Seed morphology and anatomy and its utility in recognizing subfamilies and tribes of Zingiberaceae[1]

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ABSTRACT

- *Premise of the study:* Recent phylogenetic analyses based on molecular data suggested that the monocot family Zingiberaceae be separated into four subfamilies and four tribes. Robust morphological characters to support these clades are lacking. Seeds were analyzed in a phylogenetic context to test independently the circumscription of clades and to better understand evolution of seed characters within Zingiberaceae.
- *Methods:* Seventy-five seeds from three of the four subfamilies were analyzed using synchrotron based X-ray tomographic microscopy (SRXTM) and scored for 39 morphoanatomical characters.
- *Key results:* Zingiberaceae seeds are some of the most structurally complex seeds in angiosperms. No single seed character was found to distinguish each subfamily, but combinations of characters were found to differentiate between the subfamilies. Recognition of the tribes based on seeds was possible for Globbeae, but not for Alpinieae, Riedelieae, or Zingibereae, due to considerable variation.
- *Conclusions:* SRXTM is an excellent, non-destructive tool to capture morphoanatomical variation of seeds and allows for the study of taxa with limited material available. Alpinioideae, Siphonochiloideae, Tamijioideae, and Zingiberoideae are well-supported based on both molecular and morphological data, including multiple seed characters. Globbeae are well-supported as a distinctive tribe within the Zingiberoideae, but no other tribe could be differentiated using seeds due to considerable homoplasy when compared to currently accepted relationships based on molecular data. Novel seed characters suggest tribal affinities for two currently unplaced Zingiberaceae taxa: *Siliquamomum*

may be related to Riedelieae and *Monolophus* to Zingibereae, but further work is needed before formal revision of the family.

Keywords: aril; chalaza; embryo; ginger; micropyle; monocotyledon; operculum; seed coat; synchrotron based X-ray tomographic microscopy (SRXTM); testa.

INTRODUCTION

Seeds are an integral part of a plant, but detailed information about them and their utility in phylogenetic studies is limited. In a seminal work on dicotyledonous seed anatomy, Corner (1976:vii) stated, "…classification without seed-structure is unsound and, consequently, our knowledge of the evolution of flowering plants." Along with potentially clarifying systematic relationships between taxa, data on seed morphoanatomy are also fundamental to addressing the carpological fossil record, which in turn enlightens our understanding of evolution, paleoecology, paleobiogeography, and past climate change (e.g., Manchester and Kress, 1993; Collinson and van Bergen, 2004; Chen and Manchester, 2007; Collinson et al., 2012; Herrera et al., 2014). Understanding seed structure also enhances our understanding of biological features that may facilitate dispersal, inhibit or facilitate dormancy, and survivability – information particularly relevant to germplasm banks, which strive to preserve biodiversity (e.g., Boesewinkle and Bouman, 1995; Baskin and Baskin, 2001; Wada et al., 2011), and contribute considerably to the proper identification of commercially important or potentially important plants (e.g., Vaughan, 1970; Wu et al., 2014) and food security. Thus, seed anatomical studies are important in a variety of ways.

Zingiberaceae are an economically and ecologically important family of commelinid monocots with a center of diversity in Southeast Asia (Kress et al., 2002; Larsen, 2005). It is the largest and most species-rich family within Zingiberales and currently contains 52 genera and approximately 1600 species, with an average of 13 new taxa being described a year for the past two decades (The Plant List, 2013). Seeds of Zingiberales have been studied for more than a century (Tschirch, 1891; Humphrey, 1896; Netolitzky, 1926; Mauriszon, 1936; Takhtajan, 1985).

More recently they have been studied in search of potential pharmacognostical characteristics (see Liao and Wu, 1996 for a review), but little information is available on utilizing seeds as a source of data for systematics and often these studies are limited in scope to a few species or genera (Kimura and Yoshimura, 1968; Liao and Wu, 1996; Wu et al., 2014). Furthermore, many early studies were the subject of inter-familial comparisons based often on immature seeds, which do not demonstrate anatomical differences seen in later stages of development (e.g., Humphrey, 1896; see Takhtajan, 1985 for comparisons). One potential reason for the paucity of studies on zingiberalean seeds is the presence of a hard, brittle seed coat with phytoliths, making traditional paraffin embedding and microtomy difficult (Benedict, 2012; Benedict et al., 2015). The use of synchrotron based X-ray tomographic microscopy (SRXTM) to analyze the seeds provides high-resolution detail of seed coats and seed and embryo internal morphoanatomy. In addition, SRXTM is non-destructive and requires no specimen-altering preparations (e.g., critical point drying, rehydration, coating, etc.), which provides the opportunity to analyze rare material from herbarium specimens and to standardize anatomical observations across a wide range of taxa (Smith et al., 2009; Benedict et al., 2015).

Traditional circumscription of the Zingiberaceae by Schumann (1904), Holttum (1950), Burtt and Smith (1972), and Larsen et al. (1998) included four tribes (Alpinieae, Hedychieae, Globbeae, and Zingibereae). Recent work, based on molecular data, recognizes four subfamilies (Alpinioideae, Siphonochiloideae, Tamijioideae, Zingiberoideae) with tribe Zingibereae, tribe Globbeae, and *Monolophus* (formerly *Caulokaempferia*; Mood et al., 2014) nested within Zingiberoideae, and tribe Alpinieae, tribe Riedelieae, and *Siliquamomum* nested within Alpinioideae (Table 1). Tamijioideae are monospecific and Siphonochiloideae contain two

genera (Kress et al., 2002; Table 1). All subfamilies are well-supported based on molecular and morphological data, but many speciose genera (e.g., *Alpinia, Amomum,* and *Curcuma*) have been shown to be paraphyletic and/or polyphyletic (*Amomum*: Harris et al., 2000, Xia et al., 2004; *Alpinia*: Rangsiruji et al., 2000; Zingibereae: Ngamriabsakul et al., 2004; *Etlingera*: Pedersen, 2004; *Globba*: Williams et al., 2004; Alpinioideae: Kress et al., 2005, 2007; *Curcuma*: Záveská et al., 2012; Leong-Škorničková et al., 2015).

The family as a whole is easily separated from other Zingiberales families by possessing ligulate distichous leaves, flowers with a single dithecal stamen, and an often showy petaloid labellum formed from two or four staminodes (Simpson, 2010). Within Zingiberaceae, the plane of distichy along a leafy shoot separates Alpinioideae (perpendicular to the rhizome) from the other tribes (parallel to rhizome), but many other floral and vegetative characters previously used to distinguish tribes (e.g., locule number, presence/absence of lateral staminodes) are not unique to any particular subfamily or tribe (Kress et al., 2002). A single synapomorphic character was suggested to distinguish Riedelieae from Alpinieae: the presence of extrafloral nectaries approximately 2.5 cm above the petiole on the midrib or costa of the adaxial surface of the leaf (Kress et al., 2002). This character has indeed been documented for all members of Riedelieae (Mood, 1996; Larsen and Mood, 1998; Kress et al., 2002), but it has also been observed in various species of Alpinioideae, e.g., *Amomum citrinum* (Ridl.) Holttum, *Amomum xanthophlebium* Baker, *Hornstedtia sanhan* M.F.Newman, as well in Zingiberoideae, e.g., *Zingiber singapurense* Škorničk. (Škorničková, pers. obs.) and therefore cannot be used to define Riedelieae. Unfortunately morphological characters useful for distinguishing various clades in

Zingiberaceae are exceeding rare, but are crucial for independently testing phylogenetic hypotheses based on molecular data.

To date, a single preliminary systematic treatment of Zingiberaceae seeds by Liao and Wu (2000) showed that the two main subfamilies can be distinguished based on anatomical details of the endotesta, which is sclerenchymatous in Alpinioideae and parenchymatous with often rectangular cells in Zingiberoideae. While this preliminary study was useful in documenting this distinguishing character between the two subfamilies, it only documented four characters for the 60 taxa analyzed. This is true for many previous studies on Zingiberales seeds where very few seed characters are incorporated into analyses, which limits their application in plant systematics (e.g., three seed characters analyzed and discussed in Grootjen and Bouman, 1981; four seed characters used in ordinal phylogenetic analyses in Kress, 1990; Kress et al., 2001).

A more recent study on seeds within subfamily Alpinioideae (Benedict et al., 2015) showed that Zingiberaceae seeds are structurally some of the most complicated seeds in angiosperms and documented 23 seed characters for the subfamily, many of which have not been used to categorize seeds prior. Benedict et al. (2015) found that Riedelieae and Alpinieae can be further distinguished based on endotesta structure and operculum layering, and that many of the proposed clades of *Alpinia* sensu Kress et al. (2005, 2007) are supported by seed characters. While these studies demonstrate the usefulness of seed characters in the systematics of Alpinioideae, their utility for Zingiberaceae as a whole remains to be tested. It is the aim of this paper to: 1) document novel characters not commonly described for Zingiberaceae seeds that may prove useful for future studies on angiosperm systematic and seed ecology studies within and outside of the Zingiberaceae, 2) provide details of seed morphology and anatomy of many

Zingiberaceae that have not been documented previously, 3) determine whether synapomorphic characters exist for subfamilies and tribes within Zingiberaceae, and 4) determine if seed characters can help resolve the correct placement of incertae sedis taxa within Zingiberaceae sensu Kress et al. (2002, 2005, 2007).

MATERIALS AND METHODS

Mature dried seeds of seventy-five taxa from three of the four subfamilies of Zingiberaceae were sampled from various herbaria, botanical gardens, or commercial growers (Table 2). The number of seeds studied per taxon ranged from one to more than 50. Each species was examined with light microscopy for external features and analyzed using synchrotron based X-ray tomographic microscopy (SRXTM; also referred to in some literature as synchrotron radiation X-ray computed tomography, SRXCT, or SRμCT).

Light microscopy and photography—

External features of the seeds were observed using a Leica MZ6 (Leica Microsystems Inc., Illinois, USA) or Nikon SMZ1500 (Nikon Instruments Inc., New York, USA) stereomicroscope and photographed using a Macropod (Macroscopic Solutions LLC, Coventry, CT, USA), outfitted with a Canon EOS 6D DSLR with a Macro Photo MP-E 65mm manual focus lens, MT-24EX Macro Twin Light flash, and a STKS-C StackShot Macro Rail (Cognisys Inc., Michigan, USA). Series of 20–75 images at various focal planes were obtained and stitched into a single image using Zerene Stacker version 1.04 software (Zerene Systems LLC, Washington, USA). Images were edited uniformly for contrast using Adobe Photoshop CS2 (Adobe Systems Inc., California, USA).

Synchrotron based X-ray tomographic microscopy—

Samples were mounted onto brass stubs or toothpicks using a PVA glue or epoxy and imaged using standard absorption contrast at the TOMCAT beamline at the Swiss Light Source (SLS; Stampanoni et al., 2006; Paul Scherrer Institut, Villigen, Switzerland; specimens scanned in 2009, 2010, 2011, 2013, and 2015); the 2-BM beamline at the Advanced Photon Source (APS; Argonne National Laboratory, Lemont, IL; specimens scanned during sessions in 2011 and 2012); or the 8.3.2 beamline at the Advanced Light Source (ALS; MacDowell et al., 2012; Lawrence Berkeley National Laboratory, Berkeley, California; specimens scanned during session in 2013, 2014, and 2015). Transmitted X-rays were converted into visible light using a 20 µm (SLS: 2013, 2015), 100 µm (SLS: 2013, 2015; APS), or 200 µm scintillator (SLS: 2009–2011) LAG:Ce scintillator screen (Crytur, Turnov, Czech Republic) or a 0.5 mm LuAG scintillator (Crytur, Turnov, Czech Republic; ALS).

At TOMCAT, projection data were magnified by 2×, 4×, or 20× microscope objectives and digitized by a high-resolution CCD camera (pco.2000; PCO GmbH, Kelheim, Germany; 2009–2011) or sCMOS camera (pco.edge 5.5; PCO GmbH, Kelheim, Germany; 2013). Samples were scanned using 10 or 13 keV and an exposure time per projection of 50, 125, 150 or 200 milliseconds. For each scan, a total of 1501 projections (2048×2048 pixels with PCO.2000 camera, 2560×2160 pixels with PCO.edge 5.5 camera) were acquired over 180°. Reconstruction of the tomographic data was performed on a 60-node Linux PC cluster using a highly optimized routine based on the Fourier transform method and a gridding procedure (Marone et al., 2010; Marone and Stampanoni, 2012), resulting in a theoretical pixel size of 3.7 µm at 2× and 1.85 µm at 4× (2009–2011) or 3.25 µm at 2× and 1.625 µm at 4× (2013–2015) for reconstructed images.

202 At 2-BM, 2.5×, 4×, or 5× microscope objectives were used to magnify the projection data,
203 and a Coolsnap K4 camera (Photometrics, Tucson, Arizona, 2011 and February 2012) or
204 pco.dimax high-speed camera (PCO GmbH, Kelheim, Germany, June 2012) was used to digitize
205 the data. Samples were scanned at 16.1 or 21 keV with an exposure time of 280–700 ms. For
206 each scan, a total of 1500 projections (2048×2048 pixels with Coolsnap K4, 2016×2016 for
207 PCO) were acquired over 180°. The tomographic reconstructions were conducted with a 64-node
208 cluster at APS using a gridrec reconstruction algorithm (Dowd et al., 1999). Reconstructed
209 images taken with the Coolsnap K4 had a theoretical pixel size of 3.7 µm at 2×, 2.96 µm at 2.5×,
210 1.85 µm at 4×, and 1.48 µm at 5×, and those taken with the pco.dimax had a theoretical pixel
211 size of 5.5 µm at 2×, 4.4 µm at 2.5×, 2.75 µm at 4x×, and 2.2 µm at 5×.
212 At the 8.3.2 beamline, samples were magnified with either a 2× or 5× microscope objective
213 and digitized using a sCMOS camera (pco.edge; PCO GmbH, Kelheim, Germany). Samples
214 were scanned at 15 keV with an exposure time of 90, 500, or 950 ms. For each scan, a total of
215 2049 projections (2560×2160 pixels) were acquired over 180°. Reconstruction was carried out
216 using a custom ImageJ (Rasband, 1997–2014) plugin for image preprocessing and Octopus
217 (Inside Matters, Aalst, Belgium) for tomographic reconstruction. Reconstructed images had a
218 theoretical pixel size of 3.25 µm at 2× and 1.3 µm at 5×.
219 Reconstructed images were processed at the University of Michigan using Avizo 7.0 or 8.0
220 (FEI Visualization Science Group, Burlington, Massachusetts, USA) for Windows 7. Images
221 were captured in Avizo 7.0 or 8.0 and edited uniformly for contrast using Adobe Photoshop CS2
222 or CS6 (Adobe Systems Incorporated, San Jose, California, USA).
223 ***Character evolution analyses—***

Character states that were not observable due to scanning conditions or missing data were treated as (?), and character states that were not applicable (e.g., character 14, operculum layering if no operculum was present, character 13) were treated as (–) to distinguish a lost character from a missing character in the character evolution analyses. The character matrix (Table 3) was imported into Mesquite v.3.03 (Maddison and Maddison, 2015) and characters were traced using parsimony onto a tree topology derived primarily from the results of the most recent family level study by Kress et al. (2002), which used a combined nuclear internal transcribed spacer (ITS) and plastid *trnK*/*matK* dataset. The Alpinioideae portion of the phylogeny follows that of Kress et al. (2007), based on a combined ITS/*matK* dataset, because it provided better resolution to the relationships of taxa within the subfamily. The placement of *Newmania* as sister to *Haniffia* was taken from Leong–Škorničková et al. (2011), based on a combined *trnK*/*matK* and ITS dataset as well, where the genus was first described and placed into a phylogenetic context within the family. The *Hedychium* clade topology was derived from Wood et al. (2000), which was based on an ITS1, ITS2, and 5.8S nuclear ribosomal DNA dataset.

