AZOLLA SPOROPHYTES AND SPORES FROM THE LATE CRETACEOUS AND PALEOCENE OF PATAGONIA, ARGENTINA

Elizabeth J. Hermsen[1,*]
Nathan A. Jud[†]
Facundo De Benedetti[‡]
Maria A. Gandolfo[§]

[*] Paleontological Research Institution, 1259 Trumansburg Road, Ithaca, New York 14850, USA
[†] Department of Biology, William Jewell College, 500 College Hill, Liberty, Missouri 64068, USA
[‡] Museo Paleontológico Egidio Feruglio, CONICET, Trelew, Argentina
[§] L.H. Bailey Hortorium, Plant Biology Section, School of Integrative Plant Science, Cornell University, Ithaca, New York 14853, USA
[1] Author for correspondence; e-mail: hermsen@priweb.org.

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Abstract

Premise of the Research. While *Azolla* has a rich fossil record based on dispersed megaspore apparatuses and microspore massulae, fossil sporophytes are relatively rare. In this contribution, we describe two fossil *Azolla* species represented by both sporophytes and spores from Chubut Province, Patagonia, Argentina: *Azolla coloniensis* De Benedetti & Zamaloa, emend. Hermsen et al., and *A. keuja* Jud et al., sp. nov. *Azolla coloniensis* and *A. keuja* are the first fossil species of *Azolla* to be represented by vegetative structures (i.e., leaves, stems, and roots) from both South America and the Southern Hemisphere.

Methodology. We examined sporophyte material of *A. coloniensis* from the Cañadón del Irupé locality, Upper Cretaceous, La Colonia Formation, and *A. keuja* from the Palacio de los Loros locality PL-2, Paleocene, Salamanca Formation. Spores of *A. keuja* were obtained from a sporophyte specimen and its surrounding rock matrix. Material was studied using standard light microscopy, epifluorescence microscopy, and scanning electron microscopy (SEM). Fossils are held at the Museo Paleontológico Egidio Feruglio, Trelew, Chubut Province, Argentina.

Pivotal Results. Azolla coloniensis produced many-floated megaspore apparatuses and microspore massulae with anchor-tipped glochidia, placing it in the fossil *Azolla* section *Florschuetzia*. *Azolla keuja* sporophytes are structurally similar to those produced by the extant African species *A. nilotica* and the Late Cretaceous–Paleocene North American species *A. schopfii* in overall size, growth form, leaf structure, and production of fascicled roots; while all three taxa produce similar microspore massulae, the structure of their megaspore apparatuses differ. *Azolla keuja* cannot be assigned to any section of *Azolla*.

Conclusions. Azolla coloniensis and *A. keuja* are important because they provide two new organismal concepts for extinct species of *Azolla*. Our inability to fully classify *A. keuja* to

section, in combination with the great morphological diversity of fossil *Azolla*, indicates that a comprehensive reevaluation of phylogeny and taxonomy that incorporates both extant and fossil species is needed.

Introduction

Azolla Lam. includes about six to seven extant species of floating aquatic ferns that grow in tropical and temperate climates (Tryon and Tryon 1982; Schneller 1990; Saunders and Fowler 1992, 1993; Reid et al. 2006; Metzgar et al. 2007). The sporophytes of *Azolla* are highly simplified and often very small; they consist of horizontally growing stems that branch profusely and bear alternate, imbricate, simple leaves in two rows (Fig. 1A; Strasburger 1873; Schneller 1990; Saunders and Fowler 1992). The leaves are divided into two lobes, a typically inconspicuous ventral lobe and a green dorsal lobe containing a cavity that houses nitrogen-fixing cyanobacterial symbionts (Fig. 1B; Strasburger 1873; Peters et al. 1978; Lumpkin and Plucknett 1980; Carrapiço 2010). The roots are adventitious, unbranched, and typically hang free in the water column (Fig. 1A; Strasburger 1873; Lumpkin and Plucknett 1980; Schneller 1990; Lumpkin 1993). *Azolla* is heterosporous. Sporangia are enclosed in homosporangiate, indusiate sori, which are sometimes called sporocarps (Fig. 1A, B; Nagalingum et al. 2006); each megasporangiate sorus contains one megasporangium that produces a single functional megaspore, whereas each microsporangiate sorus has multiple microsporangia that in turn produce multiple microspores (Nagalingum et al. 2006). Each megaspore is part of a complex structure called a megaspore apparatus, which includes a megaspore proper and a proximal column to which a series of floats are attached (Mettenius 1847; Strasburger 1873; Martin 1976;

Fowler and Stennett-Willson 1978; Nagalingum et al. 2006). The microspores occur in groups surrounded by a common matrix that is typically covered with glochidia (hairs); these units are known as microspore massulae (e.g., Mettenius 1847; Strasburger 1873; Tryon and Tryon 1982; Saunders and Fowler 1992).

Molecular phylogenetic studies of the extant species of *Azolla* support the division of the genus into two monophyletic sister groups that correspond to the traditionally recognized sections *Azolla* and *Rhizosperma* (Reid et al. 2006; Metzgar et al. 2007; see also a recent cluster analysis of data from morphology and molecular markers by Pereira et al. 2011). Section *Azolla* includes the New World species *A. caroliniana, A. filiculoides*, and the *A. mexicana*-*A. microphylla* complex, as well as the Asian to Australasian species *A. rubra* (Reid et al. 2006; Metzgar et al. 2007). Section *Rhizosperma* comprises the widespread *A. pinnata* species group and the African species *A. nilotica* (Reid et al. 2006; Metzgar et al. 2007). These sections can be neatly separated based on the characteristics of their spores. Section *Azolla* is characterized by megaspore apparatuses with three floats and microspore massulae with anchor-tipped glochidia, whereas *Rhizosperma* is characterized by megaspore apparatuses with nine floats and microspore massulae with either simple glochidia (i.e., glochidia lacking anchor-shaped ends) or no glochidia (Table 1; see also, e.g., Mettenius 1847; Martin 1976; Tryon and Tryon 1982; Metzgar et al. 2007). Sporophyte characters separating the sections include the absence (section *Azolla*) or presence (section *Rhizosperma*) of trichomes on the stem (Saunders and Fowler 1993; Reid et al. 2006; Metzgar et al. 2007; Pereira et al. 2011), as well as differences in sporophyte shape and leaf structure (Pereira et al. 2011).

The fossil record of *Azolla* begins in the Late Cretaceous and includes more than 50 fossil species, most of which are represented by dispersed spores (Collinson 1980; Kovach and Batten

1989; Batten and Kovach 1990; Vajda and McLoughlin 2005). These fossils suggest that the genus was widely distributed in the past and was sometimes abundant even at very high latitudes (e.g., Hall 1977; Askin 1989, 1990; Collinson 2001; Brinkhuis et al. 2006; Collinson et al. 2009; van der Burgh et al. 2013). Because extinct species often do not fit into the taxonomic framework provided by the sections that encompass extant diversity, several sections of *Azolla* have been proposed based on combinations of spore characters observed solely in fossils (Table 1).

Despite *Azolla*'s rich fossil spore record, sporophyte material is relatively rare; there are fewer than 15 species represented by sporophyte macrofossils preserving stems, roots, and/or leaves (Table 2; Fig. 2). Three of these fossil *Azolla* species are described solely from sporophyte material, whereas the remainder are also known from spores (Table 2). Sporophyte records are widespread in the Northern Hemisphere, including occurrences documented from the Indian subcontinent, Siberia, continental Europe, the British Isles, and North America (Fig. 2; Table 2). By contrast, the only fossil species from the Southern Hemisphere for which sporophyte structures are known is *Azolla boliviensis*. The specimens of this Cretaceous to Paleocene species include megasporangiate sori (indusia and megasporangia) and microsporangia, but no vegetative organs have been found (Vajda and McLoughlin 2005).

The goal of this contribution is to present two extinct *Azolla* species from Chubut Province, Patagonia, Argentina, that are known from both spore and sporophyte specimens, including vegetative and reproductive structures found in organic connection. These are among the southernmost known occurrences of modern and fossil *Azolla* and the only fossil *Azolla* sporophytes retaining vegetative structures from the Southern Hemisphere. They also add to the growing body of research documenting fossil heterosporous water fern (salvinialean) sporophytes from South America. Other reports include marsileaceous ferns from the Early Cretaceous of Colombia (Monje-Dussán et al. 2016) and the Late Cretaceous of Argentina (Cúneo et al. 2013, 2014; Gandolfo et al. 2014; Hermsen et al. 2014; Puebla et al. 2015; Vallati et al. 2017), as well as *Salvinia* (Saliviniaceae) from the Paleogene of Colombia (Pérez-Consuegra et al. 2017).

Materials and Methods

Geographic and Stratigraphic Context

The *Azolla* fossils described below were collected from the Maastrichtian (~72–66 Ma, Upper Cretaceous) La Colonia Formation and the Danian (~66–61 Ma, early Paleocene) Salamanca Formation. Both crop out in Chubut Province, Patagonia, Argentina (Fig. 2).

Plant macrofossils and associated microfossils (algae, spores, and pollen) are known from the Cañadón del Irupé, Quebrada del Helecho, and Cerro Bosta localities of the La Colonia Formation (Gandolfo and Cúneo 2005; Cúneo et al. 2013, 2014; Gallego et al. 2014; Gandolfo et al. 2014; Hermsen et al. 2014; De Benedetti et al. 2018). Although *Azolla* spores have been recovered from all of these localities (Cúneo et al. 2014; Gandolfo et al. 2014; De Benedetti et al. 2018), *Azolla* sporophyte material has been found only at the Cañadón del Irupé locality (Cúneo et al. 2014; Gandolfo et al. 2014). The La Colonia Formation is thought to be largely Campanian to Maastrichtian in age (but may also include sediments that are younger and older near the top and bottom, respectively) based on several biostratigraphic markers such as palynomorphs, foraminifera, marine invertebrates, and dinosaurs (see Pascual et al. 2000, as well as citations therein). The horizons yielding the macrofossil flora are considered to be Maastrichtian (N.R. Cúneo, pers. com.); they are flat, laminated mudstones interpreted as having been deposited in stagnant waterbodies, possibly coastal lagoons (Pascual et al. 2000; Cúneo et al. 2014). The aquatic portion of the fossil flora includes algae (e.g., *Pediastrum*, *Spirogyra*), aquatic ferns (*Azolla*, *Paleoazolla*, and several species of Marsileaceae), monocots (Typhaceae and a floating aroid), and lotus (*Nelumbo*), indicating a low-energy, shallow, freshwater environment (Cúneo et al. 2014).

Fossils were also collected from the Palacio de los Loros-2 locality (PL-2) of the Salamanca Formation in southwestern Chubut Province (Fig. 2; Iglesias et al. 2007; Clyde et al. 2014; Comer et al. 2015). The horizon yielding plant fossils at PL-2 comprises flat laminated to thin-bedded mudstone deposits in tidally-influenced fluvial swale-fill channels (Comer et al. 2015). The age assignment of these deposits is constrained to the early Danian using the recently revised chronostratigraphic framework for the Salamanca Formation in the study area, which is based on age-diagnostic foraminifera, calcareous nanofossils, dinoflagellate cysts, paleomagnetic data, and radiometric dates from units stratigraphically lower and higher than the fossil-bearing layers (Clyde et al. 2014; Comer et al. 2015). Other elements of the PL-2 assemblage described so far include araucariaceous and podocarpaceous conifers (Escapa et al. 2018; Andruchow-Colombo et al. 2019) and angiosperm reproductive structures assigned to the families Cunoniaceae and Menispermaceae (Jud et al. 2018a, b).

