MORPHOLOGY, ULTRASTRUCTURE, AND EVOLUTIONARY SIGNIFICANCE OF POLLEN IN A CHLORANTHACEOUS STAMINATE STRUCTURE FROM THE EARLY CRETACEOUS OF PORTUGAL

Maria Tekleva,[1,]* Mário Miguel Mendes,[2,]† Jiří Kvaček,[3,]‡ Peter K. Endress,[4,]§ and James A. Doyle[5,]‖

*Paleontological Institute of the Russian Academy of Sciences, Profsoyuznaya 123, Moscow 117647, Russia; †Marine and Environmental Sciences Centre, University of Coimbra, Largo Marquês de Pombal, 3030-790 Coimbra, Portugal, and Fernando Pessoa University, Praça de 9 Abril, 4249-004 Porto, Portugal; ‡National Museum Prague, Václavské náměstí 68, 115 79 Praha, Czech Republic; §Department of Systematic and Evolutionary Botany, University of Zurich, Zollikerstrasse 107, 8008 Zurich, Switzerland; and ‖Department of Evolution and Ecology, University of California, Davis, California 95616, USA

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Premise of research. Accumulating data from phylogenetic analyses of living taxa and from paleobotany (pollen, leaves, and floral structures) has greatly improved our understanding of the Cretaceous rise to dominance of the angiosperms. Relatives of the near-basal family Chloranthaceae were conspicuous in the Early Cretaceous. These include female flowers and adhering pollen (*Asteropollis*) that resemble those of the genus *Hedyosmum*, but male structures with in situ pollen are not well known. Here we focus on the morphology and ultrastructure of pollen from a spike of unistaminate, ebracteate flowers from the Aptian–Albian Catefica locality and its evolutionary implications.

Methodology. The coalified mesofossil was isolated from unconsolidated sediment by sieving and was cleaned with HCl, HF, and water. In situ pollen was studied using LM, SEM, and TEM. The phylogenetic relationships of the fossil were evaluated with parsimony analysis of a morphological data set with arrangements of living taxa based on molecular studies.

Pivotal results. The pollen aperture is often poorly defined but is most commonly a three-armed sulcus. The exine is reticulate-columellate with a nanoverrucate supratectal sculpture. The nonapertural nexine consists of a thicker foot layer and a thin but continuous endexine that thickens and becomes lamellated under the aperture; the total nexine thickness is less than in most extant Chloranthaceae. Despite some uncertainty due to the thin nexine and similarities between the staminate structure and that of *Ceratophyllum*, phylogenetic analyses are most consistent with a position attached to the stem lineage of *Hedyosmum*.

Conclusions. The variable but mainly three-armed sulcus of the Catefica fossil may represent an intermediate stage in the transformation from the ancestral simple sulcus of Chloranthaceae to the four- to six-armed sulcus of typical *Asteropollis* pollen and living *Hedyosmum*. Dispersed trichotomosulcate pollen with a chloranthaceous exine structure may be indicative of relatives of *Hedyosmum*, not only *Ascarina*, as is sometimes assumed.

Keywords: angiosperms, Chloranthaceae, pollen, ultrastructure, Early Cretaceous, Portugal.

Online enhancement: appendix.

Introduction

Fossil pollen, leaves, and floral structures related to the angiosperm family Chloranthaceae make up a prominent portion of

[1] Email: tekleva@mail.ru.
[2] Author for correspondence; email: mmmendes@mail.telepac.pt.
[3] Email: jiri_kvacek@nm.cz.
[4] Email: peter.endress@systbot.uzh.ch.
[5] Email: jadoyle@ucdavis.edu.

the record of angiosperms during their Early Cretaceous rise to dominance (Couper 1958; Walker and Walker 1984; Friis et al. 1986, 1994, 1999, 2011, 2019*a*, 2019*b*; Pons 1988; Crane et al. 1989; Pedersen et al. 1991; Eklund et al. 1997, 2004; Friis and Pedersen 2011; Doyle and Endress 2014, 2018; Doyle and Upchurch 2014; Sender et al. 2021). Today, the family is reduced to four genera (*Hedyosmum*, *Ascarina*, *Sarcandra*, and *Chloranthus*) and ca. 77 species (Endress 1987; Todzia 1993). Molecular phylogenetic studies (see Soltis et al. 2018) indicate that Chloranthaceae is one of the five clades making up the mesangiosperms (core angiosperms), which include all angiosperms except for *Amborella*,

Nymphaeales, and Austrobaileyales; the other four clades are Magnoliidae, monocots, eudicots, and *Ceratophyllum*.

The first Cretaceous fossils to be compared to Chloranthaceae were monosulcate pollen grains from the Barremian of England with what is now recognized as a reticulate-columellate exine structure; Couper (1958) described them as *Clavatipollenites hughesii* and compared them to pollen of living *Ascarina*. In subsequent SEM and TEM studies, many authors used the name *Clavatipollenites* for reticulate-columellate monosulcate pollen with a supratectal nanosculpture, a sculptured sulcus membrane, and a thick nexine consisting mainly of a homogeneous (nonlamellate) foot layer (Doyle et al. 1975; Walker and Walker 1984; Chapman 1987; Chlonova and Surova 1988; Archangelsky and Taylor 1993). This identification is uncertain, as SEM studies by Hughes et al. (1979) revealed several distinct reticulate-columellate monosulcate pollen types in Couper's type sample, and it is not clear which of these corresponds to the holotype of *C. hughesii*, which was studied with LM alone. Such pollen has been associated with fossil flowers and fruits, notably *Couperites* (Pedersen et al. 1991), which phylogenetic analyses indicate may or may not be related to Chloranthaceae (Eklund et al. 2004; Friis et al. 2011; Doyle and Endress 2014), and *Canrightiopsis*, which is securely nested within the family (Friis et al. 2015; Doyle and Endress 2018).

Among other Early Cretaceous fossils related to Chloranthaceae are dispersed pollen grains described by Hedlund and Norris (1968) as *Asteropollis asteroides*, which has a four- to six-armed sulcus, an aperture condition known only in *Hedyosmum* today, and female flowers recently named *Hedyflora* (Friis et al. 2019*b*), with one carpel and three basally united tepals that are fused to the inferior ovary, as in extant *Hedyosmum*. The exine structure of *A. asteroides* is similar to that of pollen assigned to *Clavatipollenites* (Walker and Walker 1984). Pollen adhering to *Hedyflora* flowers from different localities in Portugal (e.g., Friis et al. 2011, 2019*a*, 2019*b*) shows varying mixtures of grains with four to six aperture arms, as in the type material of *A. asteroides* (Hedlund and Norris 1968), or three aperture arms (trichotomosulcate), a type variously assigned to *Clavatipollenites*, *Asteropollis*, or *Jusinghipollis* Jansonius & Hills in the dispersed pollen record. It should be noted that low frequencies of trichotomosulcate pollen grains, along with monosulcate grains, are produced in *Ascarina* (Chapman 1987; Eklund et al. 2004; Friis et al. 2011).

By contrast, staminate structures resembling those of *Hedyosmum* are less well known as fossils. Until now, they consisted of isolated stamens and small groups of stamens containing pollen and small spikes of stamens (as in *Hedyosmum*) that contain no pollen and are presumed to be immature (Friis et al. 1994, 2011, 2019*b*).

In this article we describe pollen from a coalified spike (head) of unistaminate, ebracteate flowers from Lower Cretaceous sediments at the Catefica locality in the Lusitanian Basin of western Portugal that will be formally described in an article on the Catefica mesofossil flora by E. M. Friis and others. The fossil is more informative than previously reported *Hedyosmum*-like male structures because it is nearly complete and contains abundant well-preserved pollen. We present observations of this pollen from LM, SEM, and TEM. For a framework for evaluation of the systematic position of the fossil and the evolutionary significance of the pollen, we have conducted phylogenetic analyses based on both floral and pollen characters using an updated version of a morphological data set employed in previous studies of the phylogenetic position of Cretaceous fossils in the phylogeny of living angiosperms (e.g., Doyle and Endress 2014), as inferred mainly from molecular evidence.

Material and Methods

The fossil reported herein was collected by M. M. Mendes in a road cut close to the Catefica locality of Mendes et al. (2017), near Torres Vedras in the Estremadura region of western Portugal (lat. 39°3′16″N, long. 09°14′24″W). The staminate structure was extracted from a dark gray mudstone layer rich in plant remains in the Almargem Formation, assigned to the "Grès de Torres Vedras" unit (Zbyszewski and Assunção 1965), considered to be late Aptian–early Albian in age. Some authors have argued that sediments from this stratigraphic interval can be dated more precisely as late early Albian based on correlation with pollen successions in better-dated coastal marine sequences, particularly the presence of both reticulate and striate tricolpate pollen (Heimhofer et al. 2007; Doyle and Endress 2014; Tanrikulu et al. 2016). For more details on geology and stratigraphy, see Mendes et al. (2017).