RESULTS

All seeds were mature, dry, and possessed seed coats derived from outer integument (testa) only; often comprising exotesta, mesotesta, and endotesta (Grootjen and Bouman, 1981; Benedict et al., 2015). Seeds were analyzed for 39 internal and external seed characters (Table 3), expanded and modified from the 23 characters identified by Benedict et al. (2015), to address the large amount of variation in Zingiberaceae seeds. Characters were determined from observations of seed external morphology and internal anatomy available from digital longitudinal and transverse sections, 3-dimensional (3D) volume renderings, and movies of serial digital

longitudinal and transverse sections (between 1000–2000 sections per series). Some of the characters introduced below may be correlated, but future developmental studies are needed to determine if these correlations are developmental in nature. Digital sections and hand-colored images of digital sections of selected taxa are provided to illustrate selected seed structures discussed below (Figs. 1A–P).

Variation in Seed Structure—

1. *Natural seed color*— Zingiberaceae seeds, when mature and dry, are often tan, red, or light brown (e.g., Figs. 2A, 2F, 2K, 2P), but can also be dark brown to black (e.g., Figs. 4A, 7F) or even white (Fig. 3A). Character states are scored as follows: 0, white; 1, tan/ red/ light brown; 2, dark brown/ black.

2. Seed surface— The surfaces of Zingiberaceae seeds can be striate (e.g., Figs. 6G, 7A, 9F), or verrucose (surfaces with small bumps, e.g., Fig. 8F). Character states are scored as follows: 0, striate; 1, verrucose.

3. Trichomes— Trichomes may (Figs. 2E, 2J, 2O, 2T, 5E, at arrows) or may not be present on the surface of the seed coat or aril. Character states are scored as follows: 0, absent; 1, present.

4. Aril— Arils and various fleshy, tubular, or disk-like appendages at the micropylar region of the seed have been given various names based on the particular region of the seed or funiculus from which they derive (Kapil et al., 1980). A detailed history and alternate classification is given by Kapil et al. (1980), in which they propose — in agreement with Corner (1976) — that all aril-like structures be called arils and terms such as arilloid, arillode, false aril, hilar aril, etc., be abandoned. They also propose that funicular arils, caruncles (derived from the exotesta, also called an exostome-aril), and strophioles (derived from the raphe tissue) be retained for

descriptive purposes only. We adopt this interpretation of the definition of an aril, and choose to describe arils in terms of presence and absence without making a distinction of exact origin (integumentary, funicular, or raphe tissue) due to the limited developmental understanding of many taxa within the family. While the origin of the aril is not always clear, the extent of the aril may be useful in distinguishing between taxa. Arils may be solid structures confined to the micropylar end of the seed (e.g., Figs. 2F, 7F, 7K, 9A), may consist of many separate strands (e.g., Figs. 3F, 3K, 4P) or a few thick lobes (Fig. 8A) at the micropylar end; or may envelope more than half of a seed and be tightly adpressed (e.g., Figs. 6A, 6E, 6J, 6O) or not (Figs. 3P–Q) to the seed coat. Character states are scored as follows: 0, enveloping more than half of seed and tightly adpressed to the seed coat; 1, present only at micropylar end of seed, solid structure; 2, present only at micropylar end of seed, divided into many separate strands; 3, present only at micropylar end of seed, divided into few thick lobes; 4, present, enveloping more than half of the seed, but not tightly adpressed to the seed coat.

5. and 6. Seed shape— Seeds in Zingiberaceae (and indeed across Zingiberales) vary considerably in their shape due, in part, to frequently tight packing within the fruit (Benedict and Smith, pers. obs.; e.g., Figs. 2K, 7F). Therefore, seed shape was documented for those seeds located closest to the middle of each fruit, showing the least compression.

5. General seed shape— Character states are scored as follows: 0, ellipsoid; 1, ovoid; 2, oblate (flattened at the poles of the seed); 3, polyhedral.

6. Seed contorted from arrangement in fruit— Character states are scored as follows: 0, no contortion of seeds from tight packing in fruit; 1, seed shape contorted by tight packing in fruit.

289 *7. Seed length*— Seed length also varies with respect to location within the fruit and a
290 binary character of "at least twice as long as wide" or "less than twice as long as wide" was used
291 to generalize seed length. Character states are scored as follows: 0, less than twice as long as
292 wide; 1, at least twice as long as wide.

293 *8. Seed body taper at micropylar region*— Character states are scored as follows: 0, absent;
294 1, present.

295 *9. Seed body taper at chalazal region*— In some seeds, the body has a slight decrease in
296 width, or taper, towards the chalazal region. Character states are scored as follows: 0, absent; 1,
297 present.

298 *10. Externally visible raphe*— During maturation of the seed, the anatropous ovules of
299 Zingiberaceae taxa may produce an externally visible groove or ridge in the seed coat
300 corresponding to the position of the raphe in the mature seed (Figs. 8A, 9A). Character states are
301 scored as follows: 0, absent; 1, present.

302 *11. External chalazal indentation*— As with the externally visible raphe (10), in some
303 Zingiberaceae the chalazal region of the seed has a distinctive circular indentation, termed a
304 "sunken chalaza" in *Costus* (Grootjen and Bouman, 1981). It is unclear if this structure is
305 homologous for Zingiberaceae seeds and future developmental work is needed to understand the
306 evolution of this trait across the order. Character states are scored as follows: 0, absent; 1,
307 present

308 *12. Micropylar region shape*— In longitudinal section, the micropylar region, sometimes
309 including a hilar rim, operculum, and micropylar mesotestal proliferation of cells, can range from
310 being conical (e.g., Figs. 1A–B, 1E–H, 4B, 4G), cylindrical (e.g., Figs. 1C–D, 3B, 3R), or not

clearly defined or absent. Character states are scored as follows: 0, absent/not clearly defined; 1, conical; 2, cylindrical.

13. Operculum— An operculum is found within many Zingiberaceae and is conical to disk-shaped in longitudinal section. Character states are scored as follows: 0, absent; 1, present.

14. Operculum layering— The operculum is derived from mesotesta and/or endotesta, making them either homogeneous, or formed of two (or more) distinctive layers. In SRXTM images, the inner, endotesta-derived layer often has an outer X-ray bright layer that forms a boundary with the outer mesotestal-derived layer; this varies from being extremely thin (Figs. 1C–D) to more substantial (Figs. 1I–J). An outer layer from mesotesta tends to be formed of larger cells, sometimes with intercellular spaces, and lacks the X-ray bright nature in SRXTM images (Figs. 1G–H, 1K–L). Character states are scored as follows: (–) no operculum present; 0, more or less homogeneous; 1, multilayered.

15. Micropylar collar— The micropylar collar (labelled "mc" in figures) is a tube or cylinder of testal cells (see character 16) that expands into the embryo chamber, often creating two 'v' shapes below the operculum in longitudinal section (Figs. 1C–F, 1I–L). Some seeds appear to have shallowly or deeply infolded micropylar collars, but this character was found to be subjective in nature and thus was not included as a separate character. The apical portion of the micropylar collar is the attachment point for the operculum of many Zingiberaceae seeds and often surrounds the apical portion of the embryo (e.g., Figs. 4L, 4Q, 8D). Character states are scored as follows: 0, absent; 1, present.

16. Micropylar collar layering— The micropylar collar is formed either from the endotesta or a combination of the endotesta and mesotesta. Liao and Wu (1996) recognized three types of

micropylar collars based on large-volume mesotestal cells ("form A"), small-volume mesotestal cells ("form B") or no mesotestal cells ("form C"). In our studies, the distinction of large and small mesotestal cells was found to be based on subtle differences; therefore two are recognized here, those that are formed from mesotesta and endotesta (=forms A or B; e.g., Figs. 1C–F, 1K–L, 4L, 4Q, 7G, 7L) and those that are formed from endotesta only (=form C; e.g., 1I–J, 6K, 6P, 7B). Character states are scored as follows: (–), no micropylar collar present therefore character not applicable; 0, formed from endotesta only; 1, formed from endotesta and additional layers.

17. Thickened micropylar collar— The micropylar collar sometimes shows a distinctly thickened mesotesta in longitudinal section with respect to the rest of the seed coat (e.g., Figs. 1K–L, 4L, 7G, 7L). Character states are scored as follows: (–) no micropylar collar present therefore character not applicable; 0, absent; 1, present.

18. Recurved micropylar collar— The inner terminus of the micropylar collar ranges from being distinctly acute (strongly recurved, e.g., Figs. 1C–F, 7Q, 8D) to weakly recurved (e.g., Figs. 1K–L, 7G), in longitudinal section. Character states are scored as follows: (–) no micropylar collar present therefore character not applicable; 0, weakly recurved; 1, strongly recurved.

19. Hilar rim— A hilar rim (labelled "hr" in figures) is an elongated tube of seed coat that forms a rim at the micropylar region of the seed. In longitudinal section it was previously described as "[it] produces the appearance of a pair of horns arising from the hilar end of the seed" (Manchester and Kress, 1993:1267; e.g., Figs. 1C–D, 2B, 2G, 3B, 7B, 8D, 10A–B). In some specimens the rim recurves slightly inwards, especially when the aril has been detached (e.g., Figs. 2G, 3B). Character states are scored as follows: 0, absent; 1, present.

354 *20. Hilar rim layering*— The hilar rim can be formed from the exotesta (e.g., Fig. 7B) or a
355 combination of the exotesta and mesotesta (e.g., Figs. 1C–D, 3B, 4B, 5G). Character states are
356 scored as follows: (–) no hilar rim present therefore character not applicable; 0, formed from
357 exotesta; 1, formed from exotesta and mesotesta.

358 *21. Micropylar mesotestal proliferation*— The mesotesta of the micropylar region of the
359 seed may have a proliferation of cells to produce a mass of cells in the shape of a donut or
360 cylinder (labelled "mmp" in figures). In longitudinal section this proliferation of cells is adjacent
361 to the operculum, above and offset from the micropylar collar (e.g., Figs. 1C–D, 5L, 7B).
362 Character states are scored as follows: 0, absent; 1, present, bulbous and wide (donut shaped; e.g.,
363 Figs. 5L, 6B); 2, present, cylindrical and narrow (e.g., 3B).

364 *22–24. Chalazal modifications*— Chalazal modifications of Zingiberaceae seeds are
365 divided into two general forms: testal proliferations (masses of mesotestal cells that have
366 undergone extra periclinal divisions in the chalazal region compared to the rest of the seed coat
367 and contribute three or more rows of cells to the seed coat; characters 22 and 23) and chalazal
368 chambers (empty cavities nested within the mesotesta of seed coats; character 24). Testal
369 proliferations do not include raphe and chalazal pigment group cells. Two types of testal
370 proliferations exist: i) a simple mass of mesotestal cells (e.g., Figs. 1O–P, 2M, 2R, 3C, 3M, 7H;
371 labeled "cmp" in figures), here termed massive, and ii) a wall or column of endotestal and
372 mesotestal cells that vertically divides the lower portion of the embryo cavity into two segments,
373 termed here a columnar mesotestal proliferation (Figs. 1M–N). These two chalazal modifications
374 are not mutually exclusive and taxa can have both a chalazal chamber and a proliferation of
375 mesotestal cells.

376 *22. Massive chalazal testal proliferations*— Character states are scored as follows: 0,
377 absent; 1, present.

378 *23. Columnar chalazal testal proliferations*— Character states are scored as follows: 0,
379 absent; 1, present.

380 *24. Chalazal chamber*— Two distinct types of chalazal chambers (labelled "cc" in figures)
381 have been identified in Zingiberaceae seeds, the *Alpinia*-type and the *Amomum*-type. The
382 *Alpinia*-type is typically lens-shaped and less than 1/3 the width of the seed (e.g., Figs. 1M–N,
383 5H, 9E) whereas the *Amomum*-type is more than 1/3 the width of the seed and often connects to
384 (and becomes continuous with) the raphe canal in the seed (e.g., Figs. 3C, 6B, 6F). Character
385 states are scored as follows: 0, absent; 1, *Alpinia*-type; 2, *Amomum*-type.

386 *25. Chalazal mucro*— A chalazal mucro (labelled "cm" in figures) – an abrupt, pointed
387 termination of the seed (in contrast to character 9, which is a gradual tapering of the seed body) –
388 was reported by Ridley (1909) where he suggested the structure (termed "terminal mucro") was a
389 modification for water and wind dispersal in *Burbidgea.* It has since also been found in other
390 Alpinioideae as well (Benedict et al., 2015). The structure is composed of endotesta, mesotesta,
391 and exotesta (Figs. 7A, 7C, 8E). Character states are scored as follows: 0, absent; 1, present.

392 *26. Seed coat thickness*— The seed coat (all layers of the testa) thickness is determined in
393 transverse sections in the middle of the seed and is measured at the thinnest region of the seed
394 coat. Character states are scored as follows: 0, 1–99 µm; 1, 100–199 µm; 2, ≥200 µm.

395 *27. Exotesta cell type*— The exotesta can be made of palisade cells (e.g., Figs. 7D, 7I),
396 generally isodiametric cells (e.g., Fig. 6H), be poorly developed (e.g., 3D), or it can be absent.

Character states are scored as follows: 0, palisade; 1, more or less isodiametric or cuboidal; 2, poorly developed or destroyed in mature seed.

28. Uniform exotesta— The exotesta is most commonly composed of a homogeneous layer of cells, but it can also be heterogeneous with cells that vary in shape as a result of irregular anticlinal divisions of the exotesta (e.g., Figs. 2S, 3T). Character states are scored as follows: 0, homogeneous; 1, heterogeneous.

