovacuolate floats (f) separated by narrow zones of suprafilosum (sf); V68462 TEM block and grids. Scale bars: *A–D, H, I,* 2 μm; *E,* 1 μm; *F,* 20 μm; *G,* 10 μm.
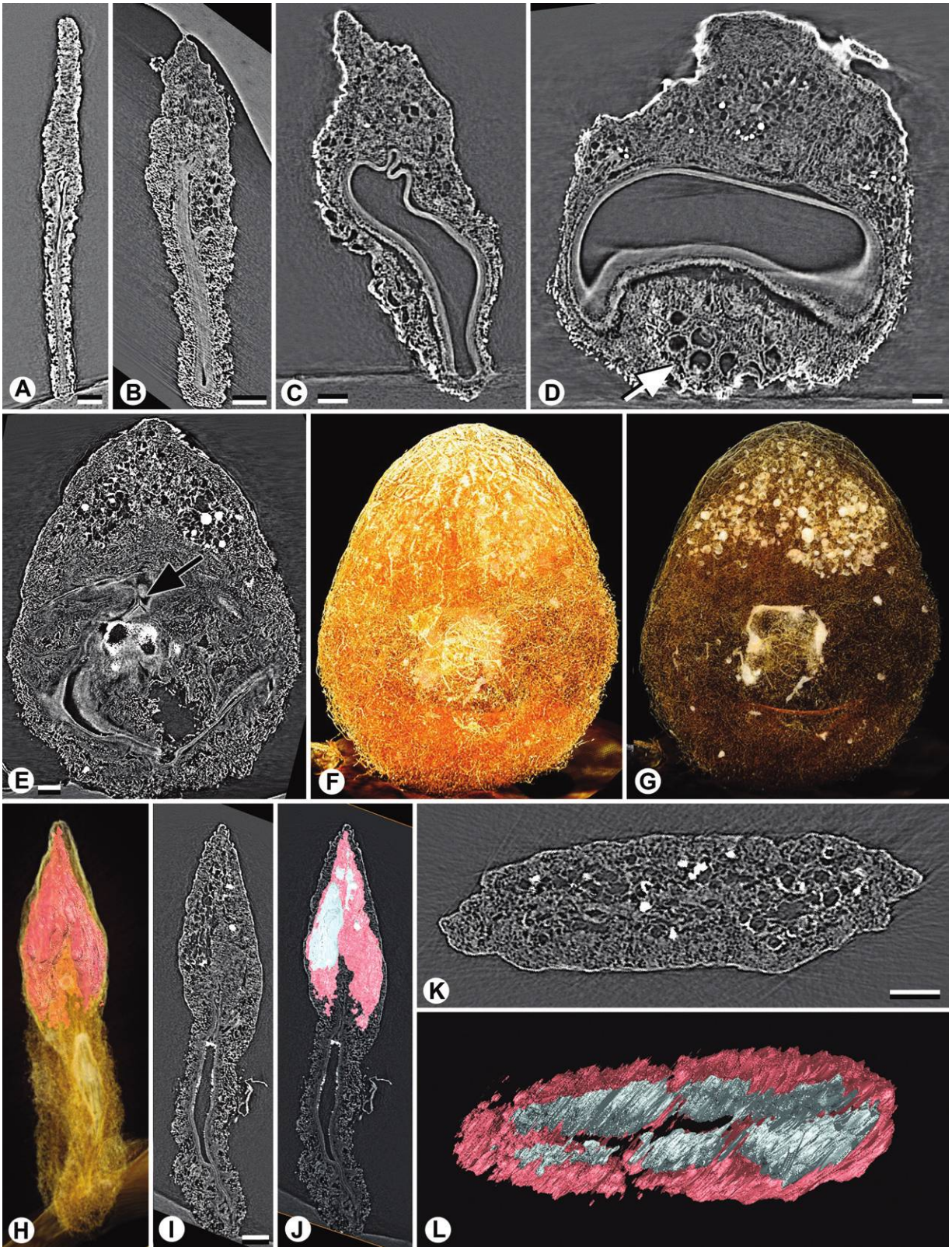
Fig. 5 SRXTM digital sections and renderings of various *Azolla* megaspore apparatuses. *A*, *Azolla primaeva* showing position of spore but no other detail. *B*, *Azolla arctica* (Collinson et al. 2009*a*) showing position of spore, filosum, and open texture of pseudovacuolate floats. *C*, *D*, *Azolla* sp. nov. 2 (Barke et al. 2012), in two longitudinal planes, showing all features seen in *B* and also two distinct layers (exine and perine) in the megaspore wall and a section through an attached microspore massula (arrow) at the base of *D*. *E–L*, *Azolla jutlandica* (Collinson et al. 2010). *E*, Longitudinal