The Catefica mesofossil flora is dominated by diverse reproductive structures of angiosperms, including flowers, fruits, and seeds. However, this flora also includes conifer twigs and seeds, selaginellaceous shoots and megaspores, and several fragments of thalloid liverworts (Friis et al. 1999, 2011, 2015; Friis and Pedersen 2011; E. M. Friis et al., unpublished data).

We isolated the staminate structure with in situ pollen from the sediment matrix by sieving it in water through a 125-μm net mesh, treating it with hydrofluoric (40% HF) and hydrochloric (10% HCl) acids, thoroughly rinsing it in water, and drying it in the air using standard methods described in earlier studies (Friis et al. 1988, 2009). The fossil specimen was mounted on an aluminum stub and coated with gold in a sputter-coater for 60 s, and the in situ pollen masses in broken stamens were examined under a Hitachi S-3700N SEM at 5 kV in the HERCULES (Cultural Heritage, Study, and Safeguard) Laboratory at the University of Évora, Portugal. The specimen and preparations made from it will be housed at the Geological Museum of Lisbon, Portugal, with the collection number P0341.

Part of one of the apical stamens was carefully removed with a scalpel and used for observations of the pollen. Glycerin slides of individual pollen grains were prepared, and the grains were photographed with a Carl Zeiss Axioplan 2 light microscope equipped with a ×100 oil immersion objective and a Leica DFC420 digital camera.

Individual pollen grains and pollen clumps were washed in hot water and ethanol and transferred to a piece of photographic film for SEM studies. The film was mounted on an aluminum stub (glued with nail polish) and was sputter-coated with gold for 8 min. The pollen grains were observed and photographed under a Tescan Vega3 XMU SEM with an accelerating voltage of 20 and 30 kV at the A. A. Borissiak Paleontological Institute, Russian Academy of Sciences.

For TEM analyses, fragments of a pollen mass were embedded in epoxy resins in the proportions described by Zavialova et al. (2018). The pollen grains were sectioned with a Leica EM UC6 ultramicrotome equipped with a diamond knife. The ultrathin sections were not stained, as they already showed

satisfactory contrast under the microscope used. The sections were examined under a Jeol 1011 TEM with an accelerating voltage of 80 kV at the Laboratory of Electronic Microscopy, Faculty of Biology, Lomonosov Moscow State University. The Jeol 1011 TEM was equipped with a side-mounted Orius SC1000W digital camera (11 megapixels, effective 8.5 megapixels); Digital-Micrograph version 2.0 (Gatan) software was used for image processing.

In describing the pollen, we generally follow the terminology of Punt et al. (2007) for morphology and Halbritter et al. (2018) for ultrastructure. In a few cases we have used earlier terms from Walker and Doyle (1975) that were not adopted by Punt et al. (2007) but that express distinctions that we consider useful.

For the phylogenetic analyses, we used the morphological data set of basal angiosperms (including basal monocots and eudicots) from Doyle and Endress (2014), with modifications and additions made by Kvaček et al. (2016) and Doyle and Endress (2018) and proposed here. We investigated the position of the fossil using a molecular scaffold approach (Springer et al. 2001), in which a morphological data set of living and fossil taxa is analyzed with the arrangement of living taxa constrained to a backbone tree based on molecular data. Chloranthaceae is one of the five clades making up the mesangiosperms, but there is persisting uncertainty about the arrangement of these clades, much of which involves the position of Chloranthaceae; we used two backbone trees that represent the range of currently viable hypotheses. In the JM (previously J/M) tree, based on analyses of plastid DNA sequences (Jansen et al. 2007; Moore et al. 2007), Chloranthaceae is sister to Magnoliidae, while *Ceratophyllum* is sister to the eudicots. In the DE (previously D&E) tree, based on a combined morphological and molecular analysis by Doyle and Endress (2000), with modifications based on more recent studies (Endress and Doyle 2009), Chloranthaceae and *Ceratophyllum* form a clade that is sister to the remaining mesangiosperms. This clade, with various positions among the three remaining mesangiosperm lines, has been found in analyses of mitochondrial genes (Duvall et al. 2006, 2008; Qiu et al. 2010; Sun et al. 2015; Xue et al., forthcoming), nuclear genes (Zhang et al. 2012; Zeng et al. 2014; Sun et al. 2015), and genes from the inverted repeat of the plastid genome (Moore et al. 2011), which have been considered more reliable in detecting ancient splitting events because of their low rates of substitution and numerous informative sites.

Scoring of the characters of the present fossil, minus vegetative and gynoecial characters (which are unknown), is presented in table 1, while the full character list and the data matrix are provided in the appendix (available online). We assume that the stamen-bearing axis is a spike of unistaminate flowers with no floral subtending bracts, as in *Hedyosmum* and *Ceratophyllum*. Earlier authors interpreted the male structures of *Ceratophyllum* to be multistaminate flowers (e.g., Endress 1994), but they are more like inflorescences in showing the delayed development of the central stamens (Endress 2004; Endress and Doyle 2009; Doyle and Endress 2014). In addition, the stamens vary greatly in number and merism and show both Fibonacci and Lucas spiral patterns (Endress 1994); the latter is very unusual in flowers but common in other kinds of shoots.

Most available floral characters are readily scored on the basis of the SEM image in figure 1A. Many floral characters are treated as unknown because their state in the female flowers is unknown

or because they are applicable only in flowers with more than one stamen.

The character that poses the most problems concerns protruding versus embedded pollen sacs (character 74). The stamens show four cylindrical pollen sacs with rounded ends, suggesting the protruding state, but this external form appears to be a result of the degradation of the outer tissues of the anther, leaving only the endothecium and pollen or only the masses of still-coherent pollen grains. Some stamens show apparent remnants of an apical cap (fig. 1A, arrowheads) and residual outer material adhering to the pollen sacs. The cap resembles the broad sterile apical area that protects the stamens in bud in most *Hedyosmum* species, in which a perianth and subtending floral bracts are absent (Endress 2008). Among similar fossils from other Portuguese localities, a group of two or three stamens from the Vale de Água mesofossil flora (fig. 3A in Friis et al. 2019b) shows much less convexity in the pollen sacs, as in the embedded state. In the Torres Vedras mesofossil flora, one isolated tetrasporangiate stamen (fig. 21a in Friis et al. 2019a) shows four cylindrical sacs, as in our specimen, but a group of tetrasporangiate stamens (fig. 21b in Friis et al. 2019a) shows connective material between adjacent sacs, suggesting that they had the embedded state. These observations are consistent with the interpretation of the protruding shape of the sacs of our fossil as an artifact, but in the absence of definitive evidence, we score character 74 as unknown. The mode of anther dehiscence (77) must also be considered unknown. However, we score the connective apex character (73) as truncated or smoothly rounded (state 1) on the basis of the small size and form of the persisting apical caps.

In studies by Doyle and Endress (2014, 2018), female flowers with associated pollen of the *Asteropollis* type, more recently described by Friis et al. (2019b) as *Hedyflora*, and associated dispersed stamens and immature male inflorescences were treated together as the "*Asteropollis* plant." To evaluate the relationship of the present fossil to *Hedyflora*, we modified the scoring of the *Asteropollis* plant by deleting inflorescence characters (only isolated female flowers are known) and characters of the staminate structures, which Friis et al. (2019b) did not formally include for *Hedyflora* (see the appendix for complete scoring). On the basis of Friis et al. (2019b), we scored the ovule as pendent (115), orthotropous (116), and bitegmic (117) and changed the exotestal and mesotestal characters (129–131) from unknown to unspecialized (0); we scored the endotesta (132) as a single endoreticulate layer (state 1), as in *Ascarina*, *Sarcandra*, and *Chloranthus*; we scored the tegmen (133) as fibrous to sclerotic exotegmen (1), as in *Ascarina* and some species of *Chloranthus* (Eklund et al. 2004); and we scored ruminations (134) as absent (0).

