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Systematic applications of pollen grain morphology and development in the acanthaceae

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SYSTEMATIC APPLICATIONS OF POLLEN GRAIN MORPHOLOGY AND DEVELOPMENT IN THE ACANTHACEAE

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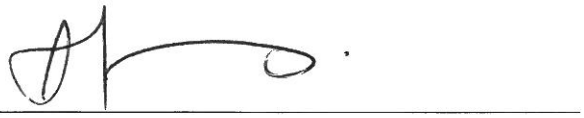
A Thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Doctor of Philosophy

October 2015

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Alisoun House)

17th day of October 2015 in Braamfontein, Johannesburg

ABSTRACT

External pollen grain morphology has been widely used in the taxonomy and systematics of flowering plants. The eurypalynous family Acanthaceae is a notable example of a group where these pollen diversities have proved useful in determining relationships between taxa. However, internal pollen wall features have received far less attention due to the difficulty of examining the underlying exine from which the external sculpturing is derived. Consequently, internal wall features have thus far not been used in formulating existing classifications. A new technique involving precise cross sectioning or slicing of pollen grains at a selected position, using a focused ion beam-scanning electron microscope (FIB-SEM), was used on 39 species of Acanthaceae to examine the internal pollen wall structure and identify features of potential systematic relevance. Five basic internal wall structures were described in this study. The study also showed that similar external pollen wall features may have distinctly different underlying structures.

Investigation of twelve species of *Justicia* confirmed that the pollen of this genus is highly variable. The internal structure was constructed from walled chambers and was not restricted to separate, individual columellae. This structure was characteristic for all the examined species.

Morphometric analyses of the genus *Justicia* and representatives of six related genera within the sub-tribe Justiciinae using a combination of external and internal wall structure characters identified in this study confirmed existing groupings of the Justiciinae broadly in line with classifications based on molecular data. Overall, inclusion of internal wall characters in cluster analyses both supports and assists in clarifying relationships which were previously obtained using external morphology alone.

A developmental study of the formation of external grain sculpturing within anthers of *Barleria obtusa* showed that the bulk of the sculptured exine appears to be present at a very early stage. The existing sculpturing then expands and stretches out over the surface of the grains as they increases in volume until maturity is reached.

The FIB-SEM afforded high resolution 3D views of internal pollen wall structure, supplementing data gained from other methods of microscopy. It was also suitable for investigating the softer tissue of the developing pollen grain walls within anthers. These additional data contribute to a better understanding of which internal wall layers and components give rise to the external features of the pollen grain wall.

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CHAPTER ONE - INTRODUCTION

The challenge with any taxonomic classification is to determine characteristics that best identify a species as a member of a specific group or taxon. Identifying plant features that have taxonomic significance is important for understanding Angiosperm systematics and phylogeny. This has proved to be problematic in the Acanthaceae, particularly when determining intrafamilial relationships such as subfamilies, tribes and even genera. The Acanthaceae are a most interesting family from a taxonomic perspective due to their worldwide distribution and diverse members, which number in the thousands. The sheer scale and diversity of this family present many challenges to producing a complete and uncontroversial classification.

One of the characteristics that has been used extensively in most existing classifications of the Acanthaceae is pollen morphology (e.g., Lindau, 1895; Bremekamp, 1953; 1965; Scotland and Vollesen, 2000). Pollen wall sculpturing is often highly distinctive and consistent within taxa. Pollen morphology is also considered to be conservative, in that fossil pollen can often be related to pollen of an extant taxon because of closely shared characteristics (Walker, 1974). These useful characteristics of pollen form the basis of pollen taxonomy and palynology.

The members of the Acanthaceae are interesting from a palynological perspective in that they encompass a wide range of pollen morphological features, from supposedly primitive right through to more advanced types. The extent of the pollen diversity therefore makes pollen morphology an attractive characteristic to investigate for its taxonomic potential. Current palynological data for this group have largely been derived from the external features of the pollen grain. One aspect of pollen morphology that has not been as thoroughly investigated (due to the difficult techniques involved), and may prove to be taxonomically important, is the internal structure of the outer pollen wall or exine. As technology has advanced, it has become possible to investigate pollen morphology in greater detail at the ultrastructural, internal level and this study aims to apply a new technology and technique specifically to the internal wall of the pollen grain in this family. Many intrafamilial relationships in the Acanthaceae still remain contentious and the application of palynological findings relating to phylogeny could be of value well beyond the confines of just this family.

As yet uninvestigated features of the internal pollen wall structure could potentially yield useful characteristics to contribute to developing an improved classification system.

General Introduction to the Acanthaceae

The Acanthaceae are a large and diverse family of dicotyledonous plants, comprising at least 221 genera with species numbering in the thousands (c. 4 000), and therefore are

among the twelve most species rich families and constitute about 1% of the described living angiosperms (Heywood et al., 2007). The family has a worldwide distribution, centred mainly in tropical and subtropical areas, rarely extending into temperate regions. The family is well represented in South and Central America, tropical Africa, Madagascar and tropical Asia, but less so in Australia. Only one genus, *Acanthus* L. is native to Europe. Relatively few species occur in temperate Asia and North America. There are about 42 genera with 350 species in southern Africa, found mainly in summer rainfall and semi-arid areas (Balkwill and Welman, 2000). This family is commonly represented in a wide range of habitats from arid areas to rain forests.

The members of this family present a morphologically diverse group of herbs, shrubs or climbers (subfamily Thunbergioideae) and even sometimes large trees (such as *Bravaisia* DC. in Mexico). Recently, the black mangroves *Avicennia* L. (eight to ten species) have also been added to the family (Schwarzbach and McDade, 2002). The leaves are simple, opposite, without stipules and occasionally spiny. Apart from the subfamilies Nelsonioideae and Thunbergioideae and in the tribe Acantheae, cystoliths are commonly present (in the tribe Ruellieae), identifiable as white streaks in the lamina of dried leaves. The family includes a wide range of floral morphologies. The inflorescence is usually spike-like (cymose or racemose) or flowers may be solitary, but conspicuous, showy bracts are often present. The flowers are always bisexual and strongly to weakly zygomorphic, usually with a two lipped corolla, or the upper lip may be lacking. The corolla forms a tube and the stamens are attached to the corolla tube. There are two or four stamens (rarely five) and anthers usually have two chambers which are often unequal (or chambers may be reduced to one per anther). Corolla aestivation patterns are imbricate or contorted (only open in *Acanthus*). There may be a wide range of pollinator relationships. The fruit is a non-fleshy, few seeded (two to eight, sometimes 16), dehiscent capsule, except in *Mendoncia* Vell. ex Vand., where the fruit is a single seeded drupe. In the subfamily Acanthoideae the seeds are borne on minute, stiff, hook-like outgrowths called retinacula. This feature is characteristic for the subfamily but is absent in the two presumed sister subfamilies, Nelsonioideae and Thunbergioideae (Clarke, 1885; Balkwill and Welman, 2000; Scotland and Vollesen, 2000) and *Avicennia*, grouped with Thunbergioideae (McDade et al., 2008).

The family is well known for the remarkable diversity of its pollen size, shape, apertures and exine structure and ornamentation. Many genera can be characterized by a distinctive pollen type, however, there is a wide range of pollen morphology within some genera e.g., *Justicia* L. (Graham, 1988; Daniel, 1998).

Classification of Acanthaceae

The exact delimitation and subdivision of the family has been controversial and resulted in a number of conflicting taxonomic groupings due to the different characters and combinations of characters used for developing the classifications (Scotland, 1992).

These characters include corolla aestivation, presence or absence of retinacula, two or four stamens, number of anther chambers and various aspects of pollen morphology. In addition, many investigations have usually focused on specific geographical regions (e.g., Balkwill and Getliffe Norris, 1988; Muller et al., 1989; Daniel, 1998; Anjum and Qaiser, 2010) and only three/four worldwide classifications have been proposed for the family, the most recent of these being that of Scotland and Vollesen (2000). The family was first subdivided on the basis of two or four stamens by de Jussieu (1789) and later, Nees (1847) published the first comprehensive classification of the family, but since then only a few major revisions have been formulated (Lindau, 1895; Bremekamp, 1965 and Scotland and Vollesen, 2000). Lindau (1895) divided the family into four subfamilies on the basis of their retinaculate fruits. He delineated the subfamily Acanthoideae as possessing retinacula, and subdivided it into two tribes based on corolla aestivation patterns. Further subdivisions of the tribes were based mainly on pollen morphology. Bremekamp (1965) reclassified the Acanthaceae, excluding genera without retinacula, and divided the remaining Acanthaceae into two subfamilies (Acanthoideae and Ruellioideae) on the basis of cystoliths, monothecate anthers and colpate pollen. Further subdivisions relied mainly on pollen morphology. The classification of Scotland and Vollesen (2000) incorporates data from previous classifications and represents a compromise between those of Lindau (1895) and Bremekamp (1965). It comprises all accepted genera of the family and is based on the synthesis of morphological and molecular data (Fig. 1.1).

These main classifications of the family, i.e. those of Lindau (1895), Bremekamp (1965) and Scotland and Vollesen (2000), have all used pollen morphology to fine-tune their classification at the genus level, as well as separating the *Acanthus* lineage from three other major lineages *Barleria* L., *Ruellia* L. and *Justicia*, the latter all having porate pollen (McDade et al., 2000b). Pollen morphology has also been used to some degree at the higher taxonomic levels of tribe and subtribe, with both Lindau (1895) and Bremekamp (1965) emphasising pollen characters at this level, notably to delimit the Acanthoideae and Ruellioideae on the basis of the Acanthoideae having colpate pollen (Bremekamp, 1965). In general, major lineages have been recognized within the Acanthaceae, but at various taxonomic levels, e.g., subfamily Ruellioideae (Bremekamp, 1965), or tribe Ruellieae, subtribe Ruelliinae (Scotland and Vollesen, 2000).

Recently much work has been done, especially by means of molecular studies, in delimiting the major tribes within the retinaculate clade of the family and investigating the relationships within these tribes (e.g., Scotland, 1991; 1993; Scotland et al., 1995; McDade et al., 2000a; McDade et al., 2005; Kiel et al., 2006; Daniel et al., 2008; McDade et al., 2008; Tripp et al., 2013). The classification of Scotland and Vollesen (2000) has more recently been revised in a comprehensive phylogenetic study across the family (McDade et al., 2008), where a few misplaced genera were identified, while Manktelow et al. (2001), has found evidence for a sixth major clade, Whitfieldieae

according to McDade et al. (2008) or Whitfieldiinae if following the terminology of Scotland and Vollesen (2000).

For the purpose of this study, the classification of Scotland and Vollesen (2000) will be utilized (Fig. 1.1), as it is the most recent analysis involving the whole family - 221 accepted genera of Acanthaceae - including species from diverse geographical locations and incorporating pollen taxonomy.

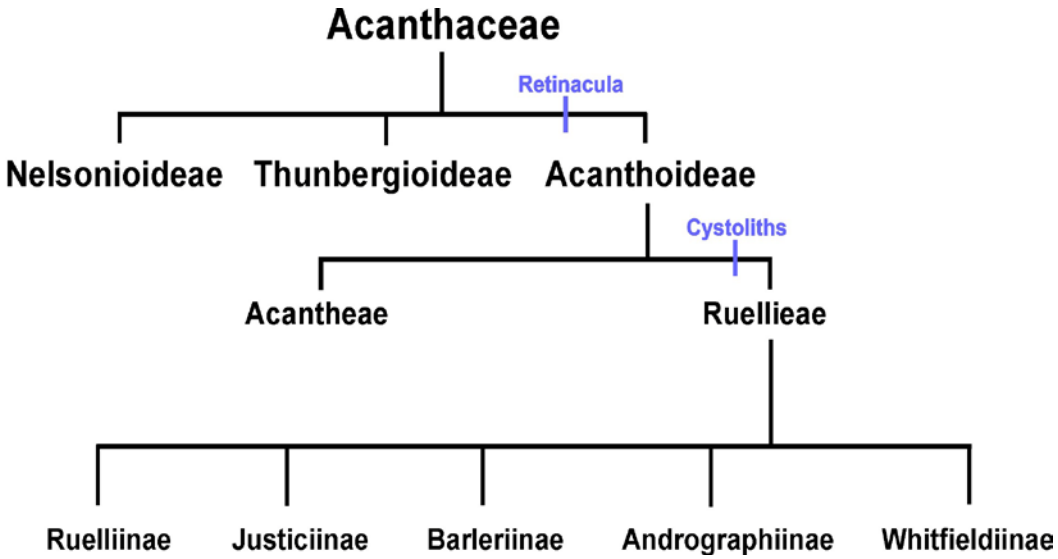

Figure 1.1 Classification of Acanthaceae *sensu* Scotland and Vollesen (2000) including Whitfieldiinae (Manktelow et al., 2001)

The larger genera are: *Justicia* comprising 600 – 700 pan-tropical species (Graham, 1988); *Strobilanthes* Blume with about 450 species in tropical Asia; *Ruellia* with approximately 350 pan-tropical species (Tripp et al., 2013); *Barleria* with more than 200 species found mostly in Africa, some in Asia, but only single species in West Africa and the Americas; *Staurogyne* Wall. with 145 pan-tropical species (Daniel and McDade, 2014); *Blepharis* Juss. consisting of 130 species in Africa and Asia; and the pan-tropical *Thunbergia* Retz. of more than 100, mostly climbing, species (Heywood et al., 2007).

In positioning the family among the Angiosperms, it has long been considered to have been derived from near the base of the Scrophulariaceae (Scotland et al., 1995). Molecular evidence confirms this and also suggests that the mangrove family Avicenniaceae has arisen within the Acanthaceae, even though they share few potential family characteristics apart from opposite leaves (McDade et al., 2008). The Acanthaceae are a classic case of a polythetic (having many, but not all characteristics in common) family with no single character defining it, but rather having the obvious similarities of its major groups holding the family together in a taxonomic sense. Recent reviews of its classification have resulted in major groupings very similar to those

recognized by specialists in the family for the last century or more.

Palynology in Taxonomy

The extensive diversity of pollen morphology makes the study of pollen, or palynology, an attractive field of investigation for pollen features which may have taxonomic significance. The following is a brief outline of pollen grain structure clarifying the terminology that will be used to describe the morphology of Acanthaceae pollen. The description and terminology generally follows that of Walker and Doyle (1975).

Most of the characteristics used in taxonomy to date have been related to either pollen grain apertures or pollen walls, but other features such as the appearance of the pollen-unit, polarity, symmetry, shape and grain size, are also used as indicators of phylogenetic relationships and higher taxonomic divisions. The apertures and pollen walls are of more importance to this study. Pollen apertures are thick or thin walled areas in the outer wall of the pollen grain (exine) through which the pollen tube usually germinates. They may also play a role in accommodating volume changes in the grain or harmomegathy (Wodehouse, 1935).

The following aperture characteristics are of taxonomic interest:

- Numbers of apertures - From a phylogenetic point of view, primitive grains are often in-aperturate, followed in order by mono-aperturate, di-aperturate, tri-aperturate and poly-aperturate (usually four, five or six pores).
- Shape of aperture - Three basic aperture shapes have been defined, elongate, furrow-like (colpate); round, pore-like (porate) and ring or band-like (zonate).
- Position - This is considered to be very important from a phylogenetic-evolutionary point of view, and aperture position is determined with respect to the equators and poles of pollen grains which are in turn determined by the position of an individual grain in the pollen tetrads produced by the pollen mother cells, even in the case of grains which are dehisced individually. Pollen developmental studies are sometimes required to verify the pole and equator areas.
- Structure (Simple/Compound) - Apertures are said to be simple or compound depending on the presence or absence of ora (specially delimited areas on the aperture membrane). Simple apertures have membranes lacking an os. Compound apertures have one or many ora.

Pollen wall features used in taxonomy relate to stratification of the wall as well as wall structure and sculpturing (Walker and Doyle, 1975). With regards to stratification, pollen walls consist of two morphologically distinct layers, the exine, or external, and the intine, or internal, layer. The exine is often made up of two chemically distinct layers called the ectexine and the endexine. The delimitation of these layers is usually only visible after applying the appropriate staining techniques for optical microscopy, or viewing with a transmission electron microscope (TEM) or FIB-SEM. As these internal

layers are chemically different and the endexine has a lower electron density than the ectexine, they are distinguishable using conventionally prepared TEM sections or with the Backscattered Electron Imaging detector when viewing with FIB-SEM. The distribution of these layers is not even over the entire surface area of the pollen walls and one layer may be thinner or thicker around apertures (Walker and Doyle, 1975). In addition, the exine itself usually consists of two morphologically distinct layers, an inner basal layer, the nexine and an outer, often sculptured layer, the sexine. In angiosperms, the sexine is often two layered with an internal layer of upright, rod-like structures called columellae, covered by another layer called the tectum. The nexine comprises the inner, unsculptured layers. The first layer of the nexine, lying directly below the sexine, is the foot layer, followed by the endexine and the intine. (Refer to Chapter Two, Fig. 1: pg.2.2/1536).

When discussing pollen walls, a distinction is made between structure and sculpturing. Structure refers to the appearance of the internal layers within the exine and sculpturing refers to any external features on the tectum, such as spines and granules. The breakdown of the tectum or the presence of pores in the tectum can blur the distinction between structure and sculpturing when, for example, columellae then form the sculpturing. Numerous sculpturing types have been identified for pollen with a definite tectum and external sculpturing. These include psilate (smooth), foveolate (pitted), fossulate (grooved), scabrate (having fine projections), verrucate (warty), baculate (rods), pilate (rods with distinctly swollen heads), gemmate (sessile pilae), echinate (spiny), rugulate (elongated structures irregularly and tangentially distributed), striate (similar to rugulate but lying more or less parallel) and reticulate (forming a network or reticulum) (Walker and Doyle, 1975).

It is possible that pollen grains with the same surface sculpturing, for example a reticulate surface, may have very different internal structures (Moore et al., 1991). In other words, pollen grains that have the same external appearance when viewed with the SEM may have distinct internal wall or exine structures (as illustrated in Fig. 1.2).

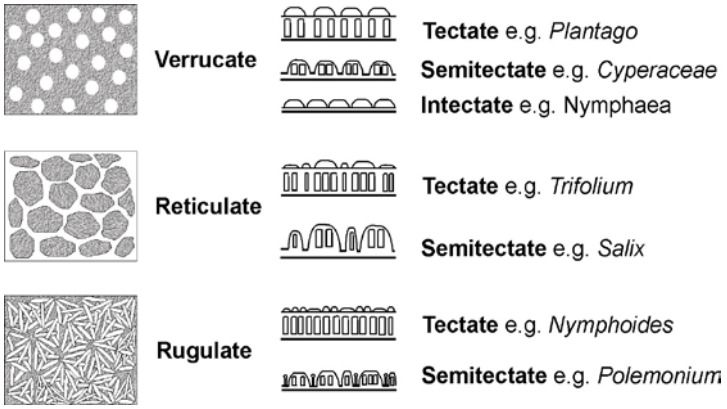

Figure 1.2 Diagrams of surface sculpturing and sections of possible underlying exine structures (redrawn from Moore, Webb and Collinson, 1991)

Acanthaceae Pollen Studies

The family is notably eurypalynous (Raj, 1961), and all palynological studies of the Acanthaceae remark on the pollen diversity. The range of pollen features includes grain size and shape; aperture number, aperture shape, structure and position as well as exine sculpturing.

The first detailed palynological studies of this family were made by Fritzsche (1832), and the taxonomic value of pollen morphology in the Acanthaceae was noted by Radlkofer (1883) who distinguished seven pollen types. These initial types were expanded by Lindau (1895), who documented eleven pollen types for the family and used pollen morphology as the basis for his sub-familial classification. He described the pollen types as Glatter, runder pollen; Spaltenpollen; Rippenpollen; Spangenpollen; Rahmenpollen; Knötchenpollen; Stachelpollen; Gürtelpollen; Daubenpollen; Wabenpollen and 'Pollen von Ander Form' (others). More recently researchers have suggested up to 26 pollen types for the family in Madagascar (Muller et al., 1989), while another study has described pollen types based on the taxa that exhibit the type of pollen sculpturing, e.g., *Barleria*-type, *Hygrophila*-type, *Strobilanthes*-type (Anjum and Qaiser, 2010).

Many members of the Acanthaceae have been the subject of detailed pollen morphological studies, especially by Balkwill and Getliffe Norris (1988), Bremekamp (1953), Daniel (1978; 1986; 1990; 1998), Furness (e.g., 1990; 1991; 1993; 1994; 1995; 1996), Graham (1988), Immelman (1989), Raj (1961), Scotland (1990; 1992; 1993), to name a few. Pollen diversity within the Acanthaceae is considered to be taxonomic rather than geographic and pollen of genera from Old World and New World regions is consistent within each genus. Although most genera have relatively homogeneous pollen, some do show pollen diversity, e.g., *Justicia* (Graham, 1988; Daniel, 1998). It has also been suggested that similar pollen morphologies are often due to convergence rather than immediate common ancestry, but some shared, derived characters (i.e. synapomorphic characters) provide evidence of relationships (Scotland, 1992).

One aspect of pollen morphological studies in the Acanthaceae that is lacking, and may prove to be taxonomically important, is a study of the internal structure of the exine. Current studies have largely focused on external features. In other families, such as the Compositae/Asteraceae, progress has been made in the study of the internal structure of the walls and the development of pollen walls within anthers (Blackmore and Barnes, 1985; 1986).

Cladistic Studies

Apart from the traditional classifications mentioned above, which are primarily based on morphology, some cladistic analyses of this family have also been undertaken. The aim of cladistics is to produce an unbiased hypothesis for the phylogenetic or

evolutionary relationships between members of a group. This is undertaken by identifying primitive (plesiomorphic) and advanced or derived (apomorphic) characteristics. A cladogram, or representation of the relationships, is drawn beginning with a universal plesiomorphic (primitive) characteristic and adding a node or branch whenever one or more apomorphic (derived) characters are present in one group and not another (Skelton et al., 2002).

Scotland and Vollesen (2000) have given several reasons for why a complete cladistic analysis of all genera of the Acanthaceae is not possible at present. These include the lack of certainty of monophyly in some genera, the extreme diversity amongst *Justicia*, the unavailability of material and incomplete knowledge of certain genera, as well as the difficulty of coding characters such as pollen morphology variation. They also mention the possible limitations of clades based purely on molecular data which excludes morphological features. The five clades of Scotland and Vollesen (2000) possessing retinaculate fruits, which have been investigated in molecular phylogenetic studies, are Acantheae (McDade et al., 2005); Justiciinae (Justicieae *sensu* McDade et al., 2000); Barleriinae (Barlerieae *sensu* McDade et al., 2008); Andrographiinae (Andrographidieae *sensu* McDade et al., 2008) and Ruelliinae (Ruellieae *sensu* Tripp et al., 2013). These investigations have served as a test for the classification of Scotland and Vollesen (2000) as they have confirmed the morphological features (including pollen morphology) used to diagnose the taxa.

A concern with cladistics is accurately determining homology, where a characteristic is common to multiple groups as a result of evolutionary descent as opposed to analogy, where the characteristic could be shared as a result of convergent evolution. Analogous features can also occur as a result of the development or growth within individuals, where the similarity is then a result of ontogeny and not phylogeny (Skelton et al., 2002). In the case of pollen morphology, certain external surface features could imply homology and yet have different underlying internal wall morphology.

As regards pollen morphological homologues in the Acanthaceae, Scotland and Vollesen (2000) accept colpate pollen as a homologue for the Acanthoideae *sensu* Bremekamp (1965), ‘Daubenpollen’ with thickened aperture margins as a homologue for Andrographideae *sensu* Lindau (1895), and a continuum of ‘Rahmen - Spangen - Knotchen - Gurtelpollen’ features in the Justicieae *sensu* Bremekamp (1965).

Microscopy Techniques used in Palynology

Palynological data for this family has to date largely focused on the external structure of the pollen grains, making use of scanning electron microscopy (SEM). One of the purposes of this study is to develop a new technique to examine the internal structure of the acanthaceous pollen walls. In the past, the pollen wall structure has been investigated using light microscopy (LM) and TEM, to give two dimensional results,

and SEM to study external features of pollen grains in three dimensions. Three dimensional (3D) reconstructions from TEM data require serial thin sectioning of a single pollen grain, which is a highly skilled technique.

Many useful and elegant microscopy studies have been performed to yield results on the features of pollen grain walls in the Acanthaceae. Raj (1961) produced a very comprehensive LM study of the pollen morphology of the family. Furness (e.g., 1990; 1991; 1993; 1994, 1995 and1996) carried out extensive SEM and TEM investigations on various taxa and many workers (e.g., Balkwill and Getliffe Norris, 1988; Daniel, 1998; Scotland and Vollesen, 2000) have looked at the pollen grain features using SEM and how they can be used to understand the phylogeny of the family.

The focused ion beam-scanning electron microscope or FIB-SEM is an instrument with a SEM column and a FIB column integrated in the same specimen chamber, like a standard SEM, but where the SEM uses a focused beam of electrons to image the sample in the chamber, the FIB uses a focused beam of gallium ions to cut or mill a cross-section while the sample remains in the chamber. Sample preparation is simple, as for standard SEM work. Specimens are pre-coated to avoid charging and increase stabilisation. Within the chamber, prior to cross-sectioning, the selected specimen may be coated with a platinum strip for extra protection. Specimen material is sputtered or milled away to a chosen depth with each pass of the beam, this allows for precise cross-sectioning of the specimen, effectively removing a layer of a predetermined thickness. One difficulty is that the sectioning process with the FIB-SEM often produces significant streaking effects on the cut surface as a result of re-deposition of milled material. This is a consequence of the action of the FIB, which creates sections by removing atoms from the surface of the specimen and depositing them behind the cutting plane. In the process, some of the removed material is also re-deposited on the exposed surface, creating streaks or amorphous blemishes that appear in most results. These unwanted deposits can be partially removed by subsequent milling or polishing with a lower beam current. While this obviously detracts from the aesthetic appearance of the results, useful data are still obtained as detailed features are still discernible. After cutting and polishing, the specimen can then be viewed with the SEM portion of the machine, to prevent further damage to the sample. In this way serial sections of an entire pollen grain can be obtained in 3D. This makes high resolution 3D investigation of pollen grain walls possible.

Although typically used for semiconductor research, FIB techniques have previously been used to investigate the ultra-structure of fossilised pollen walls with promising results (Villanueva-Amadoz et al., 2012). FIB techniques have also been used to characterise fragile and delicate biological specimens by cutting a window through the surface to observe subsurface layers (Ishitani et al., 1995), or by cutting an ultra-thin layer or lamella out of the specimen to view with a TEM (Grandfield and Engqvist,

2012), or by FIB tomography, which involves obtaining serial sections through a resin embedded specimen (Kizilyaprak et al., 2013). In these cases, the specimens are fixed and prepared in the same way as for TEM examination.