Fossil Specimen Curation and Preparation

The fossils are housed in the paleobotanical and palynological collections of the Museo Paleontológico Egidio Feruglio (MEF; repository acronyms MPEF-Pb for macrofossil collections and MPEF-PA for palynological collections), Trelew, Chubut Province, Patagonia, Argentina. Macrophotographs were taken with a Canon EOS Rebel T2i DSLR camera. Photomicrographs captured under standard illumination were taken using a Nikon SMZ1000 stereoscopic microscope with Nikon DS-Fi1 Camera Head and DS-L2 Camera Control Unit. Microsporangiate sori were manually removed from one *A. coloniensis* sporophyte (MPEF-Pb 5060) and examined using a JEOL JSM-6460 scanning electron microscope (SEM) at Aluar S.A., Puerto Madryn, Chubut Province, Argentina.

All illustrated spores of *A. keuja* were recovered from the same sample. Some of them were peeled directly from a single specimen with sporophyte remains; the rock from which peels were taken was later disaggregated to recover more spores. The disaggregated rock was treated with hydrofluoric acid (70%) and hydrochloric acid (30%). The resulting residues were decanted and washed several times, then filtered with 100 μm meshes. Megaspore apparatuses and microspore massulae were picked with needles under a stereomicroscope, cleared with a 10% sodium hypochlorite solution for 2–10 min, stained with safranin, and mounted on slides using glycerin jelly as a mounting medium. Specimens examined using an SEM were not cleared; they were mounted directly on stubs using double-sided tape. Longitudinal sections of spore material were made using a razor blade. Light microscopy (LM) observations were made with a Nikon Eclipse 80i microscope coupled with a Nikon DS-L4 camera at the MEF. SEM observations were made with a JEOL JSM-6460 SEM at Aluar S.A., Puerto Madryn, Chubut Province, Argentina.

Modern Comparative Material

The Patagonian fossils were compared with specimens of modern *Azolla* from the LH Bailey Hortorium Herbarium (BH), Plant Biology Section, School of Integrative Plant Science (SIPS), Cornell University, Ithaca, New York, USA. Fragments of shoots cleared for study were soaked for 48 hours in water with dilute detergent, then for 3–5 days in a 1:1 water and bleach solution. Afterward, the specimens were stained with Toluidine Blue O and transferred to temporary wet-mount slides. Cleared specimens were photographed with an Olympus UC90 high definition digital camera under a Nikon SMZ745 dissecting microscope and an Olympus BX60 compound microscope at the L.H. Bailey Hortorium, Plant Biology Section, SIPS, Cornell University. Other specimens of *Azolla* were photographed using a Nikon D7100 DSLR camera at the University of Florida Herbarium (FLAS), Florida Museum of Natural History, Gainesville, Florida, USA.

Terminology

The term “sporocarp” has been widely used in the literature for the thin-walled structures that surround the sporangia of *Azolla.* The term “microsporocarp” has typically been used for the structure that surrounds multiple microsporangia, each of which contains several microspore massulae; the term “megasporocarp” has been used for the structure that surrounds one megasporangium, which in turn contains one megaspore apparatus (e.g., Foster and Gifford 1974; Schneller 1990; Nagalingum et al. 2006). Nagalingum et al. (2006), however, argued that the term sporocarp as traditionally used suggests equivalence between structures that are not of the same evolutionary or developmental origin in Marsileaceae and Salviniaceae within water ferns (Salviniales). In Salviniaceae (*Azolla* and *Salvinia*) the structure called a “sporocarp” is equivalent to a single indusiate sorus (Foster and Gifford 1974; Nagalingum et al. 2006). Herein, we thus use the terms “microsporangiate sorus” and “megasporangiate sorus” for the sporangium-enclosing structures of *Azolla* (see fig. 1D in Nagalingum et al. 2006), as these terms are more precise.

Terminology applied to the megaspore apparatus and microspore massula varies, and in some cases is defined inconsistently; terminology in this paper follows the usage in De Benedetti et al. (2018) and references cited therein. Brief definitions of selected terms for reproductive features as used in this paper are given in Appendix 1 for reference.

Results

Order—Salviniales Link

Family—Salviniaceae Martinov

*Genus—*Azolla *Lam.*

*Section—*Florschuetzia *Kempf*

*Species—*Azolla coloniensis *De Benedetti & Zamaloa, emend. Hermsen, Jud & Gandolfo*

Fig. 3A–H

Original species description. *Azolla coloniensis* De Benedetti & Zamaloa in De Benedetti et al. (2018), pgs. 363 and 366, plates I–II, and fig. 15.2 *in* M. Krings, C.J. Harper, N.R. Cúneo, G.W. Rothwell, eds. Transformative paleobotany. Academic Press, London and Oxford, U.K., and San Diego and Cambridge, U.S.A.

Synonyms. *Azolla*-like megaspore, Cúneo et al. (2014), fig. 4A; *Azolla* microspore massula, Cúneo et al. (2014), fig. 4B; *Azolla* sporophytes, Cúneo et al. (2014), fig. 5B, D; *Palaeoazolla* [sic] *patagonica*, Gandolfo et al. (2014), fig. 2a; *Azolla* sp., Gandolfo et al. (2014), fig. 3a, b.

Emended diagnosis. Sporophyte stem with at least one dichotomy, sinuous in course, glabrous, bearing alternately to suboppositely arranged lateral branches; lateral branches bearing upturned, imbricate leaves; each leaf obdeltoid in shape, apex bilobed; adventitious roots solitary. Fertile sporophytes with megasporangiate sori or both mega- and microsporangiate sori. Megasporangiate sori ovoid, borne singly or in groups of up to 3; microsporangiate sori borne in groups of two. Megaspore apparatus ovoid, surface of exoperine reticulate, collar absent, columella dome-shaped, floats 18–21 (or more) in three (to four) tiers, cap absent; exoperine, columella, and floats with hairs (i.e., infrafilosum and suprafilosum present). Microspore massula with anchor-tipped, aseptate glochidia; about 16 massulae per microsporangium, about 4 microspores per massula.

Lectotype designated here. MPEF-PA 80, megaspore apparatus illustrated by De Benedetti et al. (2018), plate I, fig. 1. Held at the Museo Paleontológico Egidio Feruglio, Trelew, Chubut Province, Argentina.

Paratypes. Other specimens of MPEF-PA 80; MPEF-PA 81–86, 92–95.

Sporophyte specimens examined. MPEF-Pb 5060a, b; 5061; 5081; 5082.

Type locality. Cañadón del Irupé locality, Chubut Province, Patagonia, Argentina (see Cúneo et al. 2014; Gandolfo et al. 2014; De Benedetti et al. 2018).

Stratigraphic position and age. Upper Cretaceous La Colonia Formation, macrofossil flora thought to be Maastrichtian in age (see Cúneo et al. 2014).

Description. Sporophytes are small and usually preserved as fragments (Fig. 3A–D). Stems are up to 13.5 mm long, 0.2–0.5 mm in width, and have a slightly sinuous course (Fig. 3A–C). Main stems, which bear either no or few leaves, dichotomize at least once (Fig. 3C) and give rise to alternately to suboppositely arranged leafy lateral branches up to 2.5 mm in length (Fig. 3A–C). Some stems clearly show a central vascular strand (Fig. 3A, B); no stem trichomes were observed. Leaves are alternate and imbricate, each leaf simple, sessile, and obdeltoid in shape with a subtly bilobed, upturned apex (Fig. 3A, B). Leaves are ca. 0.5–1.0 mm long by 0.3–0.4 mm wide and fed by a single vein. No evidence of large root fascicles was found, and roots are interpreted as being borne singly on stems (Fig. 3B, D). Roots are up to 2.2 mm long, show a vascular strand, and do not branch (Fig. 3B, D).

Fertile sporophytes bear megasporangiate sori or both mega- and microsporangiate sori (Fig. 3C–F). Megasporangiate sori are ovoid, ca. 0.4–0.6 mm long by 0.2–0.4 mm wide, and borne on short stalks; they occur singly or in groups of up to three (Fig. 3C, E). Microsporangiate sori are larger and bear multiple microspore massulae; on the single specimen on which they have been observed, they are apparently born in a pair (Fig. 3D, F). The microsporangiate sori found attached to a sporophyte are ca. 0.7–0.9 mm in diameter (Fig. 3D, F, G); individual microsporangia in these sori are about 130–190 μm in diameter (Fig. 3G, H).

The megaspore apparatuses and microspore massulae were described as *Azolla coloniensis* De Benedetti & Zamaloa by De Benedetti et al. (2018) based on dispersed structures.

Remarks. Several types of salviniaceous spores are known from the La Colonia Formation: *Azollopsis*, *Azolla coloniensis*, an unnamed species of *Azolla*, and the monotypic taxon *Paleoazolla patagonica* (Archangelsky et al. 1999; Cúneo et al. 2014; Gandolfo et al. 2014; De Benedetti et al. 2018, 2019a). Of these, two have been recovered from sediments at the Cañadón del Irupé locality: *Azolla coloniensis* (De Benedetti et al. 2018) and *Paleoazolla patagonica* (Fig. 3I). Although no spores were macerated directly from the La Colonia Formation sporophyte specimens, *Paleoazolla* can readily be eliminated as corresponding to the La Colonia *Azolla* sporophytes. According to a recent reinvestigation of *Paleoazolla patagonica* by De Benedetti et al. (2019b), *Paleoazolla* spores occur in heterosporangiate sori. Each heterosporangiate sorus includes an oblong megasporangium associated with a group of several microsporangia (Fig. 3I; De Benedetti et al. 2019b), making it distinct from the ovoid megasporangiate sorus and roughly circular microsporangiate sorus found on the La Colonia Formation *Azolla* sporophytes (Fig. 3E–G).

Furthermore, there is correspondence between the dispersed spores of *Azolla coloniensis* and the sporangia on the La Colonia Formation *Azolla* sporophytes. The megasporangiate sori attached to *Azolla* sporophytes are ovoid in shape (Fig. 3C, E), which corresponds to the shape of the *A. coloniensis* megaspore apparatuses (De Benedetti et al. 2018). The overall size of the megasporangiate sori (0.4–0.6 mm long by 0.2–0.4 mm wide) also corresponds to the size of dispersed *A. coloniensis* megaspore apparatuses (360–520 µm long by 260–360 µm wide; De Benedetti et al. 2018). The *A. coloniensis* microspore massulae are considered to have been produced by the La Colonia Formation *Azolla* sporophytes because dispersed *A. coloniensis* microspore massulae are found attached to, and thus correspond to, *A. coloniensis* megaspore apparatuses (see De Benedetti et al. 2018). The diameter of microsporangia found *in situ* on a sporophyte (up to about 190 μm; Fig. 3D, F, G) is comparable to the diameter of dispersed *A. coloniensis* microsporangia (up to 195 μm; De Benedetti et al. 2018).

De Benedetti et al. (2018) presented the comparative spore morphology and justification for recognizing *A. coloniensis* as a distinct species of *Azolla* on the basis of its spore structure. *Azolla coloniensis* is typified by a megaspore apparatus, MPEF-PA 80 (De Benedetti et al. 2018). This number refers to more than one megaspore apparatus mounted on a single SEM stub; two specimens from MPEF-PA 80 were figured with the original description of *Azolla coloniensis*, although neither was clearly labelled as the holotype. Thus, we have here designated a lectotype to serve as the type for the species.