We have made two changes to the definitions of the characters used by Doyle and Endress (2018). For the sulcus branching character (87), we explicitly define the several-armed state (1) to include grains with three (trichotomosulcate) or more sulcus arms, both of which occur in the present fossil, while excluding cases in which low frequencies of trichotomosulcate grains co-occur with typical monosulcate grains, as in *Ascarina* (Chapman 1987; Eklund et al. 2004). We did not add trichotomosulcate as a new state because it co-occurs with either an unbranched or several-branched sulcus in all taxa in the data set (not counting the unique "syntricolpate" condition of *Illicium* and *Schisandra*, a state of character 85). For the previous nonapertural nexine character (95), state 0 was defined as "foot layer, not consistently foliated, distinctly

Table 1

Scoring of the Catefica Fossil for Phylogenetic Analyses

Character(s)	Description
1–41	Vegetative characters: unknown
42	Inflorescence: (0) solitary flower, (1) botryoid, panicle, or thyrsoid, *(2) raceme, spike, or thyrse*
43–45 and 48–62	Not scored because female structures are unknown
46	Floral subtending bracts: (0) present, *(1) present in female, absent in male flowers, (2) absent in all flowers*
47	Sex of flowers: (0) bisexual, *(1) unisexual*
63	Stamen number: (0) more than one, *(1) one*
64–69	Inapplicable (unknown) because flowers have only one stamen
70	Glandular food bodies on stamens or staminodes: *(0) absent*, (1) present
71	Stamen base: *(0) short*, (1) long and wide, (2) long and narrow
72	Paired basal stamen glands: *(0) absent*, (1) present
73	Connective apex: (0) extended, *(1) truncated or smoothly rounded*, (2) peltate
74	Pollen sacs: (0) protruding, (1) embedded
75	Microsporangia: *(0) four*, (1) two
76	Orientation of dehiscence: (0) distinctly introrse, *(1) latrorse to slightly introrse*, (2) extrorse
77	Mode of dehiscence: (0) longitudinal slit, (1) H valvate, (2) valvate with upward-opening flaps
78–81	Stamen embryological characters: unknown.
82	Pollen unit: *(0) monads*, (1) tetrads
83	Pollen size (average): (0) large (>50 μm), (1) medium (20–50 μm), *(2) small (<20 μm)*
84	Pollen shape: (0) boat shaped, *(1) globose*, (2) triangular, angulaperturate
85	Aperture type: *(0) single or disulcate*, (1) inaperturate, (2) sulcate, (3) (syn)tricolpate with colpi arranged according to Garside's law, (4) tricolpate
86	Single aperture shape: *(0) elongate*, (1) round
87	Single aperture branching: (0) unbranched or occasionally three branched, *(1) predominantly three or more branches*
88	Infratectum: (0) granular, (1) intermediate, *(2) columellate*
89	Tectum: (0) continuous or microperforate, *(1) perforate (foveolate) to semitectate*, (2) reduced
90	Grading of reticulum: *(0) uniform*, (1) finer at ends of sulcus, (2) finer at poles
91	Striate muri: *(0) absent*, (1) present
92	Supratectal spinules: (0) absent, *(1) present*
93	Prominent spines: *(0) absent*, (1) present
94	Aperture membrane: (0) smooth, *(1) sculptured*
95	Extra-apertural nexine stratification: (0) foot layer, not consistently foliated, no distinctly staining endexine, *(1) foot layer and distinctly staining endexine or only endexine*, (2) all or in part foliated, not distinctly staining
96	Nexine thickness: (0) absent or discontinuous, *(1) thin but continuous*, (2) thick (one-third or more of total exine)
97–143	Gynoecial, fruit, and seed characters: unknown.

Note.   Italics denote the scoring used for phylogenetic analyses of the Catefica fossil. Some character definitions have been simplified. See the appendix (available online) for complete definitions.

staining endexine absent or only discontinuous traces," while state 1 was "foot layer and distinctly staining, continuous endexine, or endexine only." The definition of state 0 specifies the absence of foliations to distinguish it from state 2, "all or in part foliated, not distinctly staining," a feature of Magnoliales and Laurales; whether this foliated nexine is a foot layer or endexine is a topic of debate, depending on whether the endexine is defined on the basis of chemical distinctness or formation from lamellae (Doyle 2005). *Chloranthus* was scored as 1 and the three other genera as 0. However, Chlonova and Surova (1988) showed that *Ascarina* has a thin inner nexine layer that often separates from the outer layer, and they interpreted this inner layer as endexine. In *Hedyosmum*, Martínez et al. (2013) showed variation between a continuous and discontinuous endexine layer in different species. In some cases, the recognition of an endexine may be a result of improved staining techniques, or its discontinuity may be due to preservational factors. Because of variation in the continuity of the endexine within genera and the minimal differences between genera, we have readjusted the limit between the two states by (*a*) restricting state 0 to cases when there is no endexine at all and (*b*) removing any reference to the continuity or discon-

tinuity of the endexine from the definition of state 1; we have scored all four extant genera as having state 1.

We previously scored stamen number (63) in *Ascarina* as either more than one or one (0/1) because of variation among species. However, when the species-level data set of Eklund et al. (2004) is used, optimization of this character on the tree found in the molecular analysis of Zhang et al. (2015) indicates that more than one stamen is ancestral for *Ascarina*, so we have rescored the genus as 0. Doyle and Endress (2014, 2018) treated the stamen number (63) of *Chloranthus* as unknown because of uncertainty about the homology of the trilobed androecium attached to the dorsal side of the single carpel—whether it represents three fused stamens or one subdivided stamen. However, the fossil *Canrightiopsis*, which phylogenetic analyses indicate is sister to *Sarcandra* and *Chloranthus* (Friis and Pedersen 2011; Doyle and Endress 2018; Sender et al. 2021), has three separate stamens attached to the dorsal side of the carpel, so we have rescored *Chloranthus* as 0.

In some analyses we included two additional fossils that phylogenetic analyses have associated with Chloranthaceae and/or *Ceratophyllum*. One is *Canrightia*, recognized in almost all mesofossil floras from the Aptian–Albian of Portugal; it has bisexual
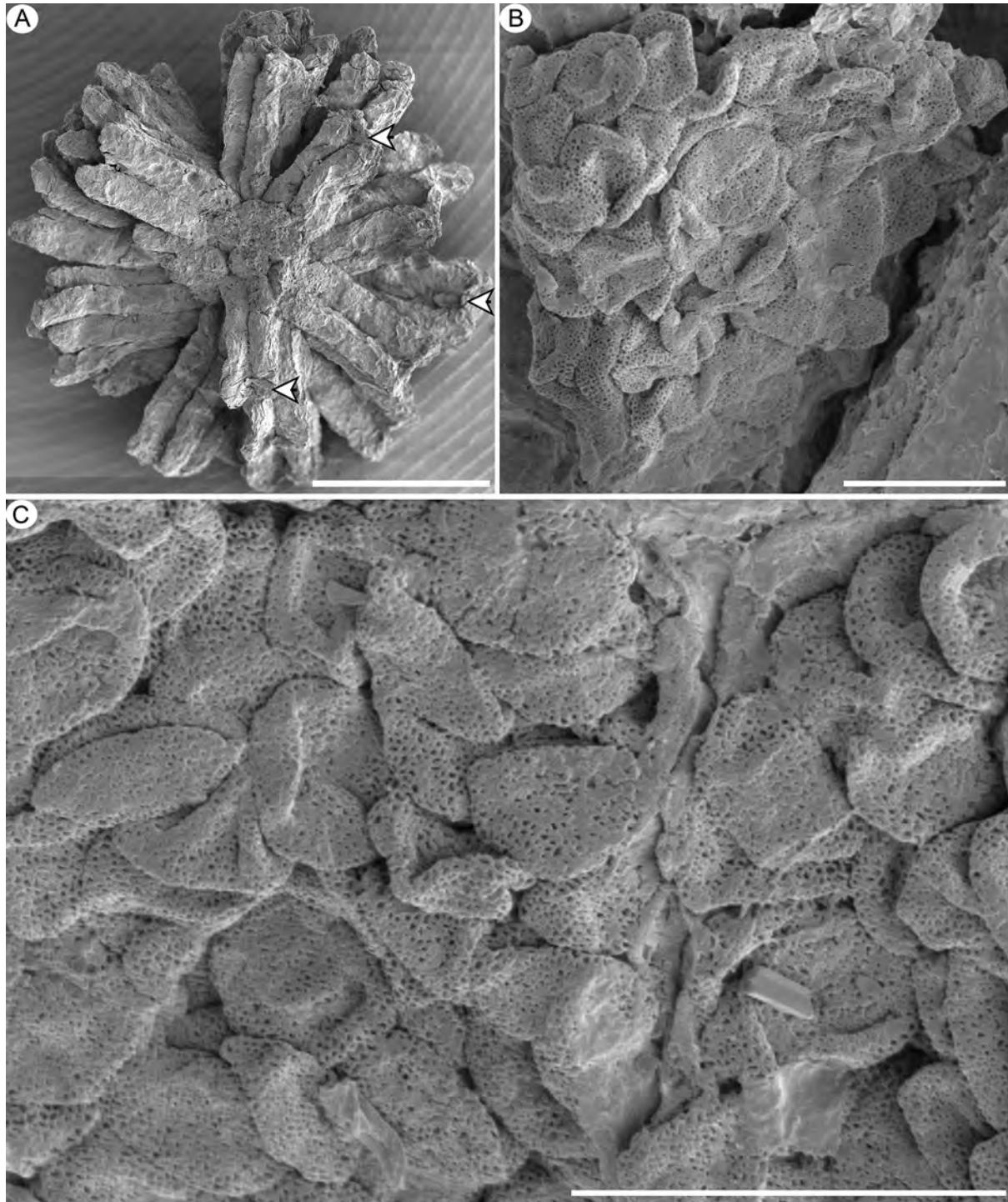
Fig. 1 SEM images of the staminate structure. *A*, Apical view of the whole structure. The arrowheads indicate the presumed remnants of the connective apex. *B*, *C*, In situ pollen masses in the stamen remnants at the center of *A*. Scale bars = 500 μm (*A*), 20 μm (*B*), 30 μm (*C*).