The outer layer of the pollen wall or exine is composed of an extremely strong and resistant substance known as sporopollenin, which due to its nature, requires complex sample preparation to obtain results from TEM. The advantage of using FIB-SEM as opposed to TEM is that with FIB sectioning, a single pollen grain may be successfully sectioned without using the extensive sample preparation required for TEM work. Sectioning pollen grains for TEM examination is a rather haphazard process where sectioning and viewing of samples are carried out on separate machines. Whereas, with the FIB technique, the exact position of the region to be sectioned can be selected and sectioning is achieved within the microscope chamber. Although this procedure is extremely time consuming, the results are immediately evident and high resolution 3D results are instantaneous. In contrast, sample preparation for TEM viewing can take weeks when considering infiltrating, embedding and obtaining serial sections of specimens. The resulting specimens can only be viewed in 2D and 3D interpretations need to be reconstructed by the researcher.

Three dimensional views of internal structure of pollen grains have been obtained by freeze fracturing. Blackmore and Barnes (1986) and Barnes (1992) developed and applied this technique, particularly to species of the Asteraceae. This method is very useful for viewing the development and arrangement of pollen grains within anthers, as well as spatial relationships between cytoplasmic organelles within the grains. However FIB-SEM is a good technique for viewing the internal pollen wall structure, the focus of this study.

AIMS AND OBJECTIVES

The aim of this study was to examine the internal structure of pollen walls in the Acanthaceae to determine their taxonomic significance by applying and evaluating a new microscopy technique (FIB-SEM). In addition, a supplementary study of the development of pollen grains within the anthers was undertaken in order to better understand the development of the pollen walls and how this could influence the taxonomic interpretation of the data.
This may be summarised into two main aims:

- **To identify new morphological characters in the pollen grain wall that may yield phylogenetic signal.**
- **To characterise the structural make-up and development of the pollen grain wall in the Acanthaceae.**

To accomplish the above, the objectives are as follows:

- Evaluate the use of FIB-SEM for investigating the detailed structure of the pollen walls in species of Acanthaceae.
- Develop a methodology for utilising FIB-SEM to investigate internal pollen wall features.
- Accumulate pollen wall data from selected genera from across the family using FIB-SEM.
- Identify internal pollen wall characteristics from the collected data and analyse the characters for taxonomic potential (or phylogenetic signal) in the family.
- To determine whether a selected species shows a similar pollen wall development pattern to documented examples, in order to examine phylogenetic signal in ontogeny.
- Discuss the relationships suggested by the internal wall structures from this study with respect to existing classifications and phylogeny based on external morphology and molecular studies.

The following key questions will be addressed:

- Do species currently accepted as being closely related share any internal pollen wall characteristics?
- Do species currently accepted as being closely related but with very different external appearance share any internal pollen wall characteristics?
- Do species with very similar external morphology, but not accepted as being closely related (divergent), have distinct or similar internal pollen wall structures?
- Do species from different tribes share any internal pollen wall characteristics or are their wall structures distinct?
- Do internal pollen wall characteristics support existing classifications of the Acanthaceae or are these characteristics not indicative of phylogeny?
- Does any discernible relationship exist between external sculpturing and internal pollen wall structure, or are they unrelated?
- Does the pattern of development of the pollen wall within anthers reveal any phylogenetic trends?
- Is it possible to use internal pollen wall characteristics for classification purposes?

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FIB-SEM: An Additional Technique for Investigating Internal Structure of Pollen Walls

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Abstract: Pollen grain morphology has been widely used in the classification of the Acanthaceae, where external pollen wall features have proved useful in determining relationships between taxa. Although detailed information has been accumulated using light microscopy, transmission electron microscopy and scanning electron microscopy (SEM) techniques, internal pollen wall features lack investigation and the techniques are cumbersome. A new technique involving precise cross sectioning or slicing of pollen grains at a selected position for examining wall ultrastructure, using a focused ion beam-scanning electron microscope (FIB-SEM), has been explored and promising results have been obtained. The FIB-SEM offers a good technique for reliable, high resolution, three-dimensional (3D) viewing of the internal structure of the pollen grain wall.

Key words: Acanthaceae, exine, focused ion beam-scanning electron microscope, FIB-SEM, palynology, pollen wall ultrastructure

Introduction

The extent of diversity in pollen morphology makes the study of pollen, or palynology, an attractive field for taxonomy, especially in groups that show a wide range of pollen surface features. These characteristics best identify a species as a member of a specific group or taxon and are therefore important for understanding Angiosperm systematics and phylogeny (Walker & Doyle, 1975).

The Acanthaceae is a notably eurypalynous family (Raj, 1961) and all palynological studies of the family remark on the pollen diversity. Consequently many members of the Acanthaceae have been the subject of detailed pollen morphological studies and a number of different pollen types have been identified for the family (e.g., Lindau, 1895; Bremekamp, 1953; Raj, 1961; Daniel 1978, 1986, 1990, 1998; Scotland, 1990, 1992, 1993; Perveen & Qaiser, 2010). Pollen morphology is one of the characteristics which have been used extensively to fine tune most existing classifications of the family Acanthaceae, especially at the genus level (e.g., Lindau, 1895; Bremekamp, 1953, 1965; Balkwill & Getliffe Norris, 1988; Immelman, 1989; McDade et al., 2000; Scotland & Vollesen, 2000). Members of this family are interesting from a palynological perspective in that they encompass a wide range of pollen morphological features, from supposedly primitive to more advanced types. Current palynological data for this group have largely been derived from the external structure of the pollen grains. Although LM and transmission electron microscopy (TEM) have provided two-dimensional (2D) images of the internal structure of the pollen grain walls, these can be difficult to interpret. This aspect of pollen morphology lacks investigation, and may prove to be taxonomically important.

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The possibility exists in which pollen grains with identical or similar surface sculpturing, for example where the surface sculpturing forms a network or reticulum (a reticulate surface), could have very different internal structures (Moore et al., 1991). In other words, pollen grains, which have the same external appearance when viewed with the scanning electron microscope (SEM), may have distinctly different internal wall or exine structures. These internal wall characteristics could prove to be taxonomically significant, and they may be particularly helpful in resolving what appear to be homoplaseous distributions of character states.

The main features of the pollen wall are shown in Figure 1, highlighting pollen wall terminology. The terminology generally complies with that of Walker and Doyle (1975).

Pollen wall structure has been investigated using LM and TEM, to give 2D results, and SEM to study external features of pollen grains in 3D. Internal views of pollen walls using SEM have to date been difficult to obtain and rely largely on luck (e.g., Graham, 1988, 564, Fig. 3B; Scotland, 1993, 485, Fig. 40; Scotland & Vollesen, 2000, 544, Fig. 25). 3D reconstructions from TEM data require serial thin sectioning of a single pollen grain, which is a highly skilled and very time-consuming technique. Nevertheless many useful and elegant studies have been performed to yield results on the features of pollen grain walls in the Acanthaceae. Raj (1961) produced a very useful comprehensive LM study of the pollen morphology of the family. Furness (1990, 1991, 1993, 1994, 1996) carried out extensive SEM and TEM investigations on various taxa in the family and many workers (e.g., Balkwill & Getliffe Norris, 1988; Daniel, 1998; Scotland & Vollesen, 2000) have looked at the pollen grain features using SEM and have used their results to understand the phylogeny of the family.

The focused ion beam-scanning electron microscope (FIB-SEM) is like an ordinary SEM, but where the SEM uses

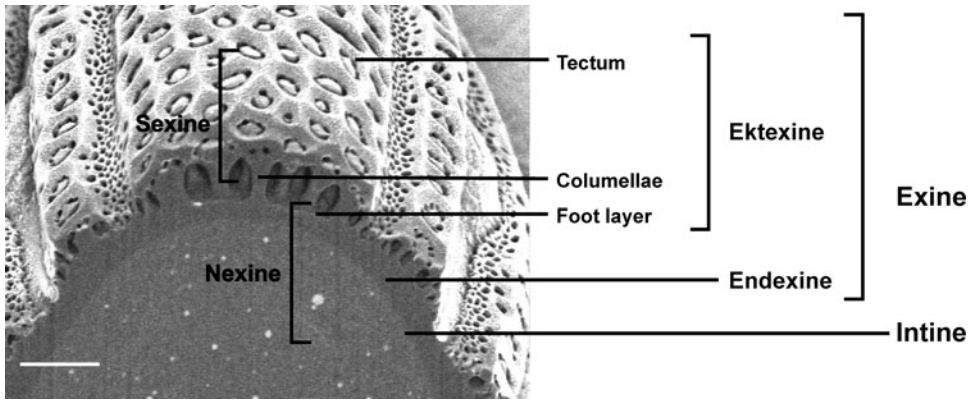

Figure 1. Cross section through a pollen grain of *Hypoestes forskaolii* (Vahl) R.Br. showing the main features of the pollen grain wall and indicating sexine (sculptured) and nexine (unsculptured) layers. Scale bar is 2 μm.

a focused beam of electrons to image the sample in the chamber, the FIB uses a focused beam of ions. This beam of ions can be used at high beam currents for site-specific sputtering or milling. Specimen material is sputtered or milled away to a chosen depth with each pass of the beam, this allows for precise sectioning of the specimen, effectively removing a layer of a predetermined thickness. After cutting, the specimen can be viewed with the SEM portion of the machine, to prevent further damage to the sample. In this way serial sections of an entire pollen grain can be obtained in 3D. This makes high resolution 3D investigation of pollen grain walls possible. This technique has recently been used to characterize the ultrastructure of pollen walls of paleopalynological remains of Cretaceous fossil pollen, with positive results (Villanueva-Amadoz et al., 2012).

An advantage of using a FIB-SEM rather than a TEM is that although one is using highly specialized equipment, the method of obtaining results does not require complex and skilled sample preparation and the preparation is simple and quick. Pollen samples do not require coating, although coating them does stabilize the sample and enhance detail. Sectioning is achieved within the microscope chamber and although this procedure takes a lot of machine time (at least 45 min for one 20–40 μm long cut), the results are immediately evident. Thus high resolution 3D results are instantaneous. In contrast, sample preparation for TEM viewing can take weeks when considering infiltrating, embedding, and obtaining serial sections of specimens. The resulting specimens can only be viewed in 2D and 3D interpretations need to be reconstructed by the researcher. The FIB-SEM provides an additional, complementary view of the internal pollen wall to that obtained with the TEM.

Materials and Methods

Test species of Acanthaceae used for this investigation:

- *Acanthus mollis* Riedel ex Nees (Subfamily: Acanthoideae; Tribe: Acantheae)
- *Sclerochiton harveyanus* Nees (Subfamily: Acanthoideae; Tribe: Acantheae)
- *Hygrophila auriculata* (Schumach.) Heine (Subfamily: Acanthoideae; Tribe: Ruellieae; Subtribe: Ruelliinae)

Pollen grains of species of *Sclerochiton*, *Hygrophila*, and *Acanthus* were selected to test the theory that grains with similar external sculpturing could have very different internal wall structures. The pollen morphology has been extensively investigated (Furness, 1991, 1994, 1996), showing them to have similar exine morphology or sculpturing of the tectum, which is finely perforated or pitted, also described as more or less finely reticulate, supported by distinct columellae (Fig. 2).

- *Barleria greenii* M. Balkwill and K. Balkwill (Subfamily: Acanthoideae; Tribe: Ruellieae; Subtribe: Barleriinae)

This species was chosen to determine whether highly sculptured walls would withstand the sectioning or collapse. These gains have very distinct, elaborate columellae supporting a vestigial tectum (Fig. 6). These species represent a variety of tribes and subtribes, but are all from the same subfamily.

Two different methods were conducted for 3D cross sectioning and analysis of pollen grains of the test species with the FIB-SEM:

1. Fresh, whole flowers with ripe anthers of species of *Acanthus* and *Barleria* were collected and immediately preserved in glacial acetic acid (GAA). Whole pollen grains were collected from anthers under a dissecting microscope and placed directly onto a carbon coated disc attached to a SEM stub. Samples were allowed to dry for at least 24 h before being viewed with a FEI FIB-SEM Nova 600 NanoLab (EDS) (Hillsboro, OR, USA). Suitable pollen grains were selected, photographed, orientated for sectioning with the FIB, and each pollen grain was sectioned from end to end, scanning and photographing the exposed surface after each cut. The FIB was operated at 30 kV and only coarse milling at 4 nA was carried out.
2. Fresh whole flowers with ripe anthers of species of *Sclerochiton* and *Hygrophila* were collected and dried in silica gel crystals. Whole pollen grains were collected from anthers under a dissecting microscope and placed di-

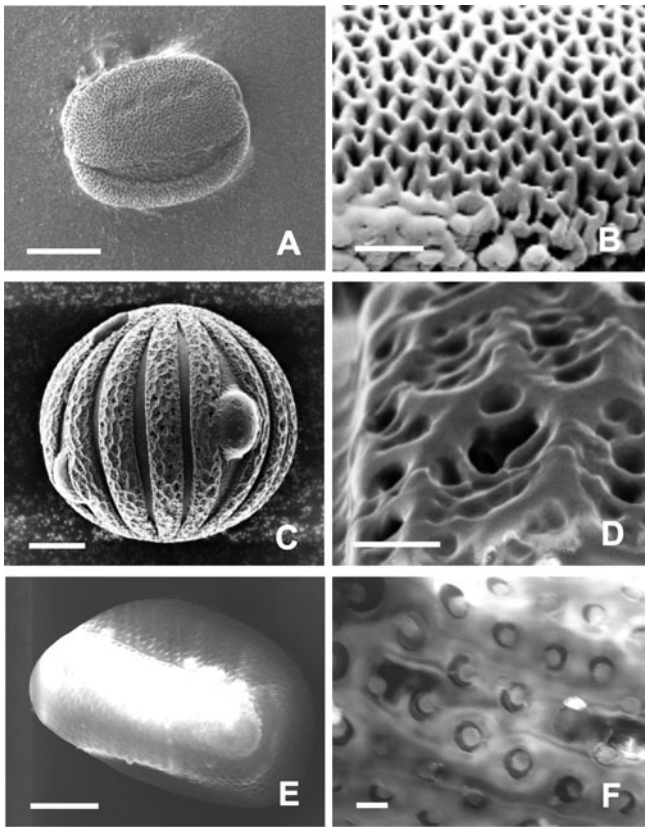

Figure 2. Electron microscopy (SEM) of whole mounts of selected pollen grains of four species for focused ion beam (FIB) sectioning, with detail of type of sculpturing for each grain. **A, B:** *Sclerochiton*. **C, D:** *Hygrophila*. **E, F:** *Acanthus*. Whole pollen grains coated with Au-Pd alloy, except for *Acanthus* (**E, F**), not coated and shows charging of an unstable specimen. All have reticulate sculpturing. Scale bar: (**A**, **C**, **E**) 10 μm, (**B**, **D**, **F**) 1 μm.

rectly onto a carbon coated disc attached to an electron microscope stub. Samples were coated with a gold–palladium (Au-Pd) alloy at 20 mA for 1 min and then viewed with a Zeiss Auriga microscope with a Crossbeam FIB workstation and Gemini FE SEM column (Oberkochen, Germany). Suitable pollen grains were selected, photographed, oriented for sectioning with the FIB, and each pollen grain was sectioned once at a selected site. This work was conducted at the CSIR, in Brummeria, East of Pretoria, Gauteng, South Africa.

The FIB was operated at 30 kV. The coarse milling current for all samples was 4 nA. In each case, a single cut took ~30 min (for grains between 30 and 40 μm in diameter).

The *Sclerochiton* sample was fine milled or polished at 600 pA to reveal more detail and remove deposition material. The *Hygrophila* sample was polished at 1 nA. This step took about 15 min.

Before milling began the selected sectioning site was coated with a platinum (Pt) strip to protect the surface and to avoid damage to the internal wall structure. This was applied at 120 pA for 5 min.

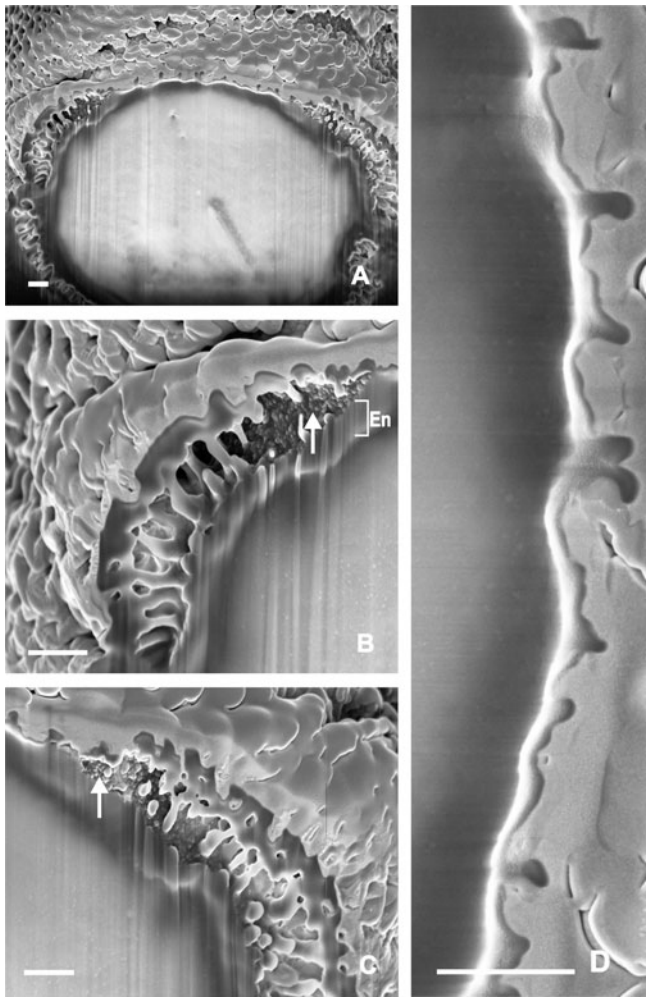

Figure 3. Cross section of a *Sclerochiton* pollen grain. **A:** Showing the whole cut surface. Cut is through the colpus and wall. The Pt coating is clearly visible and appears to mask some wall features in the colpus. **B**, **C:** Section of wall showing reticulate tectum, distinct columellae, wide spaced and some incomplete. The first layer of nexine, the foot layer (arrows), is atypically sculptured in this species and is distincly granular. The endexine (En) is distorted, but a distinct layer. **D:** Section through colpus, only tectum present, columellae absent (the white layer may be an artifact caused by re-deposition of coating material). Scale bars 1 μm.

Results

In preparing the pollen grains for FIB-SEM sectioning, the pollen grains of species of *Acanthus* and *Barleria* were not coated (Figs. 5, 6) and images included areas of charging. For this reason coating of the specimens is recommended in order to lend stability and detail to the data obtained from the pollen grains.

For specimens coated with a Au-Pd alloy followed by a Pt strip, it was noted that in instances when the microscope was not perfectly aligned and the cut did not reach the Pt layer that this did not make any difference to the results obtained. It therefore seems unnecessary to use this coating as it sometimes obscures wall features when too thick and the sporopollenin of the pollen walls appears sufficiently

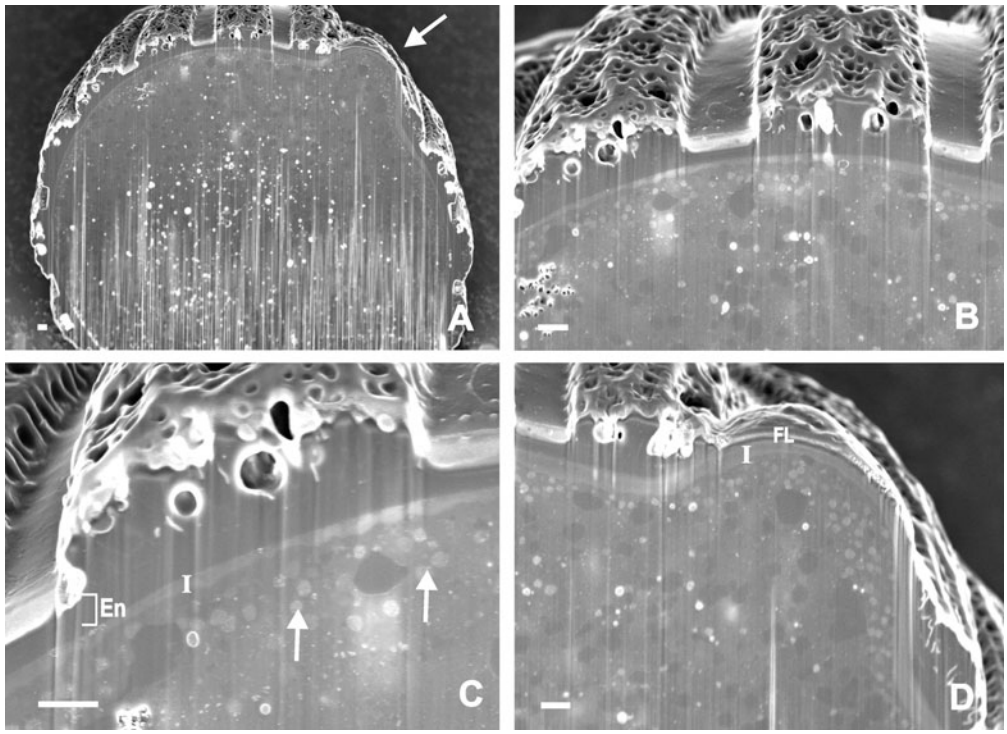

Figure 4. Cross section of a *Hygrophila* pollen grain. **A:** Section through the whole grain cut through a pore (arrow). **B**, **C:** Section of wall showing sections of coarsely reticulate or porate tectum supported by inner sexine consisting of partially "fused" columellae forming cavities within the wall, alternating with psilate strips of wall, free of tectum and columellae, making up a smooth foot layer or nexine. These strips are sometimes referred to as pseudocolpi. Underlying is a layer of endexine (En) covering a thin layer of intine (I). Adjacent to the intine is a layer of vesicles, possibly containing wall building material (arrows). **D:** Section through a pore. In this case the tectum is absent and a condensed foot layer (FL) and endexine with a thin layer of intine (I) covers the pore. A layer of vesicles is also visible. Scale bars 1 μm.

resilient to protect the wall from excessive damage during the procedures.

FIB cross sectioning of a pollen grain of *Sclerochiton* (Fig. 3) shows a finely pitted tectum supported by distinct, fairly evenly spaced columellae. Perforations are round, formed in between the neighboring columellae (Figs. 3B, 3C). Columellae may have curved edges and are thicker at the base; some are not complete and seem to not reach the tectum due to the angle of the cut. The columellae are embedded in a distinct, granular foot layer. This structure of columellae does not continue into the colpus. In the colpus the tectum seems to form overlapping plates which overlie a more finely granular foot layer (Fig. 3D). The endexine is a distinct layer, about a third as thick as the ektexine.

FIB cross sectioning of a pollen grain of *Hygrophila* (Fig. 4) shows clear bands of sculptured tectate wall interspersed with psilate bands of half the width (Fig. 4B). There is a clear foot layer at the base of each psilate strip, but this layer is not well defined within the sculptured bands (Fig. 4C). These wider strips consist of a finely reticulate tectum within a coarsely reticulate tectum, supported by a lattice of columellae forming cavities within a more solid wall. Underlying this is a layer of endexine, about the same thickness as the foot layer, and a thin intine.

Two specimens, *Acanthus* and *Barleria*, were not coated prior to viewing and sectioning with the FIB-SEM (Figs. 5, 6). Insufficient internal wall detail was observed due to lack of contrast and charging of specimens. It is possible, however, to achieve serial sections through a pollen grain (Fig. 5) and therefore at various selected sites in one grain. The FIB-SEM serial sectioning of a pollen grain of *Acanthus* (Fig. 5) shows a simple structure with cavities within a more solid exine and straight, sometimes fused columellae rather than free standing columellae.

It is evident with FIB cross sectioning of a pollen grain of *Barleria* that integrity of the pollen grains is maintained during sectioning and therefore even highly sculptured grains can be sectioned (Fig. 6).

Discussion

FIB-SEM is a good technique for high resolution, 3D viewing of the internal structure of the pollen grain wall. Dry or preserved and fixed pollen grains may be used, both giving satisfactory results. Small pollen grains as well as larger, more elaborately sculptured grains can be sectioned with a favorable outcome. Whole grains can be viewed with the SEM and a specific site, or area of interest, selected for sectioning with the FIB.

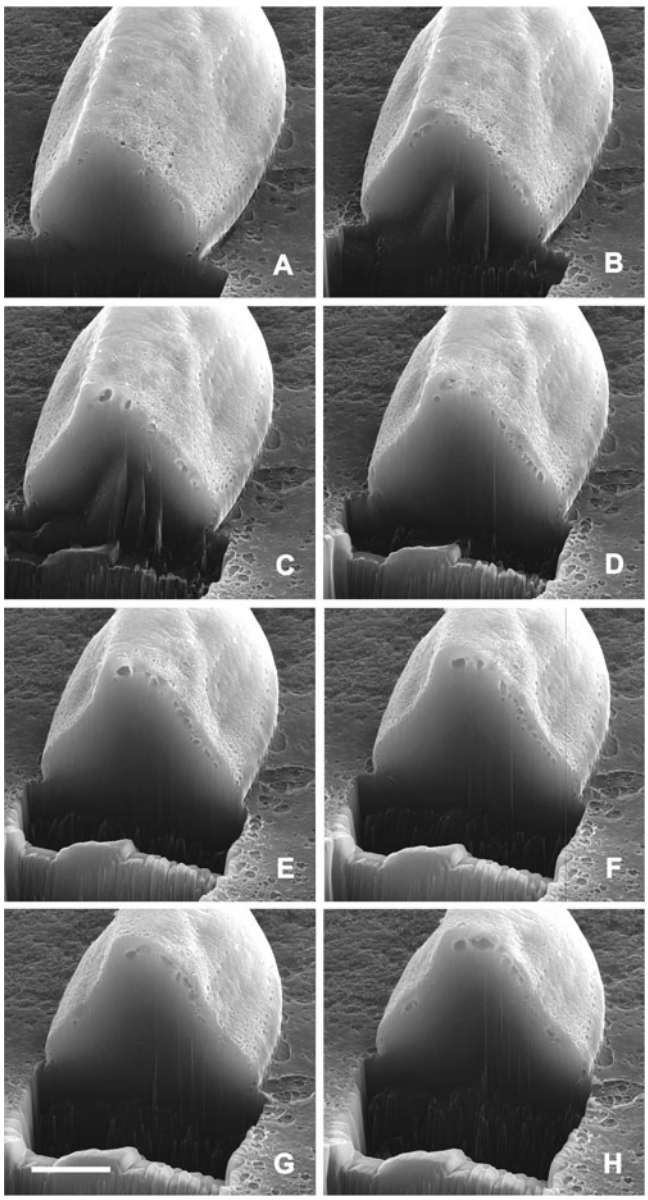

Figure 5. A–H: Serial sections through a pollen grain of *Acanthus*. Scale bar is 10 μm.