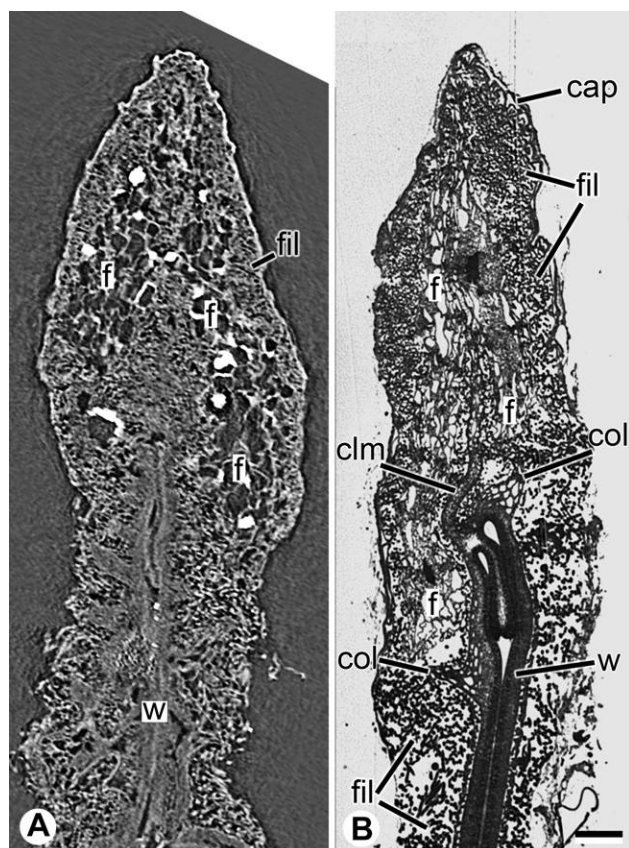
Fig. 6 Digital longitudinal section of *Azolla jutlandica* (*A*) compared with TEM section of *Azolla arctica* (*B*) at same magnification. The same basic structures are present in both species (direct TEM comparison of *A. jutlandica* was not possible; pyrite prevented sectioning). Note detailed structure in TEM not visible in digital section. f = floats, fil = filosum, col = collar, clm = columella, w = megaspore wall. *A*, M. E. Collinson's personal collection; *B*, U22875L, Laboratory of Palaeobotany and Palynology, University of Utrecht, modified from Collinson et al. (2009*a*). Scale bar = 10 μm.