29. Multiseriate exotesta— Previously described as a multiple epidermis by some authors (e.g., Wu and Liao, 1995; Liao and Wu, 2000), the exotesta is often uniseriate, but can be multiseriate with two or more cell layers (e.g., Fig. 2N). Character states are scored as follows: 0, absent; 1, present.

30. Number of types of mesotestal cells— The mesotesta, when differentiated, is composed of three cell types occurring in distinct layers in Zingiberaceae seeds (Liao and Wu, 1996, 2000). These layers have been previously described as the hypodermis (directly beneath the exotesta), the translucent cell layer (beneath the hypodermis) and the pigment layer (beneath the translucent cell layer and above the endotesta). In some taxa, the three types can be discerned (e.g., Fig. 4I), whereas in others it is either absent, a single type (e.g., Figs. 6D, 6H), or two types (e.g., Figs. 4S, 9G). Character states are scored as follows: 0, absent; 1, one type; 2, two types; 3, three types.

31. Endotestal cell thickness and shape— The endotesta is the innermost layer of cells of the seed coat in Zingiberaceae seeds. Its thickness and shape vary considerably and can range from very small square to rectangular sclerified cells that are less than 30 µm in thickness (e.g., Fig. 6N), to palisade sclerified cells 30 µm or greater in thickness (e.g., 6D, 6H), to a thin layer,

<15 µm, of parenchyma cells (e.g., Figs. 2N, 2S, 3T). Character states are scored as follows: 0, thin parenchyma (<15 µm thickness); 1, short sclerenchyma (15–30 µm in thickness); 2, elongate sclerenchyma (≥30 µm in thickness).

32. Endotestal gap location— The endotesta has a small circular to ellipsoid interruption, often in the chalazal region of seed, and typically represents the point where the raphe terminates in the seed coat. In longitudinal section, this endotestal gap is filled by the chalazal pigment group so is not seen as a true void (e.g., Figs. 1M–P, 6L, 6Q). The location of the gap varies from being at the base of the seed (Fig. 6Q) to the side of the seed (Fig. 7R). Character states are scored as follows: 0, present at the chalazal end; 1, present on the side.

33. Chalazal pigment group— As noted in previous studies (Liao and Wu, 1996, 2000), the chalazal pigment group (cpg) is a small collection of cells in the embryo cavity above the raphe and endotestal gap. Previously it was determined that members of Zingiberoideae have a discoid-shaped cpg, termed ‘crescent-shaped’ by Liao and Wu (2000; Figs. 1O–P, 5M), while members of Alpinioideae have 'trumpet-shaped' cpgs (Liao and Wu, 2000) or otherwise non-discoid cpgs (Figs. 1M–N, 6L). Character states are scored as follows: 0, discoid-shaped; 1, non-discoid-shaped.

34. Raphe canal— The raphe in mature seeds is destroyed on some taxa, leaving a canal in the seed coat from the micropyle to the chalaza (Fig. 6F; labeled “rc” in figures). In some specimens the raphe canal terminates at (and merges with) the chalazal chamber (e.g., Fig. 6F), but can be differentiated from the chalazal chamber by being slightly smaller in diameter. Character states are scored as follows: 0, absent; 1, present.

35. Embryo length— The embryos in most Zingiberaceae are elongate and extend for more than half the length of the seed, but in some taxa they are much shorter. Character states are scored as follows: 0, elongate; 1, short.

36. Embryo shape— The shape of the embryo ranges from being straight (Fig. 8C), L-shaped (with a sharp, nearly right-angled curve in the embryo that is less than 25% of the embryo length, Fig. 9B), to J-shaped (with a smooth curve in the embryo that is ca. 50% of the embryo length, Fig. 6F). Character states are scored as follows: 0, straight; 1, L-shaped; 2, J-shaped.

37. Embryo base— Independent of shape, some taxa have embryos that are enlarged (bulbous; e.g., Figs. 3M, 5Q) or forked (e.g., Fig. 6B) at the base. Character states are scored as follows: 0, not differentiated; 1, bulbous; 2, forked.

38. Embryo–testa contact— The embryo, perisperm, and sometimes endosperm occupy the embryo cavity of Zingiberaceae seeds. The embryo can either be in direct contact with the seed coat (Figs. 9E) or nested within endosperm/perisperm and not touching the innermost wall of the seed coat. Character states are scored as follows: 0, absent; 1, present.

39. Basally proliferated endosperm — Endosperm in some taxa is found in greater abundance surrounding the base of the embryo (e.g., Figs. 3S, 4M, 6F) compared to the micropylar end. In some SRXTM images perisperm is often composed of large cells (e.g., Fig. 5C) and endosperm is often darker and lacks observable cell walls (e.g., Fig. 6F). In other SRXTM images endosperm appears as small cells (perhaps nuclei; e.g., 5R) and individual perisperm cells are indistinguishable (e.g., Fig. 5R). In both cases endosperm always immediately surrounds the embryo and both the embryo and endosperm are nested within the perisperm (e.g., 2R, 5C, 7H, 9C). This character refers to the relative distribution of endosperm,

and whether it is proliferated, or more abundant, toward the chalazal end of the seed as compared to the micropylar end. Character states are scored as follows: 0, absent; 1, present, weak or minimal amount; 2, present, strong or copious amount.

Zingiberaceae seeds in a systematic context—

Results for all species studied are summarized in Table 3. All tribes of Alpinioideae and Zingiberoideae were sampled as well as the subfamily Siphonochiloideae, which was previously unknown for seed morphoanatomy. It was not possible to study the monospecific subfamily Tamijioideae as no herbarium we contacted (E, K, MO, MICH, NY, SING, US) had fruit or seed material available.

Zingiberoideae— Fourteen genera and 26 species representing both tribes were examined (Figs. 1A–F, 1O–P, 2A–T, 3A–U, 4A–T, 5A–T). The seeds of the 26 species studied from Zingiberoideae have in common 10 character states (character numbers in parentheses). They all lack an externally visible raphe (10), have no columnar chalazal testal proliferation of cells (23), no chalazal mucro (25), and their embryos are nested within nutrient tissue and do not contact the endotesta (38). All Zingiberoideae seeds have seed coats less than 100 µm in thickness (26; Figs. 2D, 2I, 2N, 2S, 3D, 3I, 3N, 3T, 4D, 4I, 4N, 4S, 5D, 5I, 5N, 5S), a hilar rim formed from exotestal and endotestal layers (19 and 20; Figs. 2B, 2G, 2L, 2Q, 3B, 3G, 3L, 3R, 4B, 4G, 4L, 4Q, 5B, 5G, 5L), a thin endotesta of parenchyma (31; Figs. 2D, 2I, 2N, 2S, 3D, 3I, 3N, 3T, 4D, 4I, 4N, 4S, 5D, 5I, 5N, 5S), an endotestal gap at the base of the seed (32; e.g., Fig. 1O–P), and a discoid chalazal pigment group (33; Figs. 1O–P, 5M).

The seven species of all three genera within Globbeae (Figs. 2A–T) have in common 16 characters. All seeds are lightly pigmented (tan, red, or light brown) (1; Figs. 2A, 2F, 2K, 2P),

have trichomes on either the surface of the seed or aril (3; Figs. 2E, 2J, 2O, 2T), an aril confined to the micropylar end of the seed that is a solid structure (4; Figs. 2F, 2K, 2P), a hilar rim formed from both the exotesta and mesotesta (19 and 20; Figs. 2B, 2G, 2L, 2Q), seed coats less than 100 µm thick (26; Figs. 2D, 2I, 2N, 2S), a thin parenchymatous endotesta (31; Figs. 2D, 2I, 2N, 2S), an endotestal gap at the base of the seed (32), a discoid-shaped chalazal pigment group (33), and elongate embryos that are not differentiated at the base and do not touch the endotesta (35, 37, 38; Figs. 1C, 1H, 1R). They all lack an externally visible raphe (10), a columnar chalazal testal proliferation of cells (23), a chalazal mucro (25), and a uniform exotesta (28; 2D, 2I, 2N, 2S). A micropylar collar (15) was found in all taxa except *Globba spathulata* Roxb. (Fig. 2Q). The combination of a weakly recurved micropylar collar (19; Figs. 2B, 2G), the absence of basally proliferated endosperm (39; Figs. 2C, 2H), and the presence of an external chalazal indentation (11) were found to unite *Hemiorchis* sp. and *Gagnepainia harmandii* (Baill.) K.Schum. and differentiate these two genera from *Globba*.

Ten genera and eighteen species were analyzed within the tribe Zingibereae (Figs. 3A–U, 4A–T, 5A–T) and found to have 13 characters in common. Seeds all have a hilar rim formed from the exotesta and mesotesta (19 and 20; Figs. 3B, 3R, 4Q), seed coats less than 100 µm in thickness (26; Figs. 3D, 3I, 3N, 3T, 4D, 4I, 4N, 4S, 5D, 5I, 5N, 5S), a thin parenchymatous endotesta (31), an endotestal gap at the base of the seed (32; Figs. 1O–P, 5M), a discoid chalazal pigment group (33; Fig. 5M), and an elongate embryo that does not contact the endotesta (35 and 38; Figs. 3M, 3S, 4H, 4M, 4R, 5M). They lack an externally visible raphe (10), columnar chalazal testal proliferations (23), a chalazal mucro (25), and a uniform or multiseriate exotesta (28 and 29). A bulbous embryo base (37) was found in *Newmania* (Fig. 3M) only, and a

micropylar collar (15) was found in all but two species, *Camptandra ovata* Ridl. (Fig. 5B) and *Cautleya gracilis* (Sm.) Dandy (Fig. 4B). The shape of the seed (5) and the embryo (36), as well as the type of cells in the exotesta (27), were all found to be quite variable in the tribe and not useful in distinguishing the tribe from other Zingiberaceae.

The seeds of *Monolophus sikkimensis* (King ex Baker) Veldkamp & Mood are the smallest described for Zingiberaceae (1.1 mm long × 0.6 mm wide) and can be distinguished from all other Zingiberaceae by the absence of a micropylar collar (15; Fig. 5Q), presence of a short, straight, bulbous embryo (35–37; Fig 5Q), and having basally proliferated endosperm that fills more than half of the embryo cavity (39; Fig. 5R).

Alpinioideae— Sixteen genera and 46 species were examined from Alpinioideae (Figs. 6A–S, 7A–T, 8A–G), representing both tribes. Two characters are shared among all alpinioid taxa: lack of a multiseriate exotesta (29; Figs. 6D, 6H, 6N, 6S, 7D, 7I, 7N, 7S, 8G), and the presence of a non-discoid chalazal pigment group (33; e.g., Figs. 1M–N, 6L, 6Q, 7H).

Eleven genera and 40 species from the tribe Alpinieae (Figs. 6A–S) were analyzed. Seven characters were found in common. All seeds of Alpinieae lack trichomes (3; Figs. 6C, 6G, 6M, 6R), have an operculum (13; Figs. 6B, 6F, 6I, 6K, 6P), have a micropylar collar (15; Figs. 6I, 6K, 6P), lack a chalazal mucro (25; Figs. 6A, 6E, 6J, 6O), and lack a multiseriate exotesta (29; Figs. 6D, 6H, 6N, 6S). Characters that were present in all Alpinieae studied were a non-discoid chalazal pigment group (33; Figs. 1M–N, 6L, 6Q), and basally proliferated endosperm (39; Figs. 6F, 6Q). A hilar rim (19) was lacking in all Alpinieae except *Aframomum* species. *Alpinia boia* Seem. was the only taxon observed to have an embryo in contact with the endotesta (38). Both

527 the presence and type of aril (4) and the shape of the embryo (36) were quite variable within the
528 tribe and not useful for distinguishing the tribe from other Zingiberaceae.

529 The five species of Riedelieae (Figs. 7A–T) that were analyzed had 12 seed characters in
530 common. All seeds of Riedelieae lacked an externally visible raphe (10), a columnar chalazal
531 chamber (23), a chalazal chamber (24), and a multiseriate exotesta (29). They all shared the
532 presence of an operculum (13; Figs. 7B, 7G, 7L, 7Q), the presence of a micropylar collar (15; 7B,
533 7G, 7L, 7Q), seed coats less than 100 µm thick (26; Figs. 7D, 7I, 7N, 7S), an exotesta of
534 isodiametric or cuboidal cells (27; Figs. 7D, 7I, 7N, 7S), a non-discoid chalazal pigment group
535 (33; Figs. 7H, 7R), an elongated embryo (35; Figs. 7M, 7R), an embryo that is not modified at
536 the base (37; Figs. 7H, 7M, 7R), and an embryo that does not touch the endotesta (38; Figs. 7H,
537 7M, 7R).

538 Many other characters were present in most, but not all, of the Riedelieae and thus are
539 potentially useful for narrower taxonomic groups but are not useful for identifying the tribe.
540 Generally the tribe had striate seeds (2), lacked trichomes (3), and had a uniform exotesta (28),
541 but in *Siamanthus siliquosus* K.Larsen & Mood verrucose seeds with trichomes (3) and a non-
542 uniform exotesta (28) were observed (Figs. 7A, 7S–T). In *Burbidgea stenantha* Ridl. (Figs. 7A–
543 E), elongate seeds (7), with a few-stranded aril (4), and chalazal mucro (25), were observed in
544 contrast to relatively short seeds, with a solid aril, and no chalazal mucro as seen in the other
545 members of Riedelieae. *Riedelia* sp. was found to have a homogeneous operculum (14), and a
546 single type of mesotestal cells (30), counter to the heterogeneous opercula and a mesotesta of
547 two distinct cell types observed in all other members of the tribe. The combination of a verrucose
548 seed surface (2), presence of trichomes (3), and a non-uniform exotesta (28) was unique to

549 *Siamanthus siliquosus* (Figs. 7P–T). A few stranded aril (4), elongate seed (7), and chalazal
550 mucro (25) in combination were found only in *Burbidgea stenantha*. The seed of
551 *Pleuranthodium* sp. (Figs. 7F–J) did not significantly taper at the chalaza (9), but had an external
552 chalazal indentation (11) and a chalazal proliferation of cells (22) resulting in a suite of character
553 states that differed from all other Riedelieae studied.

554 The large and elongate seeds of *Siliquamomum tonkinense* Baill. (Figs. 8A–G) can be
555 easily distinguished from other Zingiberaceae by the presence of an aril confined to the
556 micropylar region of the seed that is separated into two or three thick strands (4; Fig. 8A),
557 conspicuous trichomes on the aril and seed coat (3; Figs. 8A, 8F), a single externally visible
558 raphe (10; Fig. 8B), and a distinctive chalazal mucro at the base of the seed (25; Figs. 8C, 8E).

