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Fossil *Kajanthus lusitanicus* gen. et sp. nov. from Portugal: floral evidence for Early Cretaceous Lardizabalaceae (Ranunculales, basal eudicot)

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Abstract

A new fossil flower, *Kajanthus lusitanicus* gen. et sp. nov, is described from the Early Cretaceous (late Aptian–early Albian) Chicalhão site near the village of Juncal, western Portugal, based on a single coalified specimen. The flower is small, actinomorphic, trimerous and bisexual, slightly compressed and with floral organs tightly adhering. The perianth is organised in more than two whorls. The inner two whorls consist of six bulky, apparently fleshy parts. The outer perianth whorls consist of narrow parts. There are six stamens, arranged in two whorls. The filaments are thick and anthers tetrasporangiate. The pollen sacs are protruding with extrorse dehiscence. Pollen observed *in situ* is tricolpate, tectate, finely punctate-perforate, compressed and more or less spherical in polar view with a diameter of about 15 μm. The gynoecium is superior and composed of three free carpels. Non-destructive virtual sectioning of the single flower using synchrotron radiation X-ray tomographic microscopy revealed the presence of several curved ovules in each carpel, arranged in two longitudinal rows on marginal placentae. The character suite of the *Kajanthus* flower is only found in extant Lardizabalaceae (Ranunculales), where it is particularly close to *Sinofranchetia*, a monotypic genus that is now endemic to China.

Keywords: *character mapping, eudicots, Figueira da Foz Formation, fossil flowers, Lardizabalaceae, mesofossils, Ranunculales, synchrotron radiation X-ray tomographic microscopy (SRXTM), tricolpate pollen*

Most present day angiosperm diversity is comprised in the eudicot clade, a well-supported monophyletic group that according to current phylogenetic models is the last major clade of angiosperms to diverge following the divergence of basal grade angiosperms (Amborellaceae, Nymphaeales, Austrobaileyales), the Chloranthaceae, magnoliids and the monocots (APG III 2009). Fossil evidence suggests an emergence of all these groups within a narrow timeframe during the Early Cretaceous (Friis et al. 2011). The earliest records of triaperturate pollen that unify the eudicots are dispersed pollen grains from around the Barremian–Aptian boundary (Early Cretaceous, about 126 million years ago) shortly after the appearance of monoaperturate pollen with distinct angiosperm features in the Valanginian–Hauterivian (Hughes & McDougall 1990; Penny 1991; Doyle 1992; Pedersen et al. 2007; Friis et al. 2011). The fossil record suggests that Early Cretaceous eudicots all belong to basal grade eudicots (see also Drinnan et al. 1991; Crane et al. 1993; Pedersen et al. 1994, 2007; von Balthazar et al. 2005), while the major radiation of core eudicots characterises the Late Cretaceous (see references in Friis et al. 2011).

In this work, we describe the discovery of a new fossil flower, *Kajanthus lusitanicus* gen. et sp. nov., from the Early Cretaceous (late Aptian–early Albian) of the Chicalhão opencast clay pit complex

close to the village of Juncal, western Portugal, corroborating this orderly pattern of eudicot diversification. The fossil flower has tricolpate pollen *in situ* and shows features that clearly place it among the basal most eudicots in the family Lardizabalaceae (Ranunculales). The fossil flower is particularly close to flowers of extant *Sinofranchetia* (Diels) Hemsl., a monotypic genus that is now endemic to China. *Kajanthus lusitanicus* is the oldest member of the Lardizabalaceae and represents the first record of the family in the Cretaceous of Europe. It is also the oldest fossil that can be placed unambiguously in a modern ranunculalean family. *Sinofranchetia* and most other extant members of the Lardizabalaceae are twining vines typically growing under fully-humid or summer-rain (monsoon) warm-temperate climates with hot summers (Wu & Kubitzki 1993). Today, the entire family comprises 35–50 species in seven genera (five monotypic) showing an intriguing disjunction (Wu & Kubitzki 1993; Qin 1997; Chen & Shimizu 2001; Christenhusz 2012). Most genera and species are restricted to East Asia and 20 species are endemic to China (Qin 1997); two (monotypic) genera occur in South America: *Boquila* Decne. in Chile and adjacent Argentina, and *Lardizabala* Ruiz et Pav. in Chile and the Juan Fernandéz archipelago. Like their Asian counterparts, the South American species thrive in evergreen to semi-deciduous forests (Christenhusz 2012).

Material and methods

Geology and preparation

The fossil flower described here is based on a single specimen (P0093) extracted from a dark-grey mudstone collected in the Chicalhão opencast clay pit complex close to the village of Juncal, in the Estremadura region, western Portugal (39° 35′ 34.8″ N; 08° 54′ 19.2″ W). The plant bearing sediments at the Chicalhão site are assigned to the Famalicão Member of the Figueira da Foz Formation and considered to be of late Aptian–early Albian age. For a more detailed account of the geology and preparation of the Chicalhão plant fossils, we refer to Mendes et al. (2014), who also give an overview of the vegetational composition of the plant fossils recorded from the Chicalhão site. Currently, more than 60 taxa of palynomorphs and plant mesofossils have been reported. The dispersed palynoflora is dominated by conifer pollen and fern spores, while angiosperm pollen is subordinate. In contrast, the mesofossil assemblage is dominated by angiosperms. The angiosperm component is mainly dispersed fruits and seeds, but the mesofossil flora also contains a few informative angiosperm flowers (Mendes et al. 2014), including the fossil flower described here.

The fossil flower was mounted on an aluminium stub, coated with gold for 60 seconds in a sputter coater and examined using a Hitachi Field S-4300 scanning electron microscope at 2kV at the Swedish Museum of Natural History, Stockholm. After scanning electron microscopy (SEM) studies, the fossil flower was remounted on a brass stub for non-destructive synchrotron radiation X-ray tomographic microscopy (SRXTM) at the Tomography Station of the Materials Science Beamline (TOMCAT), Swiss Light Source at the Paul Scherrer Institute, Villigen, Switzerland (Stampanoni et al. 2006). The tomographic dataset was acquired using a 20× objective and coupled to a 20 µm thick LAG:Ce (lutetium aluminum garnet:cerium) scintillator screen with an isotropic pixel size of 0.325 µm. Reconstructions were made using the Avizo® software package (for further technical details see references in Friis et al. 2014). Following SRXTM, the flower was again remounted and recoated with addition 60 seconds gold coating for additional SEM examinations. The SEM-micrographs were improved using the Adobe Photoshop® software package to enhance contrast and remove stain from the background.

The specimen used for the present study is housed in the Earth Sciences Department, Technology and Sciences College, New University of Lisbon, Portugal.

Assessment of the systematic position of Kajanthus

To establish the systematic affinities of the fossil flower, we included our fossil in the morphological matrix of Doyle and Endress (2010; D&E matrix in the following). Based on the matrix, pairwise mean morphological distances were calculated using PAUP★ (Swofford 2002) after exclusion of all characters that are not observable in the fossil. A zero morphological distance (MD = 0) means that two taxa do not differ in any character, whereas a MD = 1 indicates that two taxa differ in all (scored) characters. The resultant distance matrix was then used to infer a neighbour-net splits graph (NNet, Bryant & Moulton 2002, 2004) with SplitsTree 4 (Huson & Bryant 2006). A NNet is a two-dimensional (planar) network that can depict an ambiguous signal in a given data set (Bryant & Moulton 2004; Morrison 2005). Because of the high levels of homoplasy in the D&E matrix, the signals from individual characters in the matrix are often

incompatible, which precludes relying on dichotomous trees to establish phylogenetic relationships of fossil taxa. For such cases, the NNet has provided a useful alternative (Denk & Grimm 2009; Friis et al. 2009). Given the exclusive placement of the fossil in the NNet with regard to the Lardizabalaceae (see Results section), no further analysis of this matrix was warranted.

Extant members of Lardizabalaceae for comparative studies of flowers and pollen were studied in the herbarium of the Swedish Museum of Natural History, Stockholm (S), and from literature (e.g. Wu & Kubitzki 1993; Qin 1997; Endress & Igersheim 1999; Zhang et al. 2005; Wang, Kirchoff et al. 2009; Wang, Ross Friedman et al. 2009; Christenhusz 2012; Hu et al. 2012). SEM-studies of pollen grains follow the standard procedure described for the fossil flower.

Molecular data compilation

Sequence data was harvested from NCBI GenBank by first exporting all data on Lardizabalaceae as GenBank flatfile, which was then processed with GBK2FAS (Göker et al. 2009) to extract information on sampled organisms and gene regions. Based on the data summary (Supplemental data File S1), six gene regions were selected, which cover all seven genera of the Lardizabalaceae as currently perceived: the nuclear-encoded 18S rDNA and ITS region comprising ITS1, 5.8S rDNA and ITS2, the plastid *matK*, *atpB* and *rbcL* genes, and the *trnL*/LF region comprising the *trnL* intron, the 3′ *trnL* exon, and *trnL-trnF* intergenic spacer. Data on each gene region was downloaded from GenBank and processed with GBK2FAS to generate FASTA-files. Sequences were aligned using MAFFT v. 6.935b (Katoh et al. 2005) using the option '--auto', which allows the programme to choose the optimal alignment algorithm based on the input data, and '--adjustdirection' were necessary. All alignments were visually checked, trimmed and controlled for unreliable data using MESQUITE v. 2.75 (Maddison & Maddison 2011). Full details can be found in the Supplemental data File S2. Based on the alignments, the 18S rDNA and *atpB* data were excluded from further analyses as they exhibited extremely low differentiating signals, which would be insufficient to infer phylogenetic trees (see figure in Supplemental data File S1).

Phylogenetic inferences

Phylogenetic trees were inferred under the Maximum Likelihood (ML) criterion based on the concatenated molecular sequence matrix using the latest stand-alone Windows-executable of RAxML v. 7.2.6 (Stamatakis 2006). Branch support was established using the fast implementation of non-parametric bootstrapping in RAxML (Stamatakis et al. 2008), the number of necessary bootstrap replicates determined by the extended majority rule consensus bootstop criterion (Pattengale et al. 2009). All analyses were run partitioned using up to ten partitions: (1) ITS1, (2) ITS2, (3–5) *matK* (1st, 2nd and 3rd codon position), (6–8) *rbcL*, (9) *trnL* intron, (10) *trnL-trnF* spacer. The 5.8S rDNA and 3′ *trnL* exon were excluded from all analyses due to the lack of differentiating signal.