flowers with a reduced perianth and androecium and is inferred to be sister to Chloranthaceae or to Chloranthaceae plus *Ceratophyllum* (Friis and Pedersen 2011; Mendes et al. 2014; Doyle and Endress 2018; Mendes and Friis 2018; Sender et al. 2021). The other is *Pseudoasterophyllites* from the Cenomanian of Bohemia, which was inferred by Kvaček et al. (2016) to be related

to *Ceratophyllum* but is more plesiomorphic in having bracts subtending the stamens in its staminate spikes.

Analyses of the position of the fossil were performed using PAUP (Swofford 1990), with the arrangement of living taxa (plus *Canrightia* and *Pseudoasterophyllites* when these are included) constrained to the backbone tree, with 100 replicates

of random additions of taxa, and with tree-bisection-reconnection branch swapping. To evaluate the robustness of the relationships obtained and the relative parsimony of alternative hypotheses, we searched for trees one and two steps longer than the most parsimonious tree(s) or moved the fossil manually on the backbone tree using MacClade (Maddison and Maddison 2003). We investigated the most parsimonious scenarios for character evolution and characters supporting relationships (synapomorphies) with MacClade.

Results

Morphology and Ultrastructure of Pollen Grains

The pollen grains are small, averaging 15 (13–17) μm on the basis of 30 pollen grains measured under LM and 14.5 (13–16) μm on the basis of 15 pollen grains measured under SEM. The equatorial outline (amb) is circular to slightly elliptical (figs. 1B, 1C, 2–4). The aperture condition is often difficult to determine because of the folding of the grains and the poorly defined margins of the aperture. When most clearly visible under LM and SEM, the aperture is most often a three-armed sulcus (trichotomosulcate; figs. 1B, 1C, 2E–2G), but a few grains have a sulcus with four or possibly five arms (figs. 2A–2C, 4A, 4B, 4F). The sulcus arms extend approximately two-thirds of the radius of the grain. Many grains seen in the in situ pollen masses under SEM (fig. 1B, 1C) show no clear aperture. Such grains are too common for all of them to be explained by the assumption that the aperture is on the hidden side of the grain. The fact that an aperture is usually visible under LM suggests that these grains are not inaperturate but rather cryptoaperturate (i.e., with differentiation of an aperture in the inner layers of the exine but not in the tectum).

LM indicates that the sexine has a semitectate structure with tectal muri forming a microreticulate sculpture pattern (fig. 2). In optical section the pollen wall is ca. 1 μm thick with clearly distinguished columellae. SEM confirms that the exine sculpturing is microreticulate (figs. 3, 4), with lumen size varying from 0.1 to 1 μm (most often 0.3–0.6 μm) and the width of the muri varying from 0.3 to 0.6 μm (mostly ca. 0.4 μm). The tops of the muri are rounded and bear rounded supratectal nanoverrucae (figs. 3F, 4C). The aperture membrane varies from being distinctly verrucate to consisting of a broken or distorted reticulum, in which case the aperture form is often unclear (figs. 3B, 4B, 4D–4F).

TEM observations (fig. 5) indicate that total exine thickness in the nonapertural areas is 0.9–1.1 μm. The tectum is 0.25–0.4 μm thick. The columellae are densely spaced, 0.2–0.4 μm high, and 0.2–0.4 μm wide. The total thickness of the nonapertural nexine is 0.2–0.4 μm. Most of the nexine consists of a homogeneous foot layer ca. 0.2–0.3 μm thick, but a thin (less than 0.1 μm) and homogeneous endexine, distinguished from the foot layer by its higher electron density, is consistently present. Toward the aperture on the presumed distal surface, the nexine is slightly thicker; the foot layer becomes thinner and lamellate, while the endexine becomes thicker (0.3–0.5 μm) and granular-lamellate (fig. 5C–5F). We interpret the outer apertural nexine as a foot layer rather than an endexine because it grades smoothly into the homogeneous foot layer at the aperture margins and the lamellae have the same electron density as the nonapertural ectexine; we have
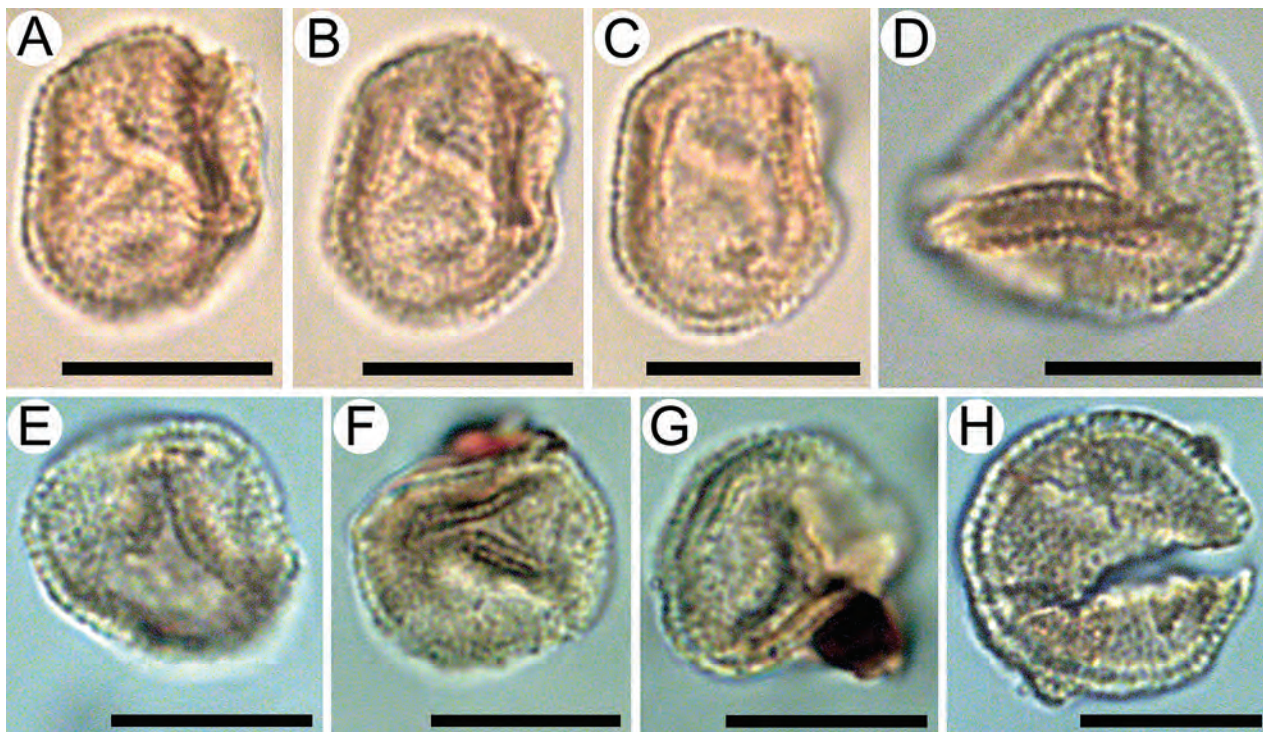