Azolla coloniensis megaspore apparatuses have 18 to 21 or more floats, and the microspore massulae have anchor-tipped glochidia (De Benedetti et al. 2018). *Azolla coloniensis* clearly falls outside of the circumscriptions of the modern sections *Azolla* and *Rhizosperma* due to the large numbers of floats on its megaspore apparatuses. The presence of anchor-tipped glochidia on its microspore massulae further excludes it from *Rhizosperma*. The combination of multifloated megaspore apparatuses and microspore massulae bearing anchor-tipped glochidia is considered diagnostic for the extinct section *Florschuetzia* (Table 1). Therefore, *Azolla coloniensis* can be referred to this section along with about 12 other extinct species of *Azolla* documented from both megaspore apparatuses and microspore massulae in the Late Cretaceous to Eocene (see De Benedetti et al. 2018, table 15.1; note that *Azolla schopfii*, discussed below, was mistakenly included in that table).

Section—Unknown

Species—Azolla keuja *Jud, De Benedetti, Gandolfo, and Hermsen, sp. nov.*

Figs. 4–7

Specific diagnosis. Larger sporophyte stems straight in course, glabrous, bearing widely spaced leaves and root fascicles. Distal shoots bearing closely spaced, imbricate leaves; each leaf deeply bilobed; dorsal leaf lobe elliptical with dichotomous venation, ventral leaf lobe elliptical with a single midvein, both lobes with a hyaline margin. Fertile sporophytes with megasporangiate sori or both mega- and microsporangiate sori borne laterally in groups of up to four. Megaspore apparatus oval to elliptical, surface of exoperine with tuberculate and rugulate excrescences, collar present; float apparatus thimble-shaped and covered by a persistent cap, inferred to be of six floats in a single tier; exoperine lacking hairs (i.e., infrafilosum absent), floats with hairs (i.e., suprafilosum present). Microspore massula with simple glochidia of spine-like or hair-like morphology; 6 or more massulae per microsporangium, 6–8 or more microspores per massula.

Holotype designated here. MPEF-Pb 9717a, b (Fig. 4A, B). Held at the Museo Paleontológico Egidio Feruglio, Trelew, Chubut Province, Argentina.

Paratypes. MPEF-Pb 9710a, b; 9711; 9712a, b; 9713; 9714; 9715a, b; 9716a, b; 9718a, b; 9720a, b; 9721; 9722a, b, c; 9724.

Spore specimens examined. MPEF-PA 1000–1009.

Type locality. Palacio de los Loros-2 (PL-2), Chubut Province, Argentina (see Clyde et al. 2014; Comer et al. 2015).

Stratigraphic position and age. Salamanca Formation, Chron C28n, early Danian (early Paleocene).

Etymology. The specific epithet keuja comes from the Tehuelche word keuj, meaning before/past.

Description. Azolla keuja sporophytes are preserved as fragments of two different types: 1. Relatively straight, robust stems bearing roots and few leaves, and 2. Leafy stems interpreted as distal shoots. The largest stems of *Azolla keuja* are fragments up to 5.85 cm long and characterized by long internodes, widely spaced dorsal leaves, and ventral root fascicles (Fig. 4C, D); they vary from 0.6–2.4 mm wide with a coalified vascular cylinder 0.1–0.5 mm wide. No trichomes were observed, and stems are interpreted as glabrous. Distal shoots are preserved as unbranched fragments up to 10.6 mm long; these have thinner stems with short internodes and alternate, imbricate leaves occurring at a frequency of 5–7 leaves per linear cm (Fig. 4A, B, E–H). Leaves are sessile, deeply bilobed, and untoothed. Dorsal leaf lobes are elliptical in shape and 1.5–2.5 mm long (measured from the base of the leaf) by 0.9–1.3 mm wide (Fig. 3A, B, E, G, H); ventral leaf lobes are elliptical in shape and 2.0–2.8 mm long and 1.0–1.6 mm wide (Fig. 3B, E, F, G). Lobe apices are obtuse and rounded and have a hyaline margin that is about 0.1 mm wide at the midpoint of the dorsal lobe (Fig. 4H) and 0.2–0.6 mm wide at the midpoint of the ventral lobe (Fig. 4F); the hyaline margin is wider toward the leaf base. Leaf venation is dichotomous. Each leaf trace diverges from the stem stele and bifurcates near the base of a leaf (Fig. 4B), with one vein branch entering the ventral lobe and the other the dorsal lobe. In the dorsal lobe, the vein dichotomizes up to three times, producing two to four freely-ending veins that widen slightly as they terminate just inside the margin (Fig. 4A, B, E, H). In the ventral lobe, the vein is unbranched and runs down the middle of the lobe, terminating near the apex just inside the margin (Fig. 4E, F). Roots are unbranched and borne in fascicles, with 9–23 roots per fascicle (Fig. 4C, D). Fascicles are spaced 5.9–10.3 mm along the stem (Fig. 4C). Roots are 0.2–0.3 mm wide, with a coalified vascular cylinder 0.03–0.06 mm wide (Fig. 4D). Root fragments are up to 38.2 mm long. Root hairs were not observed.

The reproductive structures are borne on distal shoot fragments. Sori occur on short stalks in homosporangiate or heterosporangiate groups of 2–4, and they are associated with leaves (Fig. 5A–D). Megasporangiate sori are 250–410 μm long by 150–340 μm wide, whereas microsporangiate sori are 470–740 μm across.

The megaspore apparatus is oval to elliptical in outline, composed of a megaspore with a float system situated on its proximal pole (Fig. 6A–H). When observed on the sporophyte, the megaspore apparatus is oriented so that the megaspore is at the base of the megasporangiate sorus and the float system is near the apex (Fig. 5A, D). The megaspore apparatus is 245–410 μm long (average=313 μm, n=36) by 147–340 μm wide (average=216 μm, n=36); the float system is 116–195 μm long (average=148 μm, n=21), and the megaspore is 135–260 μm wide (average=198 μm, n=6). The megaspore is spherical to sub-spherical when uncompressed, with a wall composed of an exine and a two-layered perine (Fig. 6R). The megaspore surface is covered by numerous tuberculate and rugulate excrescences of spongy appearance; these are irregularly distributed and sometimes fused, forming conspicuous folds (Figs. 5D; 6A–H). The excrescences are highly variable in shape and size (up to 65 μm in maximum width) and sometimes have a central depression (Fig. 6I). The excrescences are ornamented with granules or verrucae (up to 5 μm in diameter) that usually grade to smaller (up to 1.2 μm diameter) and partially fused granules at their apices, sometimes forming an irregular reticulum (Fig. 6I, J, N). Perinal hairs were not found on the surface of the megaspore; therefore, there is no infrafilosum.

Under light microscopy, the exine appears smooth and the spore body is easily distinguished from the perine (Fig. 6G, H). The perine is thick, up to 60 μm, and is composed of an endoperine and an exoperine, both involved in the formation of the excrescences (Fig. 6I–L, N). In sections under SEM (Fig. 6M, Q–S), the exine (up to 2.3 μm thick) and the exoperine are homogeneous, whereas the endoperine is very variable in thickness and has a vacuolated structure with alveoli up to 8 μm in diameter. The endoperine is more developed in the raised areas of the excrescences (Fig. 6M, Q, S).

The float system occupies the proximal two-fifths to proximal half of the megaspore apparatus. The float apparatus is thimble-shaped and in all specimens analyzed was covered by a cap (Fig. 6A–H). A collar subtends the floats, although it is sometimes poorly defined. The collar originates from the perine in the proximal region of the megaspore (Fig. 6A–H) and appears to be formed by coalescence of the perinal excrescences. The floats have a vacuolated structure (Fig. 6G, H) and are completely covered by the hairs (0.8–1.3 μm diameter) of the suprafilosum (Fig. 6O, P). Some of these perinal hairs appear to be extensions of the small granules on the surface adjacent to the proximal part of the collar. The floats appear to be attached to a columella in the central region of the float zone (Fig. 6G, H). The number of floats is uncertain, although six arranged in one tier can be inferred in some specimens based on impressions visible on the surface of the cap (Fig. 6E–G).

Microspore massulae have been found attached to the megaspore apparatuses (Fig. 7A–D). Microsporangiate sori contain at least twenty microsporangia (Fig. 7E). Microsporangia are 142–250 μm in diameter (average=182 μm, n=22) and contain six or more massulae (Fig. 7E, L).

Massulae also occur in isolation (Fig. 7F–J). Massulae are triangular, elliptical, or irregular in outline and 67–147 µm (average=107 µm, n=28) in their longest dimension (Fig. 7C, F–J). The surface of the microspore massulae has spine-like (up to 3 µm long) to hair-like (up to 5 µm long) glochidia that are sparse and irregularly distributed (Fig. 7C, M, N).

The massulae have a vacuolated structure, with each massula containing 6–8 or more microspores (Fig. 7F–J). The microspores are trilete, 15–30 µm (average=24 µm, n=80) in diameter, and have a psilate to scabrate exine (Fig. 7D, O). The laesurae extend one-third to one-half the diameter of the spore (Fig. 7J, K).

Remarks. Because some *A*. *keuja* spores were lifted directly from the sporophytes, the spores and sporophytes unequivocally belong to the same taxon. Furthermore, the megaspore apparatuses with their distinctive, folded perine were observed on a fertile *A*. *keuja* sporophyte viewed under epifluorescence (Fig. 5D). Megaspore apparatuses of *A. keuja* have an apical cap. Although no floats have been directly observed as part of the apparatus, impressions on the cap suggest the occurrence of six elongated floats (Fig. 6E–G). The number of floats excludes *A. keuja* from all fossil and extant sections of *Azolla* (see Table 1).

Discussion

The two Patagonian species described herein are represented by sporophytes having attached megasporangiate and microsporangiate sori and associated dispersed megaspore apparatuses and microspore massulae. Both species were found in the same general region (Chubut Province, Argentina), on either side of the Cretaceous-Paleogene (K-Pg) boundary.

They are heterosporous ferns assigned to the genus *Azolla* based on sporophytes with simple and imbricate leaves, unbranched roots, and separate mega- and microsporangiate sori; megaspore apparatuses bearing a proximal float system; and microspores grouped into glochidiate massulae. Although it might be tempting to conclude that *A. coloniensis* and *A. keuja* provide evidence that a single lineage of *Azolla* survived the end-Cretaceous mass extinction in Patagonia, the species show significant differences from each other in sporophyte and spore structure (Table 3). These differences suggest that they are not closely related.

The two species may also have been ecologically distinct. Although both were deposited in marginal marine environments, the paleocommunities that they inhabited were very different. *Azolla coloniensis* grew in freshwater lagoons occurring on coastal plains (Cúneo et al, 2014), whereas *A. keuja* was preserved in brackish tidal flats (Clyde et al, 2014; Comer et al., 2015). The La Colonia community is characterized by autochthonous taxa that are salt intolerant, indicating a low-energy, freshwater environment with no seawater influence (Cúneo et al. 2014). These include freshwater green algae (e.g., *Botryococcus*, *Pediastrum*, and *Spirogyra*) and a macrofossil flora rich in aquatic to semi-aquatic macrophytes, such as several types of water ferns (Salviniales), *Nelumbo*, and the extinct floating monocot *Aquaephyllum* (Gandolfo and Cúneo 2005; Cúneo et al. 2013, 2014; Gallego et al. 2014; Gandolfo et al. 2014; Hermsen et al. 2014). The Palacio de los Loros palynoflora includes freshwater algae (the green algae *Pediastrum* and Zygnemataceae) and dinoflagellate cysts (Clyde et al. 2014); the macrofossil flora is dominated by terrestrial angiosperm taxa (Iglesias et al. 2007; Jud et al. 2018a, b). Comer et al. (2015) interpreted the macrofossil flora of the Palacio de los Loros PL-2 locality, which yielded the *A. keuja* sporophyte fragments, as parautochthonous, representing vegetation occurring inland from the site of deposition and carried a short distance downstream.