Two sets of analyses were done: one based on species-consensus data and a second set of analyses based on genus-consensus data. For both cases, strict consensus sequences were used based on all available accessions of a species and genus, respectively. Each set of analyses included a comprehensive analysis of the concatenated data, six analyses with one gene region eliminated, 15 analyses with any possible combination of two gene regions eliminated, and six single-gene analyses. All input and output files are provided in Supplemental data File S2.

Character plot

Thirteen morphological traits that are variable within extant Lardizabalaceae and that can be observed in the fossil material have been scored. The Ancestral State Reconstruction Package implemented in MESQUITE was used to reconstruct possible ancestral states along the genus-consensus molecular tree under maximum parsimony (MP) with the standard parsimony model (all characters treated as unordered; details not shown) and under ML using Lewis' one-parameter Markov-model (Lewis 2001; see Results section). The ML framework allows establishing the proportional probability that a certain character-state could be found in the (hypothetical) ancestor of a given clade while considering not only the terminal states (like under MP), but also the genetic distances between the (hypothetical) ancestors and terminal taxa, which are notable in the case of Lardizabalaceae.

The phylogenetic position of the fossil was further investigated by linking the fossil to all possible branches of the genus-consensus molecular tree and recalculating the number of required parsimony changes (steps) for each alternative tree (see Friis et al. 2009).
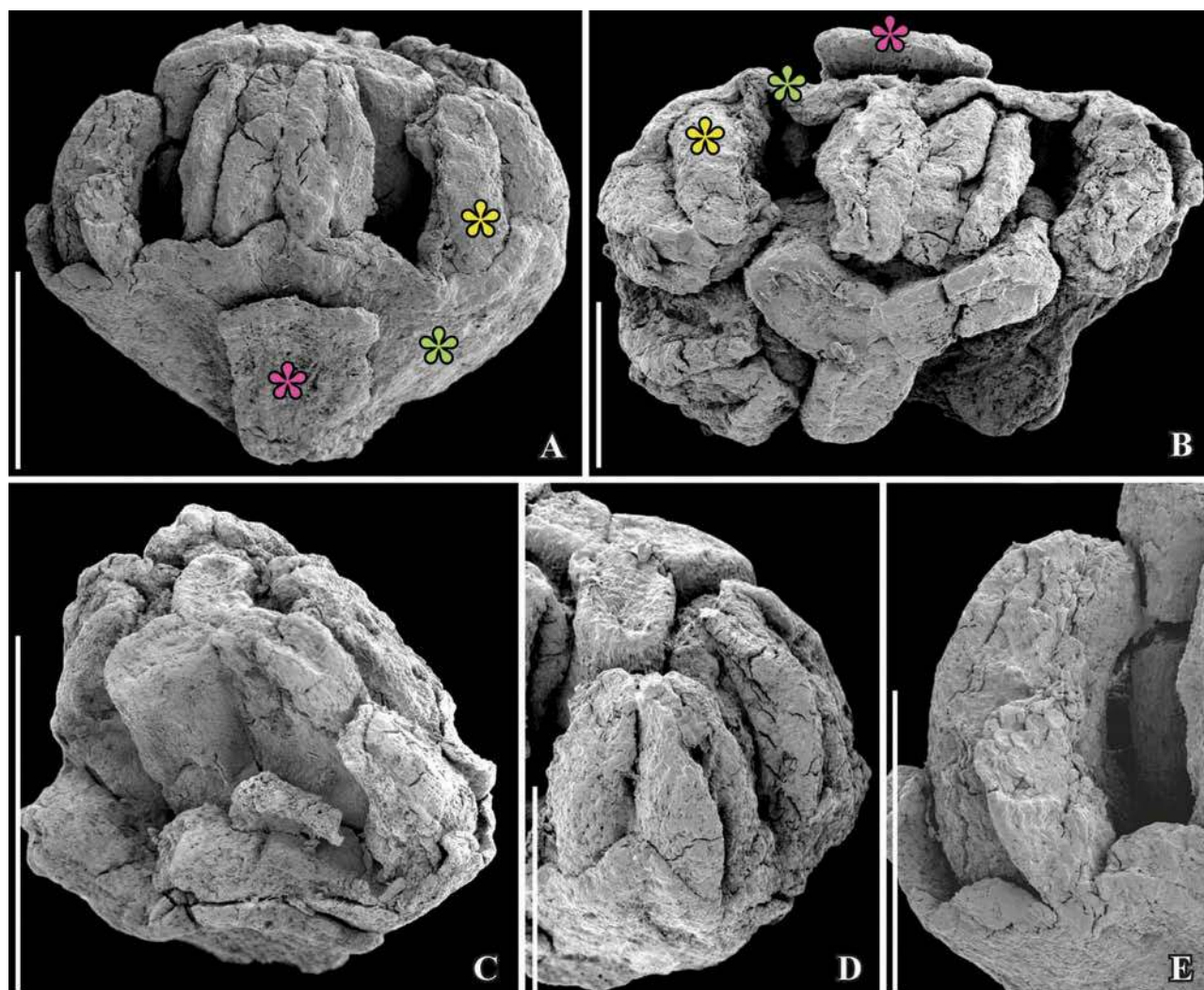
Figure 1. *Kajanthus lusitanicus* gen. et sp. nov. from the Early Cretaceous Chicalhão site, Juncal village, Portugal; holotype (P0093). SEM micrographs of flower morphology. **A.** Lateral view of flower showing one narrow perianth part of the outer perianth whorls (*purple asterisk*) followed by the tightly adpressed parts of the inner perianth whorls (*green asterisk*) enclosing the stamens and carpels. **B.** Apical view of flower showing the three free carpels closely adhering to each other along the ventral faces and the four entire stamens surrounded by the several whorls of perianth parts (*green and purple asterisks*). **C.** Oblique-lateral view of flower showing broken stamens and the three carpels; note that width and breadth of carpels are almost the same for the whole length of the carpels. **D.** Lateral view of flower showing the tetrasporangiate anthers with the adaxially placed pollen sacs. **E.** Lateral view of fragmented pollen sac showing the recurved shape of thecae; pollen grains can be observed where part of a pollen sac is broken. Scale bars – 250 μm (A, D), 200 μm (B, E), 500 μm (C).

Results

Systematic description

<div align="center">

Order Ranunculales
Family Lardizabalaceae
Genus *Kajanthus* gen. nov.

</div>

Derivation of generic name. — In honour of Kaj Raunsgaard Pedersen for his contribution to the study of the Early Cretaceous floras in Portugal.