the discontinuous vacuole linings in the fossil record and hence combines some characters that are segregated, and potentially diagnostic at infrageneric level, in Neogene *Salvinia* species based on spores.

The detailed SEM and TEM observations reported here for *S. cobhamii* from the type locality will form the basis for future detailed comparisons of other *Salvinia* from earliest Eocene deposits in the United Kingdom and North America. *Salvinia* was widespread in southern England during the PETM warm-climate interval but is lacking in all other meso-

fossil floras that have been studied in the UK Paleogene (summarized in Collinson and Cleal 2001*a*, 2001*b*, 2001*c*). By contrast, *Azolla* occurs in several UK floras through the Paleocene and Eocene (see below). Therefore, warm climates may have been a key factor favoring the presence of *Salvinia* in the United Kingdom during the PETM. Notably, undescribed *Salvinia* (vegetative remains and associated sori and spores) first occur in the United States in the PETM interval (S. Wing and M. E. Collinson, personal observations, 2012), also possibly suggesting a link with warm climates.

Azolla anglica significance. Martin (1976*a*, 1976*b*) described *A. anglica* as having maniculae (finger- or hooklike processes) as a float-retention mechanism. The same mechanism was judged to be present in *Azolla teschiana* (Florschütz) emend. Batten & Collinson and *Azolla velus* (Dijkstra) Jain & Hall emend. Batten & Collinson (Batten and Collinson 2001), as reported by Martin (1976*b*). Batten and Collinson (2001) used TEM observations of *A. velus* to demonstrate that the hooklike "maniculae" were merely sections through suprafilosal elements at the junction with the perine. The new TEM data on *A. anglica* also show similar sections through suprafilosum elements (fig. 3*E*, 3*G*) and strongly support the conclusion of Batten and Collinson (2001, p. 26) that "maniculae cannot be sustained as a method of float retention" in *Azolla*. The floats in these species, as in many other species, are retained by being enmeshed in suprafilosum that arises from modified exoperine in the collar and columella. New SEM data show that sparse infrafilosum, arising from the exoperine in other areas of the megaspore wall (fig. 2*B*, 2*C*), may assist in float retention when supra- and infrafilosum become intertwined.

Azolla anglica occurred during the warm climates of the early PETM in the southern United Kingdom, while *Azolla jutlandica* is recorded during the warm climates at the end of the Early Eocene climatic optimum (EECO) in sediments from the southern North Sea and Denmark (Collinson et al. 2010; Barke et al. 2012). Four other species of *Azolla* are recorded from the Paleogene in onshore United Kingdom and nearby continental Europe. *Azolla teschiana* occurs in the late Paleocene (Thanet Beds, United Kingdom) and late Paleocene or Paleocene-Eocene transitional strata of the Netherlands and Belgium (Batten and Collinson 2001). *Azolla colwellensis* Collinson and *Azolla prisca* Reid & Chandler emend. Fowler both occur in late Eocene deposits of the Solent Group, United Kingdom (Collinson 1980). *Azolla nikitinii* Dorofeev emend. Friis occurs in the Oligocene of Belgium (Vanhoorne 1992).

The new information on *A. anglica* confirms that it is morphologically distinct from all of these geographically or stratigraphically similar species. Notable distinguishing fea-

section parallel to plane of compaction showing all features seen in *B* and also the trilete laesura on the megaspore (arrow). *F*, Voltex rendering; note cellular pattern in the apical cap and filosum covering distal part of apparatus. *G*, Transparent voltex rendering; note scattered pyrite crystals (white) concentrated in the float zone and a patch of probable microcrystalline pyrite within the spore. *H–J*, Views in plane of compression. *H*, Transparent voltex containing rendering of floats showing that floats have been distinguished from outer filosum during labeling. *I*, Median longitudinal section showing features seen in *B* (spiny dinoflagellate attached at right). *J*, Same section as in *I* with sectioned rendering of floats showing two floats (red), one on either side of midline, one of which (blue gray) is sectioned in this plane. *K*, Transverse section through float zone showing pseudovacuolae of floats. *L*, Rendering of floats (red) cut at the same plane as *K*, indicative of six sectioned floats (blue gray), some poorly distinguished. All specimens from M. E. Collinson's personal collection. Scale bars = 25 μm.

tures include the large megaspore wall excrescences in *A. teschiana*; the nine floats in *A. prisca*; the narrow coalescing rugulae and open spaces in the exoperine of *A. colwellensis*; the megaspore wall excrescences, nodular masses of the exoperine, and hairs on the microspore massula surface in *A. jutlandica*; and the megaspore wall excrescences, mostly laterally continuous exoperine, and nine floats in *A. nikitinii*. Further distinguishing morphological details of all species may be found in the references cited above.