The ability to select a single pollen grain for sectioning is advantageous as it cuts down on the milling time considerably. Sectioning a single grain about 20–40 μm in diameter takes ~45 min to complete, including rough cutting and polishing. In contrast, a cluster of grains or a microspore mass 300–400 μm long could take up to 14 h (Villanueva-Amadoz et al., 2012). Since this latter time was based on fossil samples, it could potentially have taken longer than comparable extant material, as the organic matter would have been replaced by minerals during the fossilization process.

On a technical note, it is clear from the results that coating the specimens is beneficial. This procedure allows for better resolution and greater wall detail to be seen. It also stabilizes the specimen and minimizes charging in the microscope chamber.

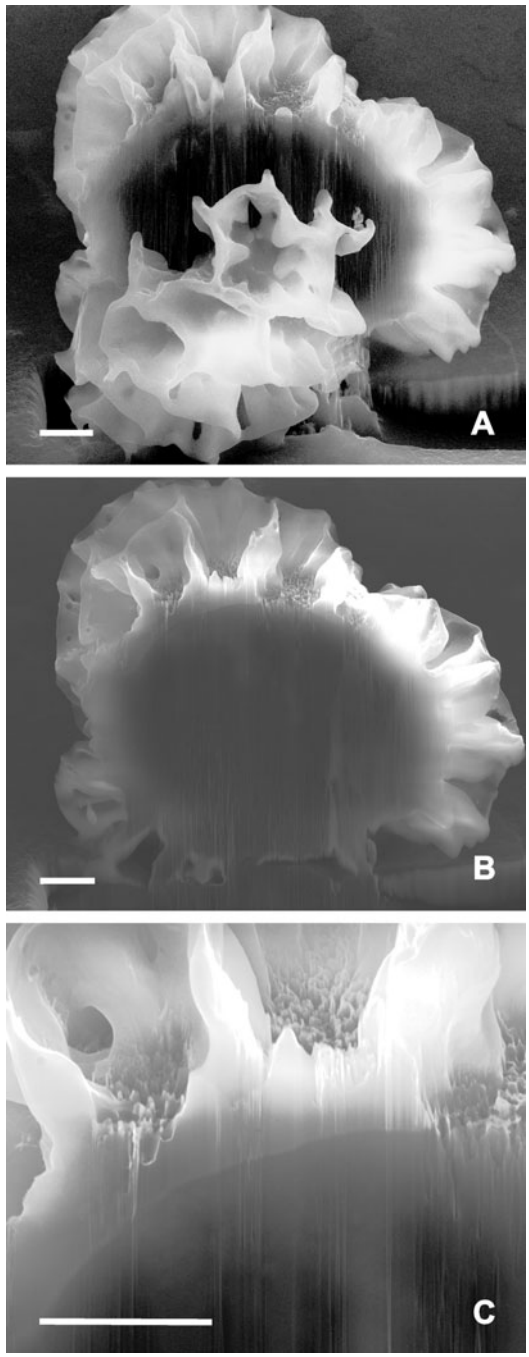

Figure 6. Cross sections through a pollen grain of *Barleria*. **A**: First cut through the pollen grain. Elaborate sculpturing and columellae remain intact. **B:** Cross section of cut surface. **C:** Detail of pollen wall and sculpturing. Scale bars 10 μm.

The milling process can often result in significant streaking effects on the samples which can sometimes be reduced by polishing or adjusting the milling current. While these obviously detract from the aesthetic appearance of the results, useful data may still be obtained as many features are still discernible in good detail.

The results confirm what has been observed with other methods of microscopy, as in the case of *S. harveyanus*. The pollen of *Sclerochiton* is said to be fairly uniform, and there

is only one pollen type for the genus (Furness, 1991), tricolpate, prolate to spheroidal grains. They are described as 'Spaltenpollen' by Lindau (1893), meaning fissured pollen with a finely pitted exine. Raj (1961) described the exine as punctitegillate, as the tectum has minute perforations. Furness (1991) carried out a detailed investigation of the pollen, using light, SEM and TEM techniques (Furness, 1991, 55, Figs. 1a–1g). She described the sculpturing as usually finely reticulate, occasionally perforate; the columellae as long, straight and widely spaced, and acknowledges the presence of a foot layer. The colpi are described as having granular membranes and she identifies the polar depression as possibly the most distinctive feature of *Sclerochiton*. The FIB cross sectioning of the pollen grain of *Sclerochiton* shows a number of typical features as well as providing more detail of the internal wall structure and highlighting features, such as the granular foot layer and curved structure of the columellae.

The pollen grain of *Hygrophila* has been described as a four aperturate, polypseudocolpate pollen (Raj, 1961; Scotland, 1993; Furness, 1994; Daniel, 1998) with sculpturing of a fine reticulum within a coarse reticulum (Scotland, 1993). The grain is divided into alternating bands of sculptured and psilate regions. Furness (1994) carried out a detailed investigation of the pollen of 34 species of *Hygrophila*, and described a *H. auriculata*—type pollen morphology (Furness, 1994, 276, Figs. 1 and 2; 277, Fig. 1; 278, Figs. 1, 2, and 8). The results correlate well with past data and in addition show the internal wall structure clearly.

Furness (1996) investigated the structure of the pollen grain wall of 15 species of *Acanthus*. She describes the pollen as tricolpate, with long narrow colpi and sculpturing in the form of a hexagonal reticulum, overlying simple columellae, a distinct foot layer and a thick endexine. In contrast, the FIB cross sections through an *Acanthus* pollen grain show cavities within a more solid wall of fused columellae rather than free standing columellae.

New procedures and techniques are worth exploring as they can yield useful results, additional data, and provide a different perspective. FIB cross sectioning data confirms that pollen grains with similar external features have distinct internal wall morphology. With greater detail and better knowledge of internal pollen wall structure, more accurate assessment of homologies can be made. This will provide more accurate data from which to reconstruct phylogenies based on pollen characters.

Based on the results obtained the following procedure is recommended:

- Collect fresh flowers if possible, place into small envelopes, store, and dry in silica gel crystals or transfer immediately into GAA.
- Harvest pollen directly from the anthers, using fine insect pins and transfer onto a carbon coated disc attached to a SEM stub.
- Lightly coat samples preferably with a Au-Pd alloy at 20 mA for 1 min or with carbon for no more than two pulses.
- View sample with SEM, identify a suitably oriented pollen grain and select the sectioning site. For smaller grains (diameter $< 40\ \mu$m) a cross section through the entire grain is feasible, but for larger pollen grains milling a small section from the wall, to save milling time, may be sufficient.
- Operate the FIB at 30 kV. A coarse milling current of 4 nA was found to be optimal for pollen grains.
- Fine milling or polishing at 600 pA was found to give the best results. This step may need to be repeated several times to reveal more detail and remove deposition material.
- Image with SEM.

Conclusion

The FIB-SEM affords a different perspective and detailed 3D information for investigating internal pollen wall structure, which supplements the information gained from other methods of microscopy. These additional data contribute to a better understanding of which internal wall layers and components give rise to the external features of the pollen grain wall. This in turn allows for a more accurate assessment of homologies and thus better use of character states in phylogenetic analyses.

Acknowledgments

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CHAPTER THREE – LABYRINTHS, COLUMNS AND CAVITIES: NEW INTERNAL FEATURES OF POLLEN GRAIN WALLS IN THE ACANTHACEAE DETECTED BY FIB-SEM

Abstract
External pollen grain morphology has been widely used in the taxonomy and systematics of flowering plants, especially the Acanthaceae which are noted for pollen diversity. However internal pollen wall features have received far less attention due to the difficulty of examining the wall structure. Advancing technology in the field of microscopy has made it possible, with the use of a focused ion beam-scanning electron microscope (FIB-SEM), to view the structure of pollen grain walls in far greater detail and in three dimensions. In this study the wall structures of 13 species from the Acanthaceae were investigated for features of potential systematic relevance. FIB-SEM was applied to obtain precise cross-sections of pollen grains at selected positions for examining the wall ultrastructure. Exploratory studies of the exine have thus far identified five basic structural types. The investigations also show that similar external pollen wall features may have a distinctly different internal structure. FIB-SEM studies have revealed diverse internal pollen wall features which may now be investigated for their systematic and functional significance.

Keywords ectexine, endexine, exine, FIB-SEM, internal pollen wall ultrastructure, palynology

INTRODUCTION

The variation and diversity in pollen morphology in the Acanthaceae makes it possible to use external pollen grain characteristics to investigate relationships among taxa and these characteristics have been used extensively in formulating their classification systems (e.g., Lindau, 1895; Bremekamp, 1953, 1965; Scotland and Vollesen, 2000). The family is notably eurypalynous (Raj, 1961) and all palynological studies of the Acanthaceae remark on the pollen diversity within the family.

The first detailed palynological studies of the Acanthaceae were made by Fritzsche (1832), and the taxonomic value of pollen morphology in the Acanthaceae was noted by Radlkofer (1883) who distinguished seven pollen types. These initial types were expanded by Lindau (1895), who documented 11 pollen types for the family and used pollen morphology as the basis for his sub-familial classification. More recently it has been suggested that up to 26 pollen types exist in the family in Madagascar (Muller et al., 1989). Even more recently, pollen types have been described based on the taxa that exhibit the particular pollen sculpturing, e.g., *Barleria*-type, *Hygrophila*-type, *Strobilanthes*-type (Anjum and Qaiser, 2010).

Many members of the Acanthaceae have been the subject of detailed pollen morphological studies, especially by Balkwill and Getliffe Norris (1988); Bremekamp (1953); Daniel (1986, 1990, 1998); Furness (e.g., 1990, 1991, 1993, 1994, 1995, 1996); Immelman (1989); Raj (1961); Scotland (1990, 1992b, 1993). Many researchers have studied external pollen features of the whole family at a global level **(**e.g., Raj, 1961**;** Scotland and Vollesen, 2000**)**, at a regional level e.g., Balkwill and Getliffe Norris (1988) in **s**outhern Africa; Muller et al. (1989) in Madagascar and other Indian Ocean islands; Daniel (1998) in Mexico; Anjum and Qaiser (2010) in Pakistan; at the tribal level e.g., Andrographideae (Scotland, 1992b) and also at the genus level e.g., *Justicia* (Graham, 1988); Furness studied pollen morphology of *Crossandra* (1990), *Sclerochiton* (1991), *Hygrophila* (1994), *Dyschoriste* (1995) and *Acanthus* (1996), to name a few.

Pollen diversity within the Acanthaceae is considered to be taxonomic rather than geographic (Daniel, 1998) and pollen morphology of genera from Old World and New World regions is consistent within each genus. The point has however been made that corresponding or similar morphologies do not always prove close relationships, as similarities could also exist as a consequence of convergence (Scotland, 1992a; 1993). Although most Acanthaceae genera have relatively homogeneous pollen, some, such as *Justicia* L. do show pollen diversity (Graham, 1988; Daniel, 1998). Pollen morphology can be used to place anomalous species (Balkwill and Getliffe Norris, 1988), but should not be used to develop classification systems alone, and should be used in conjunction with other plant features (Scotland, 1992a; 1993).

Due to the well documented diversity of external pollen features, the Acanthaceae are an ideal family for investigating internal wall characteristics which may also be of taxonomic (House and Balkwill, 2013) and phylogenetic (Walker, 1974; Walker and Skvarla, 1975; Doyle, 2009) value. It is also possible that pollen grains with the same surface sculpturing, for example a reticulate surface most common in the Acanthaceae, may have very different internal structures (Moore et al., 1991). In other words, pollen grains which have the same external appearance when viewed with the scanning electron microscope may have a distinctly different internal wall structure.

In contrast to the external features, internal pollen wall (notably exine) construction has been formerly difficult to view and is therefore far less well documented. Light microscopy and transmission electron microscopy (TEM) have provided two dimensional images of the internal pollen grain wall, but more recently, FIB-SEM has afforded three dimensional viewing, offering a different perspective (House and Balkwill, 2013). For example, in a TEM (two dimensional, ultra-thin) section, walls of adjacent tunnels or those surrounding cavities or chambers, may give the appearance of being distinct, individual columellae. A three dimensional cross-section viewed with the FIB-SEM, however, affords an unambiguous view in which one can clearly distinguish

between a columella and the cross-section of a solid wall. Three dimensional views of internal structure of pollen grains have been obtained by freeze fracturing. Blackmore and Barnes (1986) and Barnes (1992) developed and applied this technique, particularly to species of the Asteraceae. This method is more useful for viewing the development and arrangement of pollen grains within anthers, as well as cytoplasmic organelles within the grains. However FIB-SEM is a good technique for viewing the internal pollen wall structure, the focus of this study.

With FIB-SEM, individual pollen grains which best represent the sample, as well as the exact site of sectioning on the grain may be selected, whereas a fixed TEM sample is sectioned all at once. Difficulties with TEM may therefore arise due to contamination with grains from other species and uncertainty as to the maturity and representativeness of the grain found in any particular section. Abnormal pollen grains have previously been described in genera of Acanthaceae (Furness, 1997), but the potential problems they may cause, can be avoided with FIB-SEM.

Some recent studies of internal wall structures by Doyle and co-workers in gymnosperms and primitive angiosperms has led them to propose that certain internal features may be indicative of phylogeny in these groups (Doyle, 2009; Doyle and Le Thomas, 2012). This paper aims to describe characteristics observed in the internal structure of pollen walls in the Acanthaceae using FIB-SEM and to open the discussion as to the relevance of these features to taxonomy and phylogeny.

For practical purposes, the terminology used in this paper generally complies with that of Walker and Doyle (1975). The main features of pollen walls, as seen with the FIB-SEM, are highlighted in Figure 3.1.

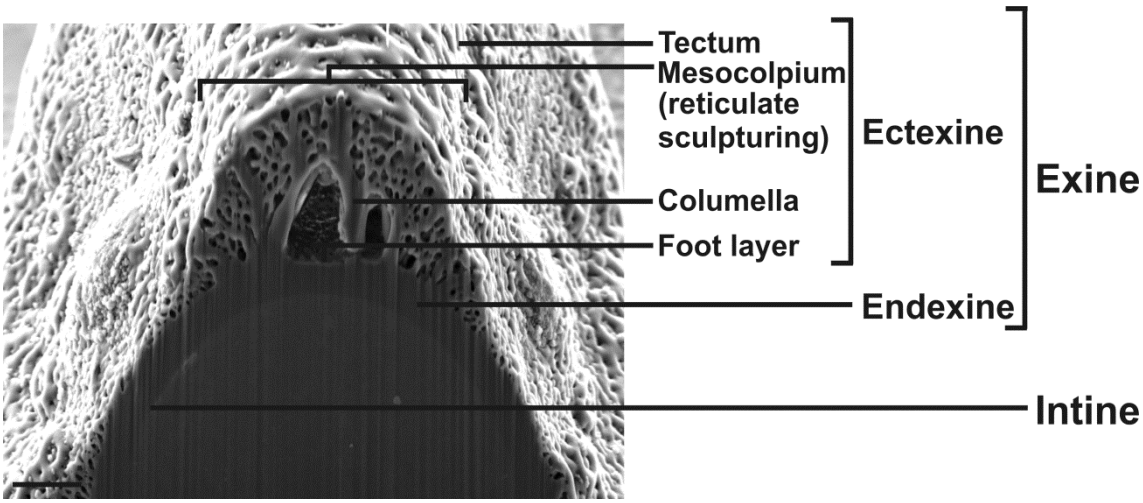

Figure 3.1 A FIB-SEM cross-section through a pollen grain of *Justicia flava* (Vahl) Vahl showing the main external and internal pollen wall features
Scale bar is 2 µm

MATERIALS AND METHODS

Pollen was collected from 32 taxa of Acanthaceae and examined using the FIB-SEM. From the original samples 13 species were chosen, for the purposes of publication, which best represented the internal wall features identified in this study, not to imply any taxonomic or phylogenetic relationship. The remaining data are included in Appendix I (Quick-Guide: pg. 7.1). Fresh flowers were collected, some were placed in small envelopes and stored in dry silica gel crystals and others were transferred immediately into vials with Glacial Acetic Acid (99%). Voucher specimens from the fieldwork have been deposited in the C.E Moss Herbarium (J) at the University of the Witwatersrand, Johannesburg.

The taxa examined here are:

Thunbergia atriplicifolia E.Mey. ex Nees (J96496); *Blepharis stainbankiae* C.B.Clarke (J96692); *Acanthus mollis* Riedel ex Nees (J96498); *Dyschoriste fischeri* Lindau (J96526); *Petalidium oblongifolium* C.B.Clarke (J96520); *Ruellia cordata* Thunb. (J96578); *Justicia flava* (Vahl) Vahl (J96517); *Justicia carnea* Lindl. (J96716); *Hypoestes forskaolii* (Vahl) R.Br. (J96717); *Rhinacanthus gracilis* Klotzsch var. *latilabiatus* K.Balkwill (J96715); *Isoglossa ovata* (Nees) Lindau (J94883); *Barleria rotundifolia* Oberm. (J96539); *Crabbea hirsuta* Harv. (J96518)

Pollen was harvested directly from the anthers, using fine insect pins and transferred onto carbon coated discs attached to electron microscope stubs. Samples were either lightly coated with Au-Pd alloy at 20 mA for one minute or with carbon for no more than two pulses. The sample of *Dyschoriste fischeri* was viewed with a FEI Nova NanoLab 600 FIB-SEM at the Microscopy and Microanalysis Unit of the University of the Witwatersrand, Johannesburg. All other samples were viewed with a Zeiss Auriga microscope with a Crossbeam FIB workstation and Gemini FE SEM column at the Council for Scientific and Industrial Research (CSIR), Brummeria, Pretoria.
The FIB was operated at 30 kV. Each pollen sample was viewed with the SEM in order to identify an individual pollen grain of average size and external morphology, typical for the species. Suitable pollen grains for each species were selected, photographed, orientated for sectioning with the FIB, and sectioned once at a selected site. The sectioning site was determined by the position of external features, such as spines and pores, which were desirable for examination. Smaller grains (up to 40 µm in length) were sectioned in the region of the equator, whereas larger grains were sectioned between the equator and pole, where the most diverse external features were located. Each sample was viewed and imaged in the Secondary Electron mode (SE2) as well as with the Backscatter Electron Imaging (ESB) detector. Analysis of this kind identifies areas of different chemical composition and electron density making it possible to distinguish the various internal layers of the pollen wall which are represented in different shades of grey. For a detailed discussion of the method refer to Chapter Two.

RESULTS

The sectioning process with the FIB-SEM often produces significant streaking effects on the cut surface as a result of re-deposition of milled material. While this obviously detracts from the aesthetic appearance of the results, useful data are still obtained as detailed features are still discernible.
The external morphology and sculpturing, and the internal structure of the pollen grain wall of the test species are described below:

Thunbergia atriplicifolia E.Mey. ex Nees (Subfamily: Thunbergioideae) (Figure 3.2): Grains are more or less globose-spherical (spherical to subprolate) with an equatorial diameter of 65 µm; grains have a coiled, spiral arrangement with linear-spiral apertures (spiraperturate), dividing each grain into five or six spiral bands, 10 – 15 µm wide (Fig. 3.2a), or six or seven lobes in cross-section, 10 – 30 µm wide (Fig. 3.2b). **Exine**: The tectum is tectate-imperforate, finely, but densely granulate-rugulate, 0.3 µm thick. The ectexine is 1.5 – 2 µm wide and is sponge-like in appearance, perforated by irregularly arranged, ramified, small cavities. This arrangement has been described as alveolate and is common in gymnosperms and basal angiosperms (Walker and Doyle, 1975; Doyle and Le Thomas, 2012). The linear-spiral aperture cuts through the exine to the intine (Fig. 3.2c). The foot layer is apparently absent. The endexine is an indistinct layer, approximately 0.5 µm wide. **Intine**: The intine is a fine layer, 0.1 µm wide (Fig. 3.2d).

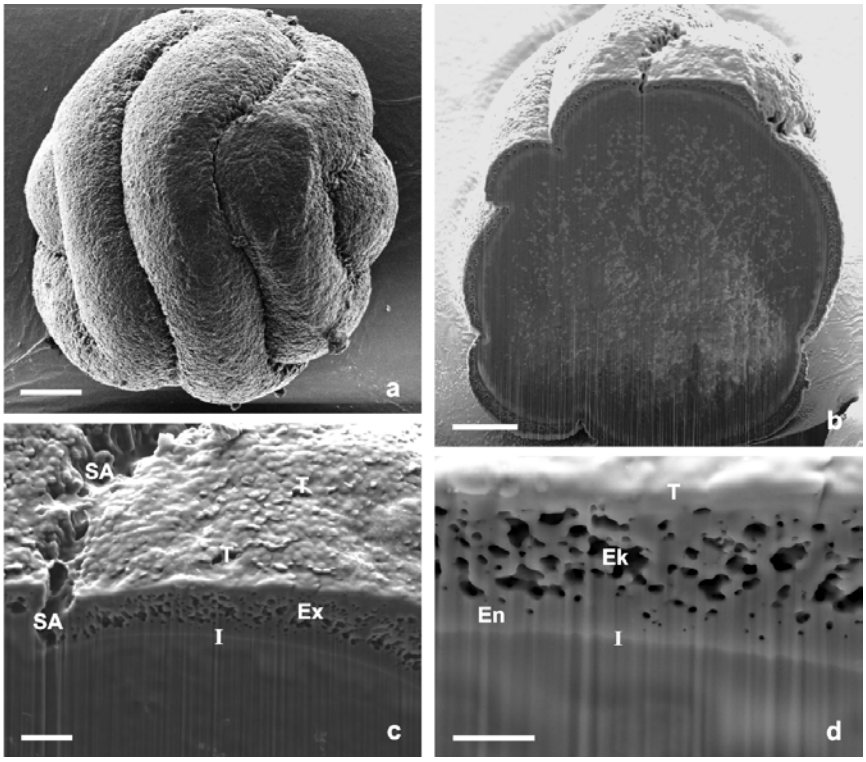

Figure 3.2 FIB-SEM micrographs of *Thunbergia atriplicifolia*: **a** SEM of a whole pollen grain showing the coiled, spiral form **b** Cross-section through the pollen grain showing the whole cut surface. The streaking effect is as a result of FIB processes, but internal wall detail can still be seen **c** Cross-section showing detail of the spiral aperture and internal wall **d** High power showing detail of internal wall structure

Ek-ectexine; En-endexine; Ex-exine; I-intine; SA-spiral aperture; T-tectum
Scale bars: **a**-10 µm; **b**-10 µm; **c**-2 µm; **d**-1 µm

Blepharis stainbankiae C.B.Clarke (Subfamily: Acanthoideae; Tribe: Acantheae) (Figure 3.3):
Grains are prolate, 25 – 35 µm long with an equatorial diameter of 15 – 25 µm. Grains are tricolpate. A narrow colpus (an elongate, furrow-like aperture) extends from pole to pole on each side of a mesocolpium (Fig. 3.3a). **Exine**: The tectum is semitectate and is arranged in a uniform hexagonal reticulum covering the grain. Straight muri, 0.2 µm thick, form the hexagonal pattern and delimit the cup-shaped lumina, 1.3 µm across. In a central hollow at the base of each lumen is a rounded disc or plug (a gemmate structure) (Fig. 3.3b). The ectexine is a much reduced, relatively thin layer, 0.1 – 0.5 µm thick, reduced to just the tectum with no free standing columellae, cavities or tunnels, and is fused onto a thick solid endexine. The endexine is a distinct, wide layer, 1 – 1.5 µm thick. **Intine**: A very fine, uniform layer (Fig. 3.3c and 3.3d).

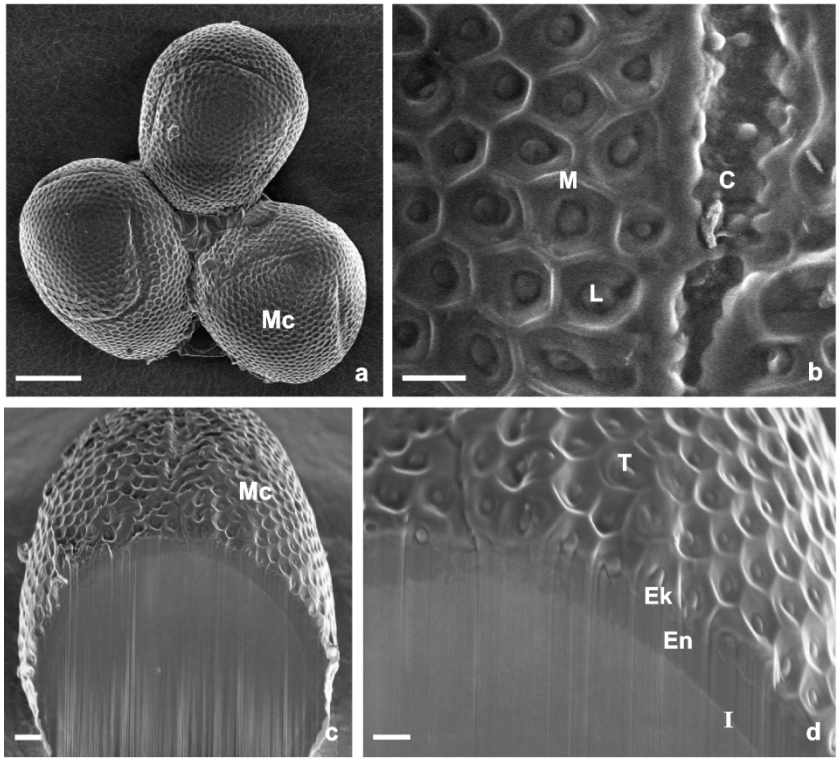

Figure 3.3 FIB-SEM micrographs of *Blepharis stainbankiae*: **a** SEM of a random cluster of pollen grains (Grains top-centre and left are abnormal with irregular colpi, but grains suitable for sectioning are easily identified with FIB-SEM) **b** Detail of hexagonal reticulum and colpus **c** Cross-section showing whole cut surface (streaking due to FIB process) **d** Detail of internal wall showing reduced, solid ectexine and endexine

C-colpus; Ek-ectexine; En-endexine; I-intine; L-lumen, M-muri; Mc mesocolpium; T-tectum
Scale bars: **a**-10 µm; **b**-1 µm; **c**-2 µm; **d**-1 µm

Acanthus mollis Riedel ex Nees (Subfamily: Acanthoideae; Tribe: Acantheae) (Figure 3.4):
Grains are prolate, 50 – 70 µm long with an equatorial diameter of 25 – 30 µm. Grains are tricolpate, with narrow colpi extending the length of the grain (Fig. 3.4a). **Exine**: The tectum is tectate-perforate to semitectate, and is arranged in a uniform hexagonal reticulum covering the grain. In the centre of each lumen is a flat rounded disc or plug (Fig. 3.4b). The ectexine, 1 – 1.5 µm thick, is more or less solid, with randomly dispersed cavities or chambers carved into the wall, with no free standing columellae

(Fig. 3.4c and 3.4d). The endexine is a distinct layer, 1.5 µm thick (Fig. 3.4b). **Intine**: The intine is a very fine layer (Fig. 3.4b).