Generic diagnosis. — Flower small, trimerous, with staminate and pistillate organs in the same flower. Floral parts tightly packed. Perianth composed of six bulky perianth parts arranged in two whorls surrounded by narrow perianth parts, apparently also in two whorls. Androecium of six stamens in two whorls on the same radii as the bulky perianth parts. Stamen base broad and bulky. Anther broadly attached to the stamen base. Anthers tetrasporangiate with projecting theca. Dehiscence extrorse by longitudinal slits. Pollen tricolpate with finely punctate tectum. Gynoecium superior composed of three free carpels closely adhering along the ventral faces. Carpels of almost the same breadth and width for their full length. Placentation marginal with many slightly curved ovules arranged in two longitudinal rows. Stigmatic surface at top of carpels undifferentiated.
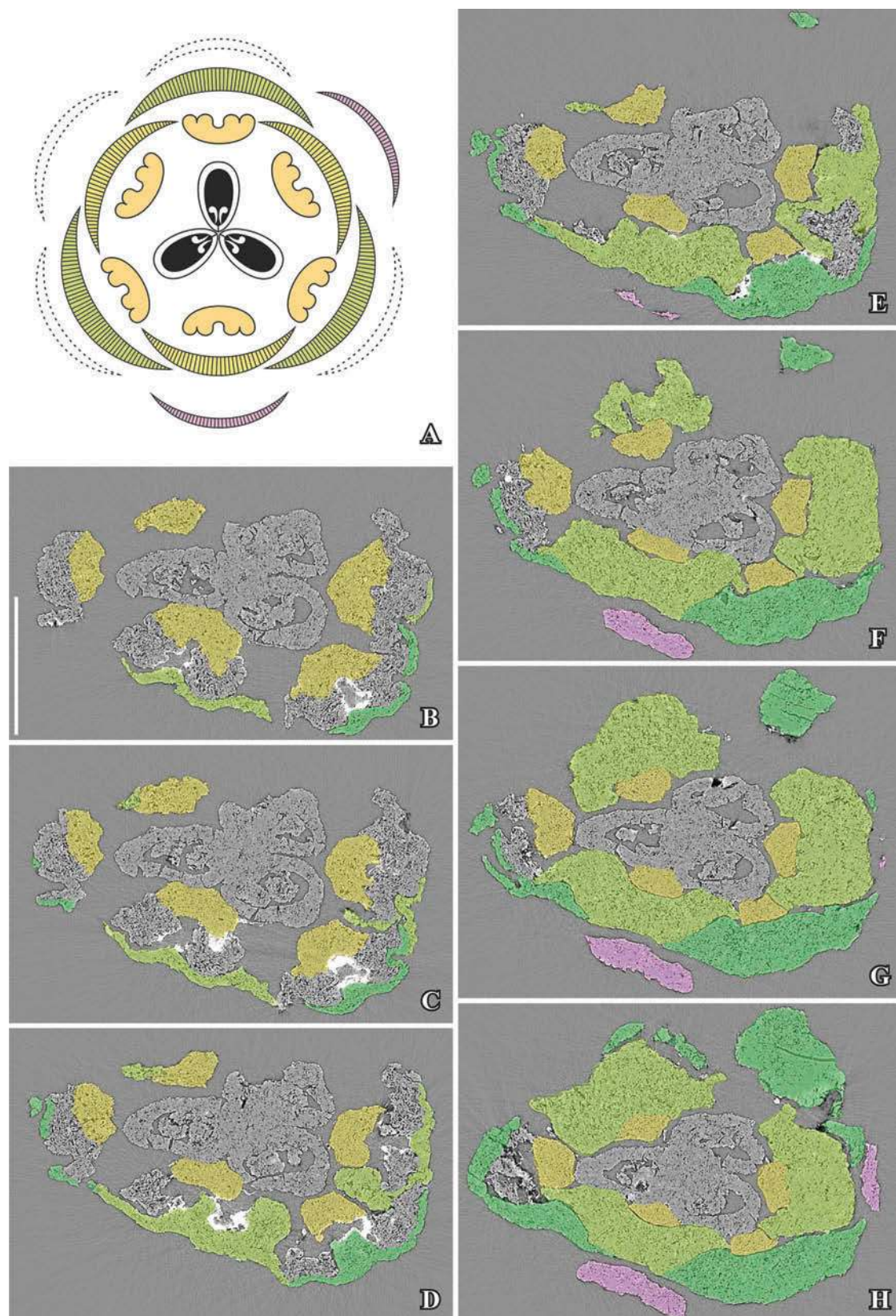
Figure 2. **A.** Floral diagram of *Kajanthus lusitanicus*. **B–H.** *Kajanthus lusitanicus* gen. et sp. nov. from the Early Cretaceous Chicalhão site, Juncal village, Portugal; holotype (P0093). SRXTM reconstructions (orthoslices in transverse sections) from apical part (**B**) to basal part of flower (**H**); outer perianth parts are marked with purple, the two inner whorls of perianth parts with green, connective of stamens with yellow; the extrose and protruding pollen sacs as well as the three carpels are not coloured. Scale bar – 250 μm.
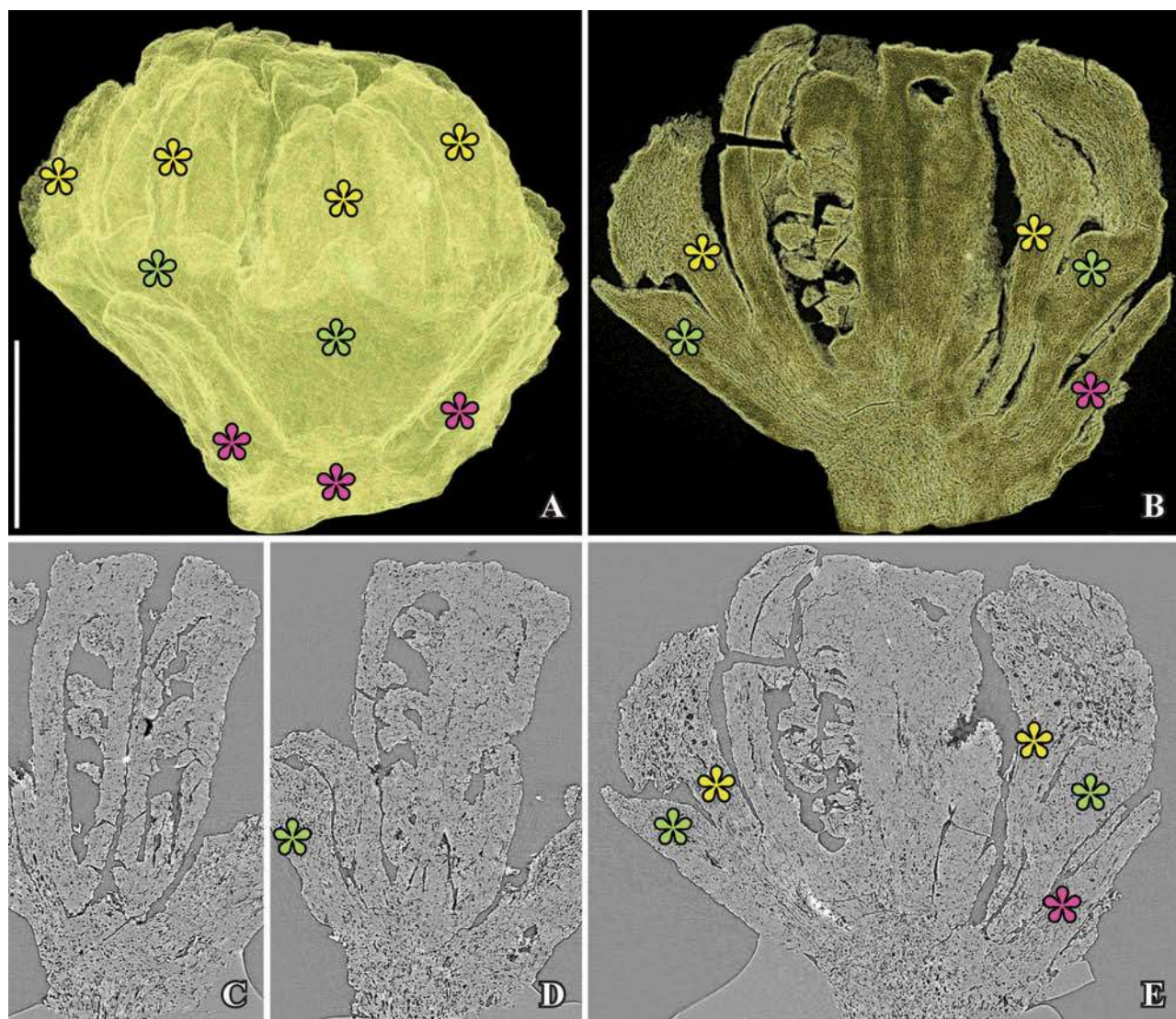
Figure 3. *Kajanthus lusitanicus* gen. et sp. nov. from the Early Cretaceous Chicalhão site, Juncal village, Portugal; holotype (P0093). SRXTM reconstructions. **A.** Transparent voltex rendering of flower showing remnants and scar from outer perianth parts (*purple asterisk*) surrounding the bulky stamens (*yellow asterisk*). **B.** Cut longitudinal voltex reconstructions (transparent renderings) through central part of flower showing two of the three free carpels in the centre, followed towards the outside of two stamens (*yellow asterisk*) with strongly protruding pollen sac, bulky perianth parts of inner perianth whorls (*green asterisk*) and outer parts (*purple asterisk*) and shape of inner perianth parts (*green asterisk*). **C, D.** Orthoslices in longitudinal section through two carpels showing the curved ovules and the flattened apex of the carpels. **E.** Orthoslices in longitudinal section through central part of flower showing carpels, stamens and perianth parts (see text to Figure 3B). Scale bar – 250 μm.

Kajanthus lusitanicus sp. nov.

Records. — Flower 4 (P0093); Mendes et al. 2014, p. 70, plate III, 5–6.

Specific diagnosis. — As for the genus.

Dimensions. — Flower about 0.85 mm long and 0.9 mm wide; stamen filament about 0.28 mm long and about 37 μm wide; anther about 0.26 mm; carpel about 0.45 mm long and 0.14 mm wide. Pollen about 15 μm in equatorial diameter.

Derivation of the specific epithet. — The specific epithet *lusitanicus* is derived from Lusitania, the name for the imperial Roman province covering the central part of Portugal, where the specimen was collected.

Holotype. — P0093, figured here in Figures 1–4.

*Type locality.* — Chicalhão opencast clay pit complex close to the village of Juncal, Estremadura region, western Portugal (39° 35′ 34.8″ N 08° 54′ 19.2″ W).
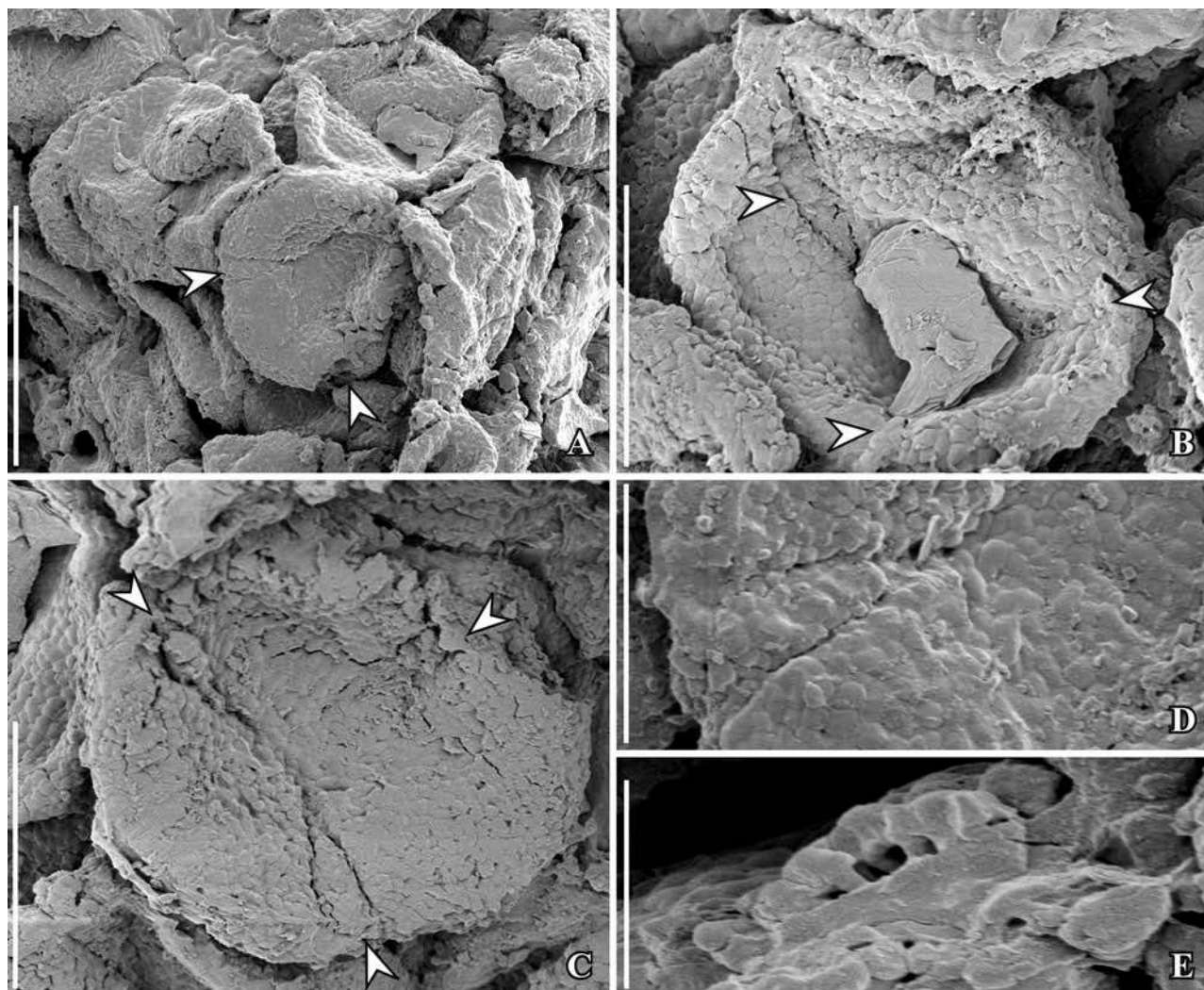
Figure 4. *Kajanthus lusitanicus* gen. et sp. nov. from the Early Cretaceous Chicalhão site, Juncal village, Portugal; holotype (P0093). SEM micrographs of *in situ* tricolpate pollen grains. **A.** Group of strongly flattened and somewhat folded pollen grains inside the broken pollen sac; two apertures in one grain are marked with arrowheads. **B, C.** Pollen grains in polar view showing the circular shape, triaperturate organisation, perforate-punctate pollen wall and verrucate aperture membrane; apertures are marked with arrowheads. **D.** Detail of pollen grain showing the verrucate colpus membrane and perforate-punctate, non-striate surface of the pollen wall. **E.** Fragmented pollen grain showing tectum supported by scattered columellae. Scale bars – 20 μm (A), 10 μm (B, C), 5 μm (D), 2 μm (E).