Fig. 2 LM images of pollen. *A–C*, Same pollen grain at different focal levels, showing the exine sculpture and columellae (*A*, *B*) and the four-armed sulcus (*C*). *D–H*, Different pollen grains, showing the exine sculpture and columellae (*D*, *H*) and the three-armed sulcus (*E–G*). Scale bars = 10 μm.
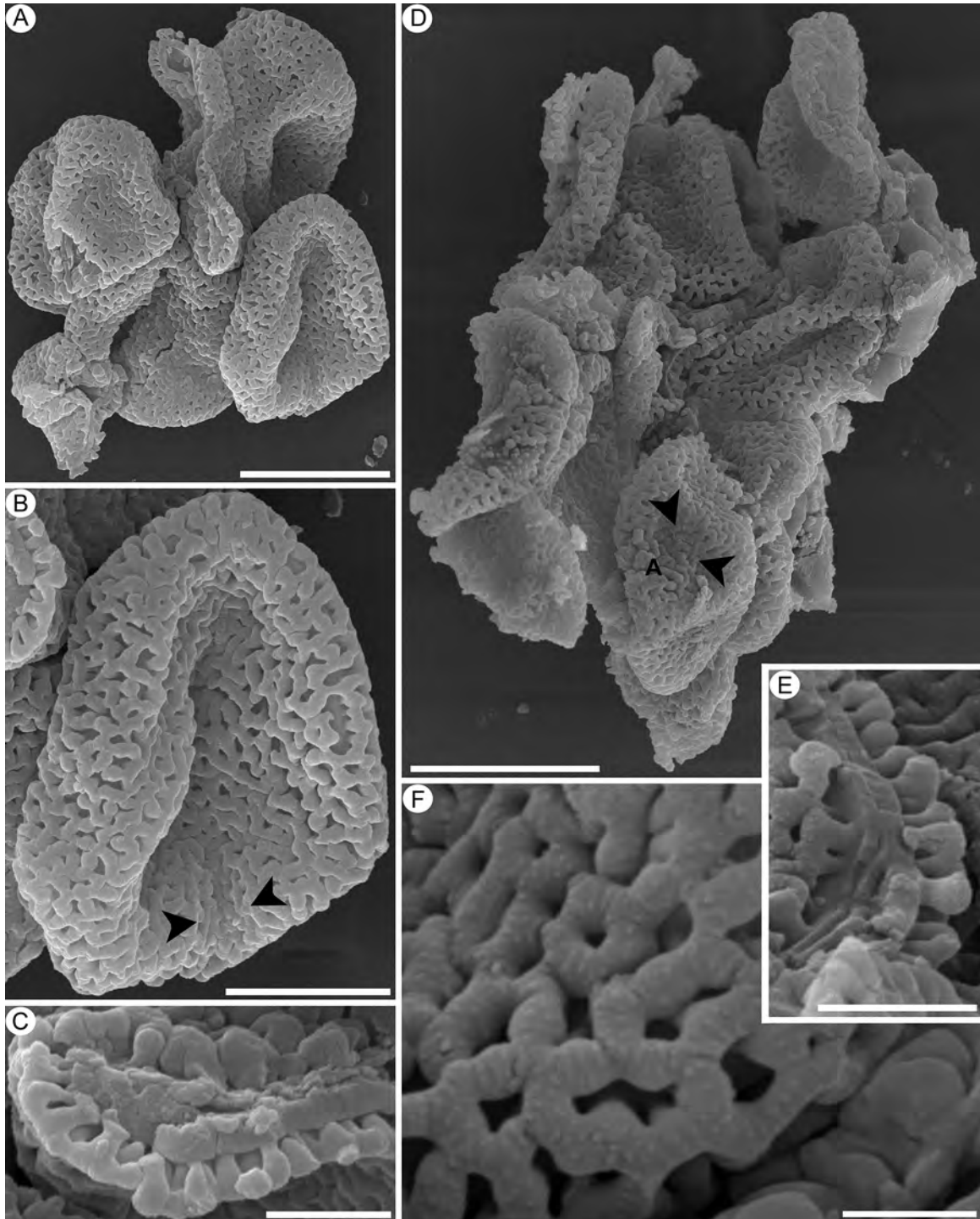
Fig. 3   SEM images of pollen. *A*, Pollen clump. *B*, Close-up of a pollen grain in *A* (*lower right*) with an aperture marked by a distorted tectal reticulum. *C*, Close-up of a broken pollen grain in *A*. *D*, Pollen clump. The arrowheads indicate aperture margins. A = aperture. *E*, Close-up of a broken pollen grain in *D*. *F*, Close-up showing the exine sculpture of a pollen grain in *D* (*upper middle*). Scale bars = 10 μm (*A*, *D*), 5 μm (*B*), 2 μm (*C*, *E*), 1 μm (*F*).

observed this structure in sections of several grains. In the apertural region the ectexine is represented by the foot layer and pillarlike elements that correspond to the verrucae and dislocated tectum seen under SEM. Ubisch bodies are 0.4–0.7 μm in diameter, with a hollow core ca. 0.2–0.3 μm in diameter (fig. 5A, 5B).

Phylogenetic Analyses

In the JM backbone tree, in which Chloranthaceae and *Ceratophyllum* are widely separated (fig. 6A), the single most parsimonious position of the Catefica fossil (1029 steps) is as
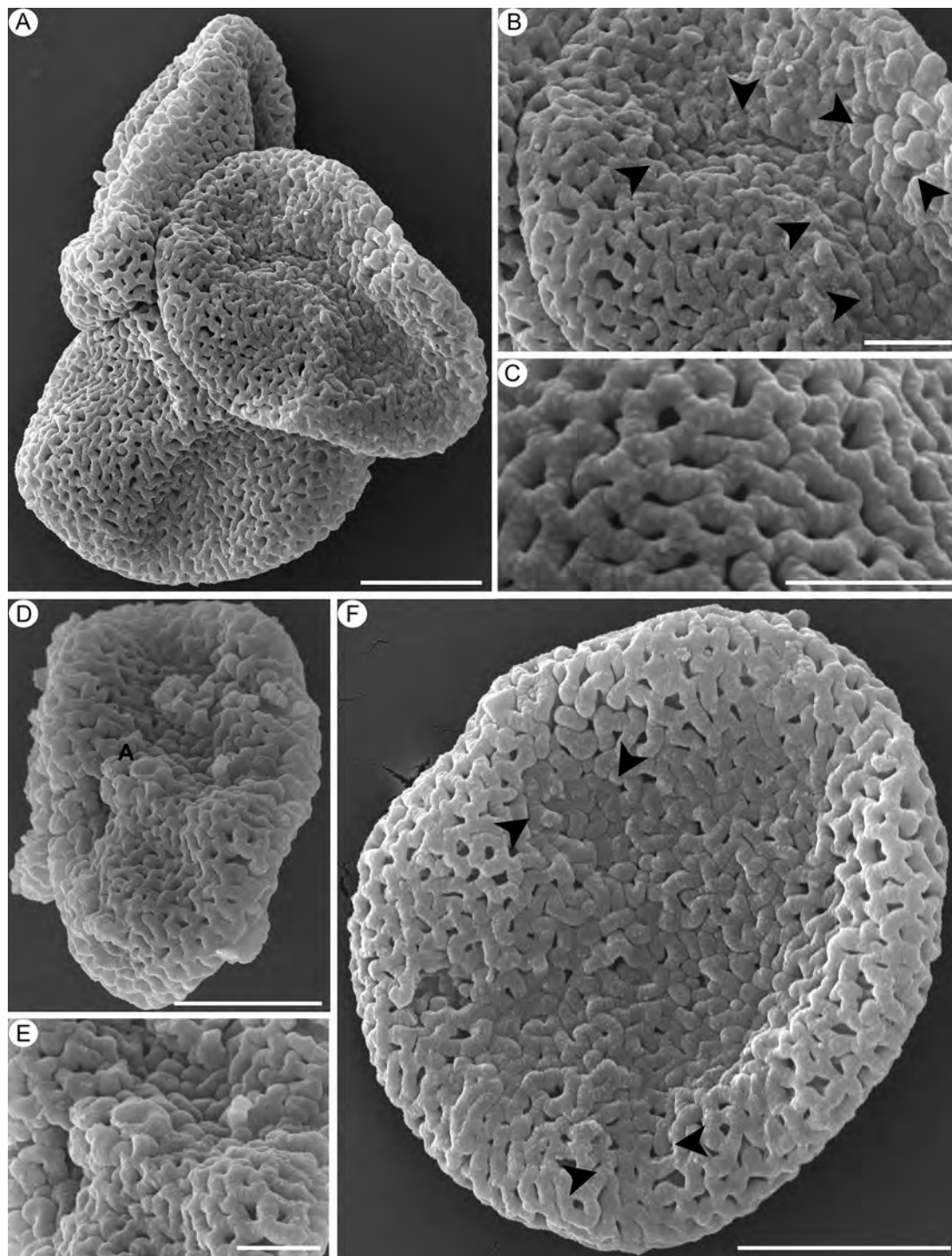
Fig. 4 SEM images of pollen. *A*, Pollen clump. *B*, Close-up of a pollen grain in *A* (*right*), showing details of the aperture membrane of a four-armed sulcus with a verrucate tectum. *C*, Close-up showing the exine sculpture of a pollen grain in *A* (*lower left*). *D*, Pollen grain, equatorial view. A = aperture. *E*, Close-up of *D*, showing an aperture membrane similar to that in *B*. *F*, Pollen grain, polar view, showing four or possibly five indistinct sulcus arms marked by a condensed reticulum. The arrowheads indicate aperture margins. Scale bars = 5 μm (*A*, *D*, *F*), 2 μm (*B*, *C*, *E*).