559 Finally, three characters in particular were found to have considerable variation within the
560 Riedelieae: the overall shape of the seed (5), and the thickness (17), and recurvature (18) of the
561 micropylar collar.

562 *Siphonochiloideae*— Two genera and three species were analyzed for Siphonochiloideae
563 (Figs. 9A–G). They can be distinguished from all other Zingiberaceae by a combination of
564 characters that includes a solid aril confined to the micropyle of the seed (4; Fig. 9A, 9D), the
565 absence of a micropylar collar (15; Fig. 9D), and the presence of a distinct externally visible
566 raphe from the micropyle to the chalaza of the seed (10; Fig. 9A).

567

568 ## DISCUSSION

569 The complexity of key aspects of the Zingiberaceae seeds (notably in micropylar, hilar and
570 chalazal regions; but also in presence of both perisperm and endosperm and variation in embryo

shape and testa) enables recognition of multiple characters and character states not available in plant groups with simpler seed organization. In addition, the use of non-destructive SRXTM substantially increases confidence in the assessment of characters because artifacts of physical sectioning (such as tears within tissues caused by sectioning, spaces linked to shrinkage during embedding, and distortion linked to different tissue response to sectioning or embedding media) are all avoided (a point emphasized by Smith et al., 2009). The only artifacts that need to be taken into account are those of shrinkage and distortion during seed drying naturally or for herbarium preparation, which were accounted for as all scanned seeds were dry prior to scanning. Furthermore, the ability to examine multiple planes of section in SRXTM datasets reinforces character state documentation. Thus, in combination, seed complexity and observation by SRXTM provide a powerful tool for phylogenetic analyses by yielding multiple characters and character states that can be applied in evaluating relationships within Zingiberaceae.

While no single seed character state was found to be unique to any single subfamily, combinations of seed character states were found that can be used to distinguish between the three subfamilies Alpinioideae [absence of a multiseriate exotesta (29), short to elongate sclerified endotesta (31), and a non-discoid chalazal pigment group (33)], Siphonochiloideae [a solid aril (4), an externally visible raphe (10), and lack of a micropylar collar (15)], and Zingiberoideae [lack of an externally visible raphe (10), a hilar rim of exotesta and mesotesta (19 and 20), no columnar chalazal proliferations (23), no chalazal mucro (25), seed coat 100–199 µm in thickness (26), an endotesta of thin parenchyma (31), an endotestal gap at the base of the seed (32), a discoid chalazal pigment group (33), and embryos that do not contact the testa (38)] (Table 3; Fig. 10A–B). In contrast, at the tribal level, the only tribe with a unique combination of

character states not possessed by any other taxon outside of the tribe was Globbeae; Alpinieae, Riedelieae, and Zingibereae were found to have distinctive characters to support the tribes, but these characters or character states were also occasionally found in taxa outside the tribe.

Of the 39 characters analyzed, 22 were found to be informative for distinguishing the subfamilies and tribes as currently recognized, whereas 17 were found to be variable at both the subfamily and tribal level and are not useful for distinguishing tribes or subfamilies (Table 3). The informative characters that allowed for differentiation of the tribes and subfamilies are: seed color (1), trichomes on seed coat or aril (3), aril type (4), an externally visible raphe (10), an external chalazal indentation (11), an operculum (13), a micropylar collar (15), a hilar rim (19), the layering of the hilar rim (20), a columnar chalazal testal proliferation (23), a chalazal chamber (24), a chalazal mucro (25), the thickness of the testa (26), a uniform exotesta (28), a multiseriate exotesta (29), the shape of the endotestal cells (31), the location of an endotestal gap (32), a chalazal pigment group (33), the length of the embryo (35), differentiation of the embryo base (37), contact of the embryo with the endotesta (38), and basally proliferated endosperm (39). Uninformative characters that were either too variable within a group or commonly found among different groups are: the surface of the seed (2), the shape of the seed (5), seed contortion (6), seed length (7), tapering of the seed body at the micropyle (8), tapering of the seed at the chalaza (9), the shape of the micropylar region (12), the layering of the operculum (14), the layering of the micropylar collar (16), a thickened micropylar collar (17), a recurved micropylar collar (18), a micropylar mesotesta proliferation of cells (21), massive chalazal testal proliferations (22), the type of exotestal cells (27), the number of types of mesotestal cells (30), the raphe canal (34), and the shape of the embryo (36).

615 ***Siphonochiloideae—***

616 The two genera of Siphonochiloideae shared a mosaic of character states with members of
617 Alpinioideae and Zingiberoideae, which is not surprising as they are sister to the rest of the
618 Zingiberaceae (Kress et al., 2002; Fig. 10B). Some members of both Siphonochiloideae and
619 Alpinioideae have a distinctive externally visible raphe (10) and embryos that touch the
620 endotesta (38), two characters lacking in all Zingiberoideae. All members of Siphonochiloideae
621 analyzed and some Zingiberoideae have a parenchymatous endotesta (31), a discoid-shaped
622 chalazal pigment group (33), and are lacking any evidence of a micropylar collar (15), which is
623 in direct contrast to Alpinioideae, where not a single member lacks a micropylar collar (15) and
624 all members have a sclerenchymatous endotesta (31) and trumped-shaped chalazal pigment
625 group (33).

626 ***Zingiberoideae—***

627 Zingiberoideae seeds show a unique combination of 10 character states (Table 3). Two of
628 these character states, an endotesta of parenchymatous cells (31) and a discoid-shaped chalazal
629 pigment group (33) have been previously used to unite the subfamily (Liao and Wu, 2000), and
630 are reported here for the first time in the previously unstudied genera *Boesenbergia*, *Camptandra*,
631 *Distichochlamys*, *Newmania*, *Gagnepainia*, and *Hemiorchis* (Liao and Wu, 2000). Interestingly,
632 the two aforementioned character states are also found within the earliest diverging lineage of
633 Zingiberaceae, Siphonochiloideae. The other ten character states, introduced here for the first
634 time, help reinforce the relationships of members of the Zingiberoideae.

635 The Globbeae were found to possess a unique combination of 16 character states in the
636 seven species analyzed, and a combination of three character states — a light colored seed (1),

637 the presence of trichomes (3), and an undifferentiated embryo base (37) — can be used to
638 distinguish the tribe from other Zingiberaceae. *Boesenbergia curtisii* (Baker) Schltr. was very
639 similar to Globbeae members, but differed in having a white seed (36), where all Globbeae seeds
640 are either red or tan and never white or black in color. *Newmania* and *Monolophus sikkimensis*
641 were also similar to Globbeae, but differed in having a bulbously differentiated embryo base, a
642 character not seen in Globbeae. It was reported previously that Zingibereae (then separated into
643 Hedychieae and Zingibereae) can be distinguished from Globbeae on the basis that *Globba*
644 *racemosa* Sm. has a multiseriate exotesta (Liao and Wu, 2000), but in our expanded sampling of
645 the tribe including all three genera, it was found that a multiseriate exotesta (29) is lacking in
646 *Globba spathulata*, *Gagnepainia harmandii*, and *Hemiorchis* sp., thus eliminating the utility of
647 this character to separate the Globbeae from the Zingibereae.

648 The Zingibereae share 13 character states in common for the 18 species analyzed (Table 3),
649 but these character states are not unique to Zingibereae, as *Globba spathulata* has an identical
650 combination of character states for the same 13 characters. The other members of Globbeae
651 differ from Zingibereae in either possessing an external chalazal indentation (*Hemiorchis* and
652 *Gagnepainia harmandii*) or a multiseriate exotesta (*Globba pendula*, *G. sessiliflora* Sims, *G.*
653 *aurea* Elmer, and *G. maculata* Blume).

654 ***Alpinioideae—***

655 Seed morphoanatomy is extraordinarily diverse (see Benedict et al., 2015 for discussion),
656 but a combination of two character states unites the subfamily: a uniseriate exotesta (29), and a
657 non-discoid chalazal pigment group (33). A non-discoid chalazal pigment group (33) and
658 sclerenchymatous endotesta (31) were originally reported by Liao and Wu (2000) in five genera

(*Alpinia*, *Amomum*, *Etlingera*, *Hornstedtia*, and *Plagiostachys*) and 43 species to unite the subfamily and corroborated by Benedict et al. (2015) in a broader analysis of the subfamily that included *Aframomum* spp., *Burbidgea stenantha*, *Geocharis aurantiaca* Ridl., *Geostachys densiflora* Ridl., *Pleuranthodium* sp., *Renealmia* spp., *Siamanthus siliquosus*, *Siliquamomum tonkinense*, and *Vanoverberghia sepulchrei* Merr. We have since sampled more Alpinioideae (introduced here) including more species of *Alpinia*, *Aframomum,* and *Hornstedtia*, and *Elettariopsis unifolia* (Gagnep.) M.F.Newman, and have found the characters mentioned above to be consistent in all Alpinioideae examined. It is important to note that our endotesta character (31) includes thickness, cell shape, and cell type, and is not directly equivalent to Liao and Wu's (2000) character. It is consistent with respect to a sclerenchymatous or parenchymatous cell type, however cells vary in thickness within the subfamily.

The Alpinieae share seven character states among the 40 species analyzed (Table 3), but the combination of these character states is not unique to the tribe. *Pleuranthodium* (Riedelieae) is identical with respect to these character states, while *Siamanthus siliquosus* (Riedelieae) is similar to Alpinieae taxa but is easily distinguished by conspicuous trichomes (3) on the exotesta, a character lacking in all Alpinieae. *Riedelia* spp. and *Burbidgea stenantha* (Riedelieae) are also similar in morphoanatomy with Alpinieae, but lack a well-formed basally proliferated endosperm (39).

Five species representing the four genera of Riedelieae were analyzed and are found to share 12 character states. However, the combination of these characters is not unique to Riedelieae, and is also found in *Vanoverberghia sepulchrei* (Alpinieae). It is notable that all

studied members of Riedelieae lack a chalazal chamber (24), which is often present in seeds of Alpinieae.

***Unplaced taxa:* Siliquamomum tonkinense *and* Monolophus sikkimensis—**

In recent studies based on molecular data *Siliquamomum tonkinense* was placed as either sister to the *Alpinia rafflesiana* clade, which was then sister to the remaining Alpinieae (Kress et al., 2005) or in a polytomy with Riedelieae and the rest of Alpinieae (sensu Kress et al., 2007), or with low bootstrap support (<50%) as the earliest diverging lineage sister to the rest of Riedelieae (sensu Kress et al., 2002). When seed morphoanatomical character states of *Siliquamomum tonkinense* were analyzed, one character state, an externally visible raphe (10), was found in some members of Alpinieae, but not in any Riedelieae, and three character states [the presence of trichomes (3), a chalazal mucro (25), and weak basally proliferated endosperm (39)] were found to ally it with members of Riedelieae. Additionally, when *Siliquamomum tonkinense* was compared to Alpinioideae taxa surveyed here, it was found to be most similar to *Burbidgea stenantha*, sharing 31 of the 39 seed character states analyzed. In parsimony analyses, both with and without added characters from DNA sequence data, *Siliquamomum tonkinense* is shown to be closely related to *Burbidgea* and never sister to *Alpinia rafflesiana*, as suggested by previous authors (data not shown). In fact, only 17 of the 39 seed characters are shared between the latter two taxa, the lowest number of characters shared between any Alpinioideae and *Siliquamomum tonkinense* (Table 3). Based on the available morphological and molecular data it is most parsimonious to conclude that *Siliquamomum* be included as a member of Riedelieae, but more morphological and molecular data are needed confirm its relationship with either tribe.

Monolophus is the only genus currently unplaced in the Zingiberoideae based on a combined ITS and *mat*K dataset (Kress et al., 2002). Larsen and Smith (1972) postulated a close relationship with *Camptandra* and *Boesenbergia*, but that was not supported based on molecular work and the genus remains unplaced (Kress et al., 2002). Seeds of *Monolophus sikkimensis* were analyzed and compared to other Zingiberoideae, and found to have one character state unique to Globbeae, a single type of mesotestal cells (30), and two character states indicative of Zingibereae, a poorly developed exotesta (27) and a bulbous embryo (37). Although further information is needed to make a formal placement of *Monolophus*, it may be more closely related to Zingibereae based on our reported seed characters.

Notable character state changes in Zingiberaceae—

Certain characteristics of Zingiberaceae seeds have many character state reversals within the family, creating a large number of homoplasious characters and character states, however other characters show less homoplasy and are useful in separating formally recognized clades (Table 3). The most useful characters for supporting currently recognized formal and informal clades are those relating to the endotestal cells (31) and the type of chalazal pigment group (33), also derived from the endotesta (Fig. 11). The endotesta has a single shift from thin and parenchymatous in Siphonochiloideae and Zingiberoideae, to sclerified of various thicknesses in Alpinioideae with no reversals to parenchymatous cells (Fig. 11). The chalazal pigment group (33) are discoid in Siphonochiloideae and Zingiberoideae, but are non-discoid in Alpinioideae, suggesting a single shift in Alpinioideae (Fig. 11). Trichomes on the seed coat or aril (3) have perhaps been gained twice in Alpinioideae (*Siamanthus siliquosus* and *Siliquamomum tonkinense*), and have been lost in at least three lineages in the Zingiberoideae (Fig. 12). Larsen

(2003) suggested that the trichomes found on some *Monolophus* create air pockets so they can be abiotically dispersed by water, which would be an interesting ecological explanation for the multiple originations of trichomes in Zingiberaceae seeds. The micropylar collar (15) was previously shown by Liao and Wu (2000) to be lost in *Cautleya gracilis* and *Monolophus coenobialis* Hance (as *Caulokaempferia coenobialis* (Hance) K.Larsen), which we confirm, and we have documented additional instances of loss of micropylar collar (Fig. 12). It was lost at least once in Globbeae (*Globba spathulata*), again in Zingibereae (*Camptandra ovata*), and in Siphonochiloideae (Fig. 12). Interestingly all these seeds, except those of members of Siphonochiloideae, are very small (1–2 mm), so the loss of the micropylar collar could be attributed to reduction in seed size, but its absence in the large seeds of *Siphonochilus aethiopicus* (>5 mm) refutes this idea. Loss of micropylar collar also does not correlate with the loss of an operculum, because all taxa studied here without a micropylar collar still possess an operculum. Further investigations into the functional roles of trichomes and the micropylar collar are needed.