Type stratum and age. — Famalicão Member of the Figueira da Foz Formation; Early Cretaceous (late Aptian–early Albian).

Description and remarks on the species. — The species is based on a single fossil flower. The flower was found isolated in the sample without remnants of stalk or any indication on how the flower was borne on the plant. It is slightly compressed. The floral organs, particularly the perianth parts, are tightly coherent and the basal part of the flower is not completely preserved obscuring the outer series of the perianth. Further, the specimen is coalified with very few details preserved at the cellular level. Despite the preservational nature of the specimen,

critical details of organisation are clear from SRXTM reconstructions and SEM observations such as arrangement and number of floral parts, placentation and position of ovule, allowing a reconstruction of the new flower.

The flower is small, about 0.85 mm long and 0.9 mm wide, trimerous, actinomorphic and bisexual with both stamens and carpels present (Figures 1–3). The floral organs are arranged in whorls with more than two whorls of perianth parts, two whorls of stamens and one whorl of carpels (Figure 2). The flower is probably preserved in a pre-anthetic or early anthetic stage implied by the closed stamens containing pollen that appears mature.

The perianth is composed of several series of perianth parts. The perianth parts of the two innermost whorls are bulky and appear fleshy with very thick lamina that becomes thinner towards the apex and margins (Figures 1A, B, D, 2B–H, 3A–E). They are broadly ovoid with a broad attachment and slightly pointed apex (Figure 1A, D). The outer perianth parts are poorly preserved, but two broken parts show a narrow elongate shape (Figure 3A, B). Scars from other parts (Figure 3A) indicate the presence of six outer organs, apparently in two whorls. We first interpreted these organs as bracts (Mendes et al. 2014), but the position of the organs and their scars show that they are instead perianth parts.

The androecium consists of six stamens arranged in two whorls alternating with the perianth whorls (Figure 2A–H). Two of the six stamens are fragmentarily preserved with only the basalmost part present, while the remaining four stamens are entire. The stamens are about 0.48 mm long with a stout, apparently fleshy base, about 0.28 mm long. The stamen base is almost square in transverse section in the lower part and dorsi-ventrally flattened towards the junction with the anther. There is no distinct joint between stamen base and anther. The anthers are broad and inflexed, about 0.33 mm long and about 0.12 mm wide, more or less basifixed or slightly ventrifixed. They are tetrasporangiate, with the two thecae in an abaxial position and for their full length protruding beyond their junction with the connective. The four pollen sacs are relatively large. Dehiscence is extrorse by longitudinal slits. The connective between the two pairs of pollen sacs is massive and shallowly U-shaped in transverse section with the two pairs of pollen sacs widely separated (Figure 2B, C). Small hollow spaces are observed in the connective (Figure 2B–D).

SRXTM revealed pollen grains in all pollen sacs, but the resolution is too low to provide any details of apertures and pollen wall. Pollen grains are also seen in a fragmented pollen sac (Figure 4). All grains are strongly compressed. Many are folded and the apertures are typically difficult to observe and the pollen was first described as monoaperturate (Mendes et al. 2014). Additional SEM, however, documents a triaperturate organisation of the grains with elongated, broad colpi that have sharp pointed ends (Figure 4B, C). The colpus membrane is sculptured by low verrucae (Figure 4A–D). The grains are circular in polar view and about 15 μm in diameter. The pollen wall is tectate, finely perforated or punctate, non-striate. The outer surface of the exine shows a pattern of closely spaced, low and polygonal verrucae that are probably a result of shrinkage of the pollen wall. Fragmented grains show a tectum about 0.4 μm thick, supported by scattered and low collumellae, about 0.2 μm high. The structure and thickness of the inner layers of the pollen wall including the foot layer are not clear from the fragments.

The gynoecium is apocarpous and the ovary superior (Figure 3B–E) consisting of three free carpels (Figures 1B, C, 2A–H). The carpels are tightly adhering to each other along the ventral faces (Figures 1B, C, 2A–H, 3B, D, E). The carpels are about 0.45 mm long and of almost the same dimension in width and breadth for their whole length, up to about 0.2 mm wide (dorsi-ventral extension) and 0.15 mm broad (lateral extension). The carpel wall is about 0.035 mm thick with a smooth inner and outer surface. The apex of the carpel is flat.

There is no style and the stigma is sessile on the flat apex of the carpels, almost as wide and broad as the carpel in the ovary region (Figure 1B, C). The margins of the stigma are slightly raised leaving a low, flattened depression in the centre. The surface is covered by a secretion-like substance and the stigma may have been wet and non-papillate.

The ovules are small, not filling the locules (Figures 2B–H, 3C–E). There are about 15 ovules per carpel arranged in two rows close to the carpel margin extending from the base of the ovarial cavity to immediately below the stigma. The ovules are curved and have the appearance of anatropous/ campylotropous organisation.

Character scoring and phylogenetic analysis

The presence of tricolpate pollen unequivocally places *Kajanthus* among eudicot angiosperms. The character suite observed in the fossil flower and the *in situ* pollen shows a close match to flowers and pollen of extant Lardizabalaceae. In the scored D&E matrix characters, *Kajanthus* is virtually indistinct from Lardizabalaceae (Figure 5). Since the Lardizabalaceae are markedly distinct from all other taxa in this matrix, *Kajanthus* can unequivocally be considered an Early Cretaceous member of the family.

A molecular-phylogenetic framework for discussing the position of *Kajanthus* within the Lardizabalaceae has been compiled (Figure 6). We follow the systematic treatment of Christenhusz (2012), who included *Holboellia* Wall. in *Stauntonia* DC. Extant Lardizabalaceae falls into four genetically distinct sub-lineages: the monotypic East Asian *Sargentodoxa* Rehder et E.H. Wilson, *Decaisnea* Hook.f. et Thomson, *Sinofranchetia*, and a South American–East Asian 'crown clade' comprising the remaining four genera: *Akebia* Decne., *Boquila*, *Lardizabala* and *Stauntonia*. The two sub-clades
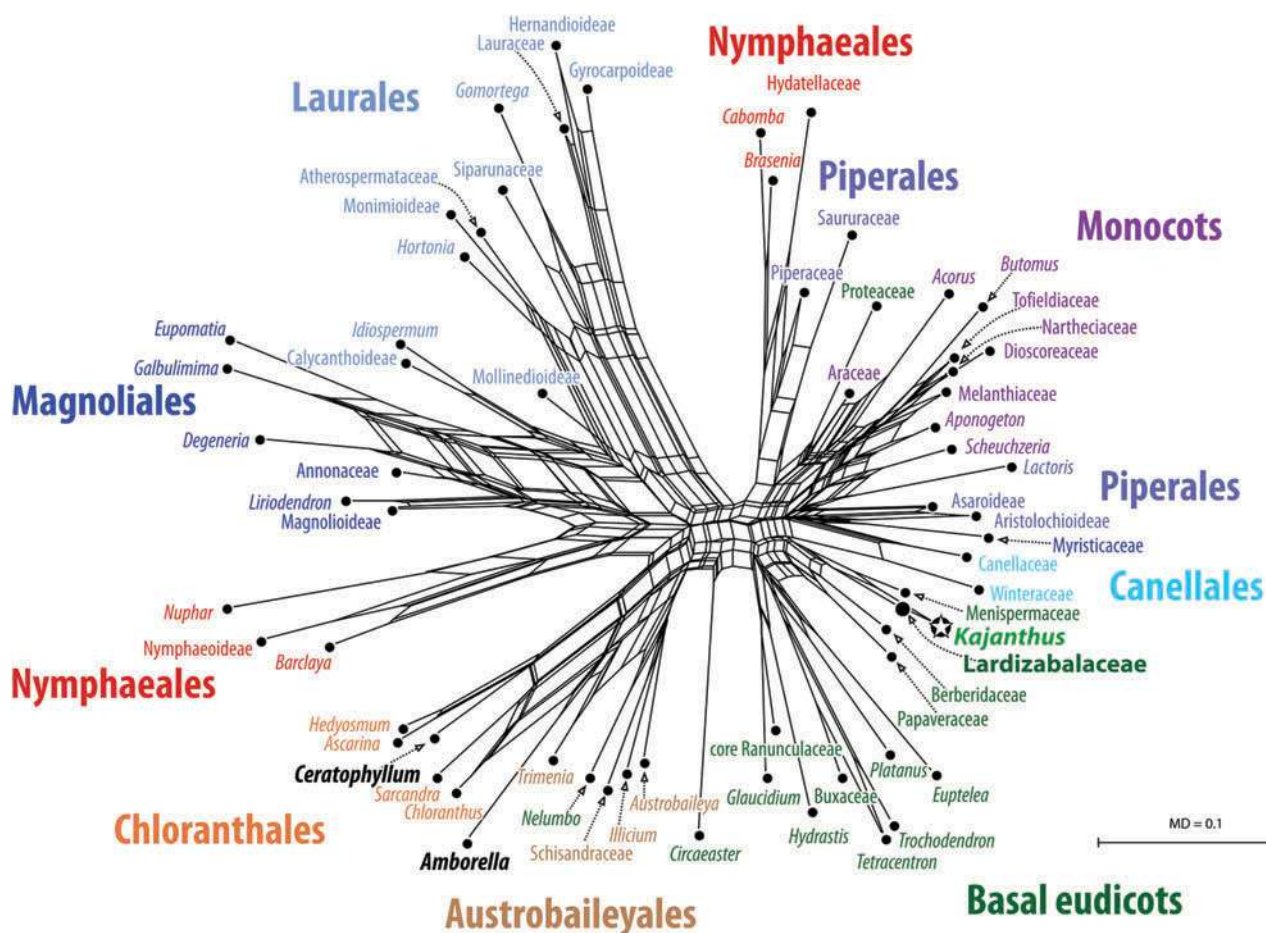
Figure 5. Neighbour-net splits graph based on mean morphological pairwise distances (MD) showing the systematic affinity of *Kajanthus* to the Lardizabalaceae. Pairwise distances were calculated based on characters in the matrix of Doyle and Endress (2010) that could be observed in *Kajanthus*. The signal in these characters is strong enough to differentiate between major groups of (basal) angiosperms (annotated in the graph by corresponding colours). The graph also shows that the Lardizabalaceae are distinct from all other taxon in the matrix and highly similar to *Kajanthus*.