Comparative sporophyte morphology of Azolla coloniensis

Apart from *A. coloniensis*, sporophyte organs are known for only three other species of *Azolla* that produced multifloated megaspore apparatuses: *A. schopfii* from the Late Cretaceous–Paleocene of North America (Sweet and Chandrasekharam 1973; McIver and Basinger 1993), *A. stanleyi* from the Paleocene of North America (Melchior and Hall 1983; Hoffman and Stockey 1994), and *A. velus* from the Paleocene of North America (McIver and Basinger 1993; however, see Batten and Collinson 2001 regarding the number of floats on the megaspore apparatuses). An additional, unnamed species was also reported from the Wind River Formation of Wyoming, USA (Collinson 1991; Stuart and Erwin 2006). Of these, *A. stanleyi, A. velus*, and the unnamed species also have microspore massulae with anchor-tipped glochidia, i.e., can be placed in section *Florschuetzia* (Jain and Hall 1969; Sweet and Hills 1976; Hoffman and Stockey 1994; McIver and Basinger 1993; Stuart and Erwin 2006). Like sporophytes of *A. coloniensis*, sporophytes of *A. stanleyi* and *A. velus* are relatively small (up to ca. 2.25 cm and 2.1 cm long, respectively) and show alternate branching; figured specimens show that at least some axes have a sinuous or zig-zag course (McIver and Basinger 1993; Hoffman and Stockey 1994). The leaves of *A. coloniensis* and *A. stanleyi* are clearly imbricate; however, *A. stanleyi* differs from *A. coloniensis* in having a deltoid growth form and megasporangiate sori that occur frequently on lateral branches (Hoffman and Stockey 1994). *A. velus* differs from *A. coloniensis* in having relatively long lateral branches and in lacking imbricate leaves (McIver & Basinger 1993). Sporophytes of the unnamed Wind River *Azolla* are much larger than those of *A. coloniensis* (6 cm or more in length), and sori occur throughout the lateral branches (Stuart and Erwin 2006; see also fig. 7.6g in Collinson 1991).

Comparative morphology of Azolla keuja

Most extant species of *Azolla* are small, with sporophytes ranging from less than 1 cm up to ca. 4 cm long (e.g., Sweet and Hills 1971; Saunders and Fowler 1992; Lumpkin 1993); several fossil species are also known from similarly small specimens (Brown 1934; Hills and Gopal 1967; Bůžek et al. 1988; McIver and Basinger 1993; Hoffman and Stockey 1994). The robust nature of *A. keuja* axes suggests that whole sporophytes were much larger than their maximum observed length of 5.8 cm (Fig. 4C). The only extant species with similarly large sporophytes is *A. nilotica* (section *Rhizosperma*), which can be up to 40 cm long (Saunders and Fowler 1992). Other fossil species represented by sporophyte fragments up to 5 cm or more in length include *A. schopfii* from the Late Cretaceous–Paleocene of North America (Sweet and Chandrasekharam 1973), *A. vera* from the Paleogene of Russia (Krystofovich 1952), and an undescribed species from the Eocene Wind River Formation of Wyoming (Stuart and Erwin 2006). Sweet and Chandrasekharam (1973) estimated that whole sporophytes of *A. schopfii* could have reached 15 cm in length, Krystofovich (1952) estimated that *A. vera* sporophytes may have reached 8 to 10 cm long, and the unnamed Wind River *Azolla* species is minimally 6 cm long (Stuart and Erwin 2006).

Azolla keuja is characterized by deeply bilobed leaves with dorsal (upper) and ventral (lower) lobes (Fig. 6E), similar to extant species of *Azolla* (Fig. 1B; Strasburger 1873; Peters et al. 1978; Schneller 1990). In extant species of *Azolla*, the dorsal leaf lobe is chlorophyllous and has a single vein that encircles a cavity harboring the cyanobacterial symbiont *Nostoc* (or *Anabaena*) *azollae* (Fig. 1B; Strasburger 1873; Demalsy 1953; Peters et al. 1978; Lumpkin and Plucknett 1980; Eily et al. 2019). The ventral leaf lobe is typically thinner than the dorsal lobe, has a rounded and obtuse apex, and is achlorophyllous except near its base (Lumpkin and Plucknett 1980; Schneller 1990; Saunders and Fowler 1992). In *A. nilotica*, however, the ventral leaf lobe is more robust, has an acuminate apex, a central chlorophyllous zone, and a broad hyaline (i.e., achlorophyllous) margin (Fig. 1B; Demalsy 1953; Saunders and Fowler 1992, 1993). *Azolla keuja* differs from all living species in that the venation of its dorsal leaf lobe is dichotomous (Fig. 4A, B, E, G, H), as also occurs in the fossil species *A. schopfii* (Sweet and Chandrasekharam 1973, see esp. their fig. 6). The ventral leaf lobe in both *A. keuja* (Fig. 4E–G, Fig. 5C) and *A. schopfii* (Sweet and Chandrasekharam 1973; table 2 in Saunders and Fowler 1993) is structurally similar to the ventral leaf lobe in *A. nilotica* (Fig. 1B). Saunders and Fowler (1992, 1993) suggested that the unusually robust, chlorophyllous ventral leaf lobe of *A. nilotica* is linked to the relatively ascendant form of its sporophyte, which exposes the ventral leaf lobe to sunlight.

Fascicled roots are documented only in the extant species *A. nilotica* and the fossil species *A. keuja* and *A. schopfii* (Fig. 4C, D; Sweet and Chandrasekharam 1973; Saunders and Fowler 1992, 1993). All other extant *Azolla* have roots borne singly (Fig. 1A; Saunders and Fowler 1992, 1993), as do other fossil species in which this character is documented (e.g., *Azolla primaeva*: Hills and Gopal 1967). Saunders and Fowler (1992) reported that *A. nilotica* plants may have up to 10 roots per fascicle, but we observed many more tangled roots per fascicle on some herbarium sheets (Jud, pers. obs.), and Singh and Singh (1987) reported up to 20. *Azolla* *schopfii* specimens have 8 to 15 roots per fascicle, although sometimes fewer (Sweet and Chandrasekharam 1973). *Azolla keuja* has 9 to 23 roots per fascicle (Fig. 4C, D).

Azolla keuja sporophytes are unusual in the they bear their sori in groups of up to four. Most extant species of *Azolla* produce their sori in pairs, although *A. nilotica* produces them in tetrads (Saunders and Fowler 1992, 1993). Most other fossil species in which this character has been documented have a maximum of two or three sori per group (e.g., *A. stanleyi*; Hoffman and Stockey 1994), although the Miocene taxon *Azolla* aff. *A. ventricosa* also has sori in groups of up to four (Bůžek et al. 1988). (The number of sori per group is unknown in *A. schopfii*.)

Despite the marked morphological similarities in sporophyte structure among *A. keuja*, *A. schopfii*, and *A. nilotica*, the three species have diagnostic differences in the structure of their megaspore apparatuses. The megaspore apparatus of *A. keuja* has a poorly defined collar and a persistent cap that covers the floats (Fig. 6A–H); we infer that at least six floats occur in a single tier based on float impressions seen on the cap (Fig. 6E). The megaspore apparatus of *A. nilotica* has nine floats in two tiers (an apical tier of three floats and a second tier of six floats), a short cap, and a collar (Martin 1976; Saunders and Fowler 1993). The megaspore apparatus of *A. schopfii* has 15 to 26 floats in three tiers, no cap, and no collar (Dijkstra 1961 as *Triletes schopfii*; Sweet and Chandrasekharam 1973; McIver and Basinger 1993; Batten and Collinson, 2001). The structure of the megaspore apparatus in *A. keuja* is unique among fossil and extant *Azolla* both in the number and arrangement of the floats and ornamentation of the spore body.

Azolla keuja has microspore massulae with spinose or hair-like glochidia (Fig. 7C, M, N). Most *Azolla* species have glochidia with anchor-shaped tips on their microspore massulae; those that do not may have coiled, filamentous/hair-like, or spinose glochidia (Table 1; see also, e.g., Collinson 1980; Saunders and Fowler 1992). Extant members of *Azolla* section *Rhizosperma* (*A.*

nilotica and *A. pinnata*) have spinose or twisted glochidia that lack anchor-shaped ends, although glochidia may be absent on massulae of *A. nilotica* (Saunders and Fowler 1992, 1993; Metzgar et al. 2007). Fossil *Azolla* species characterized by microspore massulae lacking anchor-tipped glochidia first appear in the Late Cretaceous (e.g., Hall 1968; Collinson 1980; Vajda and McLoughlin 2005). Some such fossil species (e.g., *A. nana*, *A. turgaica*, *A. ventricosa*) are assigned to section *Rhizosperma* (Dorofeev 1959; Friis 1977). Those having filamentous glochidia with circinate tips (e.g., *A. circinata*) are assigned to the fossil section *Filifera* (Hall 1968). Others, like *A. keuja* and *A. schopfii*, cannot be assigned to a section.

Concluding remarks

Azolla and its sister taxon, *Salvinia*, are the only living genera of the floating aquatic fern family Salviniaceae, which is within Salviniales, a broader group of heterosporous ferns (e.g., Smith et al. 2006; Nagalingum et al. 2008; PPG I 2016). The fossil record of *Azolla*, which begins in the Cretaceous, demonstrates that the lineage including extant *Azolla* diverged from the *Salvinia* lineage no later than the Late Cretaceous (e.g., Pryer 1999) and that *Azolla* was widely distributed by the end of the Cretaceous (Batten and Kovach 1990; Vajda and McLoughlin 2005). Several additional dispersed spore genera from the Late Cretaceous and Paleogene, such as *Ariadnaesporites*, *Azollopsis*, *Glomerisporites*, *Hallisporites*, *Paleoazolla,* and *Parazolla*, may also belong to the *Azolla* stem lineage or elsewhere in the phylogeny of Salviniales (Hall 1969b, 1975; Collinson 1991, 1992, 1996; Batten et al. 1998; Archangelsky et al. 1999; Nowak and Lupia 2005). Unfortunately, the majority of fossil *Azolla* species and species within these putative stem genera are known solely from their spores, and only in exceptional cases are spores linked to sporophyte material. For example, *Parazolla* spores are known to have been produced by the extinct taxon *Hydropteridium pinnatum* (G.W. Rothwell & Stockey) Hermsen & Jud, comb. nov. (see Appendix 2), through discovery of *Parazolla*-type spores in the sporocarps of the *Hydropteridium* plant (Rothwell and Stockey 1994). Phylogenetic analyses suggest that *H. pinnatum* may be a stem taxon to crown Salviniaceae (*Azolla* and *Salvinia*) or crown Salviniales (Rothwell and Stockey 1994; Pryer 1999; Rothwell 1999; Yamada and Kato 2002; Rothwell and Nixon 2006). This case highlights the importance of organismal concepts based on both sporophyte and spore material for understanding the diversity and evolution of the heterosporous aquatic fern clade (see also, e.g., Nagalingum 2007; Collinson 1991, 1996; Hermsen et al. 2014).

The fossil record of *Azolla* now includes 11 species known from vegetative structures (stems, leaves, and/or roots) and spores, and another three known only from sporophyte material (Table 2). The Patagonian species *A. coloniensis* and *A. keuja* are the first fossil records of *Azolla* that represent “whole plants” from the Southern Hemisphere (Fig. 2), and, as such, substantially enrich our knowledge of the past worldwide diversity of genus. Their occurrences on either side of the Cretaceous-Paleogene boundary are congruent with the hypothesis of a major filtering of aquatic fern diversity across the K-Pg boundary, wherein *Azolla* and other extant genera crossed the boundary whereas most fossil spore genera (e.g., *Paleoazolla*) did not (Collinson 2001; Collinson et al. 2013). Nevertheless, the Patagonian *Azolla* are apparently unrelated species that occurred in roughly the same geographic region on opposite sides of the K-Pg boundary. The Late Cretaceous species *Azolla coloniensis* is typical of the archaic Late Cretaceous to Eocene species of *Azolla* in the morphology of its megaspore apparatus, which is many-floated (De Benedetti et al. 2018). It is one of only a few fossil species with many-floated megaspore apparatuses that is also documented from sporophytes preserving vegetative structures. The

Paleocene species *Azolla keuja* has a unique combination of spore and sporophyte character states that make both its classification and affinities difficult ascertain, although it shows some intriguing similarities to the African species *A. nilotica* and the Late Cretaceous to Paleocene North American species *A. schopfii*.