Conclusions—

Zingiberaceae seeds are morphologically and anatomically diverse and possess a large number of systematically significant characters. Many of the characters used here are novel and have the potential to be applied to other seed bearing plants with similar structural complexity. The use of non-destructive SRXTM substantially increases confidence in the assessment of characters because complications and artifacts arising from physical sectioning are avoided. SRXTM also provides the ability to investigate rare and/or endangered taxa from herbaria, which is useful for future studies centered on seed or fruit morphoanatomy as these are sometimes less

common in collections as compared with flowering specimens. Thirty-nine characters were analyzed for 75 species within Zingiberaceae and 22 characters were found to be useful for differentiating the subfamilies and tribes as currently described. Using a combination of characters, the subfamilies Alpinioideae, Zingiberoideae, and Siphonochiloideae could each be distinguished using seed morphoanatomy. Globbeae were the only tribe found to possess a unique combination of character states not seen in any species outside the tribe. The lack of seed character states that unite the other tribes may be due to a significant amount of homoplasy, but seed features are still useful in combination with other morphological characters to determine synapomorphies for the various clades, documenting the importance of widely surveying plant groups for novel characters not previously used in classification studies. The seed character states of currently unplaced genera within Zingiberaceae, *Monolophus* and *Siliquamomum*, have been compared to those of other taxa within the family and suggest that *Siliquamomum* may be related to Riedelieae, and *Monolophus* to Zingibereae. However, more data are needed in order to formally revise the family. The Zingiberaceae are a large family with considerable morphological and anatomical variation in both reproductive and vegetative characters. The research presented here demonstrates the utility of using seed characters to independently test hypotheses of evolutionary relationships. Further, morphological studies like this are critical to understanding long-term evolutionary patterns where the fossil record will be considered, as no DNA data are available for these extinct taxa.

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Table 1. Currently recognized subfamilies, tribes, and genera within Zingiberaceae (after Kress et al., 2002; Takano and Nagamasu, 2007; Kress et al., 2010; Leong-Škorničková et al., 2011; Mood et al., 2014). Numbers in parentheses indicate currently accepted number of species reported on The Plant List (2013), IPNI (2015), in Mood et al. (2014), or Leong-Škorničková et al. (2015).(*) denotes genera described since Kress et al. (2002).

Subfamily **Siphonochiloideae**	Subfamily **Tamijioideae**	Subfamily **Alpinioideae**	Subfamily **Zingiberoideae**
Tribe **Siphonochileae**	Tribe **Tamijieae**	Tribe **Alpinieae**	Tribe **Zingibereae**
Aulotandra (6)	*Tamijia* (1)	*Aframomum* (56)	*Boesenbergia* (69)
Siphonochilus (11)		*Alpinia* (247)	*Camptandra* (4)
		Amomum (180)	*Cautleya* (2)
		Cyphostigma (1)	*Cornukaempferia* (3)
		Elettaria (11)	*Curcuma* (105)
		Elettariopsis (20)	*Distichochlamys* (3)
		Etlingera (100)	*Haniffia* (4)
		Geocharis (6)	*Haplochorema* (6)
		Geostachys (25)	*Hedychium* (95)
		Hornstedtia (34)	*Kaempferia* (36)
		Leptosolena (1)	*Larsenianthus** (4)
		Plagiostachys (27)	*Nanochilus* (1)
		Renealmia (87)	*Newmania** (2)
		Vanoverberghia (2)	*Myxochlamys** (2)
			Parakaempferia (1)
		Tribe **Riedelieae**	*Pommereschea* (2)
			Rhynchanthus (4)
			Roscoea (23)
		Burbidgea (5)	*Scaphochlamys* (34)
		Pleuranthodium (23)	*Stadiochilus* (1)
		Riedelia (75)	*Zingiber* (146)
		Siamanthus (1)	
			Tribe **Globbeae**
		Incertae Sedis	
		Siliquamomum (3)	*Gagnepainia* (3)
			Globba (106)
			Hemiorchis (3)
			Incertae Sedis
			Monolophus (25)

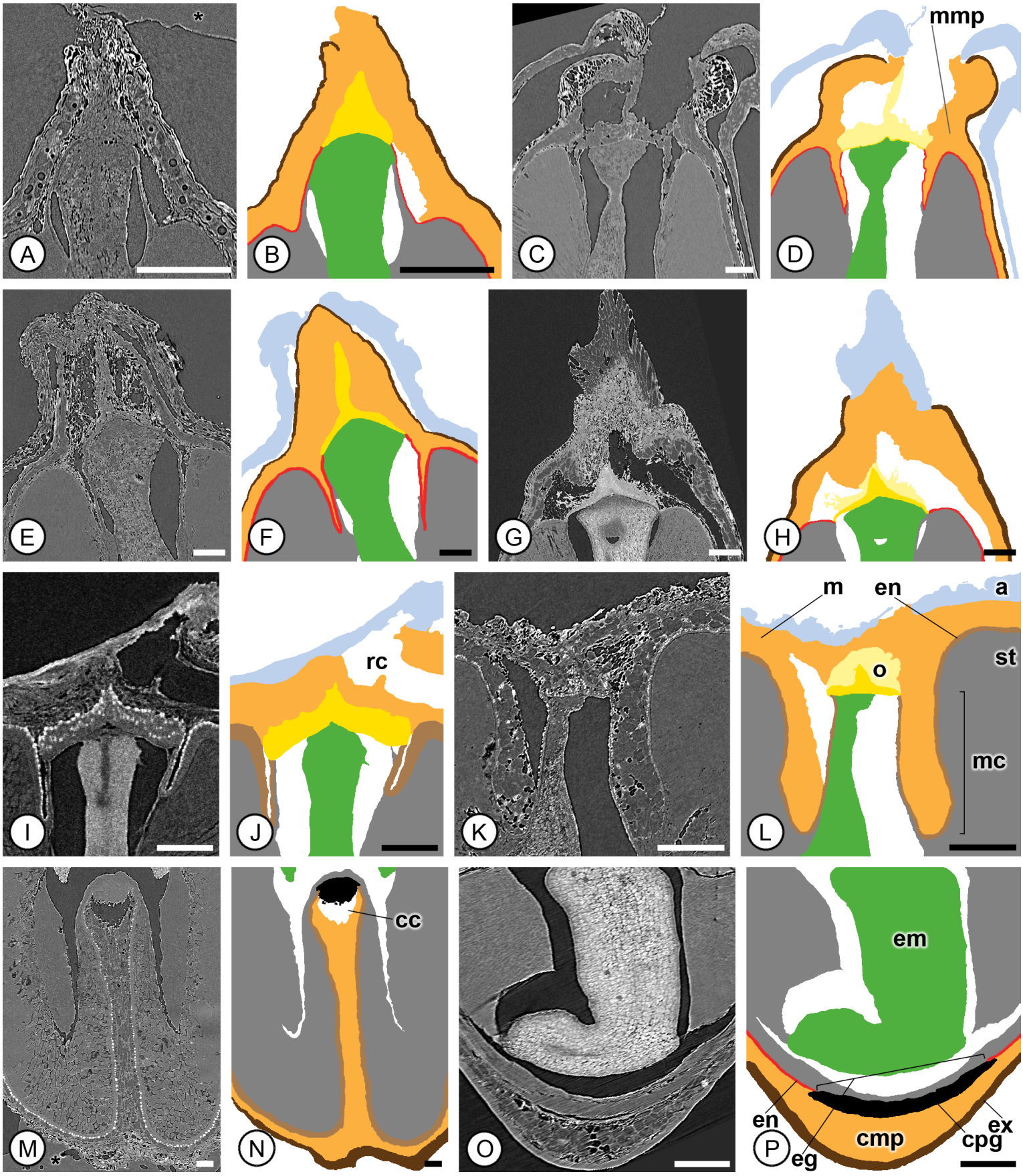
925 Table 2. List of specimens sampled and their voucher information. Herbarium abbreviations
926 follow Index Herbariorum (Thiers, continually updated). Numbers in parentheses indicate
927 number and type of specimens scanned per taxon.

Species	**Voucher Information**
Aframomum chrysanthum Lock	SING, GRC–173 (1 seed)
Aframomum daniellii (Hook.f.) K.Schum.	Delft University of Technology, JW van Loon (1 seed)
Aframomum melegueta K.Schum.	US, J. Higgins 44 (1 seed)
Alpinia aquatica (Retz.) Roscoe	SING, GRC–22, and US, WJ Kress 05–7809 (2 seeds and 1 fruit)
Alpinia boia Seem.	US, WJ Kress 79–1071, and US, AC Smith 4087 (2 seeds)
Alpinia brevilabris C.Presl	US, M Ramos 30411 (1 seed)
Alpinia caerulea (R.Br.) Benth.	SING, JLS–1660 (1 seed)
Alpinia carolinensis Koidz.	US, DH Lorence 7907 (1 seed)
Alpinia conchigera Griff.	SING, GRC–205 (1 seed)
Alpinia fax (Thwaites) B.L.Burtt & R.M.Sm.	US, AHM Jayasuriya 1217 (1 seed and 1 fruit)
Alpinia galanga (L.) Willd.	US, Shiu Ying Hu 6225 (1 seed and 1 fruit)
Alpinia haenkei C.Presl	US, ADE Elmer 17662 (1 seed)
Alpinia japonica (Thunb.) Miq.	NY, Muratailcitamura 639 (1 seed)
Alpinia javanica Blume	SING, Umbai and Millard 1430 (1 seed)
Alpinia luteocarpa Elmer	US, Kress and Li 05–7785 (1 seed)
Alpinia malaccensis (Burm.f.) Roscoe	US, C Saldanha 14771 (1 seed)

Alpinia nigra (Gaertn.) B.L.Burtt	US, WJ Kress 00–6808 (1 seed)
Alpinia oceanica Burkill	E, Stone and Streimann 10296 (1 seed)
Alpinia purpurata (Vieill.) K.Schum.	E, AN Miller NGF 38482 (1 seed)
Alpinia rafflesiana Wall. ex Baker	SING, Ridley s.n. (1 seed)
Alpinia stachyodes Hance	US, n.c., 1801 (1 seed)
Alpinia zerumbet (Pers.) B.L.Burtt & R.M.Sm.	US, Wen 9412, and US, Fosberg 38289 (2 seeds)
Amomum koenigii J.F.Gmel.	SING, VNM–B–1443 (1 seed)
Amomum lappaceum Ridl.	SING, JLS–1667 (1 seed)
Amomum ochreum Ridl.	SING, JLS–1670 (1 seed)
Amomum sericeum Roxb.	SING, JLS–1273 (1 seed)
Aulotandra trigonocarpa H.Perrier	K, M Bardot–Vaucoulon 1272 (1 fruit)
Boesenbergia curtisii (Baker) Schltr.	NY, Henderson 22874 (1 seed)
Burbidgea stenantha Ridl.	SING, GRC–88 (1 seed)
Camptandra ovata Ridl.	JLS–1669 (1 seed)
Cautleya gracilis (Sm.) Dandy	MO, K Larsen 46744 (2 seeds and 1 fruit)
Cautleya spicata (Sm.) Baker	MICH, JC Benedict s.n. (commercially purchased) (1 seed)
Curcuma montana Roxb.	SING, JLS–73474 (1 seed)
Curcuma pierreana Gagnep.	SING, Ly–489 (1 seed)
Distichochlamys citrea M.F.Newman	SING, JLS–1615 (1 seed)
Elettariopsis unifolia (Gagnep.) M.F.Newman	MO, JF Maxwell 00–390 (1 seed)
Etlingera elatior (Jack) R.M.Sm.	SING, SNG–56 (1 seed)

Etlingera linguiformis (Roxb.) R.M.Sm.	US, WJ Kress, M Bordelon, T Htum 02–7044 (1 seed)
Etlingera yunnanensis (T.L.Wu & S.J.Chen) R.M.Sm.	SING, JLS–1717 (1 seed)
Gagnepainia harmandii (Baill.) K.Schum.	SING, GRC–132 (1 seed)
Geocharis aurantiaca Ridl.	SING, Corner 32777 (1 seed)
Geostachys densiflora Ridl.	SING, JLS–1662 (1 seed)
Globba aurea Elmer	MICH, HH Bartlett 15543 (1 seed)
Globba maculata Blume	MICH, HH Bartlett 7544 (1 seed)
Globba pendula Roxb.	NYBG, Rahmat Si Toroes 3668 (1 seed)
Globba sessiliflora Sims	SING, JLS–1957 (1 seed)
Globba spathulata Roxb.	US, WJ Kress 01–6914 (1 seed)
Hedychium coronarium J.Koenig	MICH, A Shilom Tan 1771 (1 seed)
Hedychium gardnerianum Sheppard ex Ker Gawl.	MICH, SY Smith s.n. (commercially purchased) (1 seed)
Hedychium hasseltii Blume	US, T Wood 94–3700 (1 seed)
Hedychium muluense R.M.Sm.	SING, JLS–54 (1 seed)
Hemiorchis sp.	US, WJ Kress 01–6884 (1 seed)
Hornstedtia conica Ridl.	SING, SNG–35 (1 seed)
Hornstedtia leonurus (J.Koenig) Retz.	SING, SNG–174 (1 seed)
Hornstedtia scottiana (F.Muell.) K.Schum.	US, WJ Kress 80–1129 (1 seed)
Kaempferia pulchra Ridl.	K, Rabil 296 (1 seed)