within this 'crown clade' accommodate the East Asian and South American species, respectively. Within the East Asian sub-clade, the species of *Akebia* and *Stauntonia* are grouped, but intra-generic relationships are highly ambiguous. The three sequenced South American species, two of which are now considered co-specific, are genetically more divergent than their more diverse East Asian relatives.

Shared morphological and anatomical traits between *Kajanthus* and all extant Lardizabalaceae include trimerous perianth, trimerous androecium consisting of six stamens in two whorls, anthers with four protruding pollen sacs, anther dehiscence extrorse by longitudinal slits, pollen grains small to medium size, tricolpate, with tectate-perforate pollen wall, and carpels free, plicate, with sessile stigma. Additional 13 traits observed for the fossils are variable among the extant genera (see Appendix) and were used to place the fossil within the molecular-phylogenetic framework.

Rooting the genus-consensus sequence ML tree under the assumption that *Sargentodoxa* is the sister taxon to the remainder of the family (e.g. Loconte & Estes 1989; Hoot et al. 1995, 1999; Wang, Lu et al. 2009), ancestral states for (hypothetical) ancestors of two or more extant genera can be reconstructed (Figure 7). Due to the markedly long backbone and terminal branches and the character suites exhibited by the extant genera, ancestral states for most characters are ambiguous (both or all three states with equal proportional probabilities). The fossil *Kajanthus*, however, shares the character suite of *Sinofranchetia*. In the light of the ancestral state reconstructions, it appears unlikely that this identity is the result of convergent evolution. Instead, two possible evolutionary scenarios need to be considered: *Kajanthus* is an ancestor of *Sinofranchetia* and an Early Cretaceous member of the *Sinofranchetia* lineage, or the Early Cretaceous *Kajanthus* and the extant *Sinofranchetia* show the plesiomorphic
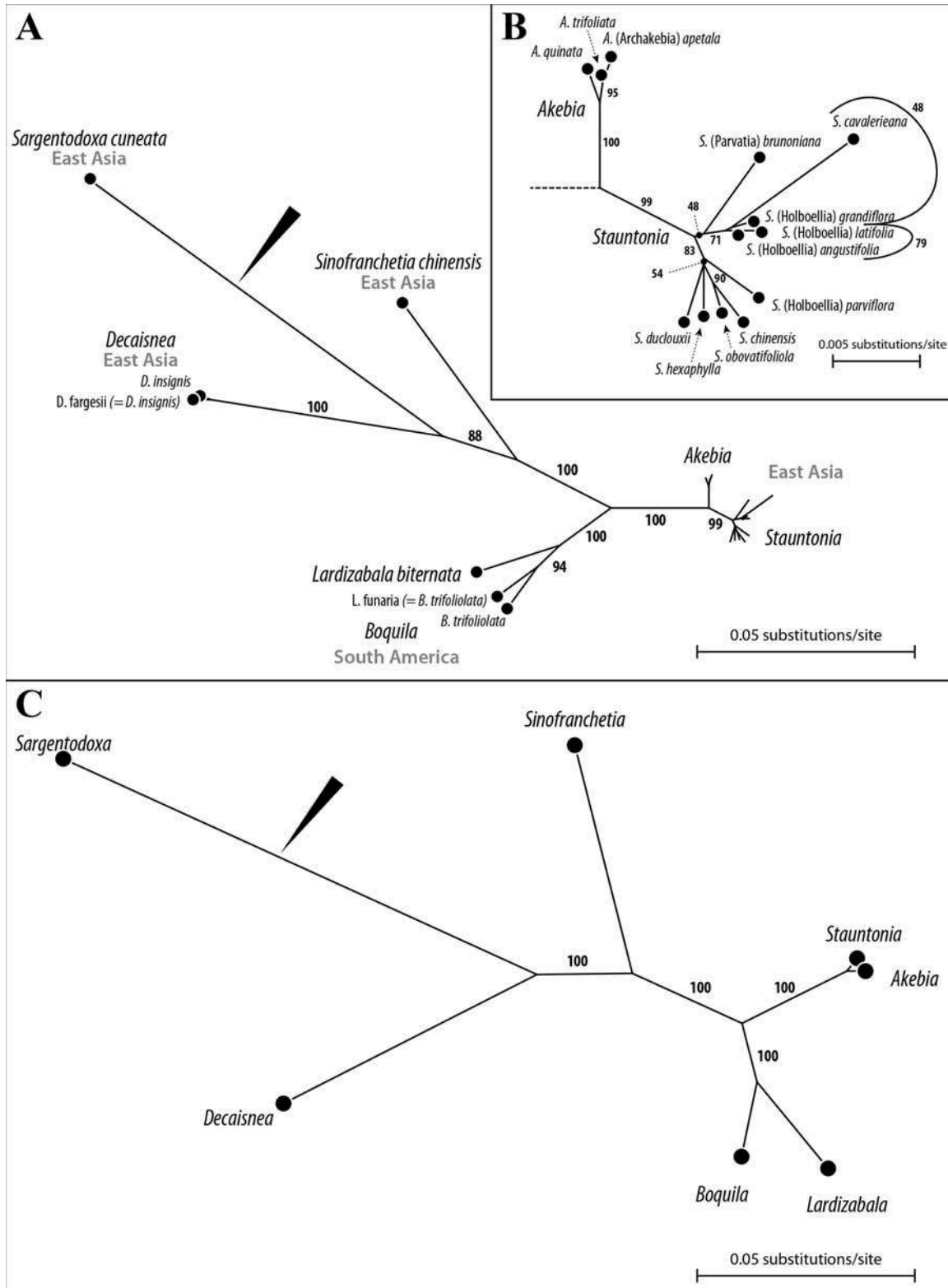
Figure 6. Maximum likelihood (ML) phylogenetic trees based on currently available family-wide molecular data of Lardizabalaceae (nuclear-encoded *ITS1* and *ITS2* rDNA spacers; plastid *matK* and *rbcL* genes, and *trnL* intron/*trnL-trnF* intergenic spacer region). **A.** Species tree illustrating the inter-generic versus intra-generic divergence in extant Lardizabalaceae; obsolete species names and genera printed in normal font (the currently valid synonym shown in italics). **B.** Close-up of the *Akebia-Stauntonia* sub-tree of (**A**); note the partly ambiguous relationships within the *Stauntonia* complex. **C.** Tree inferred based on genus-consensus sequences, which has been used to evaluate the position of Kaja*n*thus within the extant Lardizabalceae (see Figure 5 and text); note the strong resemblance with the species tree, particularly in regard to the branch-lengths, and unambiguous support along all branches. Numbers at branches indicate ML bootstrap support for each branch based on 450 (**A**) respectively 50 replicates (**C**). The currently accepted Lardizabalaceae root is indicated by black arrows.