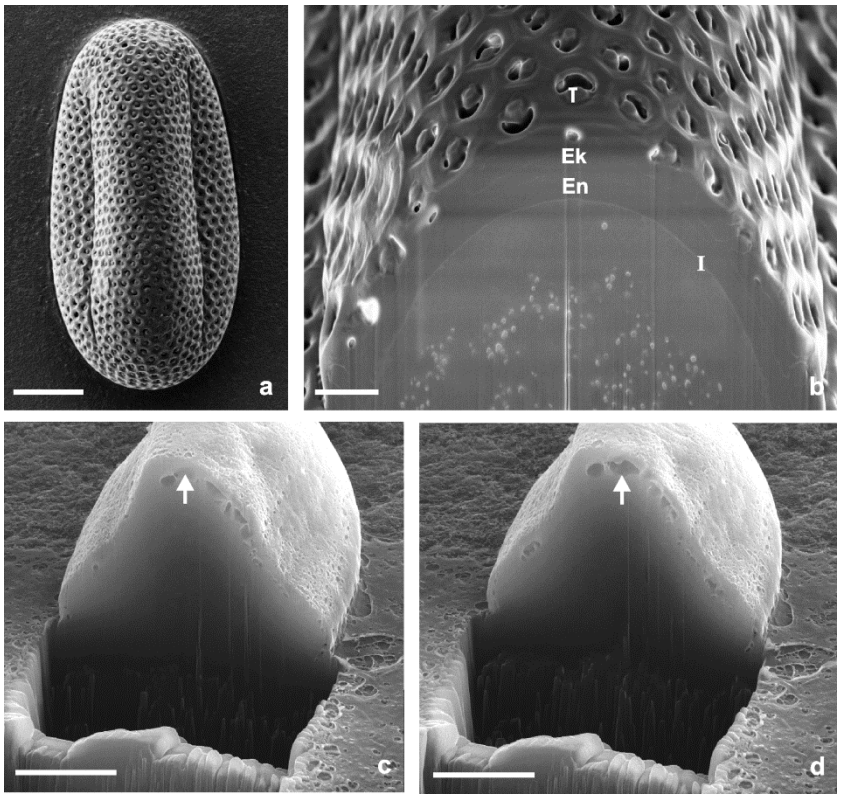

Figure 3.4 FIB-SEM micrographs of *Acanthus mollis*: **a** SEM of a whole grain. Narrow colpi extend the length of the grain **b** Cross-section showing the whole cut surface and internal layers of pollen wall **c** and **d** Serial cross-sections showing appearance of cavities/chambers in the wall. Arrows mark the same chamber (this sample was not coated prior to viewing)

Ek-ectexine; En-endexine; I-intine; T-tectum

Scale bars: **a**-10 µm; **b**-2 µm; **c**-10 µm; **d**-10 µm

Dyschoriste fischeri Lindau (Subfamily: Acanthoideae; Tribe: Ruellieae; Sub-tribe: Ruelliinae) (Figure 3.5):
Grains are prolate, 50 – 60 µm long with an equatorial diameter of approximately 40 µm. Grains are tricolporate with 6 pseudocolpi between each colpus, a narrow furrow containing an endopore. Pseudocolpus bands run from pole to pole, while colpi are shorter (Fig. 3.5a). **Exine**: The tectum covering the inter-pseudocolpal bands is perforate and sculptured with micro-echinae (Fig. 3.5b). The ectexine is 1 – 1.5 µm thick and restricted to the inter-pseudocolpal bands, each about 6 µm wide (Fig. 3.5c – 3.5f). Along the apertures the exine forms 2 lips which protrude around the endopore to form an atrium (Fig. 3.5c – 3.5e). The interior regions of the inter-pseudocolpal bands appear to contain chambers interconnected by cavities in the chamber walls (Fig. 3.5c – 3.5f). The endexine is a continuous, uniform layer, 0.6 µm thick. The pseudocolpi have exine confined to endexine, while the inter-pseudocolpal bands have exine comprising endexine and ectexine. **Intine**: The intine was not visible in these sections due to reduced contrast in specimens examined with the FEI Nova NanoLab 600 FIB-SEM (Fig. 3.5).

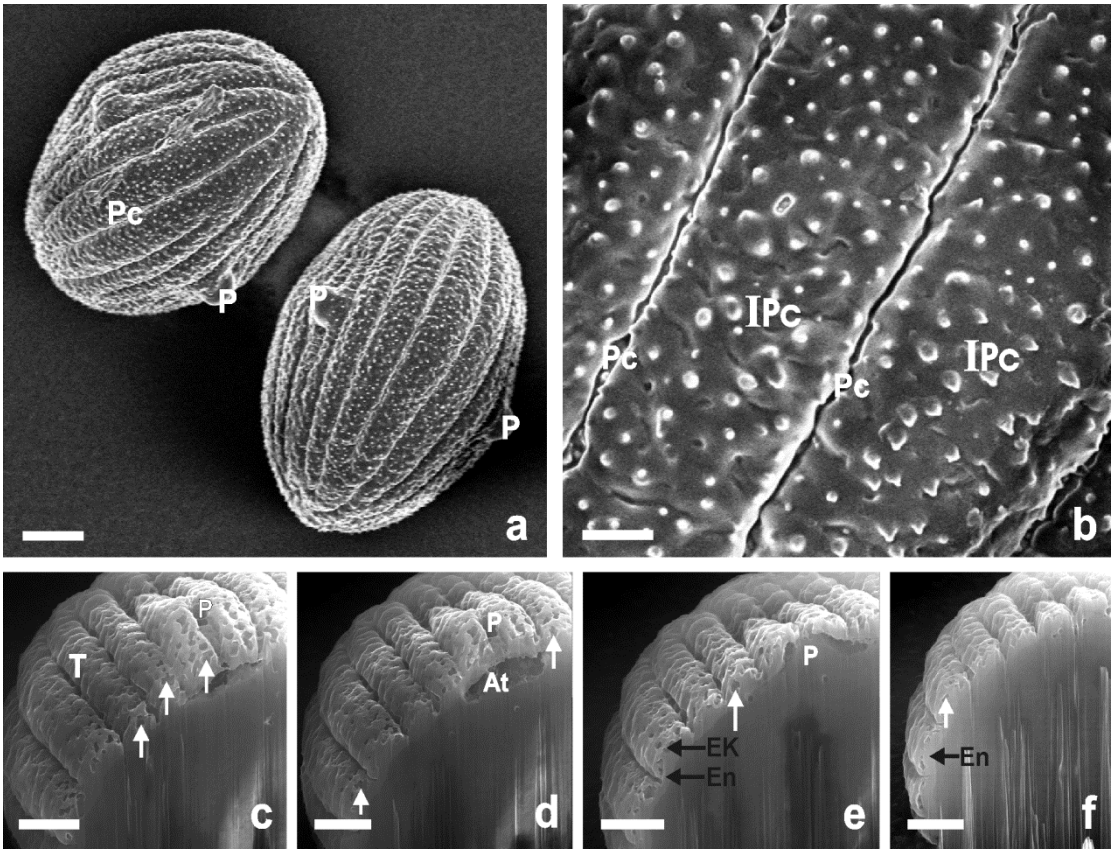

Figure 3.5 FIB-SEM micrographs of *Dyschoriste fischeri*: **a** SEM of a whole pollen grain showing the pseudocolpate arrangement **b** Detail of external sculpturing showing micro-echinae **c–f** Serial cross-sections showing internal wall structure and an endopore. White arrows indicate cavities in the ectexine (Streaking due to FIB process)
At- atrium; Ek-ectexine; En-endexine; IPc-inter-pseudocolpal band; P-endopore; Pc-pseudocolpus; T-tectum
Scale bars: **a**-10 µm; **b**-2 µm; **c**–**f**-5 µm

Petalidium oblongifolium C.B.Clarke (Subfamily: Acanthoideae; Tribe: Ruellieae; Sub-tribe: Ruelliinae) (Figure 3.6):
Grains are globose-prolate, 50 µm long with an equatorial diameter of 40 µm. Grains are triporate and apertures are surrounded by 4 raised ridges of exine. There are two pseudocolpi on each side of each aperture with an inter-pseudocolpal band in between, about 5 µm wide. Adjacent to this is a broad mesocolpium, 10 – 15 µm wide (Fig. 3.6a).
Exine: The tectum is semitectate, reticulate. The muri are supported by closely packed, regularly arranged, distinct columellae. The ectexine is approximately 3 µm thick, and is absent in the pseudocolpi. The foot layer is a thin layer, coarsely granulate, distinct in the pseudocolpi (Fig. 3.6c and 3.6d). The endexine is a uniform layer 1 µm thick.
Intine: The intine is a very fine layer, less than 0.1 µm thick (Fig. 3.6b).

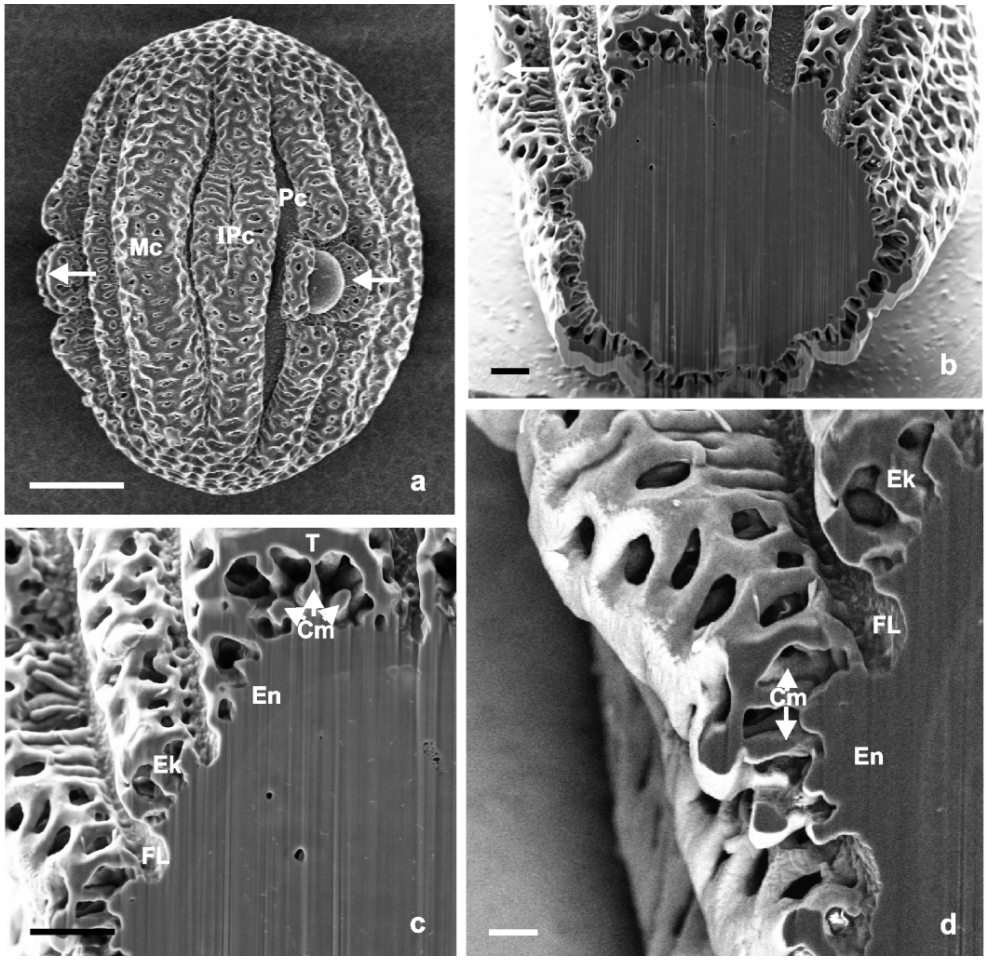

Figure 3.6 FIB-SEM micrographs of *Petalidium oblongifolium*: **a** SEM of a whole pollen grain. Arrows indicate the aperture surrounded by raised ridges of exine **b** Cross-section showing whole cut surface. The arrow indicates the aperture **c** Cross-section through the pollen wall in which part of the tectum has been removed to show supporting columellae (arrows) **d** High magnification showing detail of internal wall features
Cm-columella; Ek-ectexine; En-endexine; FL-foot layer; IPc- inter-pseudocolpal band; Mc-mesocolpium; Pc-pseudocolpus; T-tectum Scale bars: **a**-10 µm; **b**-3 µm; **c**-**3** µm; **d**-1 µm

Ruellia cordata Thunb. (Subfamily: Acanthoideae; Tribe: Ruellieae; Sub-tribe: Ruelliinae) (Figure 3.7):
Grains are globose-spherical, about 40 µm in diameter (Fig. 3.7a). Grains are triporate. **Exine**: The tectum is semitectate, with a very open reticulate pattern and is restricted to the muri, which appear ridged and surround the lumina, which are roughly circular, 2 – 6 µm in diameter and contain densely packed, fine granules on the base (Fig. 3.7b). The main feature of the ectexine is the distinct, individual columellae, 0.5 – 1.5 µm thick, rounded at the base and apex, which support the muri forming a very loose/open reticulum (Fig. 3.7d). The lumina are interconnected by the spaces between the columellae. The ectexine is about 3 µm thick and endexine is a continuous layer about 0.8 µm wide. The foot layer is granulate, best seen at the base of the lumina (Fig**.** 3.7c and 3.7d). **Intine**: The intine is a very fine, amorphous layer that is not clearly visible.

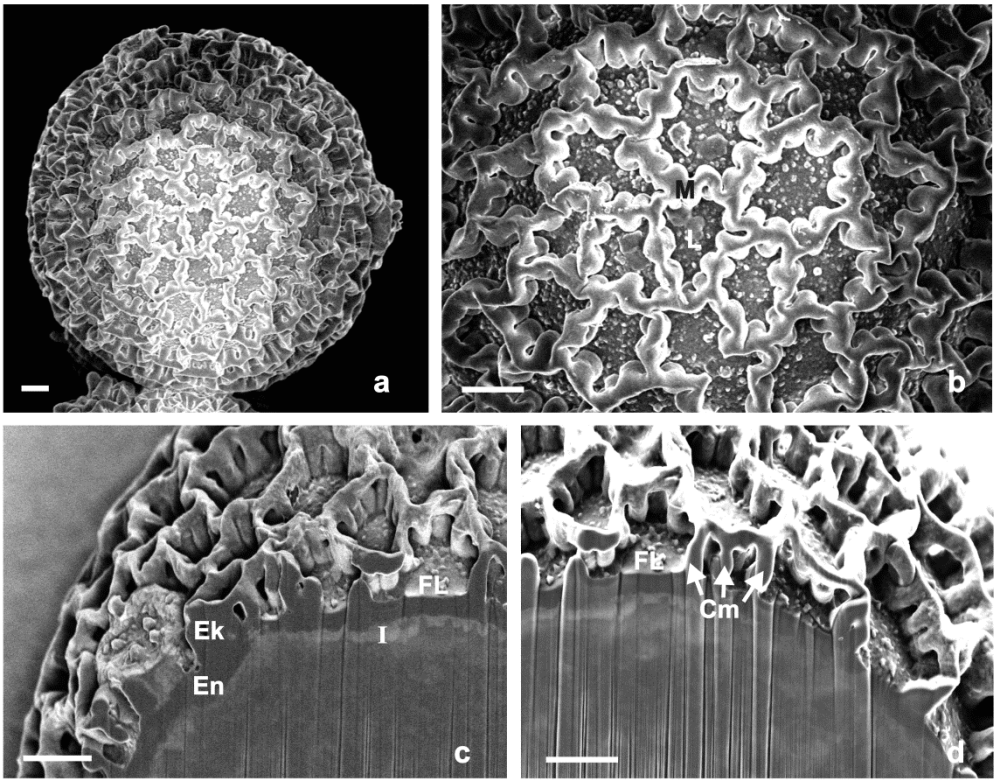

Figure 3.7 FIB-SEM micrographs of *Ruellia cordata*: **a** SEM of the whole pollen grain **b** Detail of the open reticulate pattern **c** Cross-section of wall showing internal wall structure. Intine is a fuzzy layer **d** Cross-section of the pollen wall showing columellae (arrows) supporting muri, and granular foot layer Cm-columella; Ek-ectexine; En-endexine; FL-foot layer; I-intine; L-lumen, M-muri
Scale bars: **a**, **b**, **c**, **d**-3 µm

Justicia flava (Vahl) Vahl (Subfamily: Acanthoideae; Tribe: Ruellieae; Sub-tribe: Justiciinae) (Figure 3.8):
Grains are three-sided, each side is prolate, 35 – 40 µm long, 15 – 20 µm wide (Fig. 3.8a), triangular in cross-section (Fig. 3.8b) with a well developed mesocolpium ridge between the trema areas (region surrounding the apertures). The mesocolpium is thickest at the equator, up to 4.5 µm – 5 µm thick. Grains are tricolporate. **Exine**: The tectum is semitectate; micro-reticulate to granulate in the trema area, traversed by a row of five to seven insulae on each side of the pore; reticulate over mesocolpium ridge, muri 0.2 µm wide surrounding lumina of the reticulum. The ectexine in the mesocolpium has large cavities forming tunnels, interspersed with arched ribs of columellae (ribs of wall material) which appear to branch at their apex and combine with a sponge-like, ramified area which extends out to the tectum (Fig. 3.8c). The ectexine is 0.8 – 5 µm thick in the mesocolpium, 0.1 µm thick in the colpus area, where just the tectum is present covering the endexine (Fig. 3.8b). The foot layer within the mesocolpium is finely granulate, not smooth and has a similar texture to the covering of the colpus areas (Fig. 3.8c). The endexine is a well defined, uniform layer, 1 µm thick. **Intine**: The intine is an indistinct, very fine layer, less than 0.1 µm wide.

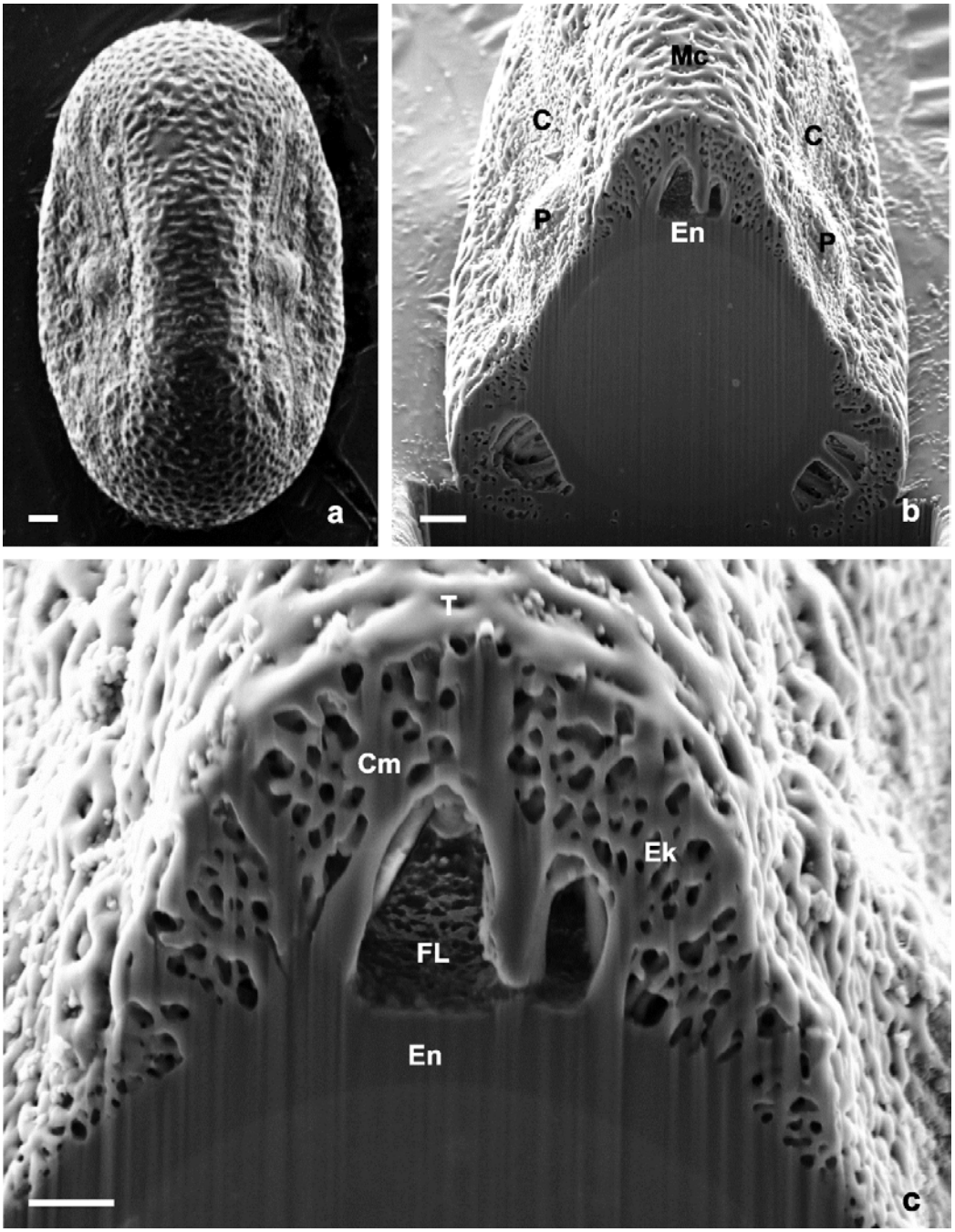

Figure 3.8 FIB-SEM micrographs of *Justicia flava*: **a** SEM of whole pollen grain showing a three sided, prolate grain **b** Cross-section through the pollen grain showing the whole cut surface **c** High power cross-section showing internal wall detail
C-colpus area; Cm-columella; Ek-ectexine; En-endexine; FL-foot layer; Mc-mesocolpium; P-pore; T-tectum Scale bars: **a**-2 µm; **b**-2 µm; **c**-1 µm

Justicia carnea Lindl. (Subfamily: Acanthoideae; Tribe: Ruellieae; Sub-tribe: Justiciinae) (Figure 3.9):

Grains are long and prolate, 70 – 75 µm in length and 25 – 30 µm in diameter. Grains are 2-colporate (Fig. 3.9a). **Exine**: The tectum is almost tectate-imperforate to tectate perforate. In the trema region (surrounding the aperture area) the tectum is micro-

reticulate and traversed by three or four rows of 10 to 12, small verrucae on each side of the colpus. Outside the trema area, laterally on the grain, the tectum is sparsely pitted by fovea (tiny 'pores'), described as foveolate. Laterally the ectexine becomes thicker, and is characterised by tunnels that pass through the wall and seem to run length-wise through the grain (Fig. 3.9b and 3.9c), but a longitudinal section would be required to confirm this. The endexine is 0.5 – 1.5 µm thick laterally, and about 0.7 µm thick in the dorsal and ventral surfaces (Fig. 3.9b). **Intine**: the intine is a very fine layer.

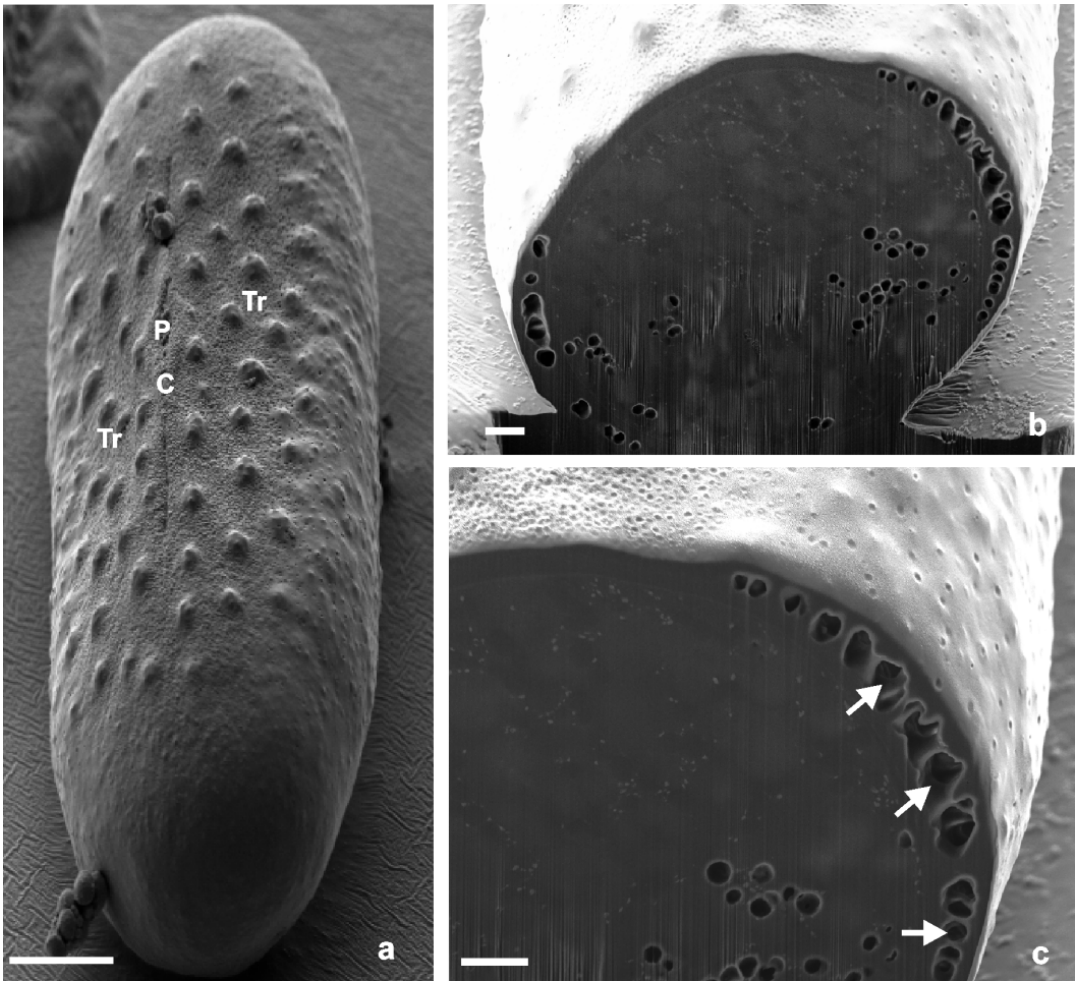

Figure 3.9 FIB-SEM micrographs of *Justicia carnea*: **a** SEM of a whole pollen grain showing a thin colpus and pore, surrounded by the trema area with verrucae **b** Cross-section showing whole cut surface **c** Detail of the ectexine in the lateral region of the pollen grain. Arrows indicate the tunnels
C-colpus; P-pore; Tr-trema area Scale bars: **a**-10 µm; **b**-2 µm; **c**-2 µm

Hypoestes forskaolii (Vahl) R.Br. (Subfamily: Acanthoideae; Tribe: Ruellieae; Sub-tribe: Justiciinae) (Figure 3.10):
Grains are prolate, 20 – 30 µm long with an equatorial diameter of 14 – 18 µm. Grains are tricolporate (Fig. 3.10a). **Exine**: On each side of the colpus is a narrow rib of reticulate exine, 1.4 µm wide. Adjacent to this is a strip of tectum, densely perforated or micro-reticulate (Fig. 3.10b). In the mesocolpium, the tectum is semitectate, reticulate,

supported by individual, evenly spaced columellae, the head of which is situated in the centre of each lumen, attached to the muri by fine rays (Fig. 3.10c). The ectexine is approximately 1.5 µm high. The foot layer is granulate, clearly seen at the base of the columellae (Fig. 3.10d). The endexine is a thin, uniform layer, less than 1 µm thick (i.e. 0.3 – 0.75 µm thick). **Intine**: The intine is barely visible in micrographs.