Molecular phylogenetic studies of *Azolla* support a simple model of relationships in which the sections *Azolla* and *Rhizosperma* are monophyletic sister groups defined by several discrete spore and sporophyte characters (Reid et al. 2006; Metzgar et al. 2007). This model does not consider the diversity of fossil species, meaning that it neglects the vast majority of morphological information available pertinent to the evolution of *Azolla*. Although the fossil sections capture some of this additional diversity, some *Azolla* fossils, like *A. keuja*, cannot be accommodated even within this expanded taxonomy. Furthermore, the fossil sections are likely artificial and may not represent monophyletic groups (Batten and Collinson 2001). Few attempts have been made to include fossil species as terminals in phylogenetic analyses (see, however, Saunders and Fowler 1993; Stuart and Erwin 2006). In the future, fossil *Azolla* species and extinct stem taxa, particularly those that are known from both sporophytes and spores, must be incorporated directly into phylogenetic hypotheses so that we can we arrive at a robust understanding of infrageneric relationships, character polarity, and character evolution in *Azolla*.

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Appendix 1

Glossary of selected terminology used in this paper. For the megaspore apparatus and microspore massula, terms and definitions follow usage of De Benedetti et al. (2018) and are adapted from Fowler and Stennett-Willson (1978) as used by, e.g., Collinson (1980) and Batten and Collinson (2001), among others (for full references, see De Benedetti et al. 2018). Terms and definitions for the spore-enclosing structures follow Nagalingum et al. (2006).

Terminology for the megaspore apparatus & megasporangiate sorus

Cap: apical structure that partially or completely covers the floats (see Collinson 1980). The cap is variously interpreted as originating from the columella (Jain 1971; Follieri 1977), the megasporangial wall (e.g., Martin 1976; Fowler and Stennett-Willson 1978; Collinson 1980), or the indusium (Tryon and Tryon 1982).

Collar: elaboration of the peripheral proximal surface of the perine forming a rim that subtends the floats. A collar is not always present.

Columella: a peg-like or cone-shaped extension of the perine located above the trilete laesura from which hairs arise that enmesh the floats.

Float: vacuolate pseudocellular structure attached to the columella or megaspore.

Float system: includes the collar (if present) and floats that are attached to the megaspore either directly or by a columella.

Infrafilosum: perinal hairs borne on the megaspore body below the collar zone.

Megaspore apparatus: includes the megaspore body and its float system ("swimming apparatus") situated on the proximal pole.

Megaspore wall: two-layered wall composed of an internal exine (= megaspore wall proper) and external perine.

Megasporangiate sorus: indusiate sorus that contains one megasporangium.

Megasporocarp = megasporangiate sorus. Term not used in this study (see Materials & Methods).

Suprafilosum: perinal hairs borne on the float system.

Terminology for the microspore massula & microsporangiate sorus

Glochidium: slender projection from the surface of a massula; may have a variety of forms (e.g., spinose, circinate/coiled, with an anchor-shaped apex, barbed, etc.).

Massula: vacuolate pseudocellular matrix that surrounds the microspores.

Microsporangiate sorus: indusiate sorus that contains multiple microsporangia.

Microsporocarp = microsporangiate sorus. Term not used in this study (see Materials & Methods).

Appendix 2

Order—Salviniales Link

Family—Hydropteridiaceae Doweld

Genus—Hydropteridium *Doweld*

Species—Hydropteridium pinnatum *(G.W. Rothwell & Stockey) Hermsen & Jud, comb. nov.*

Basionym—Hydropteris pinnata G.W. Rothwell & Stockey (1994) Amer J Bot 81: 481, figs. 1–41.

Holotype. UAPC-ALTA S36,881; held in the Paleobotanical Collection, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada (Rothwell and Stockey 1994).

Remarks. The genus name *Hydropteris* G.W. Rothwell & Stockey is a later homonym of *Hydropteris* Kondinskaja (Doweld 2001; Aulenback 2009; IFPNI International Editorial Board 2014–2018). Doweld (2001) created the replacement genus name *Hydropteridium* Doweld for *Hydropteris* G.W. Rothwell & Stockey, but never formalized a new combination for the type and sole species. Aulenback (2009) reinterpreted "*Hydropteris*" *pinnata* as a species of the existing genus *Dorfiella*, but also did not properly formalize a new combination. We are here formalizing the new combination *Hydropteridium pinnatum*, which maintains *H. pinnatum* in a distinct genus.

Figure Legends

Fig. 1 Fertile sporophytes of *Azolla*. A, *Azolla filiculoides* showing branches with imbricate leaves, unbranched roots (R), and microsporangiate sori (Mi); FLAS P9007 (Otto Degener 35,788, Hawaii. Image captured July 2013 and used with permission of the University of Florida Herbarium, joint right-of-use and copyright reserved). B, Leaf of *Azolla nilotica* cleared and stained with toluidine blue to show details of the anatomy. The leaf trace diverges from the stem (St) and immediately branches. One branch becomes the midvein of the ventral leaf lobe (VL). The other branch supplies the dorsal lobe (DL), encircling the leaf cavity and sending off an apical spur. Note the hyaline margin (HM) on both the dorsal and ventral leaf lobes as well as a megasporangiate sorus (Me); BH 000199372. Scale bars: A = 5 mm; B = 0.25 mm.

Fig. 2 Distribution map for fossil *Azolla* sporophytes with vegetative structures (roots, stems, and/or leaves) worldwide, Late Cretaceous–Neogene. Numbers on map correspond to numbers in Table 2. Note that some localities occurring in the same geographic region have been combined (e.g., Eocene southern Okanagan Highlands localities in British Columbia, Canada; Eocene Green River Formation localities in Colorado, Utah, and Wyoming, U.S.A., etc.). Point map made using Simplemappr (Shorthouse 2010).

Fig. 3 *Azolla coloniensis* De Benedetti & Zamaloa emend. Hermsen, Jud & Gandolfo and *Paleoazolla patagonica*. A, Sterile sporophyte showing sinuous stem, branching pattern, and leaf arrangement and morphology; MPEF-Pb 5061. B, Stem bearing short, leafy branches and unbranched roots; MPEF-Pb 5081. C, Sporophyte with megasporangiate sorus; MPEF-Pb 5082.

D, Sporophyte with microsporangiate sori; MPEF-Pb 5060. E, Megasporangiate sorus; MPEF-Pb 5060b. F, Microsporangiate sori; detail of specimen shown in D, MPEF-Pb 5060. Note: These microsporangiate sori were later manually removed for examination with an SEM (see G, H). G, Scanning electron photomicrograph of a microsporangiate sorus with individual microsporangia discernable (circular structures), removed from MPEF-Pb 5060 (sorus on the left in F). H, Detail of microsporangiate sorus showing circular microsporangia (arrowheads), removed from MPEF-PB 5060 (from sorus on the right in F). I, Two sori of *Paleoazolla patagonica*, each consisting of one megasporangium (Me) with attached microsporangia (Mi), MPEF-PB 5075 (these sori were later manually removed and examined under SEM). Scale bars: A–D = 2 mm; E, F, I = 1 mm; G = 200 μm; H = 100 μm.

Fig. 4 *Azolla keuja* Jud, De Benedetti, Gandolfo & Hermsen, sp. nov., vegetative structures. A, Distal leafy shoot showing imbricate leaves and dichotomous venation of dorsal leaf lobes; MPEF-Pb 9717a (holotype). B, Distal leafy shoot showing imbricate leaves and dichotomous venation of the dorsal lobes. One leaf clearly shows the bilobed leaf structure; arrow indicates visible portion of the ventral lobe; MPEF-Pb 9717b (holotype). C, Robust stem with root fascicles along ventral side (lower arrow) and leaves attached to dorsal side (upper arrow); MPEF-Pb 9722a (paratype). D, Close-up of the root fascicle attached to the main stem, showing stem stele and diverging fascicle trace; MPEF-Pb 9711 (paratype). E, Distal shoot showing dorsal and ventral leaf lobes; MPEF-Pb 9716a (paratype). F, Close-up of specimen shown in E, detail of ventral leaf lobe with a single midvein; MPEF-Pb 9716a (paratype). G, Distal shoot showing dorsal and ventral leaf lobes; MPEF-Pb 9714a (paratype). H, Close-up of a dorsal leaf lobe showing dichotomous venation and hyaline margin (at arrow); MPEF-Pb 9713 (paratype). Scale bars: A, B = 1.1 mm; C = 6 mm; D–H = 1 mm.

Fig. 5 *Azolla keuja* Jud, De Benedetti, Gandolfo & Hermsen, sp. nov., reproductive structures. A, Microsporangiate and megasporangiate (at arrow) sori attached to a distal stem fragment; MPEF-Pb 9714a (paratype). B, Microsporangiate sorus attached to distal stem fragment; MPEF-Pb 9712b (paratype). C, Shoot apex showing sori among the ventral leaf lobes; MPEF-Pb 9715a (paratype). D, Close-up of reproductive structures under epifluorescence (but not the same structures shown in B). The perinal excrescences of two megasporangiate sori fluoresce brightly in the megasporangiate sori, indicated by the right two arrows; and the larger microsporangiate sorus fluoresces more dimly, indicated by the left arrow; MPEF-Pb 9712a (paratype). Scale bars: A = 0.5 mm; B, C = 1 mm; D = 200 μm.

Fig. 6 *Azolla keuja* Jud, De Benedetti, Gandolfo & Hermsen, sp. nov.; megaspore apparatuses. A–F, Megaspore apparatuses showing the float system covered by the indusial cap and the distal megaspore with perinal excrescences; arrows indicate the collar. A, MPEF-PA 1007. B, MPEF-PA 1008. C, MPEF-PA 1009. D, MPEF-PA 1009. E, Dotted line indicates the individual floats; MPEF-PA 1009. F, MPEF-PA 1009. G–H, Megaspore apparatuses showing the vacuolated structure of the floats, the central columella of the float system, and the distal megaspore. G, Note the thick perine and the columella (arrow); MPEF-PA 1002. H, MPEF-PA 1000. I, Excrescence with a central depression; MPEF-PA 1007. J, Excrescences showing the typical ornamentation pattern with fused granules at the apex; MPEF-PA 1007. K, Zone between the indusial cap and the megaspore, note the different types of excrescences; detail from G, MPEF-

PA 1002. L, Thick perine and rounded appearance of the excrescences; detail from H, MPEF-PA 1000. M, Longitudinal section of a megaspore apparatus; MPEF-PA 1007. N, Excrescence showing the typical ornamentation pattern with fused granules at the apex; MPEF-PA 1008. O, Suprafilosum covering the vacuolated floats; detail from M, MPEF-PA 1007. P, Perinal hairs of the suprafilosum above the collar; detail from B, MPEF-PA 1008. Q, Section through megaspore wall showing the strongly vacuolated structure of the endoperine; detail from M, MPEF-PA 1007. R, Section through the megaspore wall showing the exine, endoperine, and exoperine; detail from M, MPEF-PA 1007. S, Section through the megaspore wall showing the expansion of the endoperine within an excrescence; MPEF-PA 1008. SEM micrographs: A–F, I–J, M–S; LM micrographs: G, H, K, L. Scale bars: A–F, M = 50 μm; G, H = 100 μm; I, P, Q–S = 10 μm; J = 2 μm; N = 5 μm; K, L = 40; O = 20 μm.