Figure 7. 'Balls-and-stick' graphs illustrating the phylogenetic position of *Kajanthus* with respect to morphological (terminal sets of 'balls') and genetic differentiation (br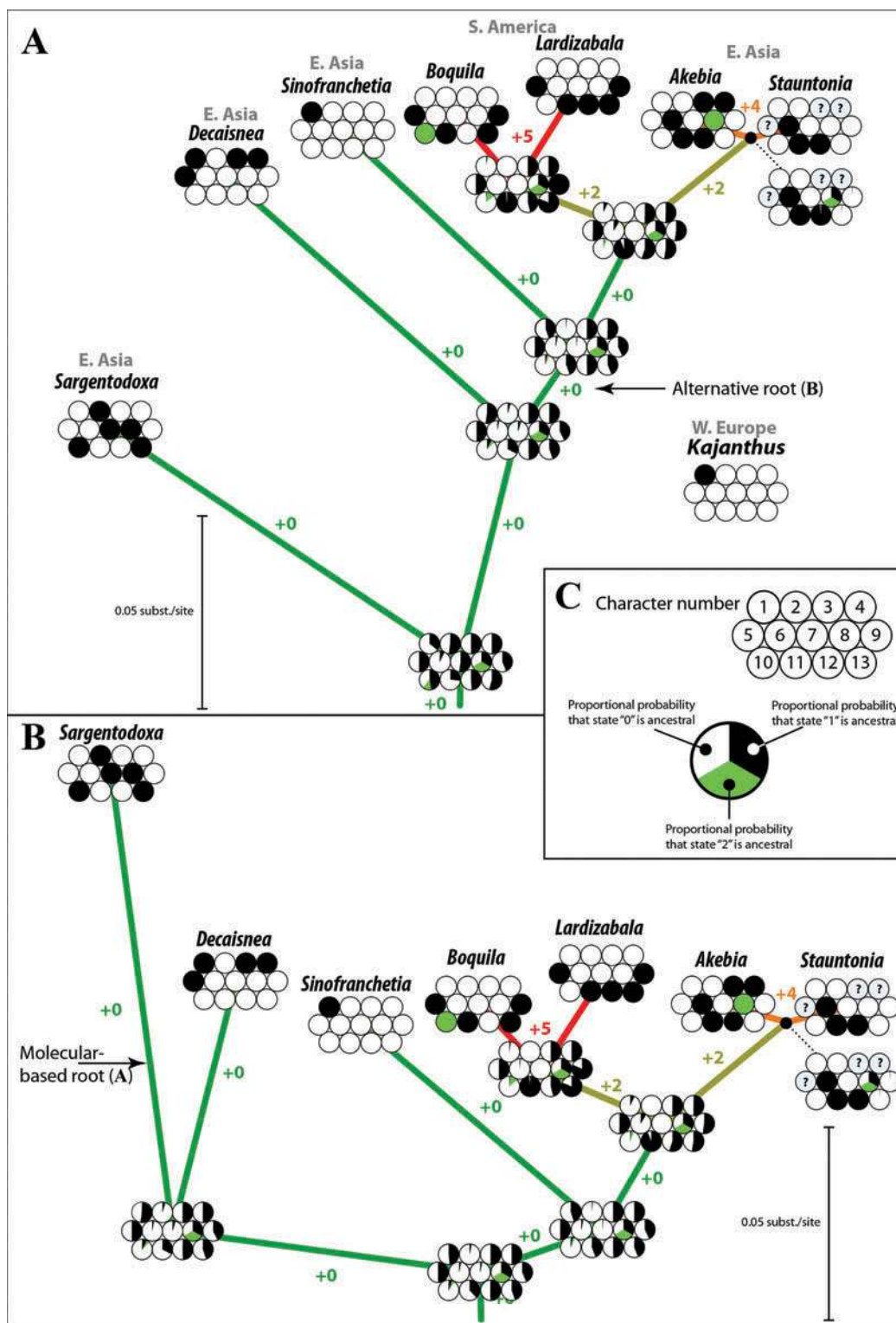anch-lengths; 'sticks') in extant Lardizabalaceae. **A.** Tree rooted using the currently accepted, outgroup-inferred Lardizabalaceae root. **B.** Alternatively rooted tree. **C.** Legend. Each character that was observed in the fossil *Kajanthus* and is variable among modern Lardizabalaceae is represented by a circle. Probable states for ancestors were reconstructed using Lewis' (2001) Maximum likelihood model for discrete characters, which estimates a probability for character state change. Due to intra-generic variability in *Stauntonia*, which now include species formerly assigned to *Hoelboellia* and *Parvatia*, three of the 13 characters (see Appendix) were treated as missing (grey 'balls' with question marks; ML ancestral state reconstruction cannot handle polymorphic terminals). Maximum parsimony optimization of characters along this rooted, molecular-based tree requires a total of 21 steps (character changes), if the fossil is excluded. Colouration of branches and numbers (+0 to +5) refers to the number of additional steps under parsimony, when the fossil is connected to the respective branch. Note that the fossil and the extant *Sinofranchetia* are identical based on the observable characters. Furthermore, the fossil can be connected to any deeper branch without increasing the number of reconstructed character changes.

morphology and anatomy of the Lardizabalaceae, from which all other morphologies are derived.

Discussion

Comments to the character suite observed in extinct Kajanthus *and extant Lardizabalaceae*

Sexuality. — Flowers of Lardizabalaceae are all functionally unisexual, typically with pistils in the functional male flowers and stamens in the functional female flowers strongly reduced. A bisexual organisation with morphologically almost identical pistillate and staminate flowers is, however, present in *Decaisnea* and *Sinofranchetia*. In the fossil flower, both stamens and carpels appear to be normally developed. Pollen grains are observed in the stamens and ovules in the carpels. Since only a single fossil flower has been discovered, it is unknown whether the *Kajanthus* plant could have produced more than one morphological type of flower. It is also difficult to assess whether the fossil flower was functionally bisexual or unisexual.

Merism. — All floral organs are arranged in trimerous whorls in the fossil flower and in all extant Lardizabalaceae except for *Sargentodoxa* that have many carpels in a spiral arrangement.

Perianth. — Flowers of *Decaisnea*, *Akebia* and some species of *Stauntonia* have one or two whorls of perianth parts. All other extant Lardizabalaceae have flowers with four whorls of perianth parts. In the fossil flower, there are two inner whorls of well-preserved perianth parts and remnants of additional outer parts. In the preliminary account of the fossil (Mendes et al. 2014), the outer parts were described as bracts. However, the position of the fragmented outer parts and scars from parts indicate the presence of six outer perianth parts, probably arranged in two whorls. Reduction in the number of perianth parts, particularly reduction or loss of petals, is a common trend in various groups of angiosperms (Endress 2011), and most likely evolved independently in the East Asian *Decaisnea* and *Akebia*/ *Stauntonia* (*s. str.*), whereas the remaining *Stauntonia* (species traditionally assigned to *Holboellia*), *Lardizabala*, *Boquila*, *Sinofranchetia* and the Cretaceous *Kajanthus* exhibit the plesiomorphic organisation.

In the extant flowers of Lardizabalaceae with four perianth whorls, the two outer whorls are usually described as sepals and the two inner whorls as petals. The petals are typically fleshy and nectariferous. In the fossil flower, the two inner whorls of perianth parts are markedly bulky, and they were probably also fleshy. The cellular preservation is, however, not sufficient to detect the presence of nectariferous tissue in the perianth parts.

Androecium. — Similar to most Lardizabalaceae, the fossil flower has stamens with fleshy or bulky stamen bases and connective, and strongly protruding pollen sacs. Stamen bases are free as in *Sargentodoxa*, *Sinofranchetia*, *Akebia* and some *Stauntonia*, while connate stamen bases occur in the other taxa.

Pollen. — Pollen of all extant genera of the Lardizabalaceae has been studied using SEM (Nowicke & Skvarla 1982; Qin 1997; Buchner and Halbritter 2000+; Halbritter 2000a+, 2000b+; Zhang et al. 2005; present study). Nowicke and Skvarla (1982) also studied the pollen wall ultrastructure of extant Lardizabalaceae (except for *Sinofranchetia*). The grains are uniform in shape, aperture configuration and structure of pollen wall. They are small to medium in size, tricolpate with tectate-perforate pollen wall (Figures 8–10). The tectum is distinct and columellae short and scattered. The tectum is smooth (non-striate) in *Sargentodoxa*, *Decaisnea*, *Sinofranchetia*, *Boquila* (Figure 8A–C) and *Lardizabala* (Figure 8D–G), while grains of *Akebia* (Figure 9) and *Stauntonia* (Figure 10) are finely or coarsely striate. The colpus membrane is finely verrucate in pollen of all extant taxa (Figures 8B, E, 9B, C, E, H, 10B, E) as it is in the fossil pollen. The grains observed in the fossil flower are identical to those of the extant taxa with non-striate tectum ornamentation. The weakly rugulate surface pattern of the fossil grains indicates some contraction, most likely due to fossilisation.

Related fossils in the Early Cretaceous

Teixeiraea lusitanica von Balthazar, Pedersen et Friis is another ranunculalean flower described from the Early Cretaceous of Portugal (von Balthazar et al. 2005). It was recovered from the Vale de Água locality close to the Chicalhão site and is of comparable age. The flower is similar to *Kajanthus* in having several series of perianth parts with both sepal-like and petal-like organs. *Teixeiraea* von Balthazar, Pedersen et Friis is, however, distinguished from *Kajanthus* in having the perianth parts in a spiral arrangement. The single flower of *Teixeiraea* is further distinguished from *Kajanthus* in being unisexual male with numerous stamens. The stamens have strongly protruding pollen sacs, but they are placed ventrally in contrast to the dorsal position of the pollen sacs in *Kajanthus*. The pollen grains of *Teixeiraea* are strikingly similar to those of *Kajanthus*, small, tricolpate, tectate-perforate with verrucate
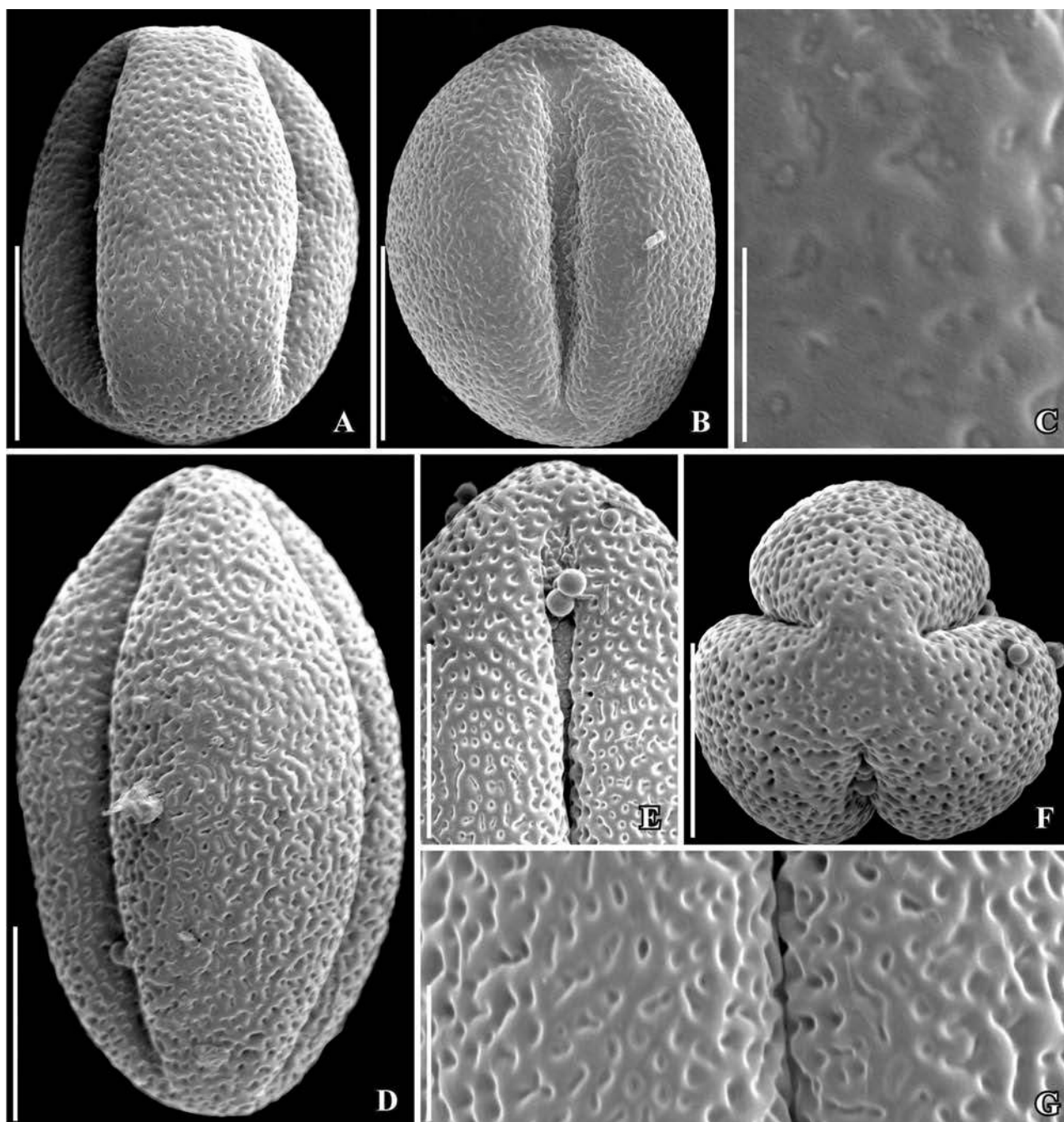
Figure 8. SEM images of dried pollen of extant Lardizabalaceae. **A–C.** *Boquila trifoliata* Decne. pollen in two different equatorial views showing general form and ornamentation of aperture membrane (**A, B**) and detail of pollen wall showing perforate-punctate non-striate tectum. **D–G.** *Lardizabala biternata* Ruiz. et Pav., pollen in two different equatorial views (**D, E**), in polar (**F**) view and detail of pollen wall showing perforate-punctate non-striate tectum (**G**). Scale bars – 10 μm (A, B, D–F), 1 μm (C) 5 μm (G).