sister to *Hedyosmum*. Unequivocal synapomorphies of the fossil and *Hedyosmum* (i.e., cases in which the position of the character state change on the tree is unambiguous) are the absence (loss) of bracts subtending the male flowers (46), one stamen (63), and

a branched sulcus (87). Its next most parsimonious positions, which are two steps worse (1031 steps), are as sister to *Trithuria* (=Hydatellaceae, in Nymphaeales), Chloranthaceae as a whole, and *Ceratophyllum*. These groups share various combinations
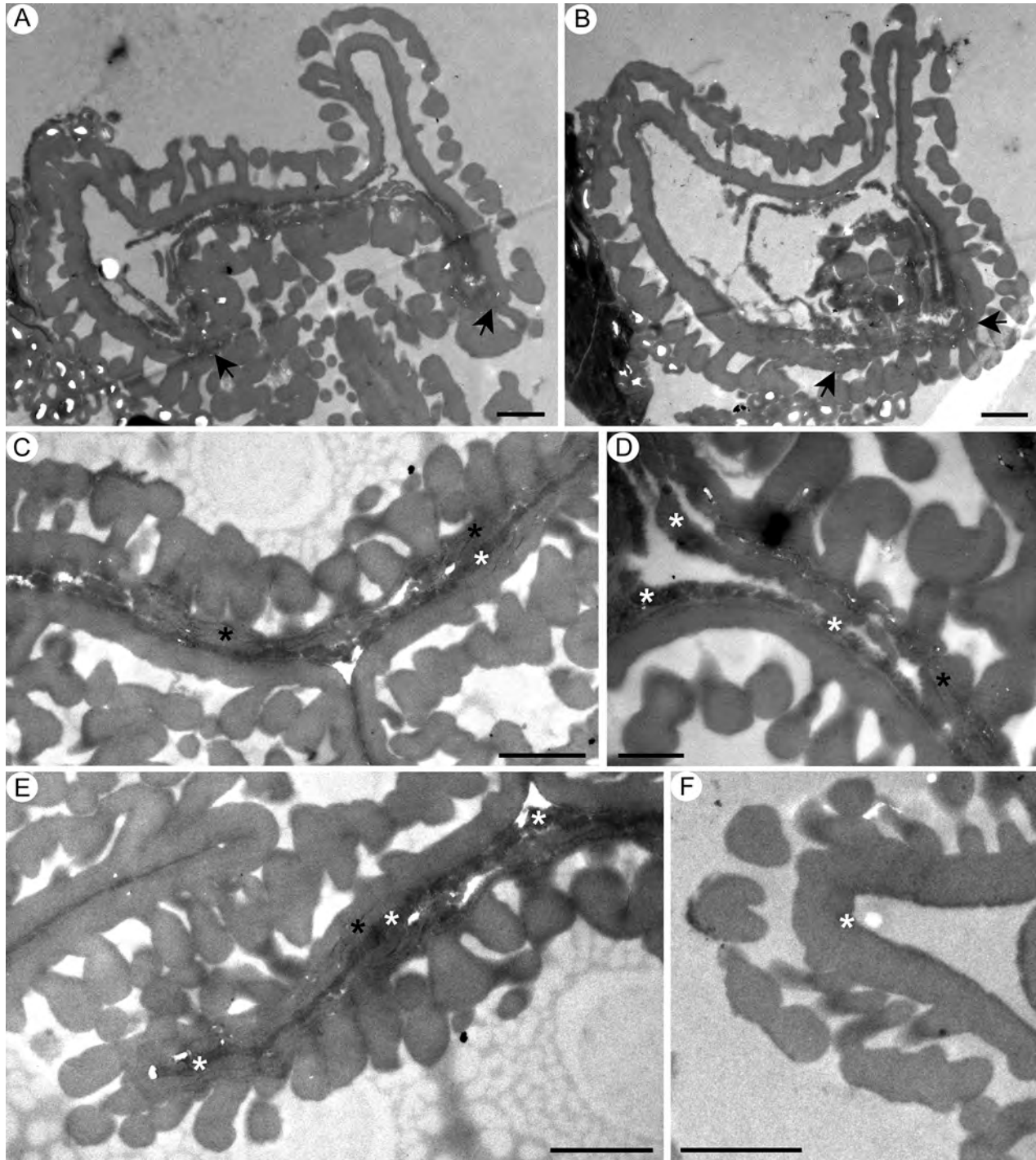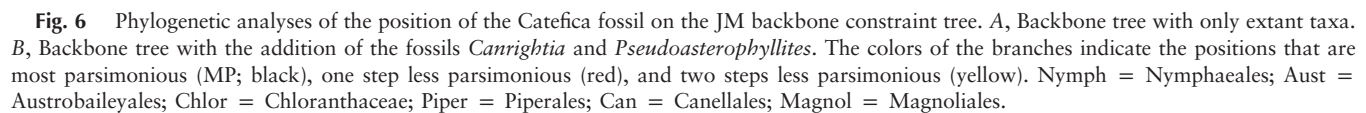
Fig. 5   TEM sections of pollen. *A*, *B*, Same pollen grain; sections through the whole grain at different levels. *C–F*, Sections showing details of the exine, with a homogeneous foot layer and continuous thin electron-dense endexine in the nonapertural regions and remnants of the tectum, lamellate foot layer, and thickened lamellate endexine in the apertural region. The arrows indicate aperture margins, black asterisks indicate the apertural foot layer, and white asterisks indicate the endexine. Scale bars = 1 μm (*A–C*, *E*, *F*), 0.5 μm (*D*).

of the loss of floral subtending bracts, unisexual flowers, one stamen, and other features with the Catefica fossil.

When the Aptian–Albian bisexual flower *Canrightia* and the Cenomanian whole plant *Pseudoasterophyllites* are added to the JM tree, as the sister groups of Chloranthaceae and *Ceratophyl-lum*, respectively (fig. 6B), the Catefica fossil has two most parsimonious positions (1043 steps): one with *Hedyosmum*, supported by the branched sulcus, the other with *Ceratophyllum*. Both positions are supported by the absence of floral subtending bracts, which were lost independently in *Hedyosmum* and

Fig. 6 Phylogenetic analyses of the position of the Catefica fossil on the JM backbone constraint tree. *A*, Backbone tree with only extant taxa. *B*, Backbone tree with the addition of the fossils *Canrightia* and *Pseudoasterophyllites*. The colors of the branches indicate the positions that are most parsimonious (MP; black), one step less parsimonious (red), and two steps less parsimonious (yellow). Nymph = Nymphaeales; Aust = Austrobaileyales; Chlor = Chloranthaceae; Piper = Piperales; Can = Canellales; Magnol = Magnoliales.

Ceratophyllum (*Pseudoasterophyllites* does have such bracts), and by parallel reductions to one stamen on the lines leading to *Hedyosmum* and to the *Ceratophyllum-Pseudoasterophyllites* clade. One character supporting the second position is the presence of