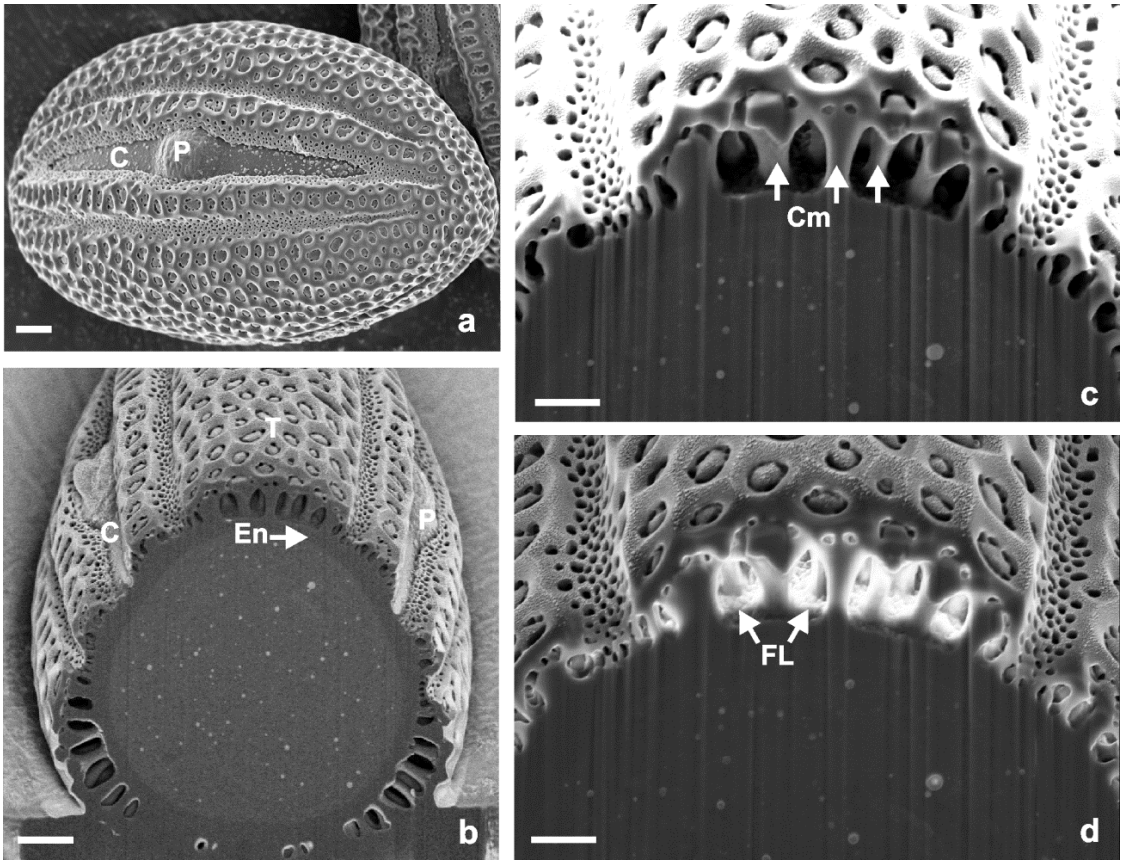

Figure 3.10 FIB-SEM micrographs of *Hypoestes forskaolii*: **a** SEM of a whole pollen grain **b** Cross-section through grain showing whole cut surface. Endexine is clearly visible as a uniform layer **c** Cross-section through the wall showing detail of internal structures. Columellae indicated by arrows **d** The same section but charging of the specimen highlights the granulate foot layer (arrows)
C-colpus area; Cm-columella; En-endexine; FL-foot layer; P-pore; T-tectum
Scale bars: **a**-**2** µm; **b**-2 µm; **c**-1 µm; **d**-1 µm

Rhinacanthus gracilis Klotzsch var. *latilabiatus* K.Balkwill (Subfamily: Acanthoideae; Tribe: Ruellieae; Sub-tribe: Justiciinae) (Figure 3.11):
Grains are globose-prolate, 30 µm long, with an equatorial diameter of 25 – 27 µm. Grains are tricolporate (Fig. 3.11a). Grains are very similar to those of *Hypoestes*, also with strips of micro-reticulate tectum on each side of a wide reticulate mesocolpium, about 12 µm wide (Fig. 3.11b). **Exine**: The ectexine is about 1.5 – 2 µm thick, straight, narrow columellae support a reticulate tectum. The foot layer is granulate, clearly seen at the base of the columellae (Fig. 3.11d). The endexine is a uniform, narrow layer, about 0.5 µm thick (Fig. 3.11c). **Intine**: This layer is very indistinct.

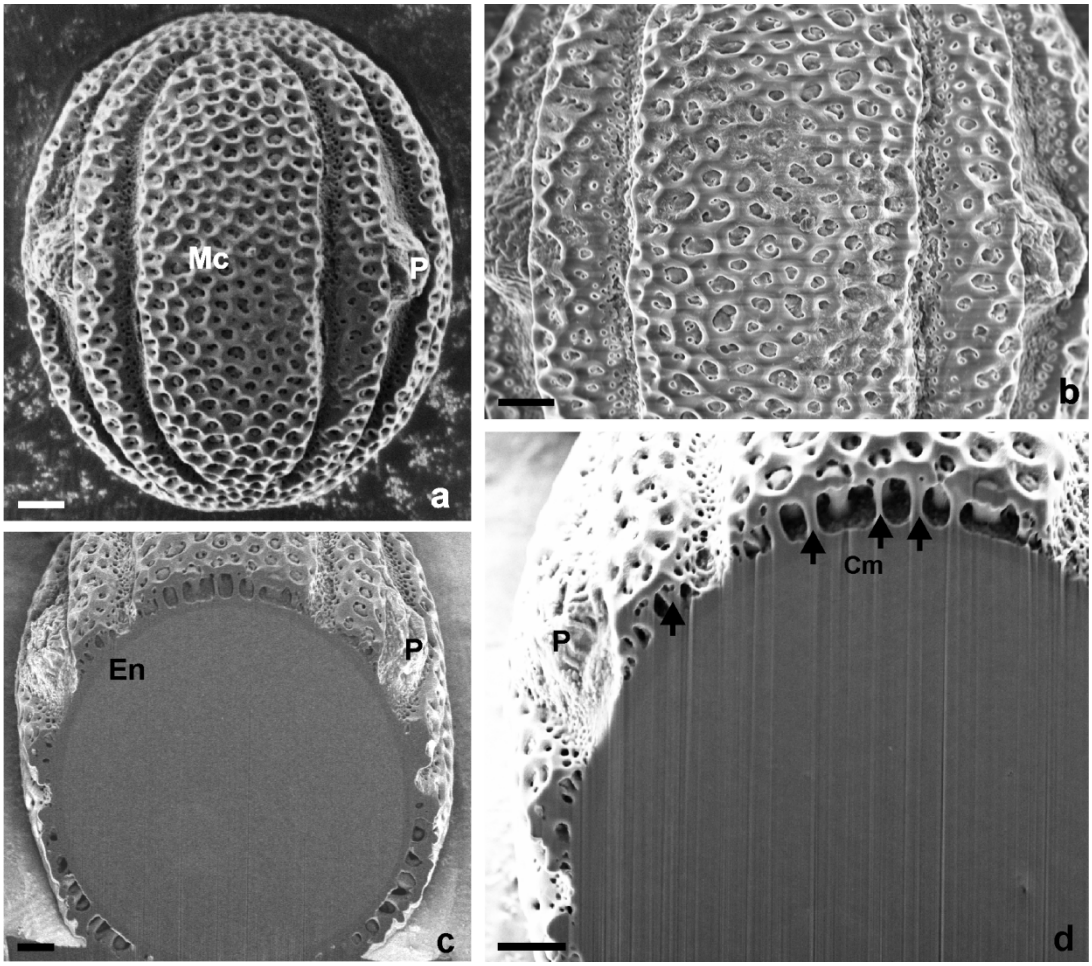

Figure 3.11 FIB-SEM micrographs of *Rhinacanthus gracilis* var. *latilabiatus*: **a** SEM of a whole pollen grain, showing 2 pores **b** Detail of reticulate sculpturing **c** Cross-section showing whole cut surface **d** Cross-section through the wall showing internal structure. Columellae indicated by arrows Cm-columella; En-endexine; Mc-mesocolpium; P-pore Scale bars: **a**-3 µm; **b**-**2** µm; **c**-2 µm; **d**-2 µm

Isoglossa ovata (Nees) Lindau (Subfamily: Acanthoideae; Tribe: Ruellieae; Sub-tribe: Justiciinae) (Figure 3.12):

Grains are spherical, oblate, about 25 µm in diameter. Grains are 2-pororate, one pore at each pole (Fig. 3.12a). The grain is divided into two areas: a central, polar, trema area surrounding each pore and a girdle area around the equatorial circumference (Fig. 3.12a and 3.12b). **Exine**: The tectum is finely pitted and granulate, with spinules scattered over the surface in the trema area (region surrounding the pore) and punctured by tiny fovea arranged in a circular pattern (micro-foveolate) in the girdle area. In the girdle area the ectexine is 1.5 – 3.5 µm thick; large, conspicuous, branching tunnels (1.5 µm wide, 2.5 µm high) with thick walls (1 µm wide), run through the ectexine. The psilate foot layer is seen at the base of the tunnels (Fig. 3.12c and 3.12d). The tectum is 0.5 – 1 µm thick, in this area. The exine is confined to a granular tectum covering the endexine, 1.5 – 2 µm thick in the polar region. The endexine in the girdle area is narrower, 0.5 – 1 µm thick. **Intine**: A very fine, uniform layer (Fig. 3.12b).

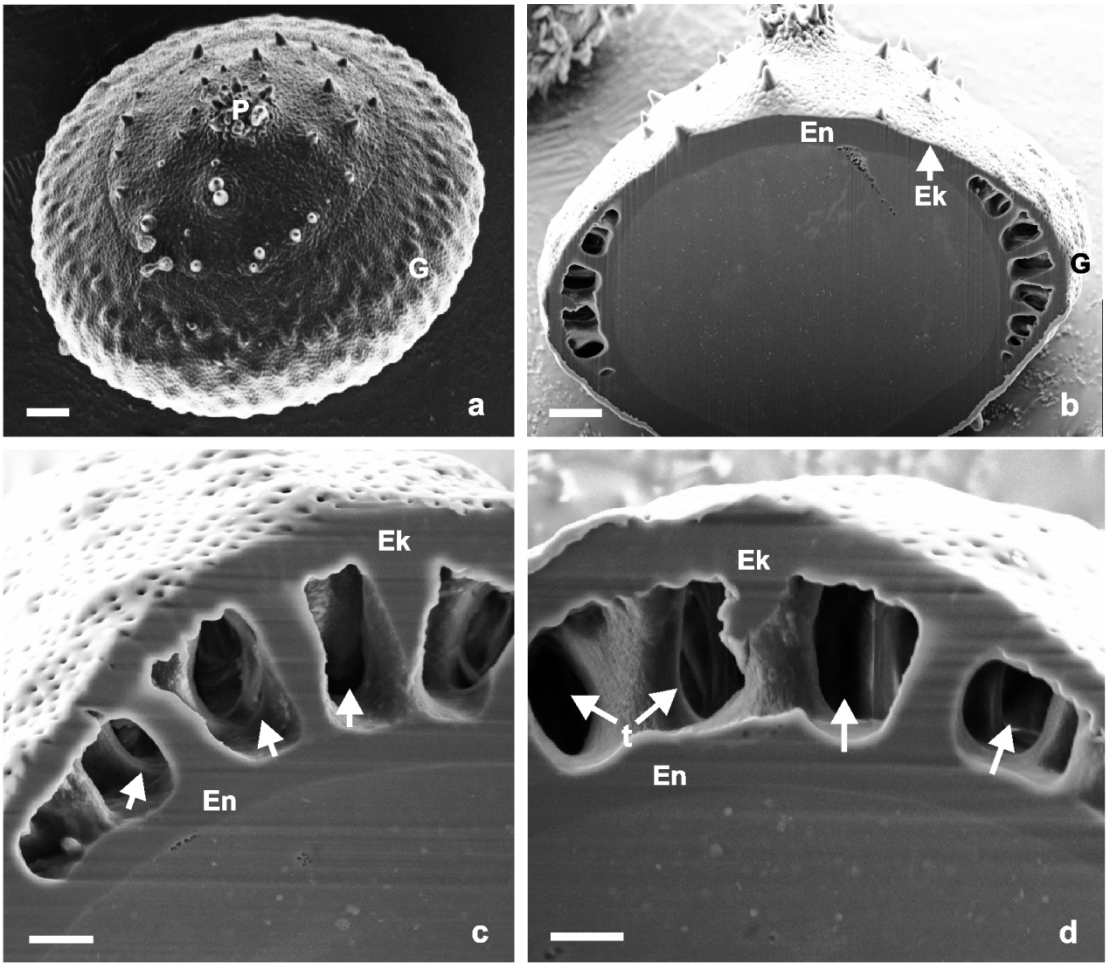

Figure 3.12 FIB-SEM micrographs of *Isoglossa ovata*: **a** SEM of a whole pollen grain showing the polar/trema and girdle areas **b** Cross-section showing whole cut surface and large thick walled tunnels in the girdle area **c** and **d** Detail of internal wall structure in the girdle area. Arrows indicate the tunnels; 't' indicates a point of branching
Ek-ectexine; En-endexine; G-girdle area; P-pore; t-branching tunnels Scale bars: **a**-3 µm; **b**-3 µm; **c**-1 µm; **d**-1 µm

Barleria rotundifolia Oberm. (Subfamily: Acanthoideae; Tribe: Ruellieae; Sub-tribe: Barleriinae) (Figure 3.13):
Grains are large, 100 µm in length with an equatorial diameter of 60 – 80 µm (Fig. 3.13a). Grains are ellipsoid in outline, 3-lobed in polar view and tricolporate. **Exine**: The tectum is semitectate, restricted to the muri which outline pentagonal or hexagonally shaped lumina, 10 – 20 µm across, forming a very open reticulum. Individual columellae, 10 µm high, 2 – 4 µm thick, support this open reticulum (Fig. 3.13b). Columellae extend from the rudimentary tectum down to the foot layer, ending in a rounded base on the foot layer. Columellae appear to be produced on both edges of the muri, which appear hollow between a thin, double wall (Fig. 3.13c and 3.13d). As a result the columellae seem to be closely packed. The ectexine is at least 10 µm thick. The endexine is a well defined layer, 1.5 – 3 µm thick; it appears to be separated into

two layers by a fine granular layer (Fig. 3.13c and 3.13d). The foot layer is psilate to verrucate, best seen at the base of the lumina. **Intine**: The intine is a very fine but well defined, continuous layer.

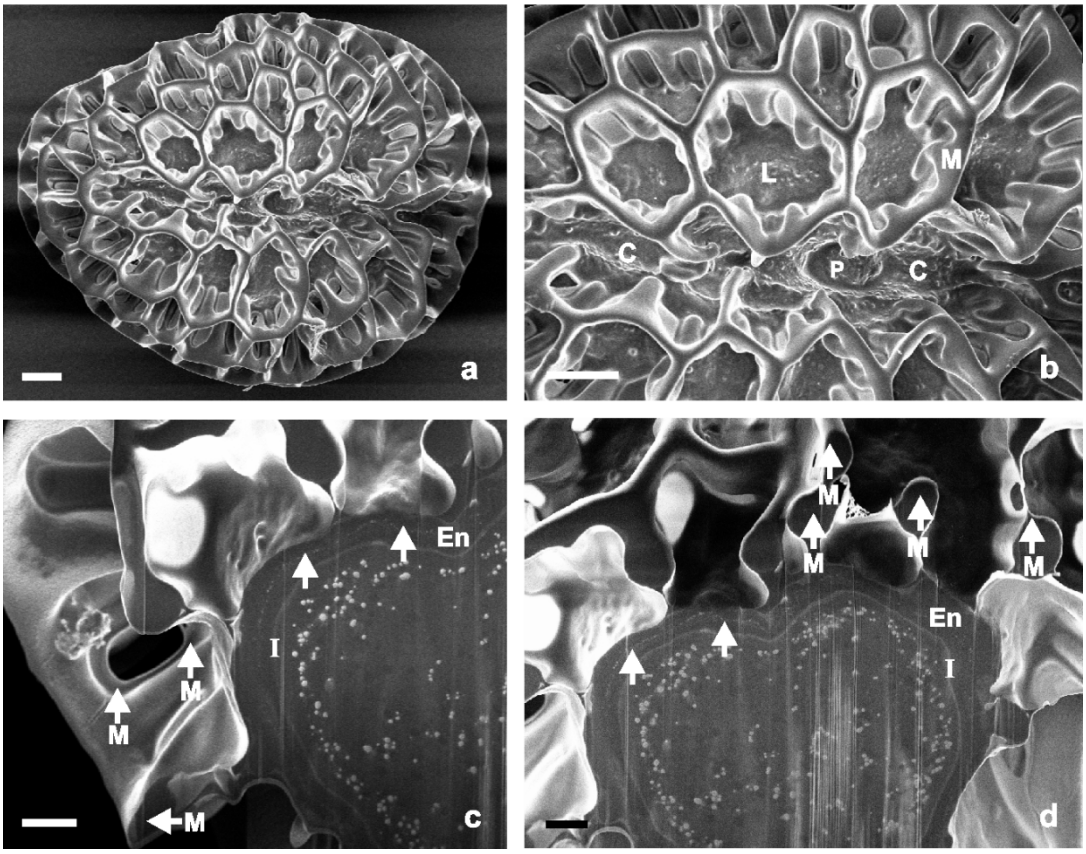

Figure 3.13 FIB-SEM micrographs of *Barleria rotundifolia*: **a** SEM of a whole pollen grain **b** Detail of sculpturing showing pentagonal/hexagonal reticulate pattern **c** Cross-section showing detail of wall structure. M-arrows indicate hollow muri **d** Cross-section through the pollen grain. Arrows indicate fine granular layer within the endexine. M-arrow indicates the hollow muri
C-colpus; En-endexine; I-intine; L-lumen, M-muri, P-pore Scale bars: **a**-10 µm; **b**-10 µm; **c**-3 µm; **d**-3 µm

Crabbea hirsuta Harv. (Subfamily: Acanthoideae; Tribe: Ruellieae; Sub-tribe: Barleriinae) (Figure 3.14):

Grains are globose-prolate, about 50 µm in length with an equatorial diameter of 42 – 45 µm, triporate, pores irregularly distributed (Fig. 3.14a). **Exine**: The tectum is confined to the muri surrounding the lumina, forming an open reticulum and grains are therefore semitectate. More or less solid, wavy walls of the muri, 1.5 – 2 µm high, divide the surface into roundish-rectangular lumina, 3 – 7 µm in diameter (Fig. 3.14b and 3.14c). In arbitrary places the lumina are connected by cavities in the walls (Fig. 3.14d). The ectexine is 2 – 2.5 µm thick. The foot layer is granulate with patches of 'pilate' sculpturing at the centre of each lumina, which may originate from the endexine (Fig. 3.14d). The endexine is a continuous layer, 1.5 µm thick. **Intine**: A thin layer.

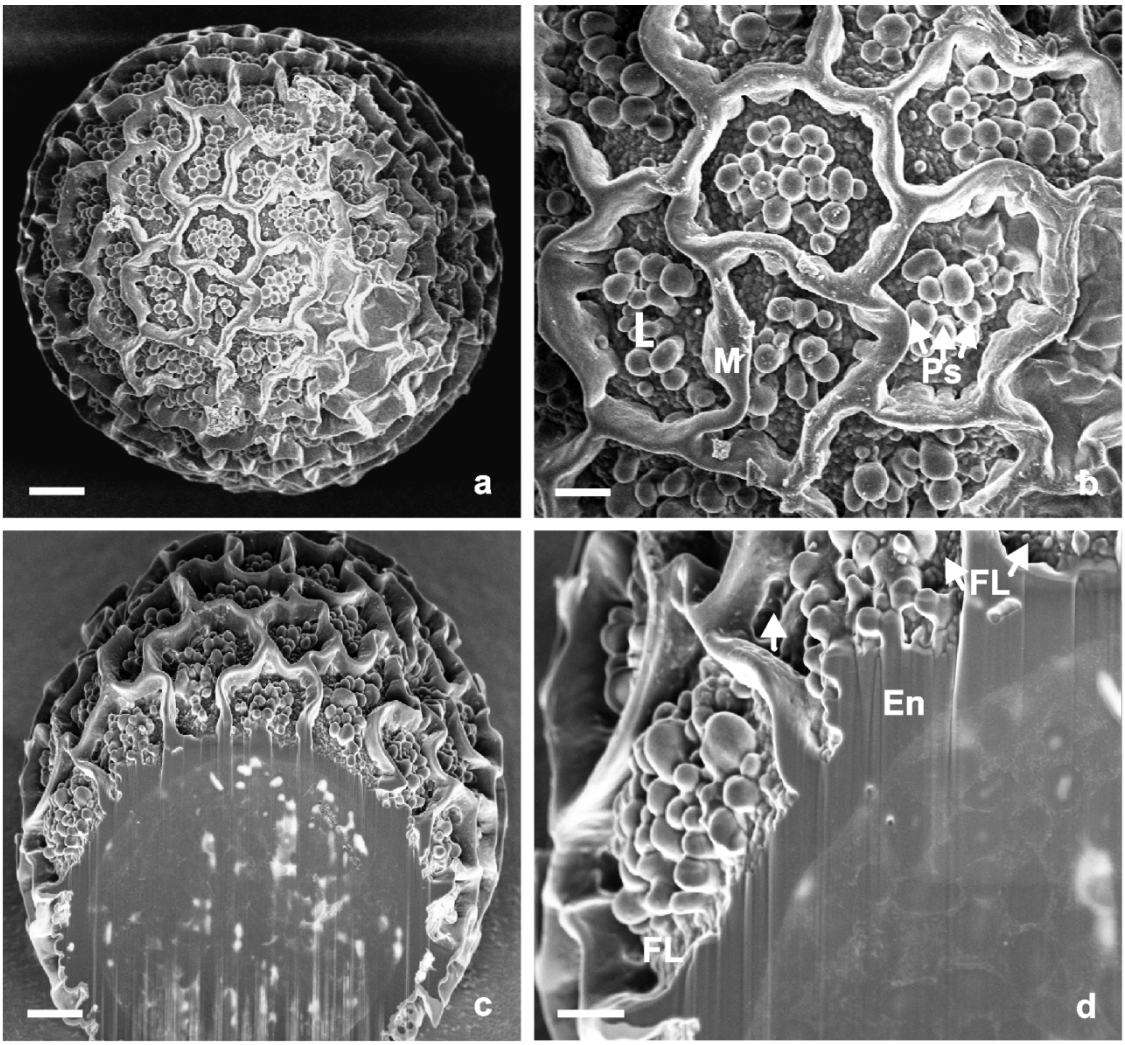

Figure 3.14 FIB-SEM micrographs of *Crabbea hirsuta*: **a** SEM of a whole pollen grain **b** Detail of reticulate sculpturing with pilate sculpturing (pila) at centre of each lumen **c** Cross-section through the pollen grain showing the whole cut surface **d** Cross-section through the wall showing detail of internal wall structure. Arrow indicates a cavity in the wall connecting two lumina. Granulate foot layer indicated. Note the pila seem to arise from the endexine

En-endexine; FL-foot layer; L-lumen, M-muri; Ps-pilate sculpturing

Scale bars: **a**-5 µm; **b**-2 µm; **c**-5 µm; **d**-2 µm

DISCUSSION

Many studies have identified and discussed diverse types of external pollen sculpturing in the Acanthaceae and their usefulness in the taxonomy of this family. In this study five discernible exine structural types were identified, from observations of the sections obtained using FIB-SEM:

1. Solid internal wall with no cavities, columellae, tunnels or other features – e.g., *Blepharis stainbankiae* (Fig. 3.3) and *Acanthus mollis* (Fig. 3.4b).

2. Porate, sponge-like in appearance – e.g., *Thunbergia atriplicifolia* (Fig. 3.2) and *Justicia flava* (in combination with Type 4) (Fig. 3.8).
3. Cavities or chambers dispersed throughout the wall – e.g., *Dyschoriste fischeri* (Fig. 3.5), *Hygrophila auriculata* (Chapter Two, Fig. 2.4: pg. 2.4/1538). The cavities sometimes seen in *Acanthus mollis* (Fig. 3.4c and 3.4d) are typically not an interconnected network of tunnels and occur discretely as they dwindle and disappear in successive sections. *Crabbea hirsuta* (Fig. 3.14) could possibly fit into this pattern if it had a more complete tectum.
4. Labyrinthine interconnected tunnels – e.g., *Isoglossa ovata* (Fig. 3.12) and *Justicia carnea* (Fig. 3.9). *Justicia flava* (Fig. 3.8) has large interconnected tunnels in conjunction with Type 2. *Barleria rotundifolia* (Fig. 3.13) could possibly fit into this pattern if it had a more complete tectum.
5. Distinct, individual, regularly arranged columellae – *e.g., Hypoestes forskaolii* (Fig. 3.10), *Rhinacanthus gracilis* var. *latilabiatus* (Fig. 3.11), *Petalidium oblongifolium* (Fig. 3.6) and *Ruellia cordata* (Fig. 3.7). The presence of distinct, individual columns distinguishes this type from Type 4 where tunnel walls in cross-section can sometimes give the appearance of columns.

It is highly likely that further investigations into the Acanthaceae and other families will yield more wall structural types.

Some of the wall structural types highlighted by this study have been described before. The porate, sponge-like type identified here, has been described by Raj (1961) in *Thunbergia* (Retz.) as a ‘thick, granular sexine’, which he considers to be an ancestral state. The work of Walker and Doyle and co-workers in gymnosperms and primitive angiosperms refers to both alveolate and granular wall structures, which they propose is indicative of phylogeny in these groups (Walker and Skvarla, 1975; Doyle, 2009; Doyle and Le Thomas, 2012). Their description of the alveolate state appears to correspond more to the structural pattern found in this study for *Thunbergia* - described here as porate or spongy - than what they describe as a granular state. The results afforded by the FIB-SEM in this species show the sculpturing to definitely have a spongy, not granular appearance, so alveolate seems a more appropriate description for these structures in this group.