colpus membrane and the combined floral and pollen features of *Teixeiraea* indicate affinity with extant members found in the Ranuculales, particularly in the clade that also encompasses the Lardizabalaceae (von Balthazar et al. 2005).

Dispersed pollen grains from Early Cretaceous palynological assemblages with a general morphology and wall structure similar to that of *Kajanthus*, *Teixeiraea* and several extant members of the Ranunculales (Lardizabalaceae, Menispermaceae and Berberidaceae) were described from the borehole Mersa Matruh 1 of Egypt as Puncttri-Fineret (Aptian–early Albian) by Penny (1991), who also referred to similar grains from contemporaneous
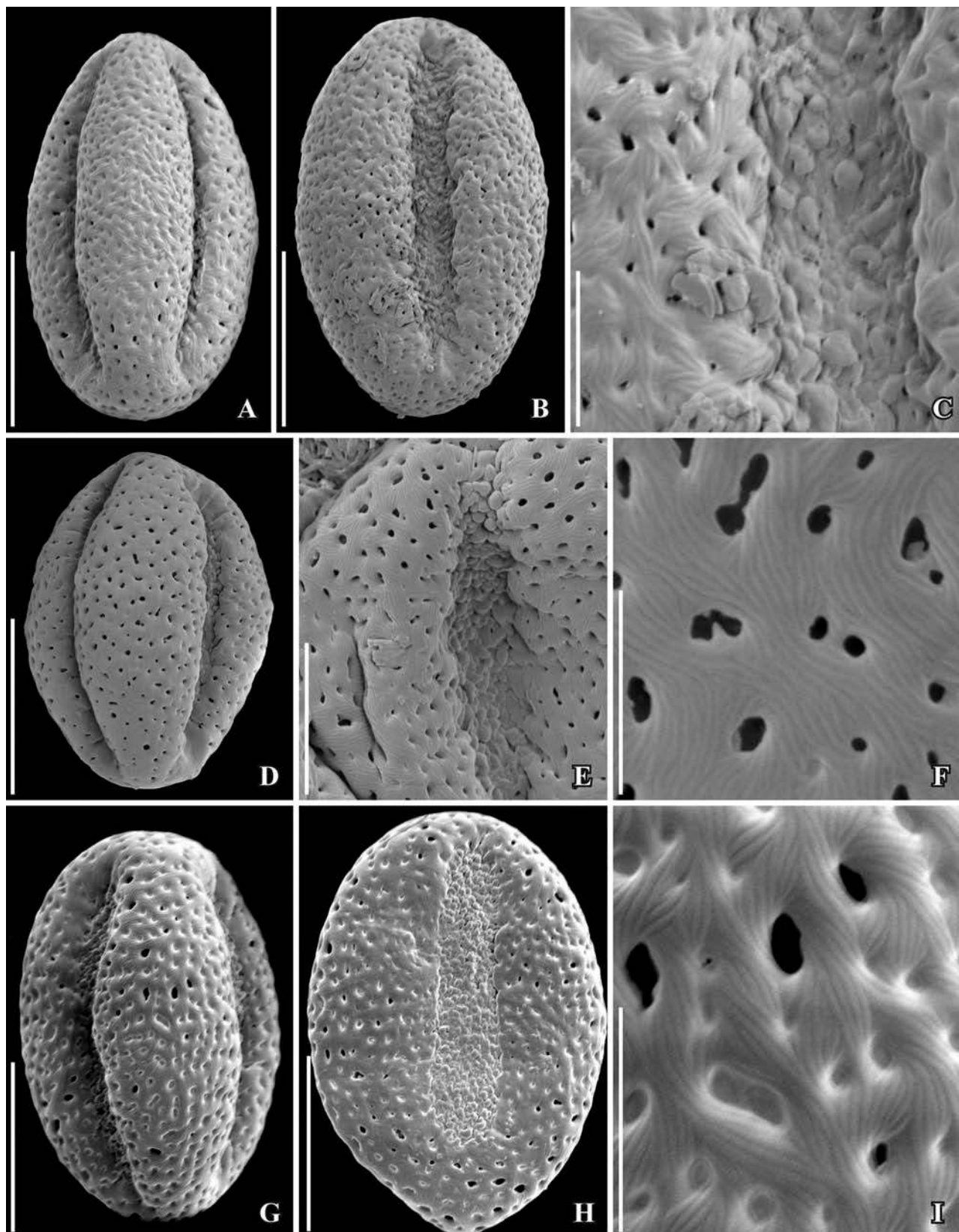
Figure 9. SEM images of dried pollen of extant Lardizabalaceae. **A–C.** *Akebia trifoliata* (Thunb.) Koidz. pollen in two different equatorial views (**A, B**) and details of pollen (**C**) showing finely perforate-punctate, striate tectum and verrucate aperture membrane. **D–F.** *Akebia* × *pentaphylla* Makino pollen in equatorial view (**D**), details of aperture with verrucate aperture membrane (**E**) and detail of pollen wall (**F**) showing finely perforate and striate tectum. **G–I.** *Akebia quinata* (Thunb. ex Houtt.) Decne. pollen in two different equatorial views (**G, H**) and detail of finely striate and punctate pollen wall (**I**). Scale bars – 10 μm (A, B, D, G, H), 2.5 μm (C, I), 5 μm (E), 2 μm (F).
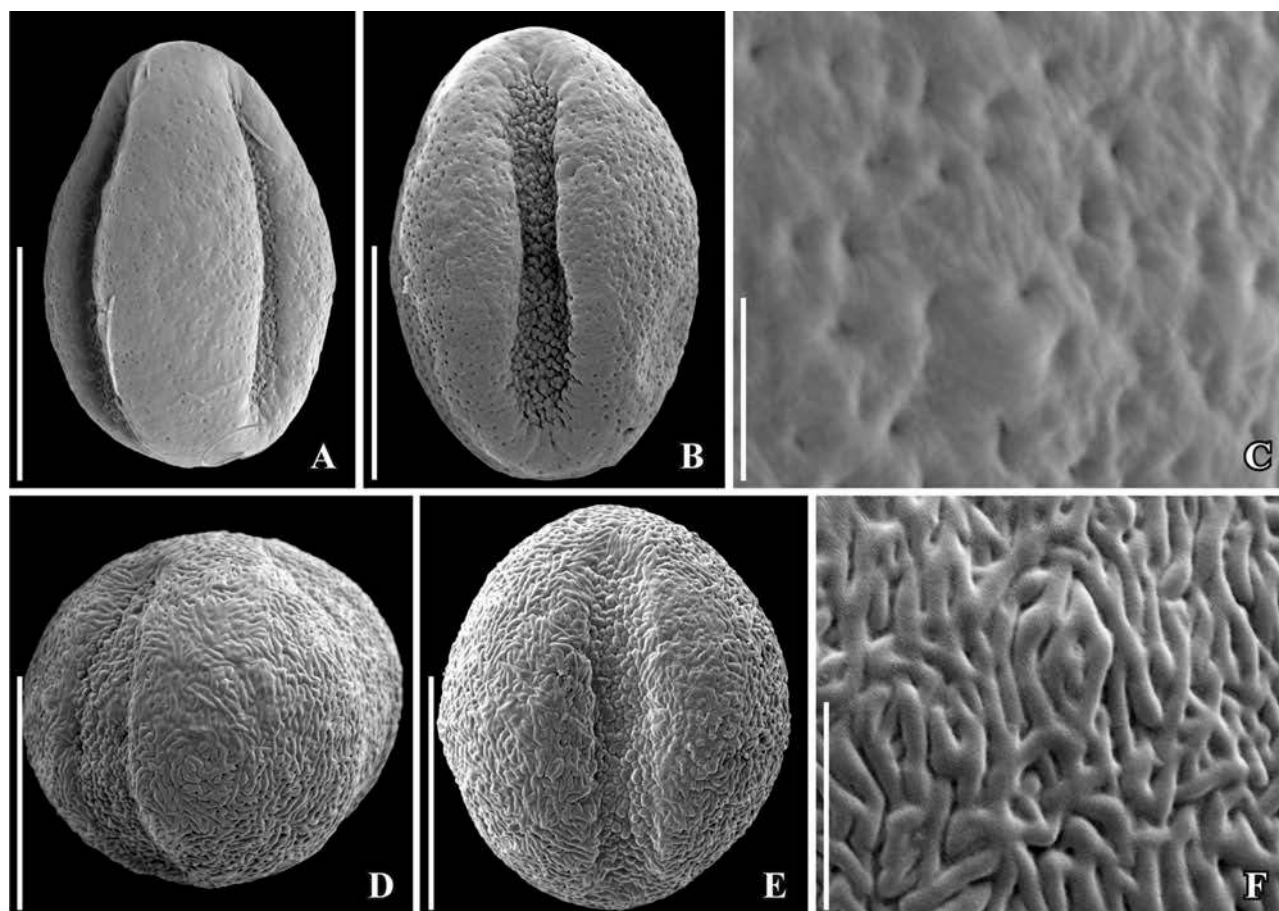
Figure 10. SEM images of dried pollen of extant Lardizabalaceae. **A–C.** *Stauntonia* (*Holboellia*) *latifolia* (Wall.) Christenh. pollen in two different equatorial views showing general form and verrucate aperture membrane (**A, B**) and details of pollen (**C**) showing finely perforate-punctate and delicate striate tectum. **D–F.** *Stauntonia hexaphylla* Decne. pollen in two different equatorial views showing general form and verrucate aperture membrane (**D, E**) and details of pollen wall (**F**) showing finely perforate-punctate and coarsely striate tectum. Scale bars – 10 μm (A, B, D, E), 1 μm (C), 2 μm (F).