"supratectal spinules" (92, including nanoverrucae and nano-echini in the terminology of Halbritter et al. [2018]) in both the Catefica fossil and *Pseudoasterophyllites*. Spinules do not support a link between the Catefica fossil and *Ceratophyllum* alone;
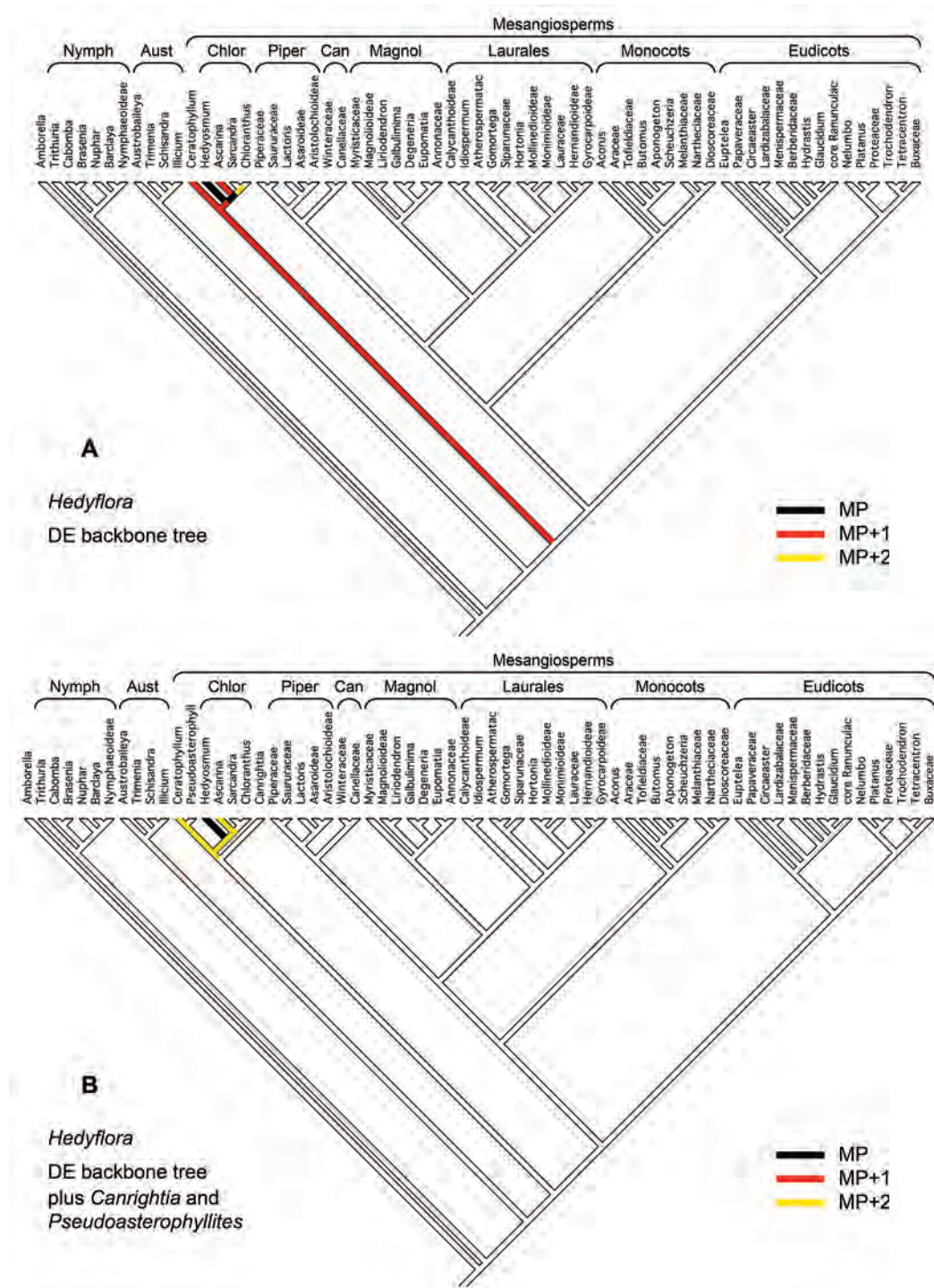
Fig. 7 Phylogenetic analyses of the position of the Catefica fossil on the DE backbone constraint tree. *A*, Backbone tree with only extant taxa. *B*, Backbone tree with the addition of the fossils *Canrightia* and *Pseudoasterophyllites*. The colors of the branches indicate the positions that are most parsimonious (MP; black), one step less parsimonious (red), and two steps less parsimonious (yellow). Nymph = Nymphaeales; Aust = Austrobaileyales; Chlor = Chloranthaceae; Piper = Piperales; Can = Canellales; Magnol = Magnoliales.

Fig. 8 Phylogenetic analyses of the position of the mesofossil *Hedyflora* on the DE backbone constraint tree. *A*, Backbone tree with only extant taxa. *B*, Backbone tree with the addition of the fossils *Canrightia* and *Pseudoasterophyllites*. The colors of the branches indicate the positions that are most parsimonious (MP; black), one step less parsimonious (red), and two steps less parsimonious (yellow). Nymph = Nymphaeales; Aust = Austrobaileyales; Chlor = Chloranthaceae; Piper = Piperales; Can = Canellales; Magnol = Magnoliales.

Ceratophyllum was scored as unknown (inapplicable) for this and most other pollen characters because of its extreme exine reduction (Takahashi 1995).

In the DE backbone tree, in which Chloranthaceae and *Ceratophyllum* form a clade (fig. 7A), the fossil has four most parsimonious positions (1019 steps): as sister to *Hedyosmum*, supported by the branched sulcus (87); to Chloranthaceae as a whole; to *Ceratophyllum*; and to the whole Chloranthaceae-*Ceratophyllum* clade. The last three positions are favored by the thin nexine (96) of the fossil versus the thick nexine of extant Chloranthaceae (an inferred synapomorphy of the crown group). Positions as sister to the *Ascarina-Sarcandra-Chloranthus* clade, to *Trithuria*, and to the clade consisting of monocots, magnoliids, and eudicots are one step less parsimonious.

The addition of *Canrightia* and *Pseudoasterophyllites* to the DE backbone, as the sister groups of the Chloranthaceae-*Ceratophyllum* clade and *Ceratophyllum*, respectively (fig. 7B), reduces the number of most parsimonious positions of the Catefica fossil (1029 steps) from four to two: it may be sister either to *Hedyosmum*, supported by the branched sulcus, or to *Ceratophyllum*. Because *Pseudoasterophyllites* has floral subtending bracts, these are lost independently in *Hedyosmum* and *Ceratophyllum*, so their absence supports these two most parsimonious placements of the Catefica fossil over more basal positions. By contrast, when only living taxa are considered, it is equally parsimonious to assume that floral subtending bracts were lost independently in *Ceratophyllum* and *Hedyosmum* or that they were lost once in the common ancestor of *Ceratophyllum* and Chloranthaceae and reappeared in the *Ascarina-Sarcandra-Chloranthus* clade.

When the *Asteropollis* plant of Doyle and Endress (2014) is stripped of its androecial characters so that it corresponds to *Hedyflora* of Friis et al. (2019b), it has the same two most parsimonious positions on both backbone trees (fig. 8A): one with *Hedyosmum*, supported by the branched sulcus, the other as sister to the *Ascarina-Sarcandra-Chloranthus* clade, supported by endotesta consisting of one layer of endoreticulate cells (character 132, state 1), which is absent in *Hedyosmum*. By contrast, when *Canrightia* and *Pseudoasterophyllites* are added to either of the two backbone trees, *Hedyflora* has one most parsimonious position, with *Hedyosmum* (fig. 8B), and other placements of *Hedyflora* are at least two steps less parsimonious. Because *Canrightia* has an endoreticulate endotesta, this character becomes equivocal as a synapomorphy of *Hedyflora* and the *Ascarina-Sarcandra-Chloranthus* clade. Furthermore, because *Canrightia*, *Pseudoasterophyllites*, *Ascarina*, *Sarcandra*, and *Chloranthus* have a sessile stigma, the presence of a style (102) in *Hedyosmum* and *Hedyflora* is an additional synapomorphy for these two taxa.

Discussion

Comparison with Living and Fossil Taxa

Extant Chloranthaceae show a moderate level of diversity in pollen characters (see data compiled by Eklund et al. 2004). Pollen size varies from small to medium; pollen shape is mainly spheroidal (globose rather than boat shaped), even in monosulcate grains. The aperture condition is monosulcate with subordinate trichotomosulcate variants in *Ascarina*, sulcate with four to six sulcus arms in *Hedyosmum*, pantoporate (polyforate, polyporate) in *Sarcandra*,

and polycolpate (with four to six short, often poorly defined longitudinal furrows) in *Chloranthus* (except for *Chloranthus erectus*, which is pantoporate). The exine has a columellate infratectal structure; its sculpture varies from perforate (foveolate) to reticulate and from homobrochate (*Hedyosmum*, *Ascarina*) to heterobrochate (*Sarcandra*, *Chloranthus*). The muri have a supratectal nanosculpture in *Hedyosmum* and *Ascarina* but are psilate in *Sarcandra* and most species of *Chloranthus* (except for *Chloranthus japonicus*). The aperture membrane has been described by various authors as verrucate, reticulate, or scabrate. In the nonapertural regions the nexine is usually thick (one-third or more of the total exine thickness), although it is thinner in *Hedyosmum orientale* and some species of *Chloranthus*; it consists of a thicker homogeneous foot layer and a thinner and sometimes discontinuous endexine (see "Material and Methods"). Toward the aperture, the foot layer is reduced, but the nexine is thicker, largely because of thickening of the endexine; lamellae have been reported in both nexine layers. Ubisch bodies (when described) have a hollow core. On the basis of parsimony optimization of character states on the well-established phylogeny, Doyle and Endress (2018) reconstructed the ancestral pollen type in the crown group as most like that of *Ascarina*, that is, monosulcate and reticulate-columellate, with supratectal nanosculpture on the muri.