Furness (1990), using ultra thin sections for TEM, has also described similar internal features to those of *Thunbergia* in pollen grains of *Crossandra* Salisb. She identifies a granular exine and a granular exine with cavities. Three dimensional data obtained from FIB-SEM sectioning, is fortunately far easier to interpret.

The columellae type has been mentioned extensively with regard to pollen wall structure in Acanthaceae species (e.g., Lindau, 1895; Raj, 1961; Graham, 1988; Furness, 1990; etc.). The distinction between actual columns and interconnected

tunnels, described here as the labyrinthine type, as well as walls interspersed with discrete chambers can only be seen by the detailed 3D sections afforded by the FIB-SEM. This type of analysis shows structural detail that is not as well observed using other techniques, e.g., TEM. Each of these exine patterns should probably be regarded as a distinct structural type.

Pollen grains with an open reticulum (a reduced tectum) do not at first glance appear to clearly exhibit any of the five wall structural types described here. In the case of *Ruellia*, *Barleria* and *Crabbea*, however, if their respective tecta were more complete, the wall structures could be matched with the described types. *Ruellia* would align with the *Hypoestes-Rhinacanthus* type (Type 5), with distinct, individual columellae supporting a reticulate tectum. *Barleria* would correspond with Type 4, as the double walled muri would give the appearance of labyrinths or tunnels intersecting the wall in cross-section. The wavy muri of *Crabbea*, with the pilate sculpturing on the foot layer, would appear as chambers or cavities within the wall in cross-section (Type 3).

Although many Acanthaceae species are known to produce sterile (lacking cytoplasm) pollen grains (Furness, 1990, 1993), there is no need to assume that the wall features described here are peculiar to sterile grains, as the external pollen wall features are entirely consistent with their viable counterparts and match the generic descriptions. Furthermore considering that the external layers are derived from the internal, underlying layers the assumption is that if the external features match those described for the species so should the internal wall structure.

Although five basic wall structural types were observed, these structures do not necessarily continue regularly around the entire circumference of individual grains and walls often change from having no features, being solid and thinner in the vicinity of the colpus to having labyrinths, columellae or cavities in other areas (e.g., Fig. 3.8, 3.9, 3.10, 3.11 and 3.12). This makes it questionable as to whether a species can be described as having a single, internal wall structural type. Some species definitely exhibit combinations. It appears that the internal wall structures which are most likely to have any phylogenetic relevance are those found in the mesocolpium or the area between the apertures. This is the area that typically has external reticulate sculpturing and exhibits the most internal structural variation.

In addition to identifying five discernible wall structural types, the results of this study provide insights to the question of the relationship between internal and external wall structures. The results show that similar external wall features or sculpturing do not always correspond to the same internal features or structure. *Dyschoriste fischeri* and *Petalidium oblongifolium* have similar external sculpturing and are both described as pseudocolpate grains where the grain is divided into sculptured inter-pseudocolpal bands of exine (sexine) alternating with pseudocolpate regions (Scotland 1993). They

are both currently allocated to the same subtribe Ruelliinae of Scotland and Vollesen (2000), or tribe Ruellieae, subtribe Petalidiinae of Tripp et al. (2013). In both species the wall underlying the pseudocolpi is solid. The internal walls of the inter-pseudocolpal bands of *Dyschoriste fischeri* contain chambers interconnected by cavities in the chamber walls (Fig. 3.5) while this region of *Petalidium oblongifolium* is filled with columellae (Fig. 3.6). This pattern is noteworthy from a taxonomic point of view as the external features of pollen are widely and successfully used as taxonomic characters in this family and they do not appear to be reliable predictors of the internal wall features. In contrast, *Hygrophila auriculata* also of tribe Ruellieae, but placed in a different subtribe Hygrophilinae (Tripp et al. 2013), consequently described as a pseudocolpate grain, differs externally from the aforementioned species as it lacks sexine lips surrounding the apertures, has broad pseudocolpi and inter-pseudocolpal bands with double reticulate sculpturing, but has a similar internal wall structure to *Dyschoriste fischeri*. In this case the internal structure may indicate relatedness, whereas the external sculpturing shows divergence.

On a phylogenetic note, *Justicia flava* exhibits the same porate/alveolate internal wall structure as observed in *Thunbergia* and *Crossandra* (Furness, 1990), but all three are allocated to different subfamilies within the Acanthaceae (Scotland and Vollesen, 2000).

Although it is too early to assess the taxonomic and phylogenetic significance of the diversity in wall structural types, the relationship between phylogeny and the types of internal pollen wall structures identified in this study is illustrated in Figure 3.15, where the study species and their wall types are listed alongside their respective groups. It is evident that Type 1 is restricted to Acantheae, Type 2 is characteristic of Thunbergioideae (but is also found in *Justicia flava* which exhibits more than one type of internal structure), while Justiceae only contains Type 4 and Type 5. With additional work and in conjunction with other taxonomic characters and existing phylogenies, it may become possible to supplement existing classifications, taxonomically assign some problematic species and infer phylogenetic relationships using these features. Moreover, the possible functional significance of the various structural types remains unknown.

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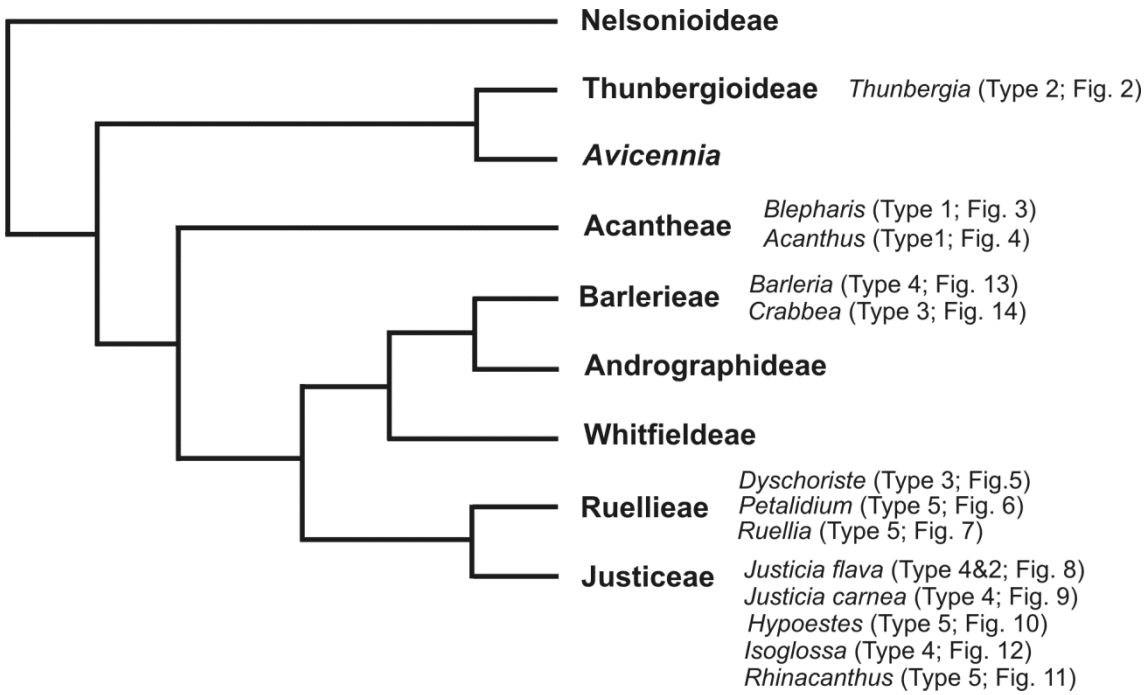

Figure 3.15 A phylogenetic tree of the Acanthaceae (Tripp et al. 2013) showing the distribution of exine types, reported in this study

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CHAPTER FOUR – TAXONOMIC SIGNIFICANCE OF THE INTERNAL POLLEN WALL STRUCTURE IN *JUSTICIA* L. (ACANTHACEAE)

Abstract

The external pollen morphology of *Justicia* is diverse and offers a wealth of characters currently used for classifying species. The pollen morphology of 12 species of *Justicia* and six species from six other related genera of Justiciinae was investigated using FIB-SEM techniques. This method allows for three dimensional examination of the internal pollen wall structure. The internal pollen wall structure can be characterised by the structure of the ectexine within the mesocolpium. This internal wall layer contains elongate, arched, walled cavities or pentagonal shaped, walled chambers, each with or without a central columella. Morphometric analyses using a combination of documented external characters and the internal wall structure characters obtained in this study were conducted. The resulting trees cluster the taxa (the 18 selected species) into separate groups broadly in line with existing classifications. Incorporating internal wall character data in cluster analyses both supports and assists in clarifying relationships within the genus which previously relied on external wall morphology alone.

INTRODUCTION

The genus *Justicia* was first established by Linnaeus in 1753 and named in honour of the Scottish horticulturist James Justice (1698–1763) (Gledhill, 2008). It belongs to the subtribe Justiciinae, in the tribe Ruellieae of the Acanthaceae (Scotland and Vollesen, 2000). The Acanthaceae are a large, pan-tropical family of great morphological diversity and *Justicia* is the largest genus of the family with approximately 700 species (McDade et al., 2000). *Justicia* is a cosmopolitan genus and exhibits extensive morphological diversity. It is not monophyletic, showing strong phylogenetic differences between Old and New World species (McDade et al., 2000). Currently *Justicia* includes over 70 generic synonyms (Scotland and Vollesen, 2000) and Index Kewensis records that more than 100 new species have been added to the genus since 1986 (Davies and Challis, 2002). The distinguishing features of the genus include simple or compound spicate inflorescences subtended by a bract and two bracteoles, a tubular, bilabiate corolla and two stamens, each with two asymmetrical anthers (Graham, 1988).

Various characters have been used for the classification of this genus, including anther and pollen morphology. Plant morphology and external pollen morphology have been extensively investigated. Many workers have studied the genus in specific geographical regions in order to develop a classification e.g., in Bolivia (Wasshausen and Wood, 2003); in Paraguay and Argentina (Ezcurra, 2002); in tropical Africa (Hedren, 1989); in Southern Africa (Immelman, 1989); in Ethiopia (Ensermu, 1990); in Central

America/Mexico (Daniel, 2004); in Thailand (Rueangswang et al., 2013). This has unfortunately resulted in a confused taxonomic classification of the genus, where some species of *Justicia* are recognised as segregate genera in one geographical region, but as members of *Justicia* in another. Very few worldwide studies of the genus have been undertaken, most significantly those of Nees (1847), Bentham (1876), Lindau (1895), and most recently Graham (1988).

Graham (1988) attempted to study as wide a sample of the genus as possible, selecting 58 species, from both the Old World and New World, to study in detail, to produce an infrageneric classification of the genus based on general as well as pollen morphology. She recognised seven Old World and nine New World sections and since this study, new sections have been added by Hilsenbeck (1990a) and Daniel (2003, 2004). As a result the circumscription of the genus has been expanded to include a number of formerly segregate genera. In her study, Graham (1988) identified 10 pollen types based on external pollen characteristics. She described pollen as generally two to three aperturate, subprolate to perprolate, having reticulate ornamentation with columellae lying beneath the lumina of the reticulum, one per lumen. The columellae branch at the apex to support the muri and the sexine is much thinner and micro-reticulate in the trema area. Lindau (1895) described the pollen of *Justicia* as 'Knötchenpollen', characterised as having two or three apertures with one to three rows of insulae on each side of the aperture area. Immelman (1983) suggested that the number of colpi per pollen grain is correlated with the inflorescence type in southern African species. Hedrén (1989) revised *Justicia* section *Harnieria*, from tropical Africa and concluded that the pollen of this section is characterised by two apertures and more than one row of insulae on each side of the aperture. Rueangswang et al. (2013) found two major groups of pollen among species in Thailand, based on aperture structure and presence or absence of insulae and pseudocolpi.

When looking at relationships among species, the challenge is to identify consistent characters that can be used to group species together into both genera and infrageneric groups. External pollen morphology is such a character which has been used extensively in formulating classification systems in the Acanthaceae. An understanding of the pollen morphology gained from previous studies has assisted the understanding of the phylogeny of the family (and consequently in the genus *Justicia*). The external features of pollen grains used in classifying species include size, shape, number of apertures and the sculpturing of the outside pollen wall layers. It has been suggested that the lesser studied internal pollen wall structure may be of taxonomic value, and that the structure of the internal wall is not dependant on the external sculpturing (Moore et al., 1991). Focused ion beam scanning electron microscopy (FIB-SEM) has proved most useful for examining the internal structure of pollen grains in three dimensions (House and Balkwill, 2013). This method was used in this study to investigate the internal structure of pollen walls in *Justicia* to determine if the relationships among the species could be

defined in terms of comparing internal pollen wall features.

Terminology: For the sake of clarity and convenience the terminology used to describe the pollen grains in this study is highlighted in a labelled micrograph (Fig. 4.1), identifying the special morphological terms used: margocolpus (Immelman, 1989), mesocolpium and trema region (Raj, 1961).

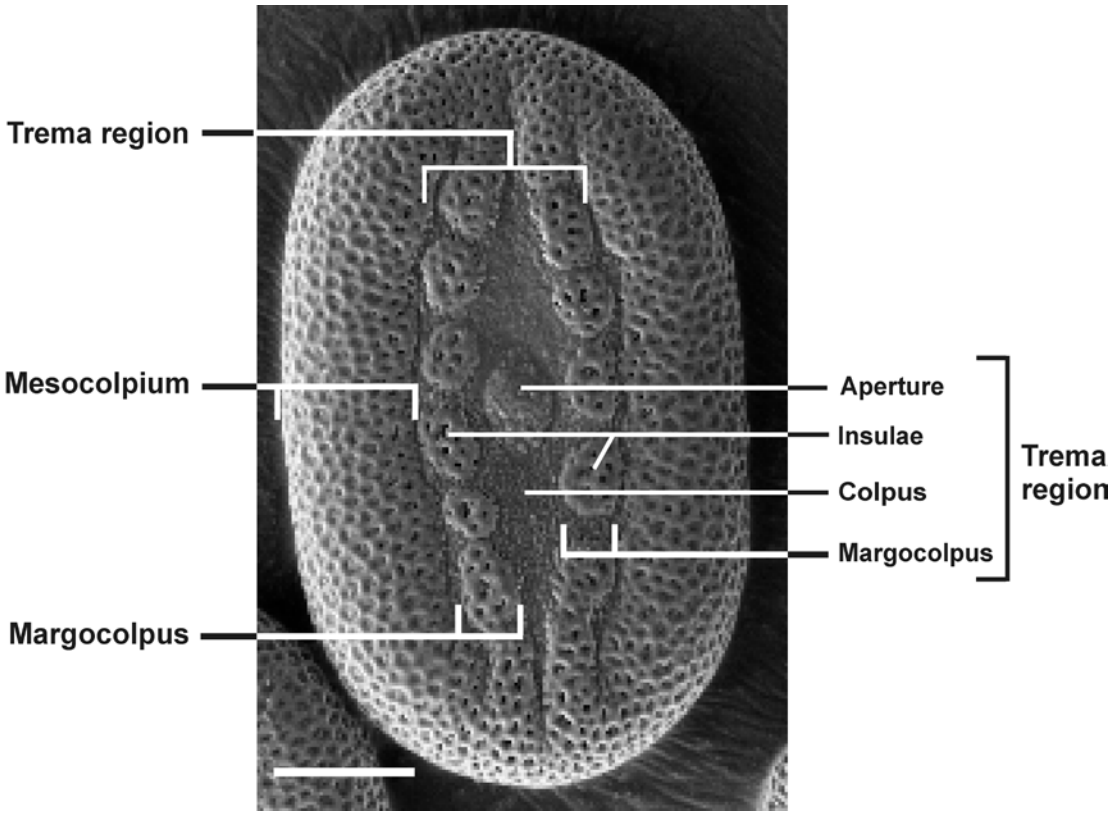

Figure 4.1 Micrograph of a whole pollen grain in equatorial view highlighting the terminology used to describe specific external pollen grain features
Scale bar 5µm

MATERIALS AND METHODS

The pollen structure of 12 species of *Justicia* L. was investigated. The species were selected to represent as many of the 10 pollen types defined by Graham (1988) as possible, and all the seven Old World sections. The species are:

J. betonica L. [Section II Betonica; pollen type 1]
J. campylostemon (Nees) T.Anders. [Section III Rhaphidospora; pollen type 1]
J. flava (Vahl) Vahl [Section IV Tyloglossa; pollen type 2]
J. petiolaris (Nees) T. Anders. [Section IV Tyloglossa; pollen type 2]
J. brandegeeana Wassh. & L.B.Sm. [Section XI Drejerella; pollen type 3]

J. aconitiflora (A.Meeuse) Cubey [*Duvernoia aconitiflora* A.Meeuse] [Section I Vasica; pollen type 4]
J. guerkeana Schinz [Section VI Justicia; pollen type 4]
J. odora (Forssk.) Vahl [Section V Harnieria; pollen type 5]
J. capensis Thunb. [Section V Harnieria; pollen type 5]
J. anagalloides (Nees) T.Anders. [Section VII Rostellularia, sub section Ansellia; pollen type 5]
J. protracta (Nees) T.Anders. [Section V Harnieria*; intermediate between pollen types 6 and 7]
J. carnea Lindl. [Section XV Cyrtanthera; pollen type 8]

*Immelman (1989) places this species into Section V Harnieria, whereas Graham (1988), due to the presence of insulae and peninsulae, assigns it to Section XIII Sarotheca – which includes several species with pollen intermediate between types 6 and 7.

The pollen of six species representing six other genera of Justiciinae was also investigated. These are:

Hypoestes forskaolii (Vahl)R.Br.
Rhinacanthus gracilis Klotzsch var. *latilabiatus* K.Balkwill
Dicliptera leistneri K.Balkwill
Mackaya bella Harv.
Metarungia longistrobus (C.B.Clarke) C.Baden
Peristrophe cernua Nees

These related taxa of the sub-tribe Justiciinae were specifically selected for their similarity in external pollen morphology to *Justicia*. Pollen was examined in order to compare the internal wall structures and better assess if the structures of *Justicia* pollen are of taxonomic relevance.

Fresh flowers were collected, some were placed in small envelopes and stored in dry silica gel crystals and others were transferred immediately into vials with 99% Glacial Acetic Acid (GAA). Both methods of storage resulted in equally favourable results.

Voucher specimens from the collections were deposited in the C.E. Moss Herbarium (J) at the University of the Witwatersrand, Johannesburg.

Pollen was harvested directly from the anthers, using fine insect pins and transferred onto carbon coated tape attached to an electron microscope stub.

Samples were lightly coated (5 nm) with Au-Pd alloy at 20mA for one minute or with

carbon for no more than two pulses (*J. flava* and *J. campylostemon*) if Au-Pd alloy was not available.

Samples were viewed with a Zeiss Auriga microscope with a crossbeam FIB workstation and Gemini FE SEM column. Suitable pollen grains were selected, photographed, orientated for sectioning with the FIB, and each pollen grain was sectioned once at a selected site. The FIB was operated at 30 kV. This work was conducted at the CSIR, Brummeria, Pretoria.

Light microscopy studies were also conducted, for measurements of the pollen grains (polar axis and equatorial diameter) and thickness of exine layers (ectexine and endexine) in the mesocolpium. Pollen grains were mounted in Fuchsin jelly on glass slides and viewed with an Olympus BX63 OM/FM microscope. This work was conducted at the Microscopy and Microanalysis Unit at the University of the Witwatersrand, Johannesburg.

Morphometric studies of the pollen using a combination of quantitative and qualitative data were carried out, based on selected external and internal pollen wall characters (Appendix: Table A and Table B). External characters that are known to be important were chosen, e.g., shape, number of apertures, sculpturing of margocolpus region. Multivariate analyses were carried out using NTSYS-pc Version 2.2 MN. Cluster analyses (CA) were conducted using the unweighted pair group method (UPGMA) in sequential agglomerative hierarchical nested (SAHN) cluster analysis.

RESULTS

Pollen Morphology

The pollen morphology data obtained from the FIB-SEM investigation of the 12 species of *Justicia* and the six related species of Justiciinae showed that although chambers and elongate, arched cavities or tunnels are present in the walls of the *Justicia* species, wall structure is never restricted to distinct, individual columellae alone. However, free standing columellae were a feature in most species from the related genera (Table 4.1). Light microscopy observations of the general pollen morphology of *Justicia* species are represented in Fig. 4.2. Specific attention was paid to viewing the internal structure of the pollen walls and not only the external sculpturing.

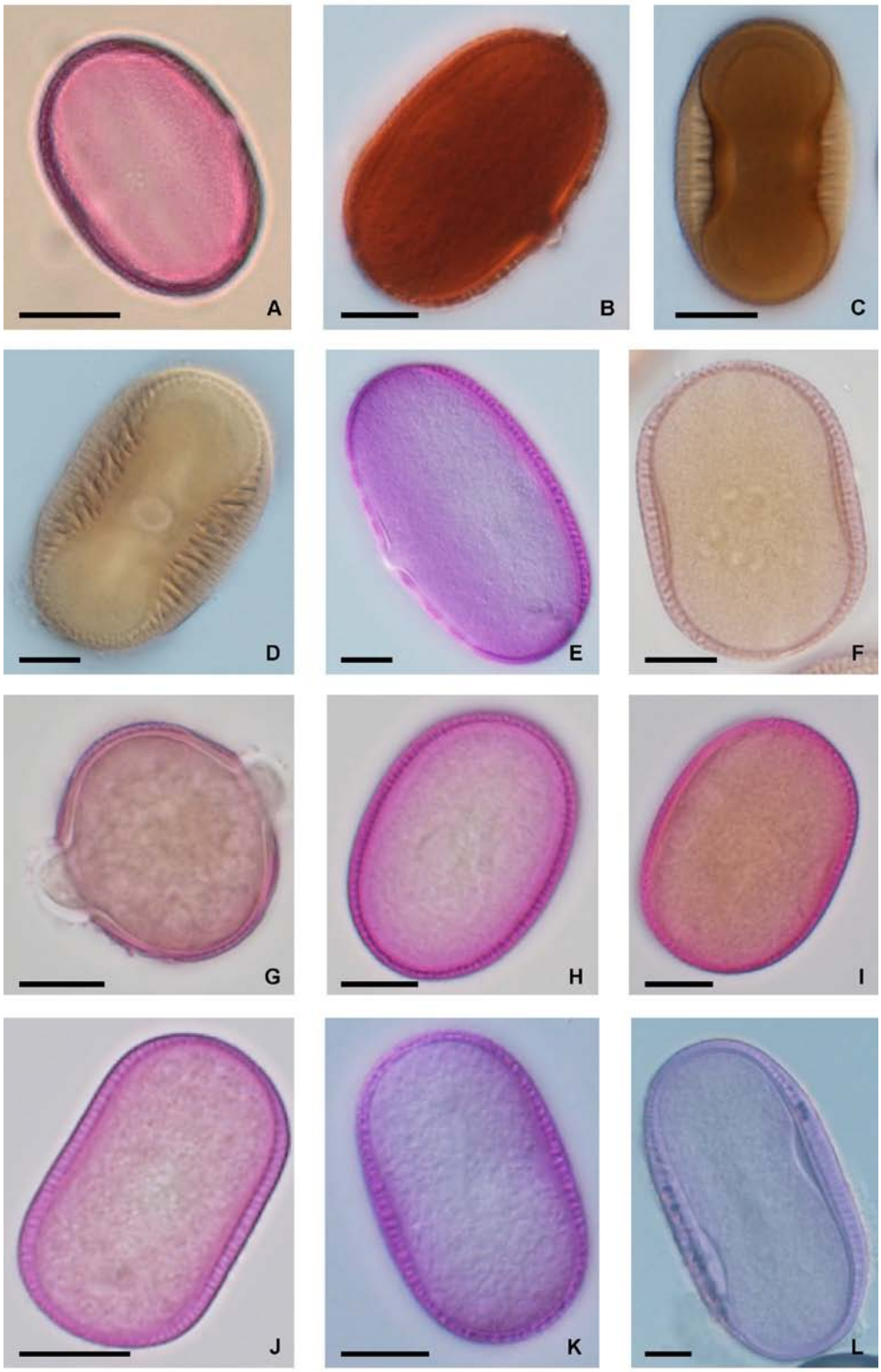

Figure 4.2 Light microscopy images of whole pollen grains of species of *Justicia* in equatorial view. **A** *J. betonica*; **B** *J. campylostemon*; **C** *J. flava*; **D** *J. petiolaris*; **E** *J. brandegeeana*; **F** *J. aconitiflora*; **G** *J. guerkeana*; **H** *J. odora*; **I** *J. capensis*; **J** *J. anagalloides*; **K** *J. protracta*; **L** *J. carnea*
Scale bars = 10 µm

Table 4.1 External and Internal Pollen Characteristics of the selected species

Taxa	Type (Graham 1988)	# apertures	shape P/E	X-section shape	Margocolpus sculpturing	Internal structure of Mesocolpium	Mesocolp. ectexine/ endexine	Meso/ Margo-colp.
J. betonica	1	3	1.41	circular	2 solid bands	chambers	1.25	3.21
J. campylostemon	1	3	1.615	circular	2 solid bands	chambers	1.25	2.68
J. flava	2	3	1.657	triangular	2 rows insulae	cavities/ arched tunnels	4.58	4.547
J. petiolaris	2	3	1.66	triangular	2 rows insulae	cavities/ arched tunnels	5.61	3.843
J. brandegeeana	3	3	1.95	triangular	2 rows insulae	chambers	2.53	3.508
J. aconitiflora	4	2	1.418	flattened	2 solid bands	chambers	4.24	3.01
J. guerkeana	4	2	1.190	circular	2 solid bands	cavities/ arched tunnels	1.13	3.445
J. odora	5	2	1.46	circular	2 rows insulae	chambers	1.85	7.372
J. capensis	5	2	1.395	circular	2 rows insulae	chambers	2.54	7.066
J. anagalloides	5	2	1.609	flattened	2 rows insulae	chambers	3.55	3.784
J. protracta	Int.6 & 7	2	1.641	flattened	4 rows insulae	chambers	3.64	5.828
J. carnea	8	2	2.15	flattened	6 rows verrucae	cavities/ arched tunnels	4.34	14.086
Hypoestes forskaolii	–	3	1.65	circular	2 solid bands	columellae	3.33	3.375
Peristrophe cernua	–	3	2.034	triangular	2 solid bands	columellae	4.12	4.25
Rhinacanthus gracilis var. *latilabiatus*	–	3	1.22	circular	2 solid bands	columellae	3.74	3.988
Dicliptera leistneri	–	3	2.45	triangular	2 solid bands	columellae	5.04	4.01
Mackaya bella	–	3	1.63	triangular	2 solid bands	chambers	2.63	2.996
Metarungia longistrobus	–	2	1.75	flattened	4 rows insulae	chambers	2.47	5.21