deposits of Portugal. These may represent early Ranunculales, but further information of pollen wall is needed for more detailed evaluations of the dispersed pollen record.

Ecological and biogeographic implications

Extant members of the Lardizabalaceae are all woody and twining vines, except for *Decaisnea* that is an erect shrub (Wu & Kubitzki 1993; Qin 1997). Despite their geographic disjunction, they thrive under the same general climate and occupy similar general habitats: evergreen to semi-deciduous, broad-leaved forests ('laurel forests'; Schroeder 1998) with sufficient (or excess) precipitation during the growing season (Cfa, Cwa climates; Köppen 1936). In the summer-dry Csa climates of central Chile or Robinson Crusoe Island, the Lardizabalaceae are confined to forest and forest margins in the Andean foothills/foreland

(100–1000 m above sea level (a.s.l.); Christenhusz 2012).

Based on the combined sedimentological, palynological and mesofossil analyses of the Chicalhão deposits, the palaeolandscape of *Kajanthus* was interpreted as vast floodplain bordering a meandering river with an active belt of channels, levees and crevasse-splays. Cheirolepidiaceous conifers and schizaeaceous ferns usually used as proxies for drier climate are common elements of the palynoflora and are thought to be prominent members of the floodplain vegetation. Angiosperms were inferred to have grown in the more moist vegetation of the active, probably disturbed habitat closer to depositional basin (Mendes et al. 2014).

The inferred phylogenetic position of *Kajanthus* close to *Sinofranchetia* implies that *Kajanthus* may have had a climbing habit. Obviously, the habit of the *Kajanthus* plant cannot be established based on the floral features or the systematic position alone.

However, there are two findings of fossil wood from the Late Cretaceous of North America that document typically features of ranuculalean climbers. These include *Lardizabaloxylon cocculoides* Page (Page 1970) from the Panoche Formation of central California and *Atli morinii* Smith, Little, Cooper, Burnham et Stockey (Smith et al. 2013) from the Campanian of British Columbia. Both species are most closely linked to the Menispermaceae that also share several wood features with the Lardizabalaceae and are included in the same ranunculalean clade as the Lardizabalaceae. Studies of extant plants of early diverging angiosperm lineages suggest that only small structural shifts are required to attain climbing habit and that lianescence may have developed early in angiosperm history (Feild & Isnard 2013).

The three 'basal' (early diverging) members of the Lardizabalaceae (monotypic *Sargentodoxa*, *Decaisnea* and *Sinofranchetia*) are deciduous, while *Stauntonia* and the South American members of the 'crown clade' are evergreen. Species of *Akebia*, the less diversified sister genus of *Stauntonia*, are deciduous to semi-(sub-)evergreen. Thus, based on the phylogenetic position of *Kajanthus*, a deciduous habit may be inferred, but also in this case the ecological signal is not unequivocal, since the distributional ranges of the deciduous and evergreen species overlap.

The fossil record of the Lardizabalaceae is poor and cannot clarify details in the biogeographic history of the family or the present disjunct distribution. The present discovery of *Kajanthus* shows unequivocally that the family is old and that it was present in Europe already in the Early Cretaceous. If the current outgroup-informed root is correct (Figure 5) and the high similarity of *Kajanthus* and *Sinofranchetia* indicates a direct evolutionary relationship, the Lardizabalaceae must have diverged before 110 Ma, rendering molecular-inferred crown group ages (Anderson et al. 2005) at least 30–60 Ma too young. Alternatively, *Kajanthus* and *Sinofranchetia* share the plesiomorphic character suite of the family exhibiting the actual ancestral states in all observed characters. This would imply that the outgroup-inferred, molecular-defined, root is misinformed (because of ingroup-outgroup long-branch attraction). In this interpretation, *Kajanthus* marks the stem age of the family corroborating previous molecular datings. Based on the character suite of *Kajanthus* and the morphological and molecular differentiation of extant Lardizabalaceae as discussed earlier, we favour the interpretation of *Kajanthus* as a stem-group taxon linked to *Sinofranchetia* by symplesiomorphic morphological

traits. However, this hypothesis would need to be further tested including explicit dating approaches based on comprehensive molecular data on extant Lardizabalaceae, which are currently not available.

Findings of fossil seeds also document that the family was present and diversified in Europe in the Cainozoic with seeds of *Sargentodoxa* reported from the late Eocene, late Oligocene and middle Miocene of Germany (Mai 2001) and from the Miocene–Pliocene of Germany, France and Italy (Marinetto 2001), seeds of *Decaisnea* reported from the late Oligocene of Germany (Mai & Walther 1991) and seeds of *Akebia* reported from the late Miocene of Germany (Mai 1995, 2001). There is, however, no record in Europe that could confirm a continuous presence of the family from the Early Cretaceous to the Late Cainozoic. The family has also been reported from the Cainozoic of North America with seeds of *Sargentodoxa* occuring in the Miocene Brandon lignite (Tiffney 1993). The fossil wood of *Lardizabaloxylon cocculoides* from the Late Cretaceous of California and is probably menispermaceous (Page 1970).

Conclusion

The discovery of *Kajanthus lusitanicus* documents for the first time the presence of the Lardizabalaceae in the Cretaceous. This finding corroborates the importance of basal lineages of eudicots in the early radiation of angiosperms. The finding also implies that the original theory of a Laurasian origin of the family and subsequent migration to South America opposing the more recently invoked long-distance dispersal theory (Christenhusz 2012; Christenhusz & Chase 2013) requires renewed attention. Furthermore, the characters suite and age of *Kajanthus* calls into question the currently accepted, molecular-defined root of the Lardizabalaceae. *Kajanthus* may well represent a stem-group taxon, which would implicate that *Sargentodoxa* is nested within the Lardizabalaceae crown group.

Pollen of extant Lardizabalaceae may be difficult to identify in light microscopic studies, but fine details, such as striation, studied in SEM and transmission electron microscopy (TEM) and pollen wall ultrastructure (Nowicke & Skvarla 1982) have potential in palynological investigation of Cretaceous angiosperms and may fill the large gaps in the biogeographic history of the Lardizabalaceae.

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Supplemental data

Supplemental data for this article can be accessed here.

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Appendix

Scoring of morphological traits that vary in *Kajanthus* and modern genera of Lardizabalaceae (Figure 6). The scored matrix is included here in the Supplemental data. In case of unique character states, the deviating taxon is given.

Character 1: Seemingly bisexual flowers – [0] absent; [1] present.

Character 2: Receptacle (female) – [0] short; [1] enlarged (*Sargentodoxa* only).

Character 3: Number of perianth whorls – [0] four; [1] (one or) two. Character unstable among *Stauntonia* spp., hence scored as missing. Species originally described as *Hoelboellia* have consistently four perianth whorls; species of *Stauntonia*, as traditionally defined, can have two or four periant whorls.

Character 4: Petals – [0] present; [1] absent. Scored as missing for *Stauntonia*. Petals are always present in species originally addressed as *Holboellia*, but absent or inconspicous in *Stauntonia s. str.*

Character 5: Stamen fusion – [0] free; [1] connate. Character unstable among *Stauntonia* spp., hence scored as missing. Stamens can be free or connate in species traditionally assigned to *Hoelboellia*, but are always connate in *Stauntonia s. str.*

Character 6: Tectum ornamentation – [0] smooth; [1] striate; shared apomorphy of *Akebia* and *Stauntonia*.

Character 7: Phyllotaxis of carpels – [0] whorled; [1] spiral (*Sargentodoxa* only).

Character 8: Maximum number of carpels – [0] three; [1] up to 13 (*Akebia*, typically 3–8); [2] many (*Sargentodoxa*; 48–123).

Character 9: Stigma – [0] restricted; [1] elongated; shared apomorphy of South American genera *Boquila* and *Lardizabala*.

Character 10: Ovule number – [0] many; [1] few (*Boquila*); [2] one per carpel (*Sargentodoxa*).

Character 11: Ovule orientation (curvature) – [0] anatropous; [1] ortho-/hemitropous; shared apomorphy of South American–East Asian core clade (*Akebia, Boquila, Lardizabala, Stauntonia*).

Character 12: Placentation – [0] marginal; [1] laminar-diffuse.

Character 13: Carpel – [0] non-stipitate; [1] stipitate.