Paleoenvironment and co-occurrence of PETM water ferns. Co-occurrence of *Salvinia* and *Azolla* in the Paleogene and Neogene record is extremely rare. Apart from this new co-occurrence in the earliest Eocene part of the Cobham Lignite Bed, Cobham, Kent, only one other example (early Miocene, Czech Republic) is known to us (Collinson 2002). The presence of both megaspores/megaspore apparatuses and microspore massulae, organically attached in *Azolla* and associated in *Salvinia*, argues strongly for local growth of the parent plants of both species. Increased rainfall during the earliest PETM is indicated by a thick clay band below the blocky lignite, the loss of the fire regime, and the change in lignite lithology (Collinson et al. 2009b). Collectively, the plant mesofossil data for the early PETM indicate a freshwater wetland habitat with open water supporting both *Azolla* and *Salvinia* plants as well as isoetalean plants and marginal herbs, including members of the Typhaceae or Sparganiaceae. Pollen of *Inaperturopollenites* Pflug and Thompson, inferred to have been produced by swamp-dwelling members of the Cupressaceae, also occurs in the blocky lignite (Collinson et al. 2009b). This vegetation was absent during deposition of the organic matter represented by the underlying laminated lignite where the pre-PETM paleoenviroment was characterized by an episodic fire regime and a fern-dominated fire-prone vegetation (Collinson et al. 2009b).

Usefulness of SRXTM

The close spacing of floats and similarity in diameter of enclosing filosum and small float vacuoles, known from TEM sections of other species (fig. 3*I*), probably explains the poor delimitation of float boundaries during labeling such that floats have merged in the rendering. In addition, compression has distorted the floats (the transverse section would probably originally have been almost circular). The SRXTM section (fig. 6*A*) of *A. jutlandica*, compared with the annotated TEM section (fig. 6*B*) of *A. arctica* Collinson et al. (2009*a*), shows that fine details in the collar, columella, and cap are not visualized by SRXTM. Nevertheless, there is clearly potential for visualization of important taxonomical characters in *Azolla*, especially if there are further technological developments in SRXTM.

Turnover at the Cretaceous-Paleogene Transition

Collinson (1991, fig. 7.7) drew attention to the fact that modern genera of the water ferns occurred in the Paleogene, whereas the modern genera and a number of extinct genera occurred in the Late Cretaceous. Our own recent work on megaspore assemblages provides typical examples supporting this pattern, with only the modern genera *Azolla* and *Salvinia*

present during the early Paleogene at Cobham (see above) but only extinct taxa present in the Maastrichtian of Spain (*Parazolla*, *Ghoshispora* Srivastava emend. Dettmann, and *Molaspora*; Batten et al. 2011b) and the mid-Cretaceous of France (*Ariadnaesporites* Potonié emend. Tschudy and *Molaspora*; Batten et al. 2010).

A large amount of other recent work on megaspore assemblages also supports this pattern. Lupia (2011) reported *Ariadnaesporites* and *Molaspora* from the Santonian of Georgia but no modern genera of heterosporous ferns. He considered Cretaceous *Balmeisporites* Cookson & Dettmann emend. Dettmann as "having close affinity to" Salviniales, providing another record of an extinct water fern taxon that occurs only in the Cretaceous (although there is some synonymy of species with those of *Ghoshispora*; Batten et al. 2011b). Lupia (2004) and Nowak and Lupia (2004) also reported only extinct genera of heterosporous ferns (e.g., *Arcellites* Miner emend. Ellis & Tschudy) in the Lower Cretaceous of Maryland. *Ghoshispora* and *Parazolla* (Batten et al. 2011b) and *Glomerisporites* Potonié (Batten et al. 1998) are all still restricted to the Cretaceous, as is the relatively new genus *Hallisporites* Nowak & Lupia (formerly a species of *Ariadnaesporites*; Nowak and Lupia 2005). *Azollopsis* Hall emend. Sweet & Hills ranges in age from late Cretaceous to Paleocene in Canada, the United States, and Argentina (Sweet and Hills 1974; Volkheimer et al. 2007; Scafati et al. 2009). *Palaeoazolla* Archangelsky, Phipps, Taylor & Taylor is another extinct genus restricted to the Cretaceous (Archangelsky et al. 1999).