Three main categories of pollen were identified in **cross-section** within the species examined: bilaterally-flattened-elliptical; obtuse-triangular and circular or round. The general morphology of the pollen categories is as follows:

Bilaterally-flattened-elliptical (Figure 4.3):

Justicia aconitiflora (Fig. 4.2F; Fig. 4.3A and 4.3B), *J. anagalloides* (Fig. 4.2J; Fig. 4.3C and4. 3D) and *J. protracta* (Fig. 4.2K; Fig. 4.3E and4. 3F) are all bilaterally-flattened-elliptical, more or less prolate and 2-aperturate. *Justicia aconitiflora* and *J. anagalloides* are almost obtusely rectangular in equatorial view (Fig. 4.2F and 4.2J). The margocolpus, situated in the trema area which surrounds the aperture, shows the most external variation among the species. The margocolpus of *J. aconitiflora* is entire, consisting of 2 raised bands, 1 on each side of the aperture (Fig. 4.3A). The

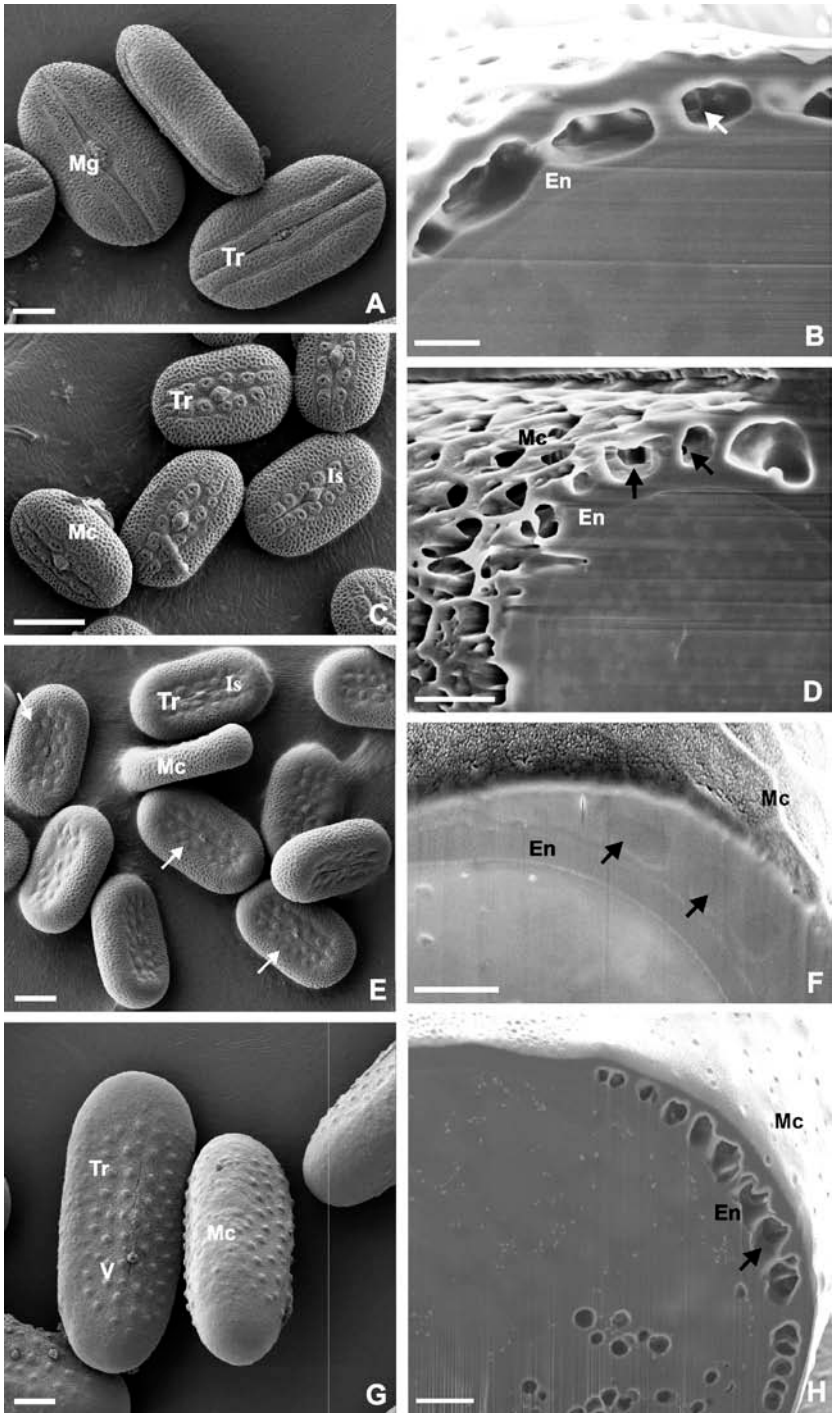

Figure 4.3 FIB-SEM micrographs of *Justicia* species of whole pollen grains showing external morphology and FIB-SEM cross-sections showing 3D detail of the internal pollen wall in the mesocolpial region

A and **B** *J. aconitiflora* (arrow indicates cavity in chamber wall) Scale bars: A=10 µm; B=1 µm

C and **D** *J. anagalloides* (arrow indicates cavity in chamber wall) Scale bars: C =10 µm; D=1 µm

E and **F** *J. protracta* (E-arrow indicates peninsulae; F-arrow indicates chamber behind lateral wall) Scale bars: E =10 µm; F=1 µm

G and **H** *J. carnea* (arrow indicates elongated chamber) Scale bars: G=10 µm; H=2 µm

En-endexine; Is-insulae; Mc-mesocolpium; Mg-margocolpus; Tr-trema region; V-verrucae

margocolpus of *J. anagalloides* is divided into 2 rows of 5 – 6 insulae (or areolae), 1 row on either side of the aperture (Fig. 4.3C), while that of *J. protracta* is divided into 4 rows of 6 – 7 insulae each, 2 rows on each side of the aperture. On the boundary between the trema area and the mesocolpium, a row of peninsulae, often still incorporated in the periphery of the mesocolpium, is visible where the mesocolpium is beginning to break down to form insulae (Fig. 4.3E). All 3 species have a mesocolpium/margocolpus ratio greater than 3 as the mesocolpia are wide in girth and cover most of the surface area of the grain with a relatively narrow trema region. Internally, the trema area only has the endexine layer covered by the tectum. The insulae or solid bands of the margocolpus contribute to the external sculpturing on the tectum. The ratio between the ectexine and endexine in the mesocolpium is greater than 3.5 in all cases, meaning that the ectexine is much wider than the endexine in this region. The lateral regions (or mesocolpia) of the grain have well developed chambers in the ectexine layer. In the mesocolpium, the tectum is made up of muri which enclose pentagonal and/or hexagonal shaped lumina, making up the reticulate pattern or sculpturing covering this area. The muri extend internally (into the ectexine) and form walled chambers in the ectexine. In *J. protracta* the internal layers appear more or less solid, as the section passes through the walls of the chamber, faintly visible behind the solid walls (Fig. 4.3F). It appears as if the grain may be immature and the wall structure and sculpturing is still developing. The chambers are connected to each other through cavities or archways in the supporting walls (Fig. 4.3D). The endexine is a solid layer, much narrower than the ectexine, and lies below the ectexine. The intine is a very fine but distinct layer.

Metarungia longistrobus (Fig. 4.8E and 4.8F) is also 2-aperturate and flattened, obtuse rectangular and has the trema region divided into at least 4 rows of insulae, 2 rows on either side of the aperture (Fig. 4.8E). A row of peninsulae is also located on the periphery of the mesocolpium. Laterally, in the mesocolpia, there is reticulate sculpturing, but the ectexine is fairly thin and the ratio between the ectexine and endexine is less than 2.5. Interconnected chambers are evident in the ectexine, with slanting rays of ectexine material within the chambers (Fig. 4.8F).

The New World species, *J. carnea* (Fig. 4.2L; Fig. 4.3G and 4.3H), is prolate-elliptical in shape, also flattened, 2-aperturate, with a thin colpoid streak and a pore in the middle of each surface. The external sculpturing of this species was very different from the other species studied. Firstly, the trema area is distinctly sculptured with multiple small verrucae in place of insulae (Fig. 4.3G). The internal wall in this area is thin and solid, consisting of endexine covered with the tectum and the verrucae form the external sculpturing on the tectum. Secondly, the mesocolpium of the grain is wide and almost forms a lateral girdle. The relationship between the small verrucae and wide mesocolpium is indicated by the high mesocolpium/margocolpus ratio of 14.086. The mesocolpium is finely and sparsely pitted with no spines, insulae or

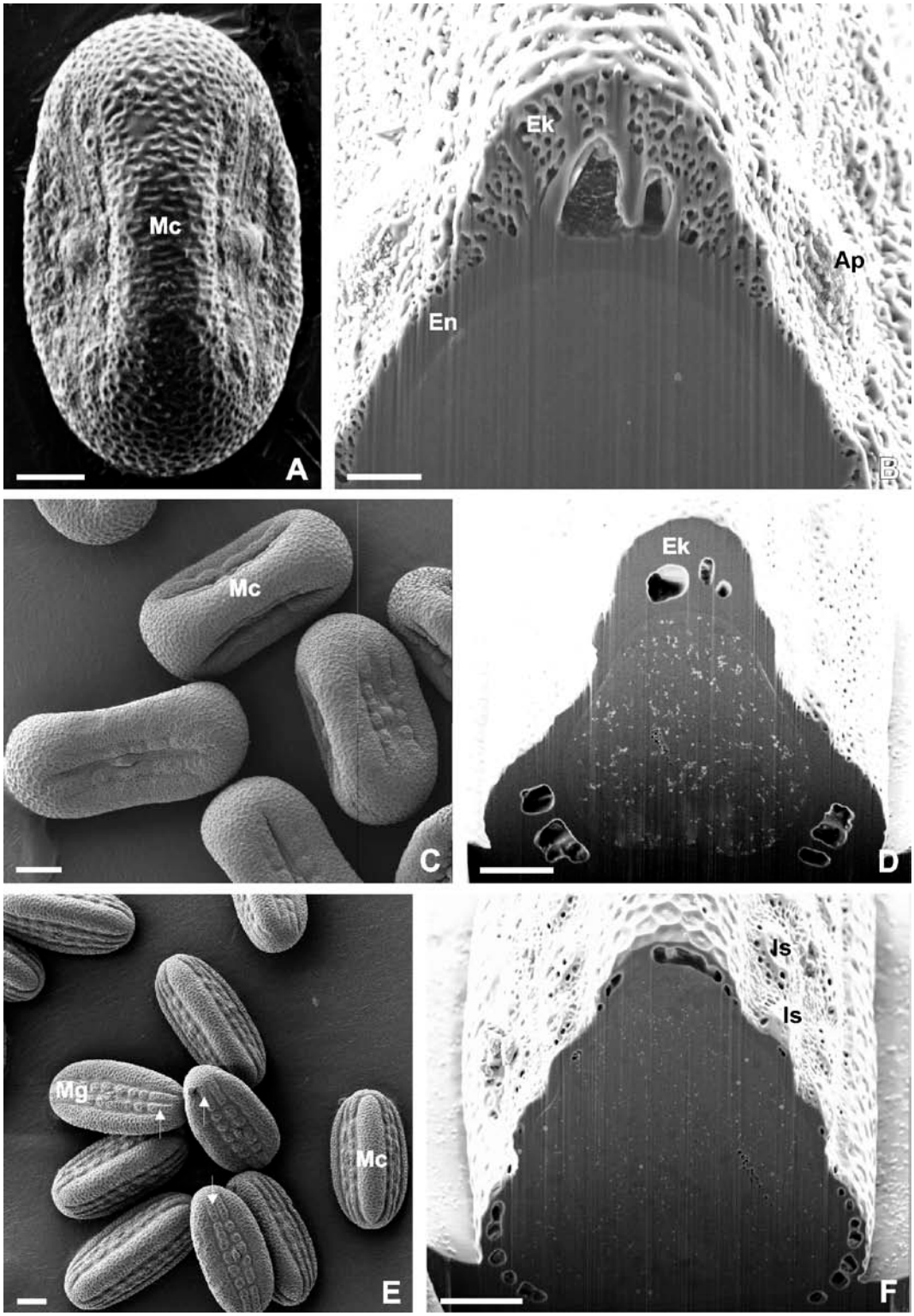

Figure 4.4 FIB-SEM micrographs of *Justicia* species of whole pollen grains showing external morphology and FIB-SEM cross-sections showing 3D detail of the internal pollen wall in the mesocolpium region

A and **B** *J. flava* Scale bars: A=5 µm; B=2 µm

C and **D** *J. petiolaris* Scale bars: C=10 µm; D=5 µm

E and **F** *J. brandegeeana* (arrow indicates solid portion of margocolpus) Scale bars: E=10 µm; F=5 µm

Ap-aperture; Ek-ectexine; En-endexine; Is-insula; Mc-mesocolpium; Mg-margocolpus

reticulate sculpturing. Internally, the wall is thicker, dissected by many elongate cavities or chambers, almost forming tunnels which decrease in size/girth towards the trema area (Fig. 4.2L and 4.3H). The ectexine is much wider than the endexine and the ectexine/endexine ratio is 4.34.

Obtuse-triangular (Figure 4.4):
Justicia flava (Fig. 4.2C; Fig. 4.4A and 4.4B), *J. petiolaris* (Fig. 4.2D; Fig. 4.4C and 4.4D) and *J. brandegeeana* (Fig. 4.2E; Fig. 4.4E and 4.4F) are all obtuse-triangular in cross-section, because they are 3-aperturate and 3-sided, each side more or less prolate in shape. In all cases the margocolpus is divided into 2 rows of 5 – 7 insulae, 1 row on each side of the aperture. In between each trema area there is a marked, well developed mesocolpium, forming the obtuse corners of the triangular shape in cross-section. In all cases the mesocolpium/margocolpus ratio is about 4, as the mesocolpia are noticeably prominent. Externally, the mesocolpium is covered with a reticulate tectum. The majority of the variation occurs internally in the mesocolpium. *Justicia flava* and *J. petiolaris* have an ectexine/endexine ratio >4.5, i.e. both have a very pronounced ectexine and a much thinner endexine. *Justicia brandegeeana* has a much narrower exine, and the ectexine is about 2.5 times thicker than the endexine (Fig. 4.4F). *Justicia flava* and *J. petiolaris* have very distinct internal, elongate chambers, almost tunnel-like, formed by arching rays of ectexine. *Justicia brandegeeana* appears to have hexagonal, internal chambers which may have a central columella (Fig. 4.4F). In *J. flava* the ectexine material has an unusual porous or alveolate structure (Fig. 4.4B), very distinct in this genus as other examples have a solid structure.

Dicliptera leistneri (Fig. 4.7A and 4.7B), *Mackaya bella* (Fig. 4.7C and 4.7D) and *Peristrophe cernua* (Fig. 4.7E and 4.7F) are all 3-aperturate and 3-sided, hence more or less triangular in cross-section. *P. cernua* and *D. leistneri* have thick columellae which extend distally from the foot layer to a central disc in each lumen in the tectum, where they branch into thin rays that reach the surrounding walls of the muri (Fig. 4.7B and 4.7F). The ectexine/endexine ratio is >4, with a much thicker ectexine than endexine. *Mackaya bella*, on the other hand, has internal walled chambers and has a thinner exine with an ectexine/endexine ratio of 2.63 (Fig. 4.7D). In all three species, the margocolpus is constructed of 2 solid bands, 1 band on each side of the aperture and the mesocolpium is much wider than the margocolpus; however the margocolpus is also well developed in *M. bella* (Fig. 4.7D).

Circular/round (Figure 4.5 and Figure 4.6):
Justicia betonica (Fig. 4.2A; Fig. 4.5A and 4.5B), *J. campylostemon* (Fig. 4.2B; Fig. 4.5C and 4.5D), *J. guerkeana* (Fig. 4.2G; Fig. 4.6A and 4.6B), *J. odora* (Fig. 4.2H; Fig. 4.6C and 4.6D) and *J. capensis* (Fig. 4.2I; Fig. 4.6E and 4.6F) are all circular in cross-section. *Justicia betonica* and *J. campylostemon* are 3-aperturate, the margocolpus is a solid band on each side of the aperture, the ectexine/endexine ratio is 1.25 and the

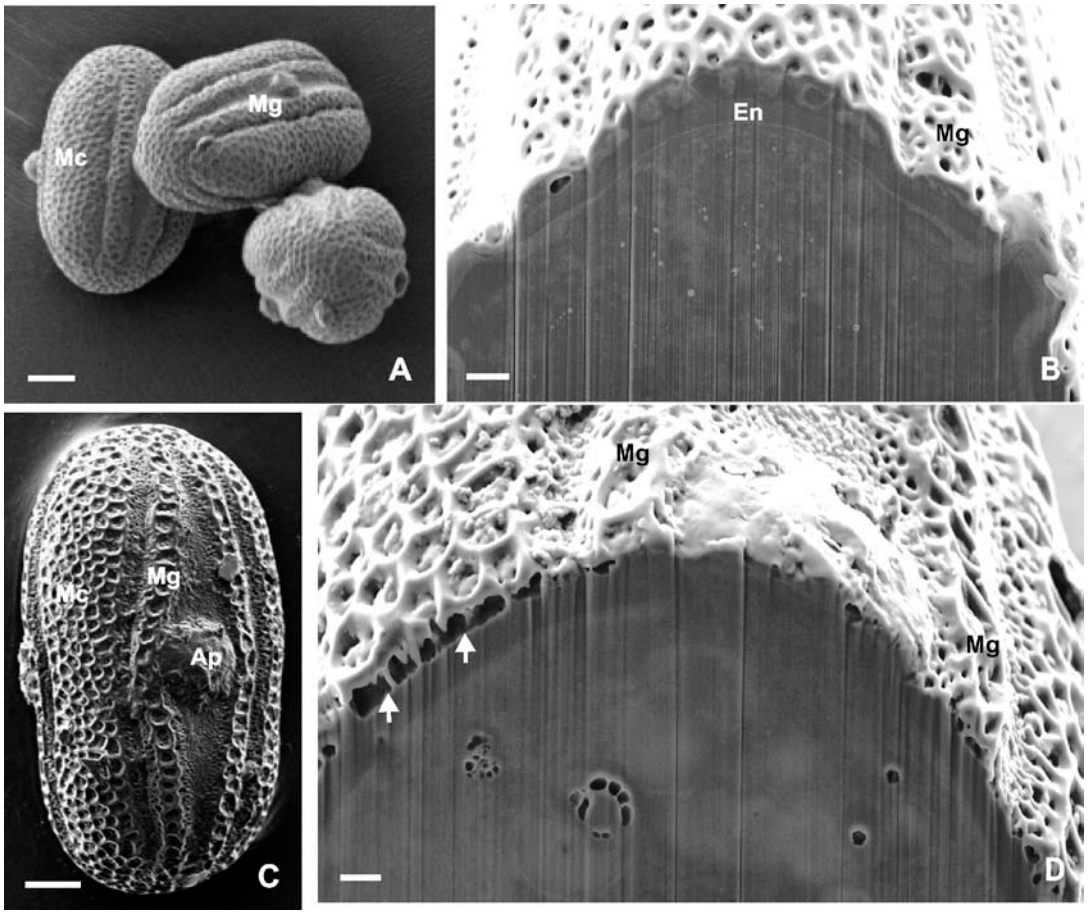

Figure 4.5 FIB-SEM micrographs of *Justicia* species of whole pollen grains showing external morphology and FIB-SEM cross-sections showing 3D detail of the internal pollen wall in the mesocolpium region

A and **B** *J. betonica* Scale bars: A=5 µm; B=1 µm

C and **D** *J. campylostemon* (arrows indicate vestigial columellae) Scale bars: C =5 µm; D=2 µm

Ap-aperture; En-endexine; Mc-mesocolpium; Mg-margocolpus

internal ectexine layer contains hexagonal chambers. Palynologically they are very similar; the only difference is that *J. campylostemon* may have the remnants of a central columella in the chamber (Fig. 4.5D). The remaining three species are all 2-aperturate. Externally, the sculpturing is a well defined reticulum. *Justicia capensis* and *J. odora* are also very palynologically similar. Both have definite trema areas with a margocolpus of 1 row of 5 – 6 insulae on each side of the aperture and internal walled chambers. Both have a very wide mesocolpium covering most of the surface area of the grain and a mesocolpium/margocolpus ratio >7. The ectexine/endexine ratio is larger in *J. capensis*, with the ectexine about 2.5 times thicker than the endexine. *Justicia guerkeana* has a solid, thick margocolpus and a well developed mesocolpium area, with internal chambers that resemble those of the obtuse-triangular category where the chambers are elongate and formed from arches of ectexine. The exine is widest over the mesocolpium region with a pronounced ectexine layer, but the endexine is almost as thick, resulting in an ectexine/endexine ratio of approximately 1.

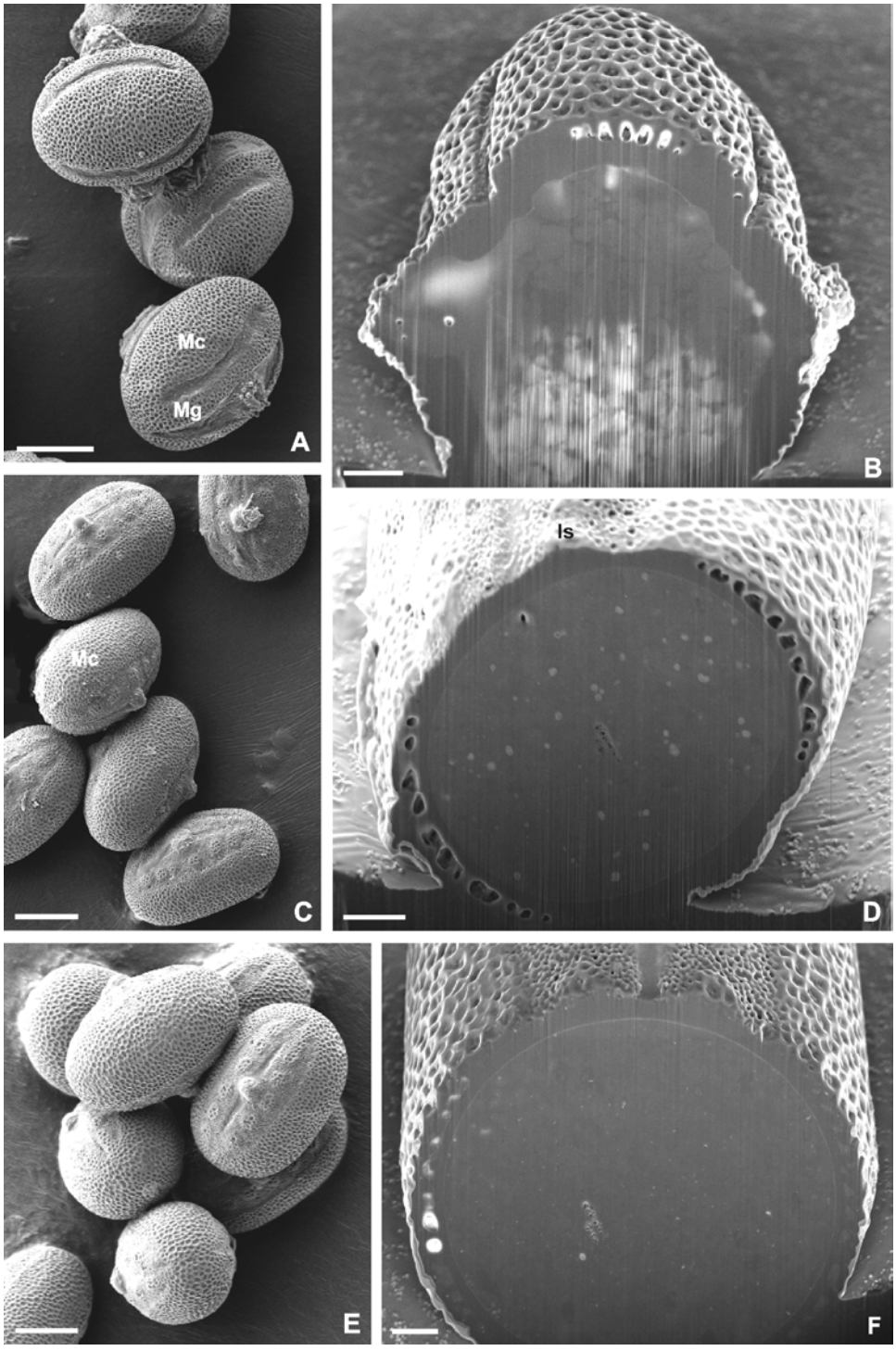

Figure 4.6 FIB-SEM micrographs of *Justicia* species of whole pollen grains showing external morphology and FIB-SEM cross-sections showing 3D detail of the internal pollen wall in the mesocolpium region

A and **B** *J. guerkeana* Scale bars: A=10 µm; B=2 µm

C and **D** *J. odora* Scale bars: C =10 µm; D=2 µm

E and **F** *J. capensis* Scale bars: E =10 µm; F=2 µm

Is- insula Mc-mesocolpium; Mg-margocolpus

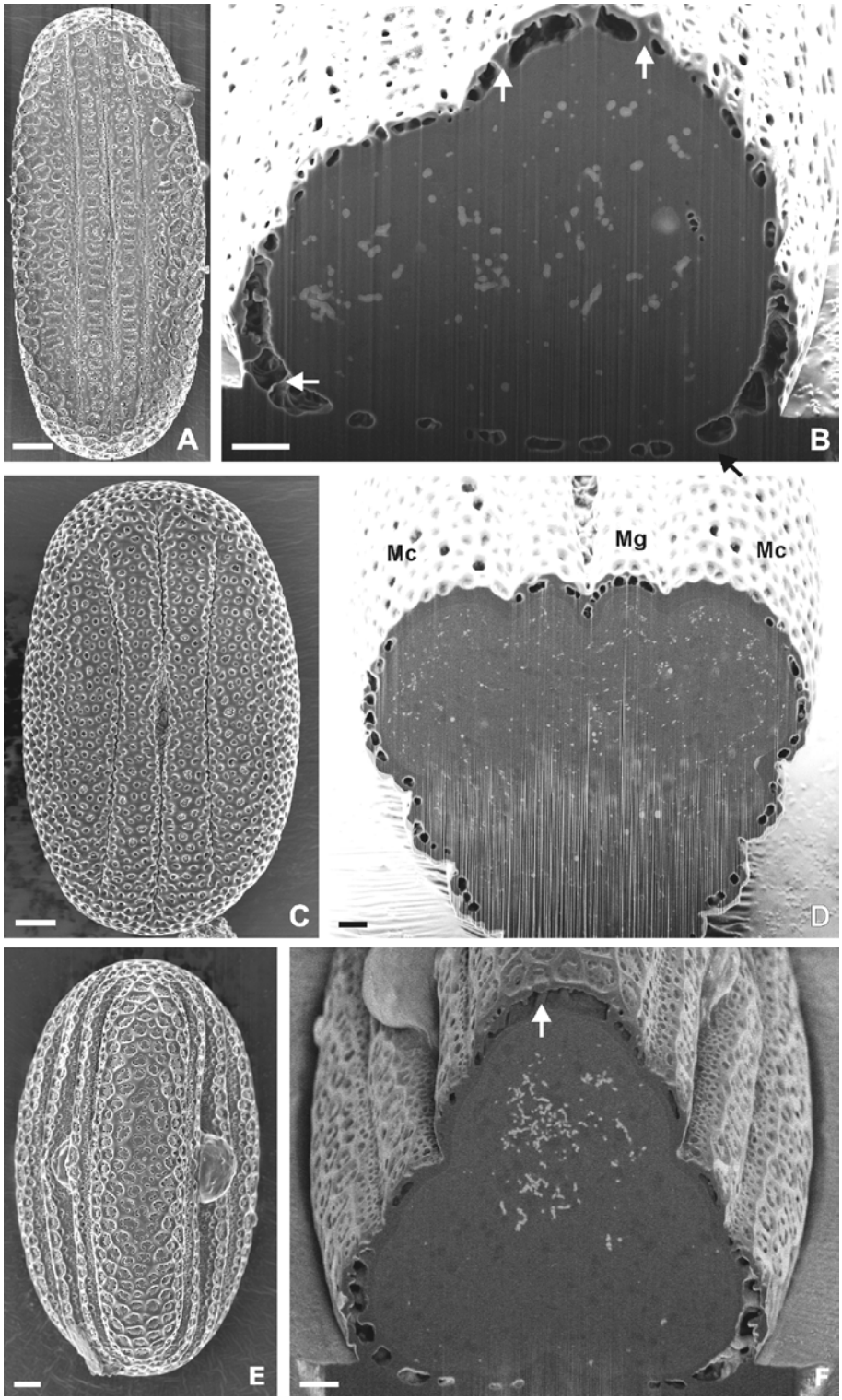

Figure 4.7 FIB-SEM micrographs of species of related genera with whole pollen grains showing external morphology and FIB-SEM cross-sections showing 3D detail of the internal pollen wall in the mesocolpium region (arrows indicate central columellae)