The Catefica fossil shows varying degrees of pollen morphological similarity to extant Chloranthaceae, chloranthaceous mesofossils, and dispersed pollen. *Ascarina*, *Canrightiopsis* (Friis et al. 2015), and dispersed pollen of the type usually identified as *Clavatipollenites* (Doyle et al. 1975; Walker and Walker 1984; Chapman 1987; Chlonova and Surova 1988; Archangelsky and Taylor 1993) have a supratectal nanosculpture but an unbranched sulcus, except for occasional trichotomosulcate grains in *Ascarina*. In *Clavatipollenites* pollen described by Chlonova and Surova (1988) and Archangelsky and Taylor (1993), the supratectal sculpture consists of more pointed elements (nanoechini), while *Canrightiopsis* differs in having a heterobrochate reticulum like that of its putative extant relatives *Sarcandra* and *Chloranthus*. The monosulcate pollen of *Zlatkocarpus* (Kvaček and Friis 2010) and *Canrightia* (Friis and Pedersen 2011) differs further in having psilate muri; this supports a phylogenetic position of these mesofossils on the stem lineage of Chloranthaceae, with or without *Ceratophyllum* (Friis and Pedersen 2011; Doyle and Endress 2014, 2018). Pollen of Late Cretaceous *Chloranthistemon* species, like that of *Sarcandra* and *Chloranthus*, differs still more in aperture condition, with a presumed distal sulcus and a second furrow perpendicular to it at the opposite pole in *Chloranthistemon alatus*, a spiral aperture resembling the seam of a tennis ball in *Chloranthistemon endressii*, and six colpi in *Chloranthistemon crossmanensis* (Crane et al. 1989; Herendeen et al. 1993; Eklund et al. 1997).

Pollen of the Catefica fossil is most like that of *Hedyosmum*, middle Albian dispersed grains of *Asteropollis asteroides* (Walker and Walker 1984), and pollen adhering to the *Hedyosmum*-like female flowers of *Hedyflora* (Friis et al. 2019b). All of these have a branched sulcus, reticulate-columellate exine structure, and supratectal nanosculpture. *Asteropollis asteroides* differs in being much larger and having more than three (four to six) sulcus arms and distinct aperture margins. Pollen adhering to *Hedyflora crystallifera* flowers and found in situ in isolated stamens from the slightly younger Buarcos locality (Friis et al. 2019b) is similar to our pollen in size and nanosculpture, but the reticulum is slightly coarser and the sulcus usually has four arms and only rarely three.

Small pollen with similar sculpture and a branched sulcus also occurs in stamens from older sediments at the Torres Vedras locality (Friis et al. 2019*a*), which also yields *Hedyflora* flowers with no adhering pollen. In some stamens the pollen has four sulcus arms (fig. 20*e*, 20*g* in Friis et al. 2019*a*) and in others mostly three arms (fig. 21*d*–21*h* in Friis et al. 2019*a*), as in the Catefica fossil. Similar dispersed pollen with a three-armed sulcus includes aff. *Clavatipollenites hughesii* of Doyle and Robbins (1977, pl. 1, figs. 20, 21) from the earliest Albian (upper zone I) of Delaware; *Asteropollis* spp. 3 and 4 of Heimhofer et al. (2007, pl. 2, figs. 6–9) from early Albian marine beds in Portugal, which show two extremes in the coarseness of the reticulum; and *Jusinghipollis ticoensis* from the late Aptian of Argentina (Llorens and Perez Loinaze 2016), which is larger and has a more open reticulum.

The nexine of the Catefica fossil is notably thinner than that of *Clavatipollenites*, *A. asteroides*, and most living Chloranthaceae, although it sometimes approaches the limit (one-third of the total exine thickness) between the two states of character 96. The fact that the pollen forms such coherent masses raises the possibility that the nexine is thin because the structure was still immature when it was shed. However, exine development had reached the phase in which the endexine was deposited on the inside of the foot layer. Information on the pollen of other mesofossils might help with evaluating the significance of this and other nexine characters, but so far these characters are unknown because such pollen has been studied only with SEM.

Phylogenetic and Evolutionary Implications

On the basis of the varied results of our analyses with the four backbone trees (figs. 6, 7), the relationship of the Catefica fossil to *Hedyosmum* is not conclusively established, but it does appear most likely. The alternatives are that it was attached to the stem lineage of Chloranthaceae, Chloranthaceae plus *Ceratophyllum*, or *Ceratophyllum*. All of these positions are favored by the thin nexine of the fossil, which contrasts with the thick nexine of most crown group Chloranthaceae. The resulting arrangements either favor or are consistent with scenarios in which reduction of the male flowers to one stamen with no subtending bract (as in *Hedyosmum* and *Ceratophyllum*) occurred on the line leading to Chloranthaceae or the Chloranthaceae-*Ceratophyllum* clade and was reversed in the clade consisting of *Ascarina*, *Sarcandra*, and *Chloranthus*. However, the single most parsimonious position of the Catefica fossil on the JM backbone tree of only extant taxa is as sister to *Hedyosmum* (fig. 6*A*), and this is one of its most parsimonious positions in all other analyses. Furthermore, the addition of *Canrightia* and *Pseudoasterophyllites* eliminates two of the four most parsimonious positions found with the DE backbone, namely those as sister to the Chloranthaceae-*Ceratophyllum* clade and to Chloranthaceae (fig. 7*B*). These results suggest that the more basal placements are the results of parallel reductions on the long branches leading to *Ceratophyllum* and *Hedyosmum* and of the paucity of characters in the highly reduced pollen of *Ceratophyllum*.

Analyses of *Hedyflora* as defined by female flowers and pollen (fig. 8) show a similar pattern, without the connections to *Ceratophyllum*, which are due mainly to features of the staminate structures. The addition of *Canrightia* and *Pseudoasterophyllites* eliminates the position as sister to the *Ascarina-Sarcandra-*

Chloranthus clade, thanks to the endoreticulate endotesta of *Canrightia*, leaving only the relationship to *Hedyosmum*. The fact that *Hedyflora* also attaches to the stem lineage of *Hedyosmum* is consistent with the hypothesis that the Catefica fossil and *Hedyflora* represent the same "transitional" stem relative of *Hedyosmum*, but they could equally well be successive branches from the stem lineage. A similar position has been inferred for the middle–late Albian leaf fossil *Todziaphyllum* from northeastern Spain (Sender et al. 2021).

Assuming that the Catefica fossil is related to *Hedyosmum*, its aperture variation may document a labile intermediate stage in the origin of the typical four- to six-armed *Asteropollis* pollen type. This suggests that it may have diverged further down the stem lineage of *Hedyosmum* than middle Albian *Asteropollis* and pollen from the early Albian *Hedyflora* mesofossils that have a predominantly four-armed sulcus. Together, these fossils also reveal considerable lability in pollen size among Early Cretaceous *Hedyosmum* relatives: from 13–17 μm in the Catefica fossil to 19–32 μm in *A. asteroides* from the middle Albian (Hedlund and Norris 1968).

Like the observations of Friis et al. (2019*b*) for *Hedyflora*, our results confirm that some trichotomosulcate pollen that might be assigned to *Jusinghipollis* is chloranthaceous and specifically is related to *Asteropollis* and *Hedyosmum*. It does not necessarily represent relatives of *Ascarina*, as is sometimes assumed. This might be taken as an argument for emending *Asteropollis* to include *Jusinghipollis*. However, the resulting genus might be too heterogeneous, given the diverse sculpture of the pollen assigned to *Jusinghipollis* and the sporadic occurrence of trichotomosulcate grains in other predominantly monosulcate extant groups with globose (spheroidal) pollen, such as Canellaceae (Wilson 1964) and Saururaceae (Smith and Stockey 2007).

There are also practical reasons to keep *Asteropollis* and *Jusinghipollis* separate. Even if pollen grains with four sulcus arms first occurred as intraspecific variants in a trichotomosulcate line, such grains may have stratigraphic value. The occurrence charts of Heimhofer et al. (2007) show *Asteropollis* as ranging through the Aptian and Albian in Portuguese marine sections, but the Aptian specimens are all trichotomosulcate, while those with a four-armed sulcus appear in the early Albian (U. Heimhofer, personal communication to Doyle and Endress [2014]). This generalization appears to hold for grains identified in the literature as *Asteropollis* from other areas (Doyle and Endress 2014, 2018). Future investigations of dispersed pollen and mesofossils with associated pollen should continue to clarify both the evolutionary and stratigraphic significance of Early Cretaceous Chloranthaceae.

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