Ariadnaesporites, with multiple species, was widespread in the Cretaceous (Collinson 1991; Batten et al. 1994; Eisawi and Schrank 2008; El Beialy et al. 2009; Batten et al. 2010; Lupia 2011), including during the latest Cretaceous in India (Samant and Mohabey 2009; references cited in Singh et al. 2011). According to work by Kovach and Batten (1989, 1993), a single species extends into the Paleocene, although this record is doubtful. *Molaspora* was widespread in and typical of the Cretaceous. However, four specimens (three from a mine shaft and one from a borehole) from Belgium are said to be Paleogene (Batten and Collinson 2001, table 1), and these are the only potential Paleogene examples. All of these Paleogene records are doubtful. It is not possible to check the single specimen from the borehole because the slide cannot be located (Batten and Collinson 2001, table 1). The Beringen mine shaft material has been previously dated to latest Paleocene or Paleocene-Eocene transition (see discussion in Batten and Collinson 2001), but there is no evidence (i.e., there are no other associated spores) to support or refute that dating. Batten et al. (2011*a*) thought it most likely that these were *Regnellidium* spores, but because only three specimens existed, none was sectioned to confirm this. As a result, all four of these *Molaspora* specimens suggested to be Paleogene have to be treated as unconfirmed records.

Therefore, with the exception of *Azollopsis* and the possible exception of single isolated records (one each of *Molaspora* and *Ariadnaesporites*), fertile material of all extinct taxa of water ferns is absent from the Paleogene. Modern genera first appear in the Cretaceous (Collinson 1991, 2001; Vajda and McLoughlin 2005), although Cretaceous *Salvinia* requires further substantiation from megaspores and microspore massulae, which are currently lacking. The modern

genera are characteristic of the Paleogene onward. This represents a major biotic turnover in water ferns across the Cretaceous-Paleogene transition.

Paleoenvironments and Freshwater Ocean Surfaces

Modern Salviniaceae (*Azolla* and *Salvinia*) are free-floating aquatic plants living on the surface of fresh waters. Recent experimental work on *Azolla* by M. van Kempen, reported in Barke et al. (2012), has shown that it does not survive above 5 psu. This supports earlier work (reviewed in Brinkhuis et al. 2006) and demonstrates that, even when artificially conditioned, *Azolla* is intolerant of all except very low salinity. *Salvinia* was shown experimentally to grow only slowly above NaCl salinity of 50 mM (~3 ppt; Jampeetong and Brix 2009). Biber (2008) reported that *Salvinia* only showed significant new growth in 0 ppt salinity, was able to maintain itself in 5 ppt, and at 10 ppt exhibited severe stress and loss of color, turgor, and tissue viability after 10 d.

Modern Marsileaceae are rhizomatous plants rooted in substrates of shallow waters and whose leaf petioles extend to the water surface on which the laminae (*Marsilea* and *Regnellidium*) may rest. *Marsilea* and *Pilularia* are typical plants of seasonal, shallow bodies of water (Keeley and Zedler 1998), whose sporocarps persist during dry phases and germinate after floods, allowing vigorous growth, possibly contributing to the survival of *Marsilea* when other aquatics are killed (Wester et al. 2006). *Regnellidium* grows in shallow waters and in surrounding wetlands with humid soil or mud areas subjected to periodic flooding (Cassanego et al. 2010; Keiling-Rubio et al. 2010).