Monolophus sikkimensis (King ex Baker) Veldkamp & Mood	SING, Wallich s.n. (1 seed)
Newmania sp.	SING, JLS–1646 (1 seed)
Plagiostachys escritorii Elmer	NY, Elmer 16216 (1 seed)
Plagiostachys philippinensis Ridl.	NY, Ramos and Edaño 75626 (1 seed)
Pleuranthodium sp.	US, TG Hartley 10989 (1 seed)
Renealmia lucida Maas	SING, JLS–1019 (1 seed)
Renealmia occidentalis (Sw.) Sweet	MICH, J Vera Santos 2513 (1 seed)
Riedelia corallina (K.Schum.) Valeton	NY, Annable 3639 (1 seed)
Riedelia sp.	SING, JLS–428 (1 seed)
Roscoea alpina Royle	AAU, 1013 (1 seed)
Siamanthus siliquosus K.Larsen & Mood	US, WJ Kress 99–6358 (1 seed)
Siliquamomum tonkinense Baill.	SING, VNM–B–1469 (1 seed)
Siphonochilus aethiopicus (Schweinf.) B.L.Burtt	MO, P Kuchar 22948 (1 seed and 1 fruit)
Siphonochilus kirkii (Hook.f.) B.L.Burtt	MO, ABKatende K1880 (2 seeds)
Vanoverberghia sepulchrei Merr.	NY, Ramos and Edaño 45045 (1 seed and 1 fruit)
Zingiber larsenii Theilade	SING, JLS–1270 (1 seed)
Zingiber officinale Roscoe	Delft University of Technology, JW van Loon (1 seed)
Zingiber spectabile Griff.	Delft University of Technology, JW van Loon (1 seed)
Zingiber thorelii Gagnep.	SING, JLS–1271 (1 seed)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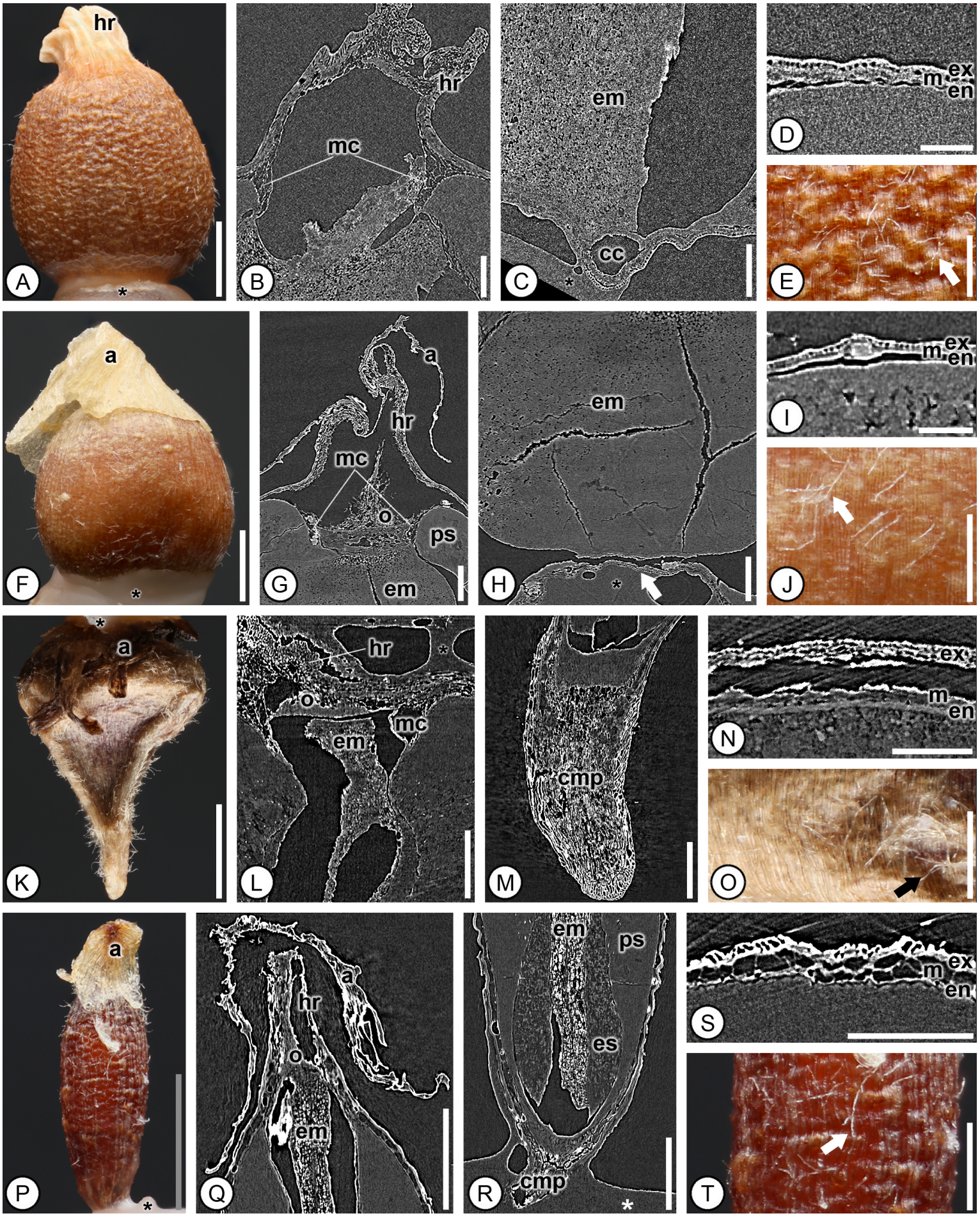
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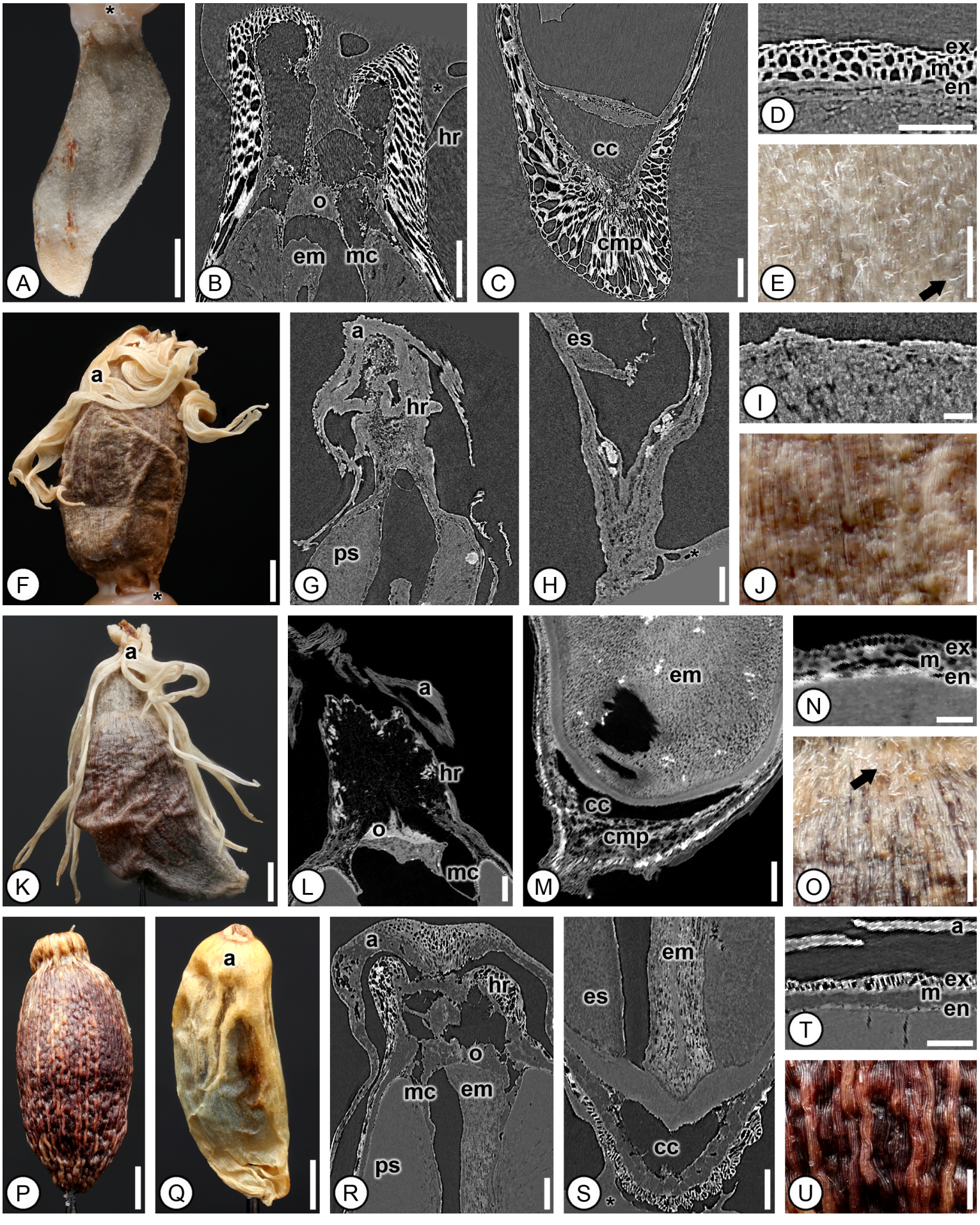
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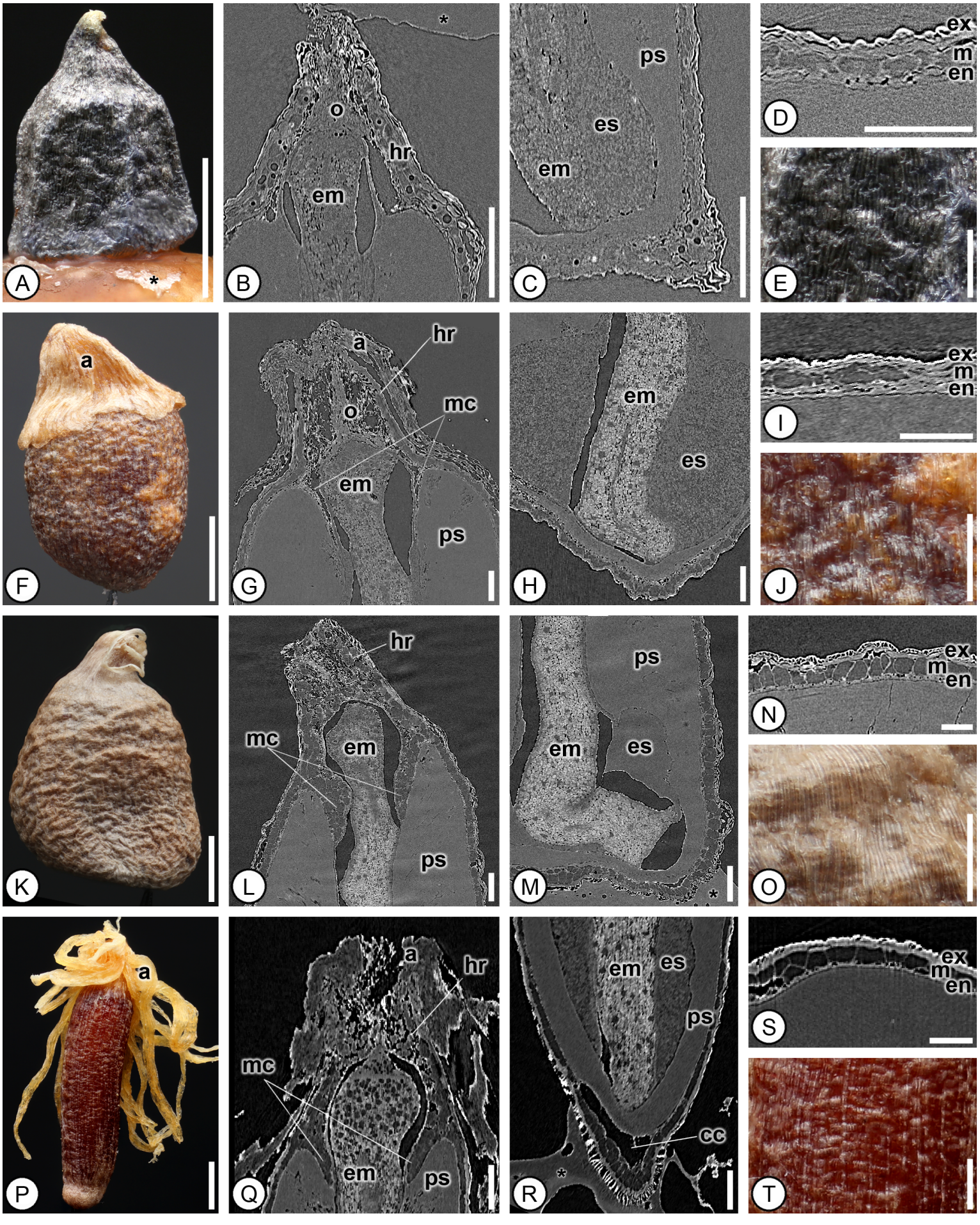
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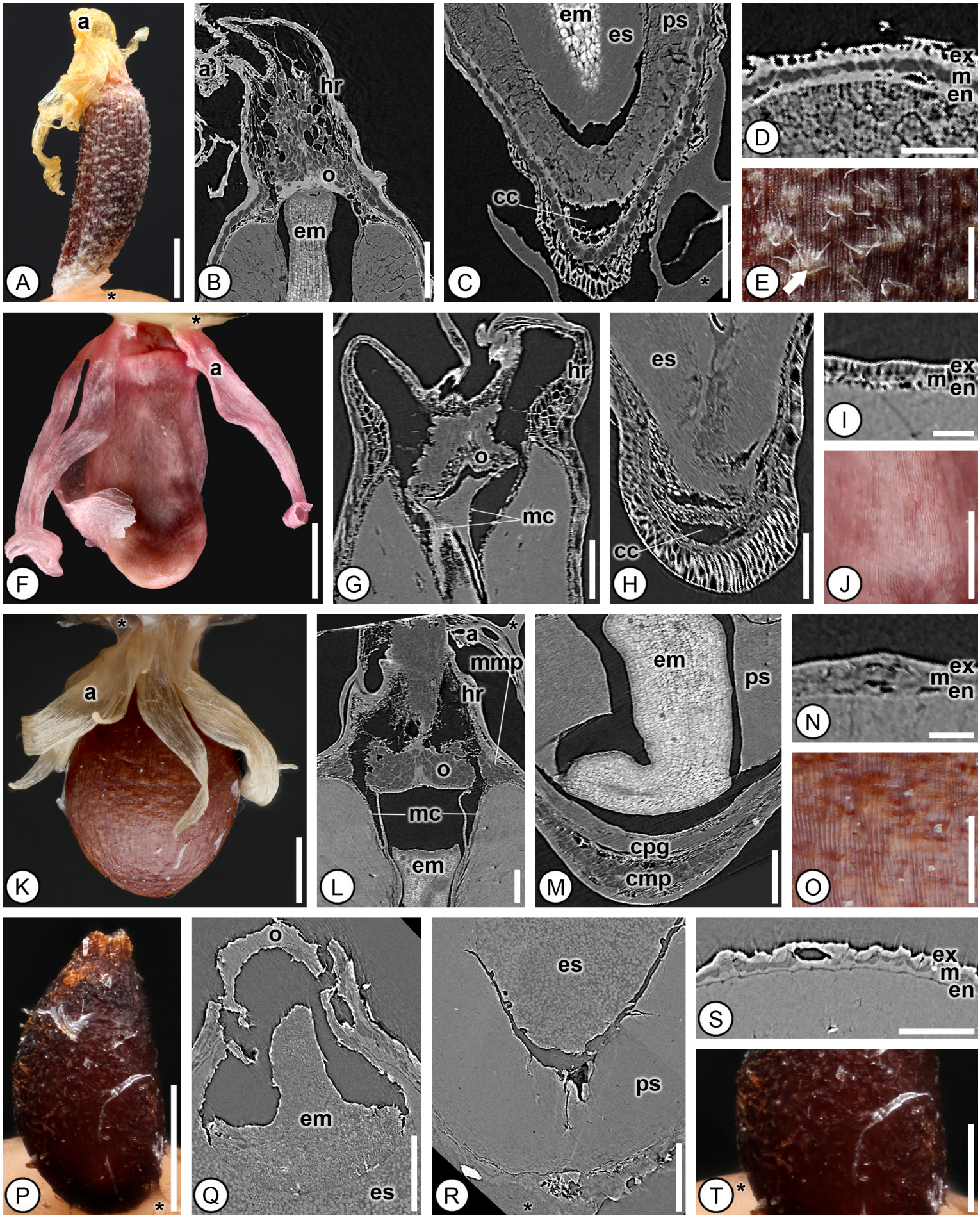
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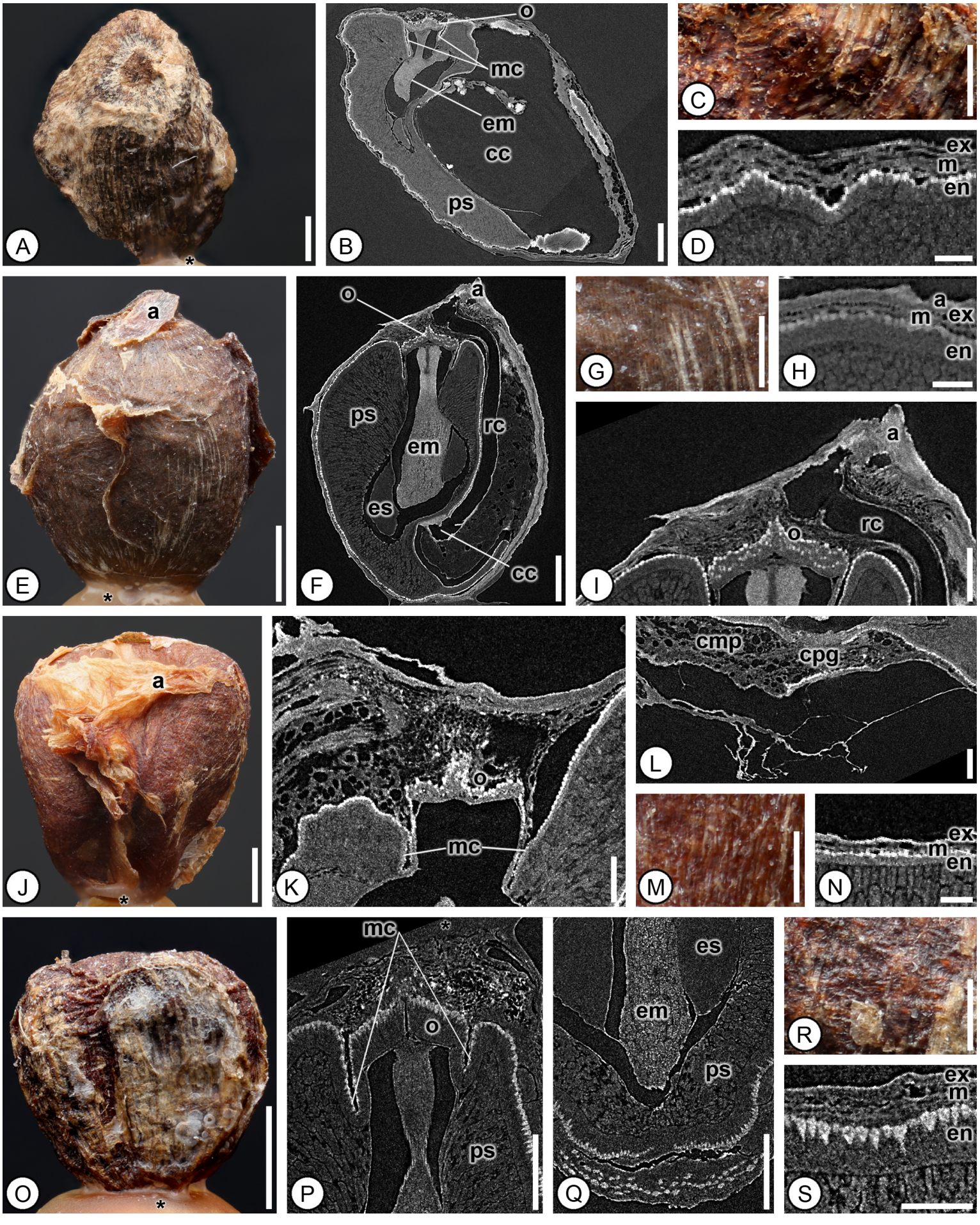
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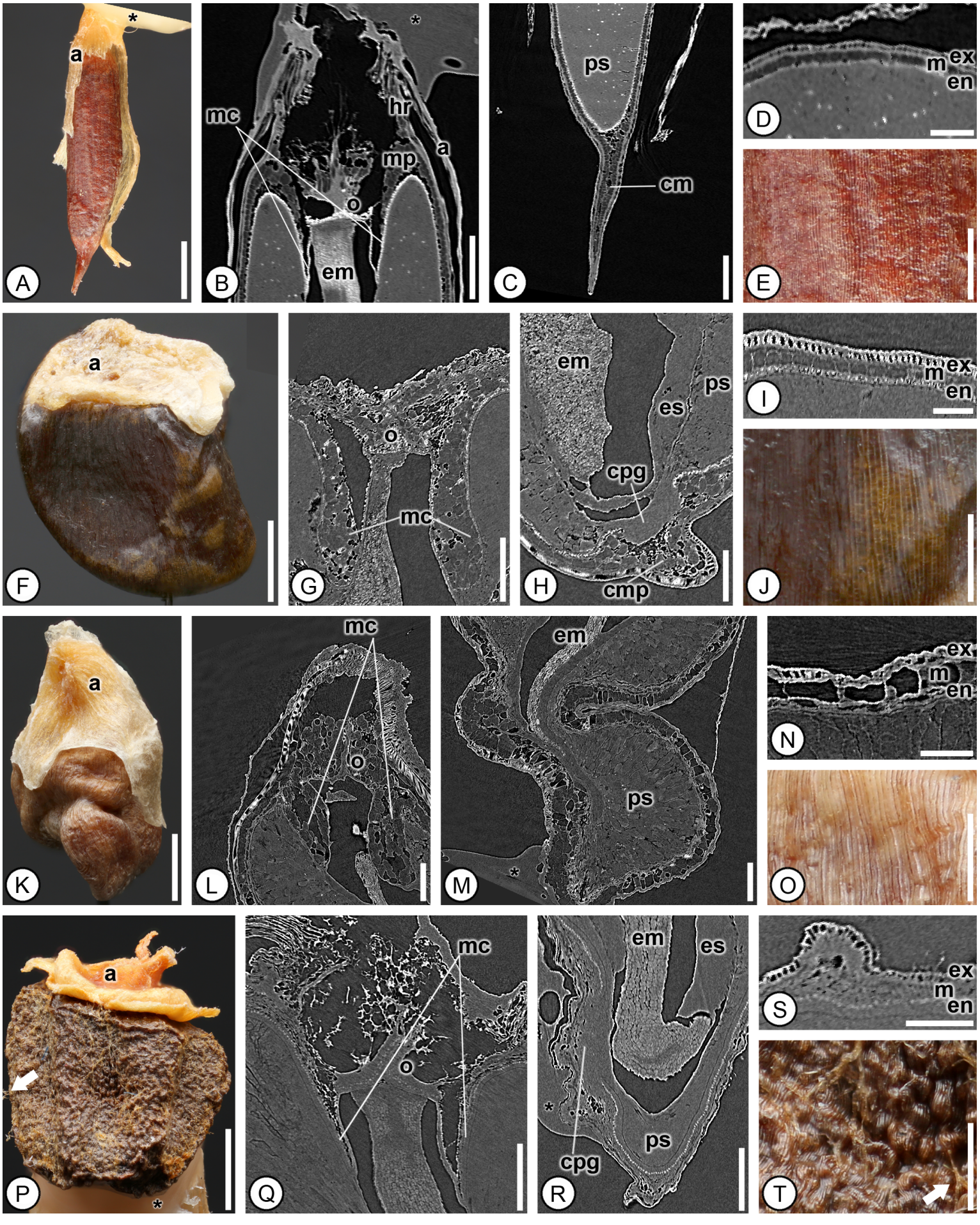
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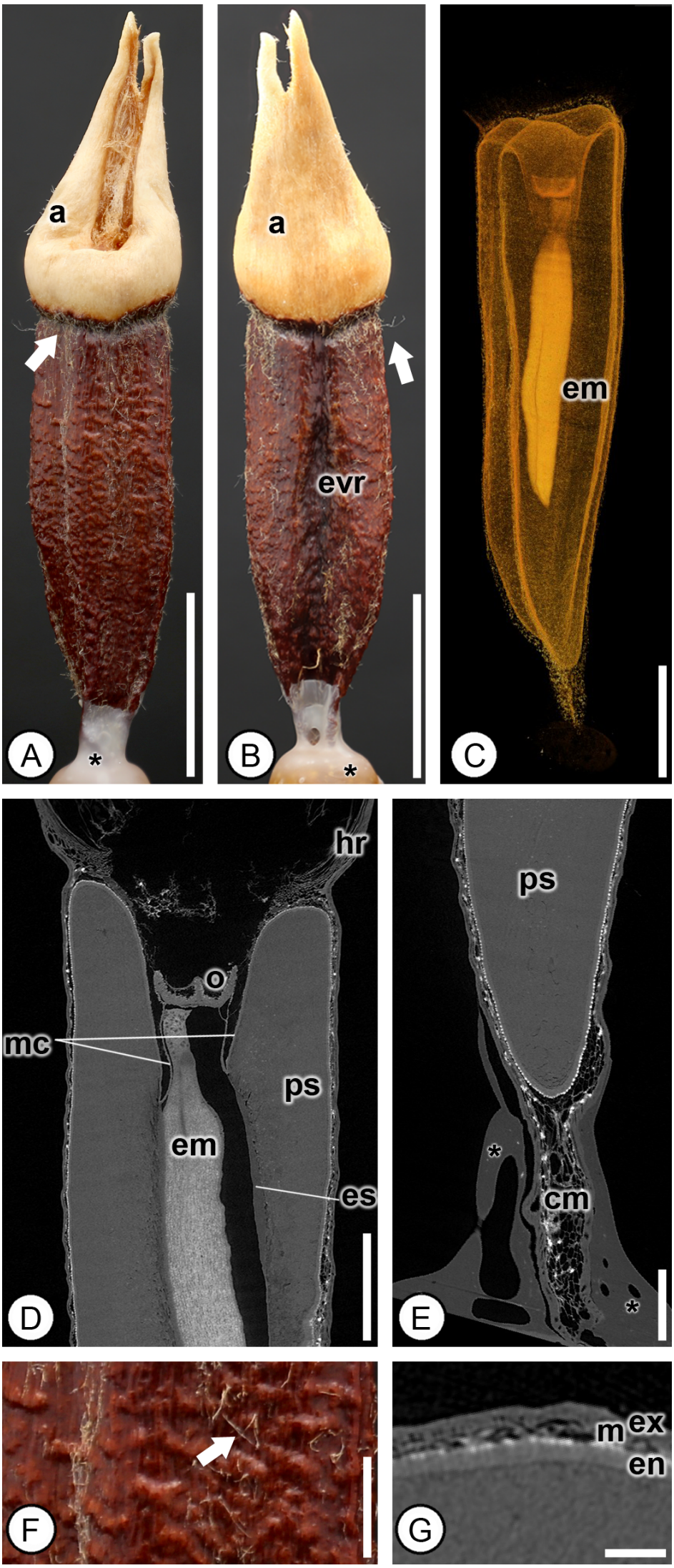
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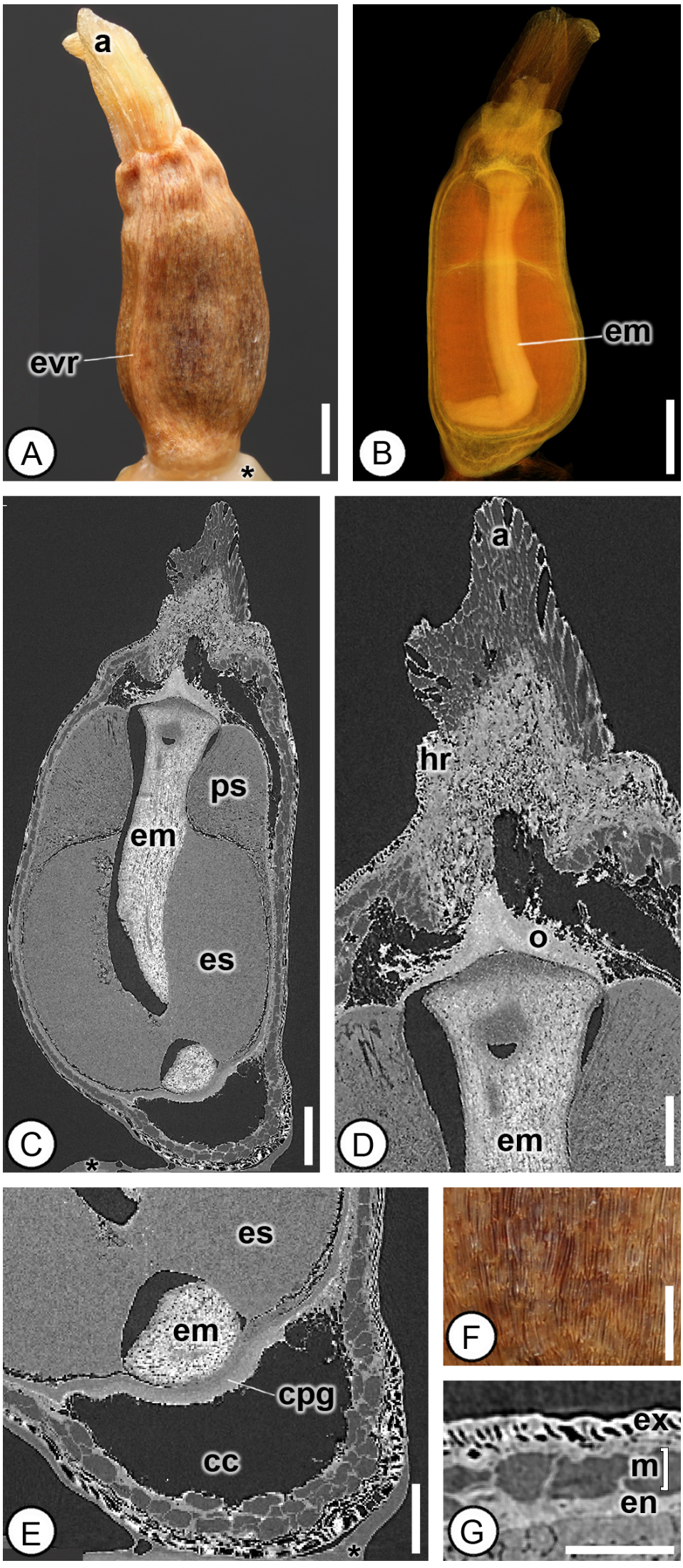
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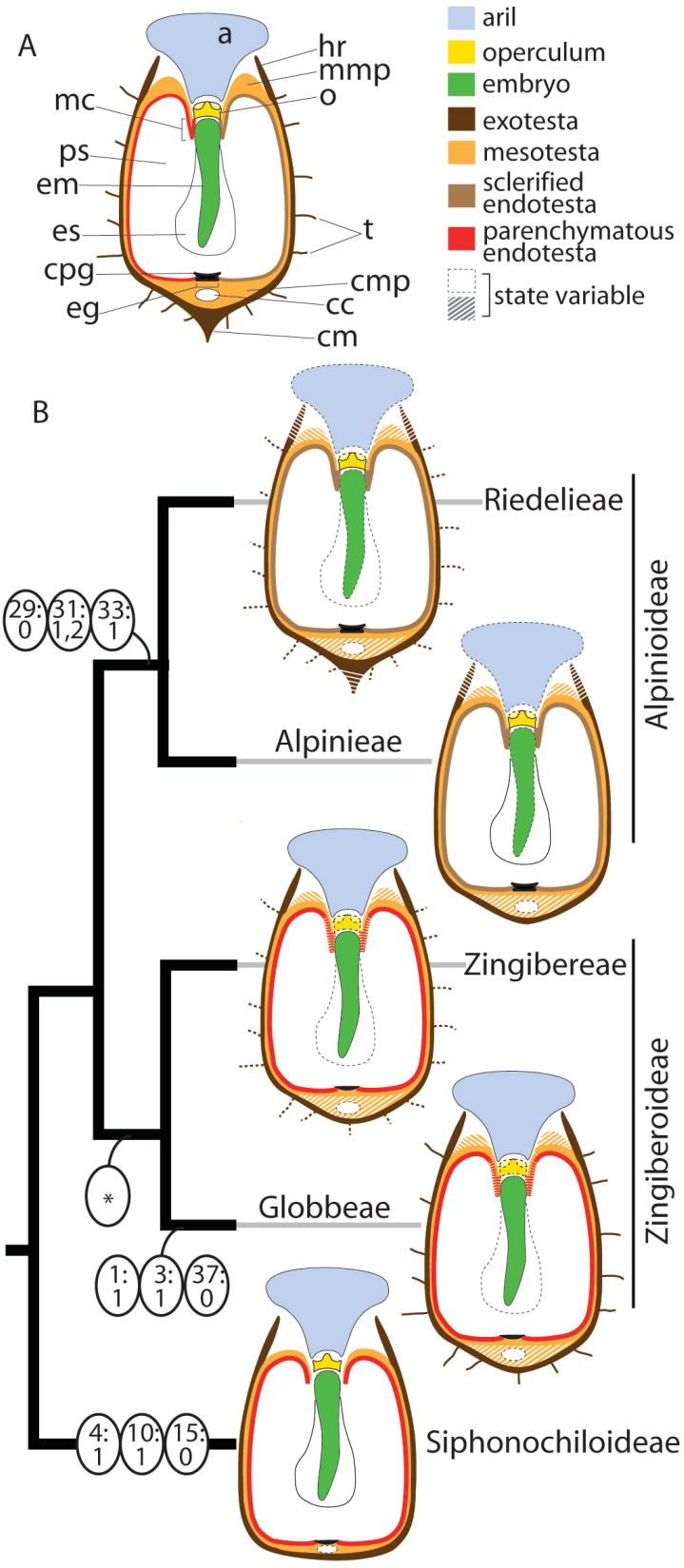
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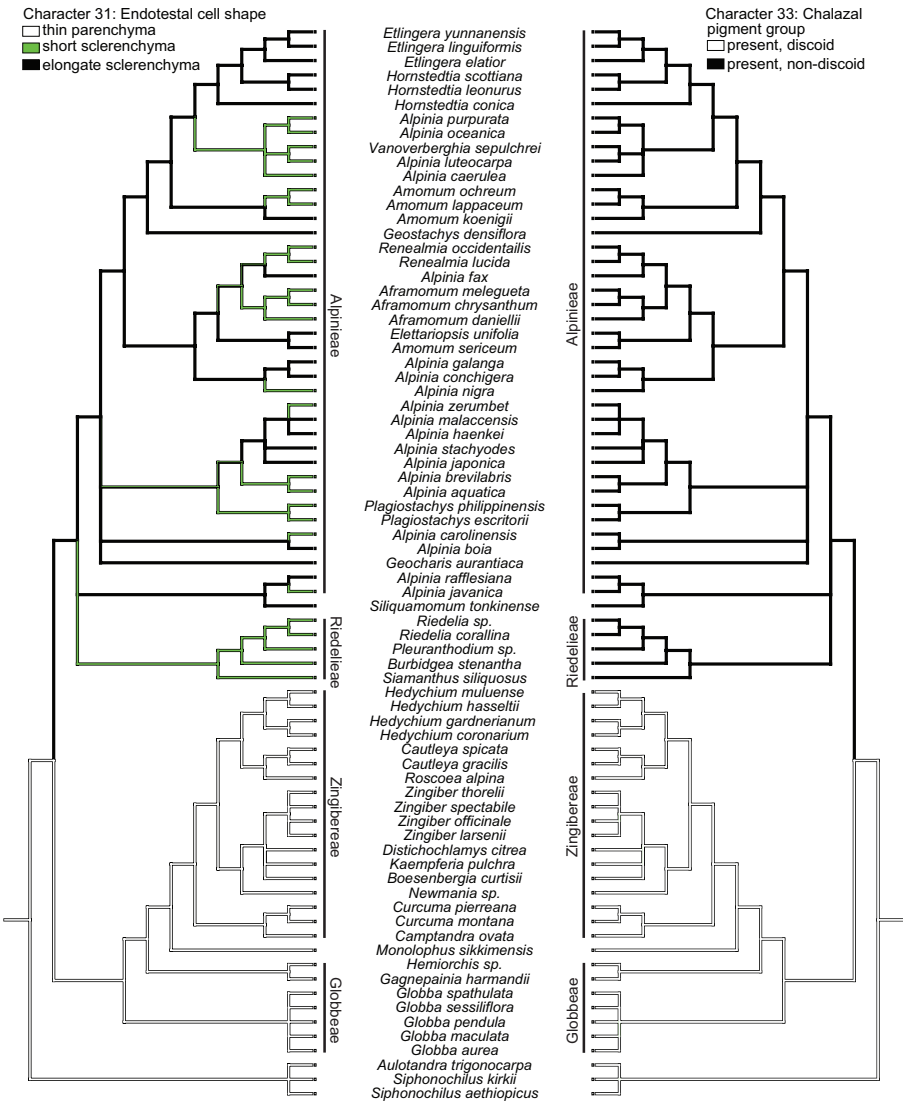
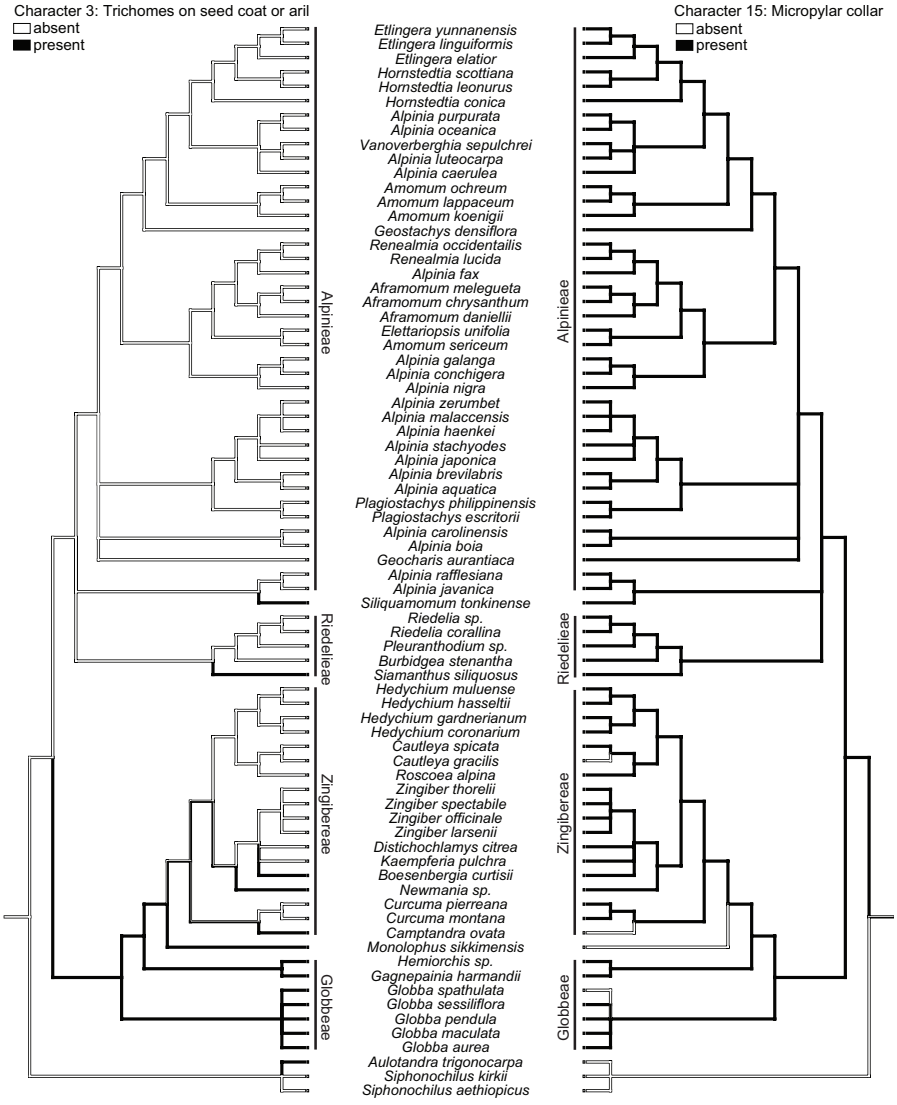
state variable
B
Riedelieae
29: 0
31: 1,2
33: 1
Alpinieae
Alpinioideae
Zingibereae
Zingiberoideae
*
Globbeae
1: 1
3: 1
37: 0
4: 1
10: 1
15: 0
Siphonochiloideae