Fig. 7 *Azolla keuja* Jud, De Benedetti, Gandolfo & Hermsen, sp. nov., megaspore apparatuses, microsporangia, and microspore massulae. A, Megaspore apparatus with two microspore massulae attached (arrow); MPEF-PA 1007. B, Megaspore apparatus with microspores and microspore massulae attached (arrows); MPEF-PA 1007. C, Triangular microspore massulae showing the spine to hair-like glochidia (left) and the internal vacuolated structure (right) attached to a megapsore apparatus; detail from A, MPEF-PA 1007. D, Psilate to scabrate microspores; detail from B, MPEF-PA 1007. E, Cluster of microspore massulae thought to represent the partial contents of a microsporangiate sorus. Each spherical to ovoid subunit probably represents the contents of one microsporangium; MPEF-PA 1007. F–J, Microspore massulae showing shape, vacuolated structure, and sparse simple (spinose) glochidia. F, Irregularly-shaped massula; MPEF-PA 1004. G, Elliptical massula; MPEF-PA 1003. H, Triangular massula; MPEF-PA 1003. I, Triangular massula; MPEF-PA 1005. J, Irregularly-shaped massula, arrow indicates microspore with trilete mark; MPEF-PA 1003. K, Trilete microspore, MPEF-PA 1004. L, Cluster of microspore massulae interpreted as representing the contents of a microsporangium; MPEF-PA 1007. M–N, Details of the surface of microspore massulae showing irregularly-distributed spinose to hair-like glochidia. M, MPEF-PA 1007. N, MPEF-PA 1007. O, Broken massula showing four psilate to scabrate microspores; detail from E, MPEF-PA 1007. SEM micrographs: A–E, L–O; LM micrographs: F–K. Scale bars: A, B, L = 50 μm; E = 100 μm; C, D, K = 10 μm; N= 2 μm; M, O = 5 μm; F–J = 20 μm.

Table 1

Comparison of sections of *Azolla*

Section	Megaspore apparatus	Glochidia on microspore massula	Age range
Azolla Lam.	3 large floats	Anchor-shaped ends	Late Cretaceous/Paleocene[1]–Recent
Rhizosperma (Meyen) Mett.	9 floats in 2 tiers (3 in upper, 6 in lower)	Spinose or filamentous, may be rare to absent	Oligocene[2]–Recent
Antiqua P.I. Dorof.	9 (or more) floats in 2 tiers (3 in upper, 6–9 in lower); cap present[3]	Unknown	Eocene–Oligocene
Filifera J.W. Hall	Unknown[4]	Filamentous/hair-like with circinate ends	Late Cretaceous
Florschuetzia Kempf[5]	More than 9 floats	Anchor-shaped ends	Late Cretaceous–Eocene
Simplicispora J.W. Hall[6]	Columella float-like or 1 float	Anchor-shaped ends	Late Cretaceous–Eocene
Trisepta K. Fowler	Triseptate columella, 9 floats in 2 tiers (3 in upper, 6 in lower)	Anchor-shaped ends	Eocene[7]

Sources. Dorofeev (1959), Hall (1968), Hall and Swanson (1968), Kempf (1968), Jain and Hall (1969), Hall (1970), Jain (1971), Fowler (1975), and Follieri (1977).

[1] Based on *Azolla indica* and *A. intertrappea* (Sahni 1941; Sahni and Rao 1943; Surange 1966; Trivedi and Verma 1971).

[2] While *Azolla deccaniana* from the Late Cretaceous–Paleocene of India was assigned to section *Rhizosperma* (Nambudiri and Chitaley 1991), its megaspore apparatus is unknown; thus, its assignment should be considered equivocal. Dispersed spores assigned to *Rhizosperma* are known beginning in the Oligocene (Dorofeev 1959, 1963; Friis 1977).

[3] Some sources (Jain 1971; Fowler 1975; Follieri 1977) indicate that this section has 6–9 floats, but that appears to be a misinterpretation of Dorofeev's (1959) description of *A. antiqua*. Section *Antiqua* could be synonymous with section *Rhizosperma* (see Martin 1976) or *Trisepta*.

[4] Fowler (1975) indicated that megaspore apparatuses in this section have more than 9 floats, but other sources (Hall 1968; Hall and Swanson 1968; Jain 1971; Follieri 1977) described the megaspore apparatuses as unknown.

[5] Synonym is *Azolla* section *Kremastospora* R.K. Jain & J.W. Hall (see Martin 1976; Collinson 1980). Both section *Florschuetzia* (originally subgenus *Florschuetzia*) and section *Kremastospora* share the same type, *A. teschiana* Florschütz (Kempf 1968; Jain and Hall 1969).

[6] Collinson (1991) considered this section invalid.

[7] Updated age after Hayes and Collinson (2014).

Table 2

Fossil *Azolla* species with vegetative sporophyte organs (i.e., roots, stems, and/or leaves)

Taxon	Spores[1]	Age	Location (number in Fig. 2)	References
Azolla coloniensis De Benedetti & Zamaloa, emend. Hermsen et al.	+ (me, mi)	Late Cretaceous	Chubut Province, Argentina (1)	Cúneo et al. (2014), Gandolfo et al. (2014), De Benedetti et al. (2018), this paper
Azolla deccaniana Nambudiri & Chitaley	+ (mi only)	Late Cretaceous–Paleocene[2]	Madhya Pradesh, India (2)	Nambudiri & Chitaley (1991)
Azolla indica Trivedi & C.L. Verma	+ (me, mi)	Late Cretaceous–Paleocene[2]	Madhya Pradesh, India (2)	Trivedi & Verma (1971)
Azolla intertrappea Sahni & H.S. Rao	+ (me, mi)	Late Cretaceous–Paleocene[2]	Madhya Pradesh, India (2)	Sahni (1941), Sahni & Rao (1943), Surange (1966), Hall (1969a)
Azolla schopfii Dijkstra	+ (me, mi)	Late Cretaceous–Paleocene[3]	Alberta (3) & Saskatchewan (4), Canada; Montana & South Dakota, USA (not mapped)	Dijkstra (1961), Jain & Hall (1969), Snead (1969), Jain (1971), Sweet & Chandrasekharam (1973), Collinson (1980), McIver & Basinger (1993),

				Batten & Collinson (2001)
Azolla keuja Jud et al.	+ (me, mi)	Paleocene	Chubut Province, Argentina (5)	This paper
Azolla stanleyi R.K. Jain & J.W. Hall	+ (me, mi)	Paleocene	Alberta (3) & Saskatchewan (not mapped), Canada; North Dakota, USA (6)	Jain & Hall (1969), Sweet & Hills (1976), Melchior & Hall (1983), Hoffman & Stockey (1994)
Azolla velus (Dijkstra) R.K. Jain & J.W. Hall	+ (me, mi)	Paleocene	Saskatchewan, Canada (4); Montana & South Dakota, USA (not mapped)	Dijkstra (1961), Jain & Hall (1969), Martin (1976), McIver & Basinger (1993), Batten & Collinson (2001)
Azolla berryi R.W. Br.	–	Eocene	Colorado, Utah, & Wyoming, USA (7)	Brown (1934), Grande (2013), Hermsen (pers. obs., 2013-2014)[4]
Azolla primaeva (Penh.) C.A. Arnold	+ (me, mi)	Eocene	British Columbia, Canada (8, 9)	Penhallow in Dawson (1890), Arnold (1955), Rouse (1962), Hills & Weiner (1965), Hills & Gopal (1967), Dillhoff et al. (2013),

				Greenwood et al. (2016), Mathewes et al. (2016)[5], Collinson et al. (2017)
Azolla prisca E. Reid & M. Chandler	+ (me, mi)	Eocene	England, UK (10)	Reid & Chandler (1926), Fowler (1975), Collinson (1980), Hayes & Collinson (2014)[6]
Azolla vera Kryst.	–[7]	Eocene–Oligocene	Omsk Oblast (11) & Tyumen Oblast (12), Russia	Kryshtofovich (1952), Akhmetiev et al. (2012), Zaporozhets & Akhmetiev (2013)
Azolla tertiaria E.W. Berry	–[7]	Miocene	Nevada, USA (13)	Berry (1927)
Azolla aff. *A. ventricosa* P. Nikitin *sensu* P.I. Dorof.	+ (me, mi)	Miocene	Ústecký, Czech Republic (14)	Bůžek et al. (1988)

Note. Localities from which only spores are documented are not mapped in Figure 2.

[1] Key: + = spores found on fertile sporophytes, or associated spores considered to be conspecific with fertile sporophytes (me = megaspore apparatuses; mi = microspores/microspore massulae); – = spores unknown.

[2] Age based on information in Smith et al. (2015).

[3] *Azolla schopfii* is known to occur in the Paleocene, although opinions vary about whether it also occurs in the Maastrichtian due to differing stratigraphic interpretations (see Batten and Collinson 2001).

[4] Utah occurrences observed: Denver Museum of Nature and Science, Denver, Colorado, USA (DMNH EPI.27589, Loc. 323); Florida Museum of Natural History, Gainesville, Florida, USA (UF 15755-23024, UF 15755-23025); Field Museum of Natural History, Chicago, Illinois, USA (PP 16743). Wyoming occurrence figured in Grande (2013) as *Azolla*, probably *A. berryi* since it occurs in the Green River Formation.

[5] Identified only as *Azolla*, but probably *A. primaeva* given locality.

[6] Identified only as *Azolla*, but probably *A. prisca* given locality.

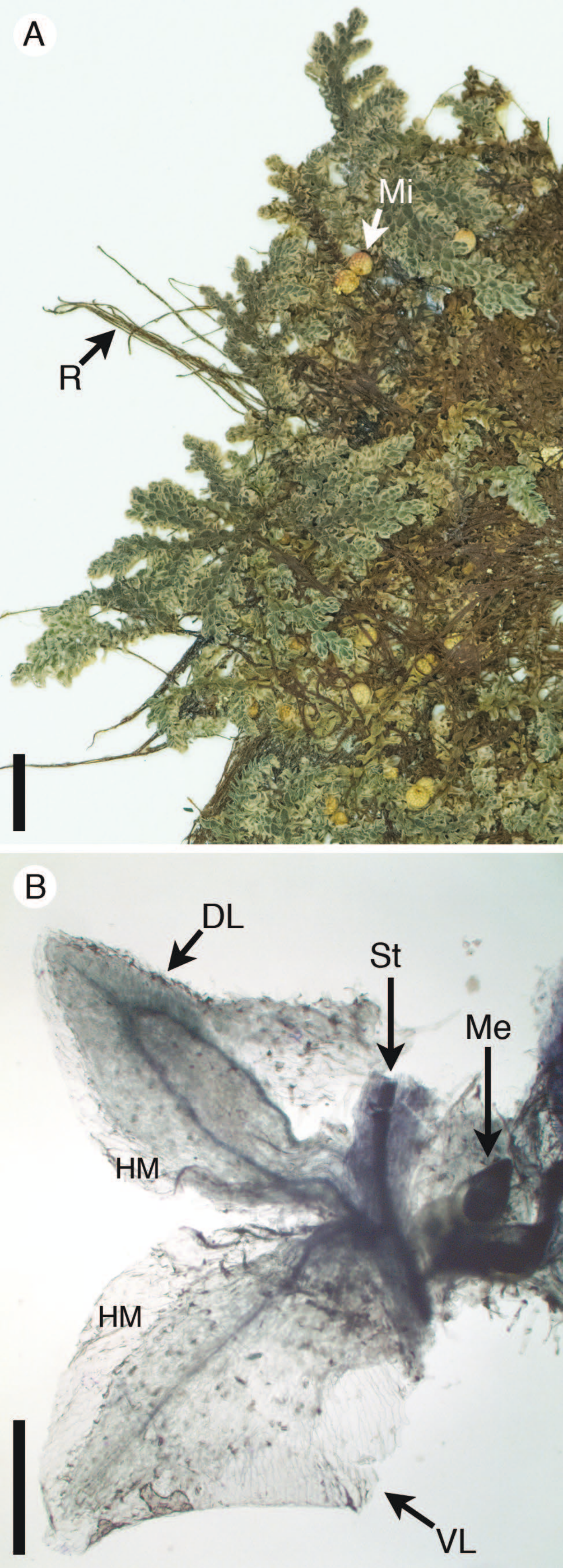
[7] Although fertile sporophyte material is known, spores have not been described.