These characteristics noted above for the living plants can be combined with evidence from facies associations of fossils indicating similar habitats (reviewed in Collinson 2002). Therefore, fossil water fern occurrences can readily be used as an indicator of fresh open waters or wetlands, as is done in most if not all of the literature on fossils cited here.

An important example of this paleoenvironmental application of water ferns comes from the latest Early to earliest Middle Eocene Arctic and Nordic *Azolla* occurrences (Brinkhuis et al. 2006; Collinson et al. 2009*a*, 2010; Barke et al. 2011, 2012). High concentrations of *Azolla* (both megaspore apparatuses and microspore massulae, fully mature and often attached to one another) are found, in the absence of terrestrial mesofossils, in sediments on the Lomonosov Ridge in the Arctic Ocean and in two areas of the Norwegian-Greenland Sea. These are associated with low-salinity-tolerant dinoflagellates and other evidence that provides corroboration of the interpretation that the *Azolla* plants were growing on freshwater "caps" on the ocean surfaces (Brinkhuis et al. 2006; Collinson et al. 2009*a*, 2010; Barke et al. 2011, 2012). Furthermore, *Azolla* also occurs in multiple other contemporaneous localities in the Arctic and Nordic Seas, but in those cases they are associated with dinoflagellates characteristic of normal marine salinity and with terrestrially derived mesofossils (such as wood and cuticle fragments). These occurrences are interpreted as indicating the growth of *Azolla* on extensive surrounding continental wetlands (Barke et al. 2012) with remains subsequently washed into ocean sediments. This short-lived (~1.2 myr) *Azolla* phase is linked to

high precipitation that prevailed in high latitudes as a consequence of the warm climates of the EECO, combined with tectonic situations and sea levels that favored isolation or partial isolation of the ocean basins. This Eocene *Azolla* event is an unexpected and unpredictable consequence of Paleogene warm climates (Barke et al. 2012).

Conclusions

Azolla and *Salvinia* co-occur in the earliest part of the PETM in the blocky lignite at Cobham, Kent, United Kingdom. Co-occurrence of these genera is an extremely rare phenomenon in the fossil record and occurs here in association with other wetland floral elements and in parallel with major environmental shifts including increased rainfall and loss of the earlier episodic fire regime. *Salvinia cobhamii* is the oldest record of *Salvinia* fully documented by crucial details (SEM, TEM) of sori, megaspores, and microspore massulae, and its occurrence at Cobham may be linked to PETM warm climates. Different species of *Azolla* characterize other Eocene warm-climate intervals in the same geographic area, but *Salvinia* is absent from those intervals. The Cobham plant mesofossil assemblage also includes a single specimen that may be a coprolite containing fragments of *Salvinia* megaspores.

SRXTM performed at the TOMCAT beamline, Swiss Light Source, has been applied to *Azolla* in an attempt to digitally render the floats that are otherwise obscured by the filosum. Filosum element diameters and smaller float vacuoles are similar in size (0.3–2.0 μm), and the theoretical pixel size is 0.35 μm (actual pixel size ~1 μm for this scan) with the $\times$ 20 objective, so this task is challenging. Many specimens are too compressed, but some success has been achieved in one species, which holds out good potential for future work.

Recent research on the spore record of water ferns confirms previous work showing a major turnover of water fern genera across the Cretaceous-Paleogene transition with modern genera characterizing the Paleogene and extinct sister taxa (with some modern genera) characterizing the Cretaceous. If extinct genera do extend into the Paleogene, they are restricted to the Paleocene.

Water ferns are excellent indicators of former freshwater wetlands and open waters, and the modern genera are intolerant of salinity above 5 psu. The recently documented example of abundant *Azolla* blooms in the Arctic and Nordic Seas, during ~1.2 myr in the latest Early to earliest Middle Eocene, shows that *Azolla* grew on freshwater ocean surfaces and was widespread in surrounding continental wetlands as a consequence of Eocene warm climates.

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