Character 31: Endotestal cell shape
thin parenchyma
short sclerenchyma
elongate sclerenchyma
Character 33: Chalazal pigment group
present, discoid
present, non-discoid
Etlingera yunnanensis
Etlingera linguiformis
Etlingera elatior
Hornstedtia scottiana
Hornstedtia leonurus
Hornstedtia conica
Alpinia purpurata
Alpinia oceanica
Vanoverberghia sepulchrei
Alpinia luteocarpa
Alpinia caerulea
Amomum ochreum
Amomum lappaceum
Amomum koenigii
Geostachys densiflora
Renealmia occidentailis
Renealmia lucida
Alpinia fax
Aframomum melegueta
Aframomum chrysanthum
Aframomum daniellii
Elettariopsis unifolia
Amomum sericeum
Alpinia galanga
Alpinia conchigera
Alpinia nigra
Alpinia zerumbet
Alpinia malaccensis
Alpinia haenkei
Alpinia stachyodes
Alpinia japonica
Alpinia brevilabris
Alpinia aquatica
Plagiostachys philippinensis
Plagiostachys escritorii
Alpinia carolinensis
Alpinia boia
Geocharis aurantiaca
Alpinia rafflesiana
Alpinia javanica
Siliquamomum tonkinense
Riedelia sp.
Riedelia corallina
Pleuranthodium sp.
Burbidgea stenantha
Siamanthus siliquosus
Hedychium muluense
Hedychium hasseltii
Hedychium gardnerianum
Hedychium coronarium
Cautleya spicata
Cautleya gracilis
Roscoea alpina
Zingiber thorelii
Zingiber spectabile
Zingiber officinale
Zingiber larsenii
Distichochlamys citrea
Kaempferia pulchra
Boesenbergia curtisii
Newmania sp.
Curcuma pierreana
Curcuma montana
Camptandra ovata
Monolophus sikkimensis
Hemiorchis sp.
Gagnepainia harmandii
Globba spathulata
Globba sessiliflora
Globba pendula
Globba maculata
Globba aurea
Aulotandra trigonocarpa
Siphonochilus kirkii
Siphonochilus aethiopicus
Alpinieae
Riedelieae
Zingibereae
Globbeae