Table 3

Comparison of morphological features of *Azolla coloniensis* and *A. keuja*

Character description	*Azolla coloniensis*	*Azolla keuja*
Sporophyte		
Longer/main stems	slightly sinuous	straight
Leaf shape	obdeltoid, apex bilobed	deeply bilobed, lobes elliptical
Root arrangement	solitary, not in fascicles	in fascicles of 9–23
Sori per group	1–3	2–4
Megaspore apparatus		
Shape	ovoid	oval to elliptical
Exoperine sculpture	reticulate	with tuberculate and rugulate excrescences
Collar	absent	present
Floats	18–21 (or more) in 3–4 tiers	6 in 1 tier?
Cap	absent	present, covers floats
Infrafilosum	present	absent
Microspore massula		
Massulae/microsporangium	~16	6 or more
Microspores per massula	~4	6–8 or more
Glochidia	anchor-shaped ends	hair-like or spine-like

Sources. De Benedetti et al. (2018), this paper.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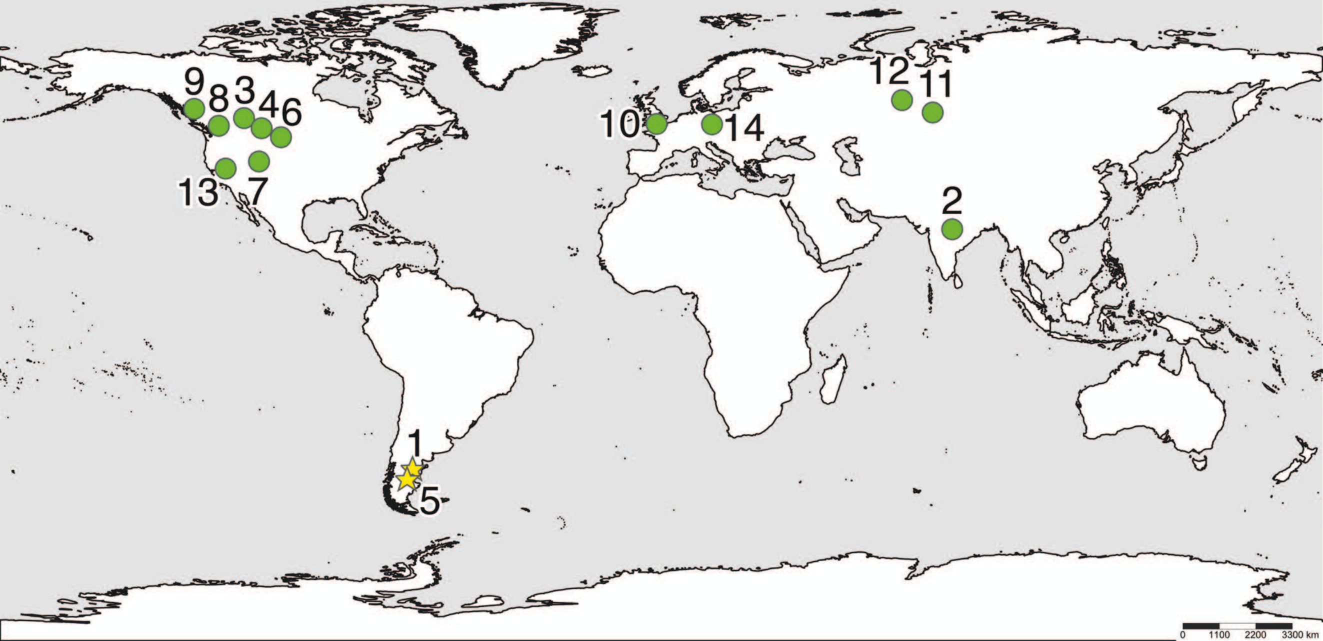
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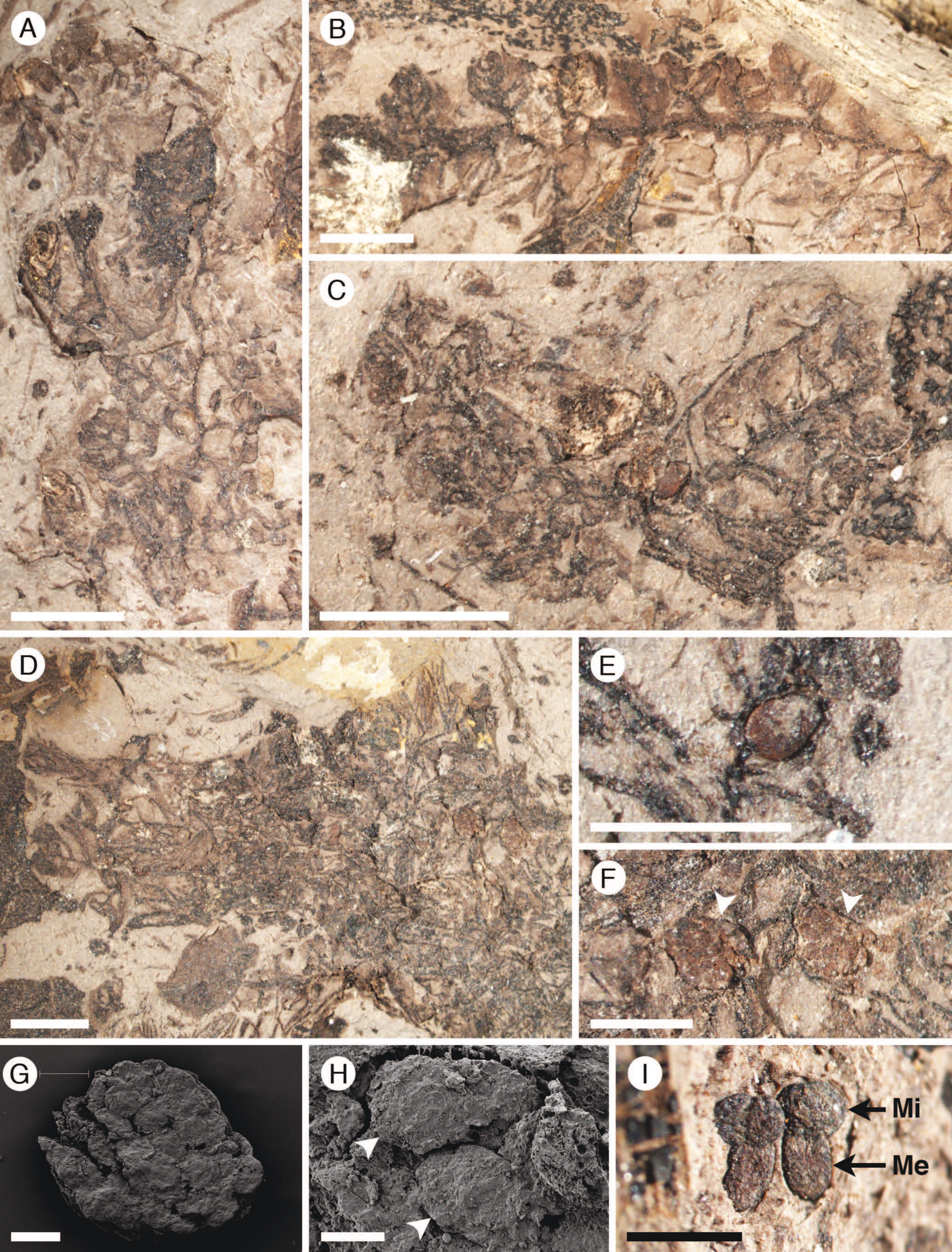
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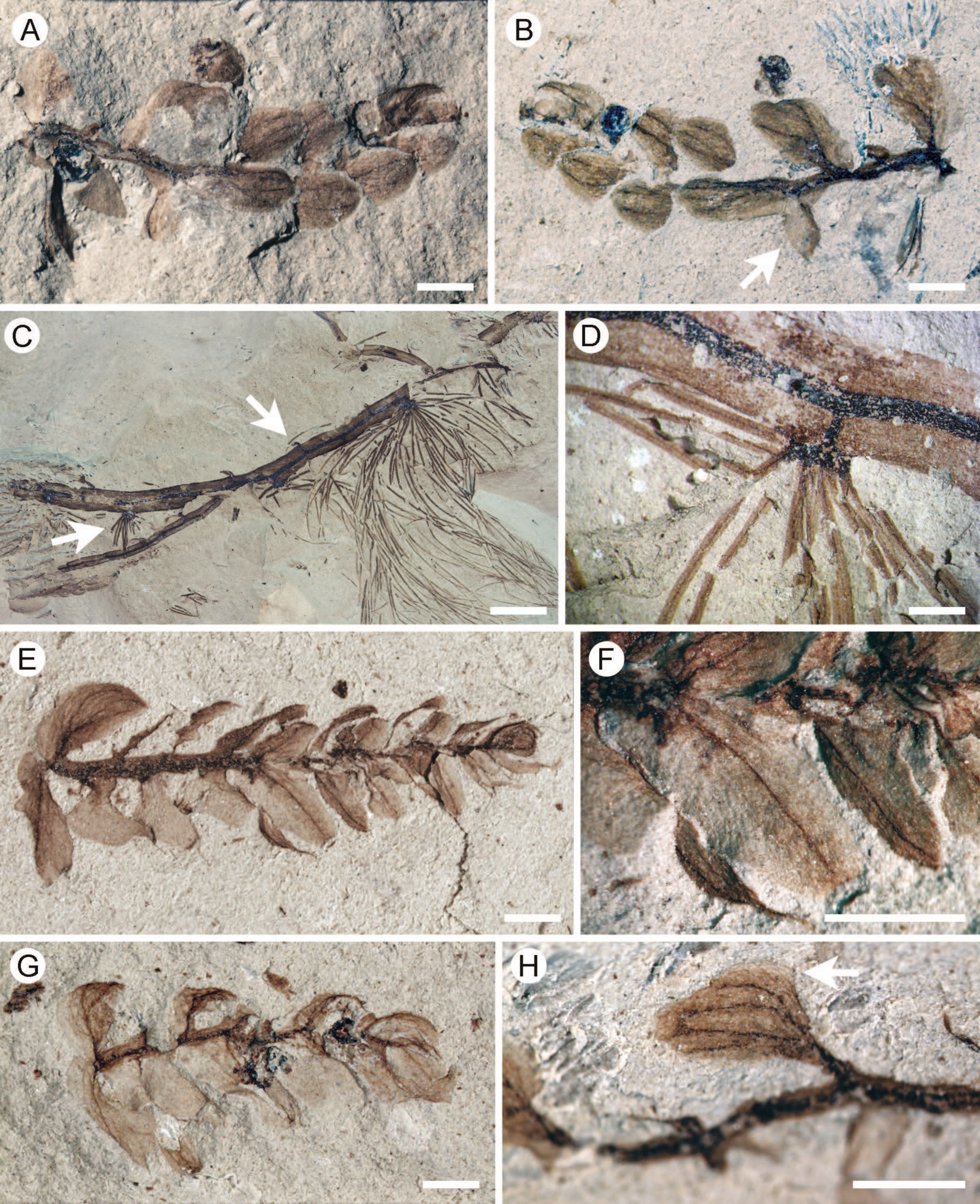
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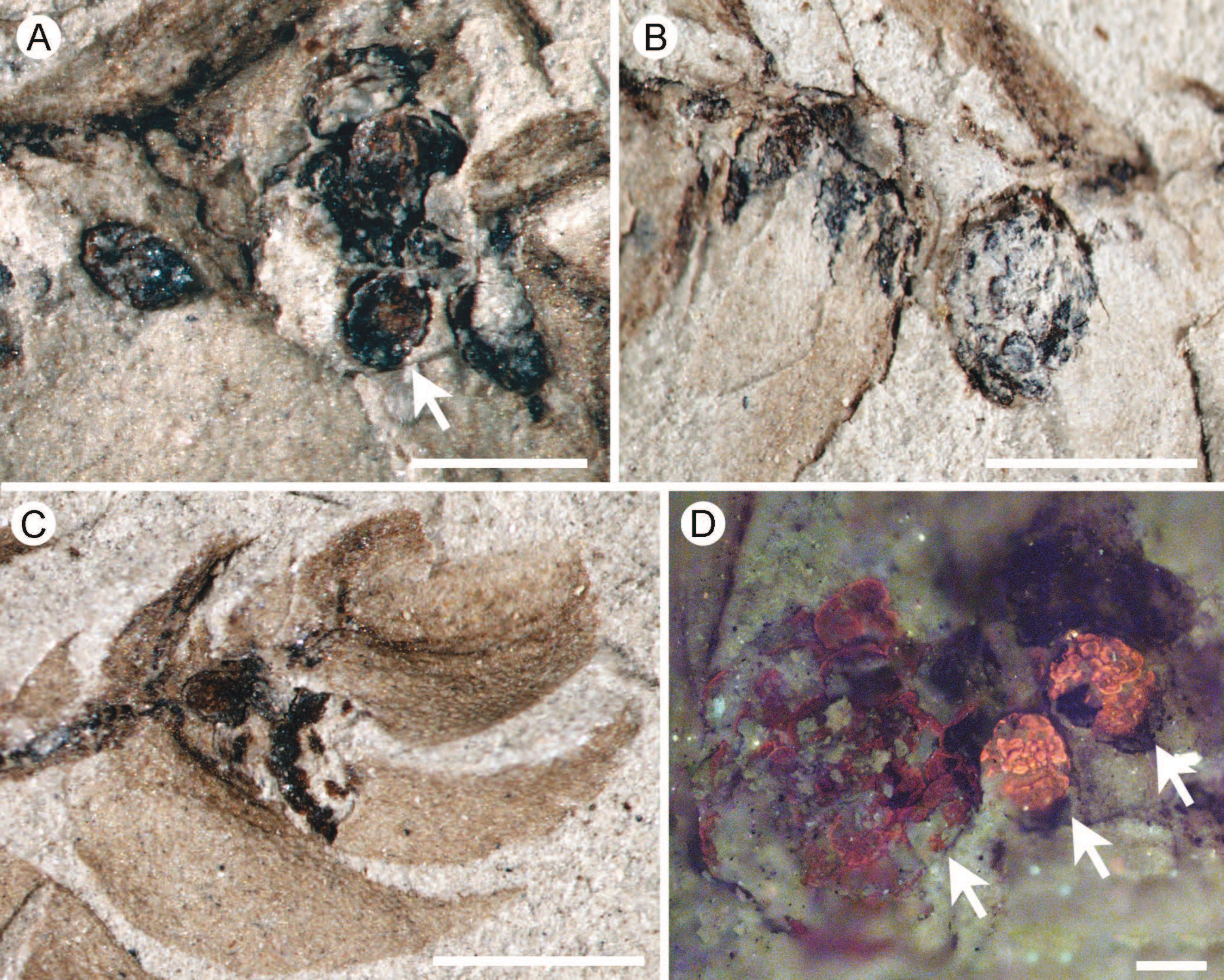
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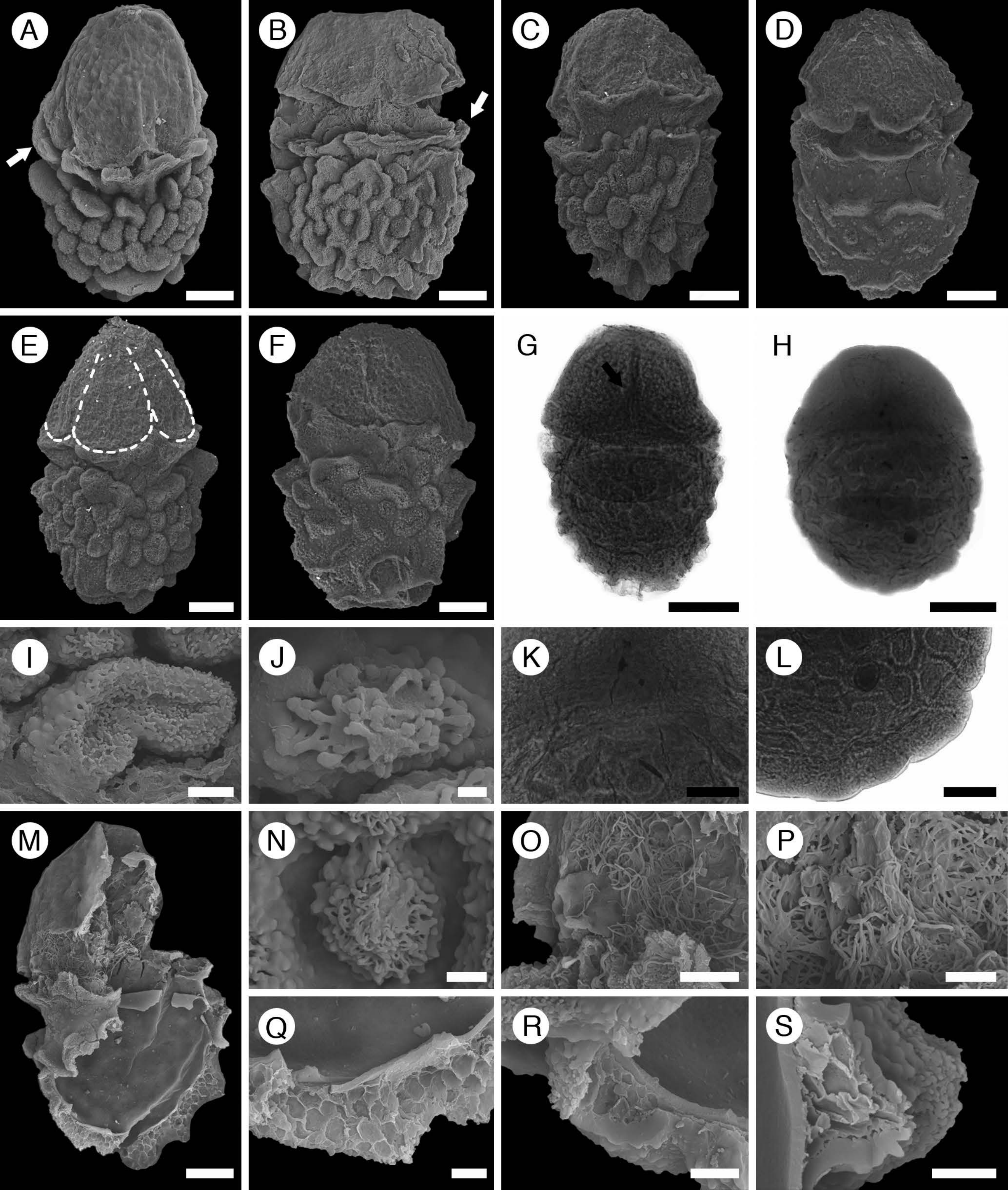
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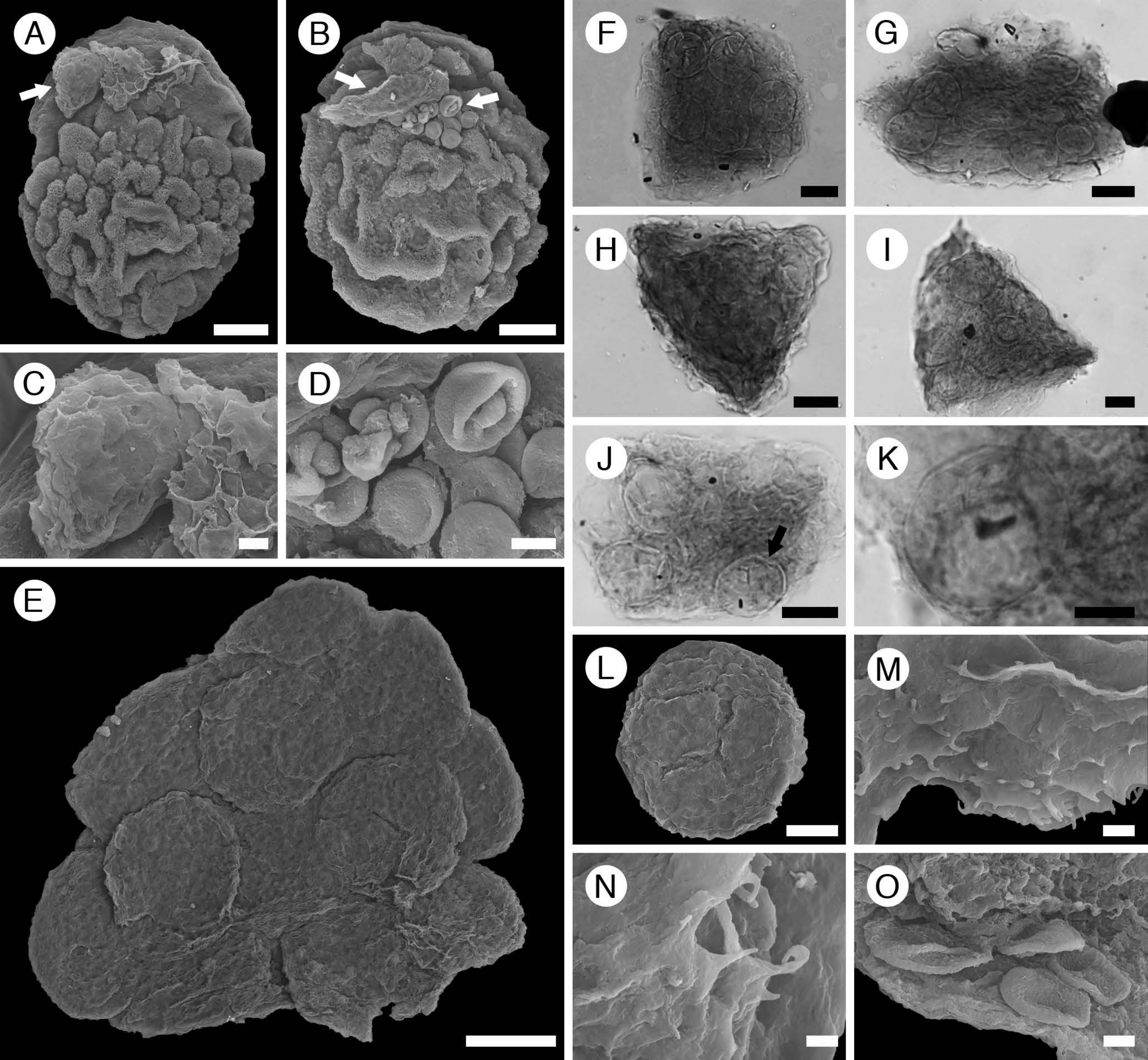
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