Character 3: Trichomes on seed coat or aril
absent
present
Character 15: Micropylar collar
absent
present
Etlingera yunnanensis
Etlingera linguiformis
Etlingera elatior
Hornstedtia scottiana
Hornstedtia leonurus
Hornstedtia conica
Alpinia purpurata
Alpinia oceanica
Vanoverberghia sepulchrei
Alpinia luteocarpa
Alpinia caerulea
Amomum ochreum
Amomum lappaceum
Amomum koenigii
Geostachys densiflora
Renealmia occidentailis
Renealmia lucida
Alpinia fax
Aframomum melegueta
Aframomum chrysanthum
Aframomum daniellii
Elettariopsis unifolia
Amomum sericeum
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Alpinia conchigera
Alpinia nigra
Alpinia zerumbet
Alpinia malaccensis
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Alpinia aquatica
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Plagiostachys escritorii
Alpinia carolinensis
Alpinia boia
Geocharis aurantiaca
Alpinia rafflesiana
Alpinia javanica
Siliquamomum tonkinense
Riedelia sp.
Riedelia corallina
Pleuranthodium sp.
Burbidgea stenantha
Siamanthus siliquosus
Hedychium muluense
Hedychium hasseltii
Hedychium gardnerianum
Hedychium coronarium
Cautleya spicata
Cautleya gracilis
Roscoea alpina
Zingiber thorelii
Zingiber spectabile
Zingiber officinale
Zingiber larsenii
Distichochlamys citrea
Kaempferia pulchra
Boesenbergia curtisii
Newmania sp.
Curcuma pierreana
Curcuma montana
Camptandra ovata
Monolophus sikkimensis
Hemiorchis sp.
Gagnepainia harmandii
Globba spathulata
Globba sessiliflora
Globba pendula
Globba maculata
Globba aurea
Aulotandra trigonocarpa
Siphonochilus kirkii
Siphonochilus aethiopicus
Alpinieae
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