A and **B** *Dicliptera leistneri* Scale bars: A=5 µm; B=2 µm

C and **D** *Mackaya bella* Scale bars: C=5 µm; D=2 µm

E and **F** *Peristrophe cernua* Scale bars: E=3 µm; F=2 µm

Mc-mesocolpium; Mg-margocolpus; arrows-individual columellae

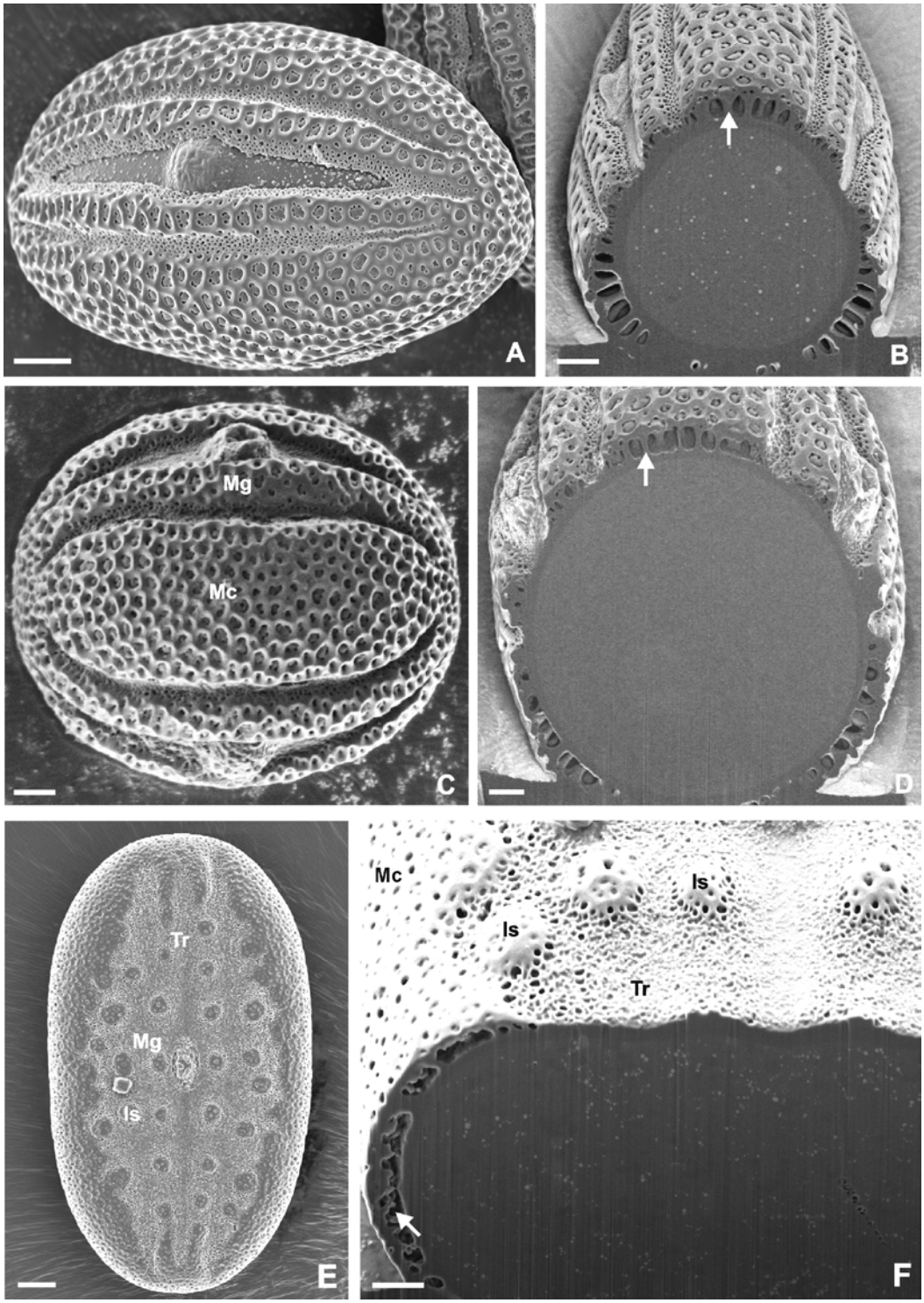

Figure 4.8 FIB-SEM micrographs of species of genera of Justiciinae with whole pollen grains showing external morphology and FIB-SEM cross-sections showing 3D detail of the internal pollen wall in the mesocolpium region

A and **B** *Hypoestes forskaolii* Scale bars: A=3 µm; B=2 µm

C and **D** *Rhinacanthus gracilis* var. *latilabiatus* Scale bars: C=3 µm; D=2 µm

E and **F** *Metarungia longistrobus* Scale bars: E=5 µm; F=2 µm

Is-insula; Mc-mesocolpium; Mg-margocolpus; Tr-trema region

Arrows indicate individual columellae

Hypoestes forskaolii (Fig. 4.8A and 4.8B) and *Rhinacanthus gracilis* var. *latilabiatus* (Fig. 4.8C and 4.8D) are both circular in equatorial cross-section (*H. forskaolii* is slightly narrower towards the poles). There is a solid margocolpus band on each side of the aperture. There are no chambers in the internal structure, instead distinct, individual columellae are found. They extend from the central disc in the lumen to the foot layer (Fig. 4.8B) or from the corners of the lumen to the foot layer (Fig. 4.8D). They both have a wider ectexine with a narrow endexine. The ectexine/endexine ratio is 3.3 in *H. forskaolii* and 3.7 in *R. gracilis* var. *latilabiatus*.

Morphometric Analyses

The first morphometric study used characters that were categorised into predetermined ranges (Appendix: Table 4.A). The mesocolpium/margocolpus ratio data were not included in these analyses. The phenogram obtained from this CA (Fig. 4.9), had a cophenetic correlation of 0.794, and therefore a high reliability. A single tree was obtained from the analysis. Two major groups were recognised. The larger group consists of eight of the 12 *Justicia* species and five of the sister taxa, and the smaller group has four of the *Justicia* species and *Metarungia longistrobus*, which is the only one of the sister group examined which is flattened and has insulae and peninsulae arranged on the trema area.

In the smaller group, (which includes *J. aconitiflora*, *J. protracta*, *J. capensis*, *J. anagalloides* and *M. longistrobus*) the important pollen characteristics are the number of apertures (two), internal structure (chambers) and cross-section shape (all are flattened, except *J. capensis* is circular).

The larger group is divided into three sub-groups (1) *Justicia flava*, *J. petiolaris*, *J. brandegeeana* and *J. carnea*. The common characteristic in this group is the internal structure of elongate cavities/tunnels, and an ectexine/endexine ratio of >4, except *J. brandegeeana* which has chambers and a ratio of 2.5. All are triangular in cross-section, except for *J. carnea* which is flattened. (2) *Hypoestes forskaolii*, *R. gracilis* var. *latilabiatus*, *P. cernua*, *D. leistneri* and *M. bella*. These are the remaining sister taxa. The external pollen characteristics are important here, all have three apertures and solid margocolpus bands. The internal wall is structured with distinct, individual columellae, except for *M. bella*, which has chambers. (3) *Justicia betonica*, *J. campylostemon*, *J. odora* and *J. guerkeana*. These are all circular in cross-section, and have an ectexine/endexine ratio of less than 2.

A second morphometric study using the same characters (Appendix: Table 4.A) was conducted, but the character 'number of apertures' was excluded from the analysis. The phenogram obtained from the CA (Fig. 4.10), had a cophenetic correlation of 0.806, and therefore also a high reliability. Once again, a single tree was obtained from the analysis and two major groups were recognised. The smaller group contains four of the *Justicia*

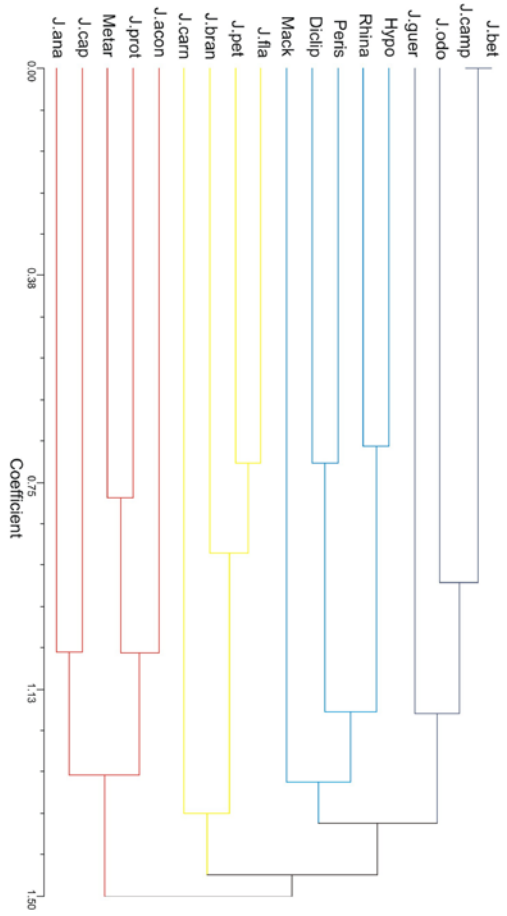

Figure 4.9 UPGMA phenogram showing the similarity of pollen wall morphology for all test species using characters from Table 4.A

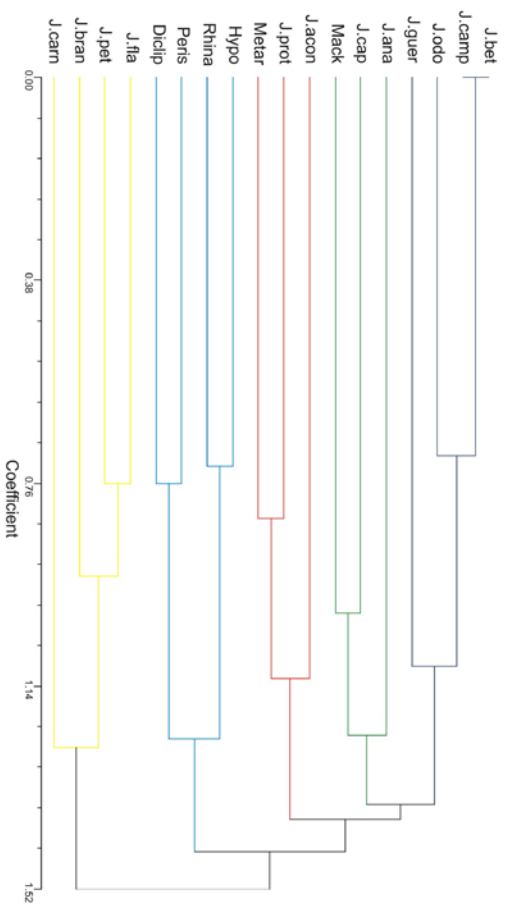

Figure 4.10 UPGMA phenogram showing the similarity of pollen wall morphology for all test species using characters from Table 4.A excluding 'number of apertures'

species and includes the two New World species, *J. brandegeeana* and *J. carnea.* Two of the 3-sided species, with an internal structure of elongate, arched cavities and an ectexine/endexine ratio of >4, *J. flava* and *J. petiolaris* are also included in this group.

The larger group is divided into four sub-groups. (1) *Hypoestes forskaolii, R. gracilis* var. *latilabiatus, P. cernua, D. leistneri*. These are the closely related sister taxa. These taxa all have distinct, individual columellae. (2) *Justicia aconitiflora, J. protracta* and *M. longistrobus* the important characteristics are an internal structure of walled chambers and all are flattened-elliptical in cross-section. *Justicia protracta* and *M. longistrobus* are grouped together and have a combination of insulae and peninsulae. (3) *Justicia anagalloides, J. capensis* and *M. bella* all have internal walled chambers. This is a contradictory placement for *M. bella* as this taxon is only distantly related to *Justicia* (McDade et al., 2000). (4) *Justicia betonica, J. campylostemon, J. odora* and *J. guerkeana*. These are all circular in cross-section, and have an ectexine/endexine ratio of less than 2.

An additional morphometric analysis was conducted using actual character values in place of predetermined ranges (Appendix: Table 4.B), in order to obtain more accurate similarity groupings. The phenogram obtained from the CA using the selected external and internal pollen wall characters (Fig. 4.11), had a cophenetic correlation of 0.859, and therefore a high reliability. A single tree was obtained from the analysis. Two major groups were recognised. The larger group consists of nine of the 12 *Justicia* species and two of the sister taxa, and the smaller group has two of the *Justicia* species and four of the sister taxa. *Justicia carnea*, a New World species which has Type 8 pollen (Graham, 1988) with verrucae instead of insulae, was separated from the groups in the CA.

The smaller group includes the sister taxa, *R. gracilis* var. *latilabiatus*, *H. forskaolii*, *P. cernua*, and *D. leistneri*. All have three apertures and solid margocolpus bands. The internal structure in the mesocolpium consists of distinct, individual columellae. The species in this smaller cluster are grouped according to their shape in cross-section. *R. gracilis* var. *latilabiatus* and *H. forskaolii* are circular in cross-section and *P. cernua* and *D. leistneri* are triangular (Fig. 4.11). The species *J. flava* and *J. petiolaris* are triangular in cross-section and form a group. The common characteristics are 1 row of 5 – 7 insulae on each side of the aperture, an internal structure of elongate, arched cavities/tunnels, and an ectexine/endexine ratio of >4.5, reflecting a very well developed mesocolpium region.

The larger group is divided into two sub-groups and separates *J. guerkeana* from the rest (Fig. 4.11) as it is the only species in this cluster that has an internal structure of arched cavities/tunnels. (1) *Justicia aconitiflora*, *J. anagalloides*, *J. protracta* and *M. longistrobus*. All have 2 apertures, internal chambers and are flattened. In this analysis *J. protracta* and *M. longistrobus* were also grouped together, as in the previous one (Fig. 4.9 and Fig. 4.10) and they share the external characteristic of a combination of insulae and peninsulae in the trema area. (2a) The New World species, *J. brandegeeana*, and *M. bella* are incongruently grouped together (Fig. 4.11). They have a number of characters in common (e.g., internal wall structure of chambers, a relatively narrow exine, triangular in cross-section) but are shown to be only distantly related in molecular studies (McDade et al., 2000) (2b) *Justicia betonica*, *J. campylostemon*, *J. odora* and *J. capensis* are all circular in cross-section, and have a relatively low ectexine/endexine ratio. They all have a similar internal wall structure. *J. odora* and *J. capensis* are grouped together and both have a high mesocolpium/margocolpus ratio (>7) as a result of a very wide mesocolpium region.

A second analysis was conducted where the character ‘number of apertures’ was removed or excluded from the analysis. The phenogram obtained from the CA (Fig. 4.12), had a cophenetic correlation of 0.873, and therefore also a high reliability. Once again, a single tree was obtained from the analysis, two major groups were recognised

and the New World species *J. carnea* was separated from the main clusters. The smaller group contains *J. flava* and *J. petiolaris*, two of the 3-sided species, with an internal structure of elongate, arched cavities and an ectexine/endexine ratio of >4. Also included are the two sister taxa, *P. cernua* and *D. leistneri*, both are also triangular in cross-section. The larger group includes nine of the *Justicia* species and the remaining four sister taxa.

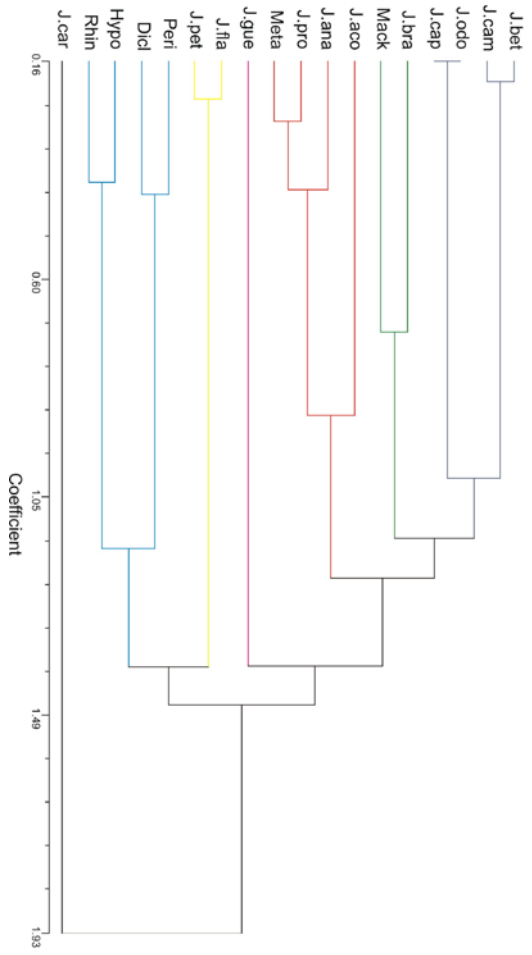

Figure 4.11 UPGMA phenogram showing the similarity of pollen wall morphology for all test species using characters from Table 4.B

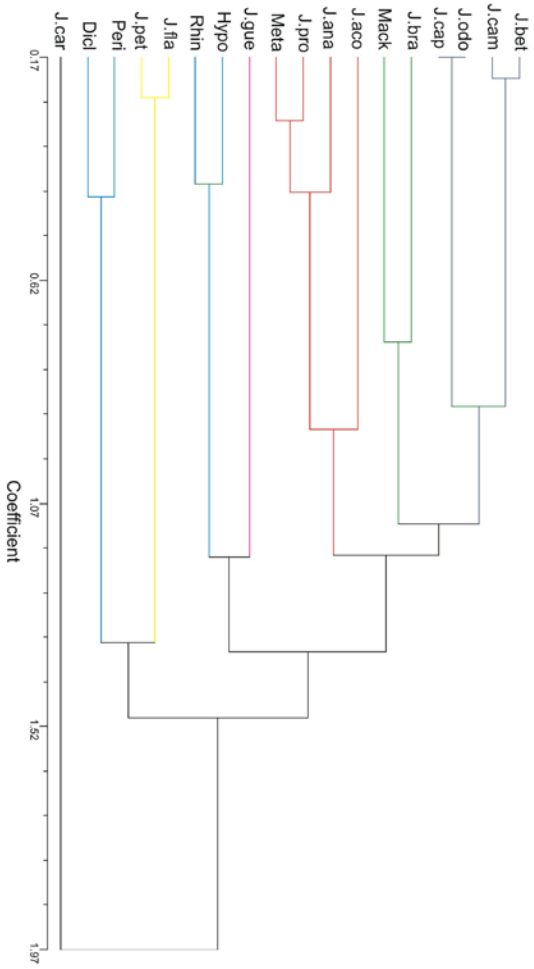

Figure 4.12 UPGMA phenogram showing the similarity of pollen wall morphology for all test species using characters from Table 4.B excluding 'number of apertures'

The larger group is divided into two sub-groups. (1) *Hypoestes forskaolii*, *R. gracilis* var. *latilabiatus* are grouped together with *J. guerkeana*. All are circular in cross-section and have an internal structure that is different from hexagonal walled chambers. (2) All taxa in this cluster have an internal structure of walled chambers. (2a) *Justicia aconitiflora*, *J. anagalloides*, *J. protracta* and *M. longistrobus* the important characteristics are an internal structure of walled chambers and all are bilaterally-flattened-elliptical in cross-section. (2b) *Justicia betonica*, *J. campylostemon*, *J. odora*

and *J. capensis*. These are all circular in cross-section, and have an ectexine/endexine ratio of less than 2 (except *J. capensis* has one of 2.5). The New world species, *J. brandegeeana* and the sister taxon *M. bella* are again incongruently grouped together, because they share a number of similar internal wall characteristics, including a narrow exine and an ectexine/endexine ratio of approximately (just over) 2.5.

A third analysis was conducted where the ectexine/endexine ratio data were excluded (Fig. 4.13) in order to try and separate *M. bella* from the *Justicia* species. The phenogram obtained from the CA had a cophenetic correlation of 0.865, and therefore also a high reliability. Once again, a single tree was obtained from the analysis, *Justicia carnea* was separated from the rest of the clusters and two major groups were recognised (Fig. 4.13).

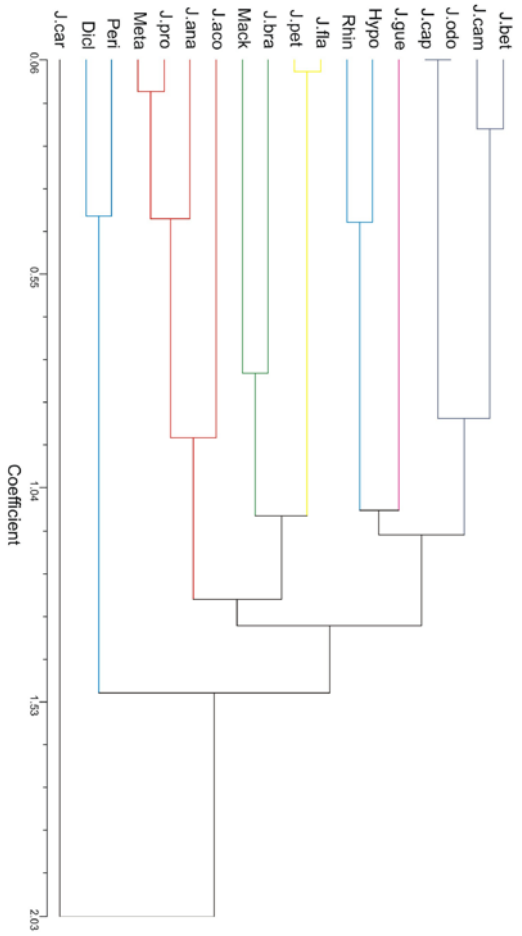

Figure 4.13 UPGMA phenogram showing the similarity of pollen wall morphology for all test species using characters from Table 4.B excluding 'number of apertures' and the 'ectexine/endexine ratio' data

The smaller group only contains two sister species, *P. cernua* and *D. leistneri*. They are closely related species with an internal pollen wall structure of individual columellae. The larger group is basically divided into two sub-groups consisting of a mixture of species and more or less the same arrangements as seen in the previous CAs. The distantly related species *J. brandegeeana* and *M. bella* are again grouped together, so removing the ectexine/endexine ratio character did not resolve this discrepancy.

DISCUSSION

The twelve species of *Justicia* examined include representatives of all of Graham's (1988) Old World sections and eight of her ten pollen types. Pollen types 9 and 10, specifically South American species, are unusual and distinct from other pollen types in *Justicia* (Graham, 1988). Several features were identified and used as characters in morphometric analyses.

The 3D FIB-SEM investigation of the sample species identified three major pollen shapes in cross-section and this was used as one of the characters in the analyses. It has been suggested that the shape of the pollen grain can be influenced by harmomegathy (Wodehouse, 1935) which is a typical infolding of pollen grains to overcome a decrease in cell volume due to dehydration. Heslop-Harrison (1987) defined this as an infolding of the pectocellulosic intine, which is more elastic and permeable to water than the rigid sporopollenin of the exine. The flexibility is thus provided by the colpi and pseudocolpi regions where the ectexine is absent, but a thickened intine and endexine is present (Volkova et al., 2013) and it has been shown that the exine provides resistance to compression as the intine folds inwards, by also stretching gradually, so that the whole pollen wall is involved in stretching and contracting during hydration or dehydration. This would allow the pollen grain to maintain its cross-sectional shape, especially for those with a thickened exine in the mesocolpium region. There may be some shrinkage over the colpoid regions, and indenting may occur. Nevertheless, with examination of SEM micrographs and LM observations, infolding of the intine was not observed in the specimens used for this study, and as a result, there is no reason to suggest that the interpretation of the shapes observed in cross-section were compromised by harmomegathy.

In order to best interpret the results obtained by FIB-SEM sectioning, it is important to ensure that a cut perpendicular to the polar axis is made through the grains and an actual cross-section is achieved (Fig. 4.8B and 4.8D). If grains are lying at an oblique angle to the cutting beam (Fig. 4.7B), or are not lying level on the sample stub (Fig. 4.6D and Fig. 4.7F), it is far more difficult to accurately determine the structure of the walls.

The wall morphology of the *Justicia* species typically exhibited internal walled chambers, most commonly pentagonal, but occasionally elongate, forming arched, tunnel-like structures. For the species of this genus that were sampled, distinct, individual columellae were not observed. (This internal structure of walled chambers, never restricted to individual columellae alone, is characteristic and seen in all *Justicia* species studied with FIB-SEM). An internal structure of distinct, individual columellae was however found in the sister taxa (*H. forskaolii*, *R. gracilis* var. *latilabiatus*, *P. cernua* and *D. leistneri*). These taxa appear to form a cohesive group (Fig. 4.9, Fig. 4.10 and Fig. 4.11), that have been similarly linked in molecular studies (McDade et al., 2000).

Hilsenbeck (1990b) proposed a hypothetical scheme of pollen evolution as observed in the genus *Siphonoglossa* s.l. Oerst. A similar range of the external pollen wall sculpturing described by Hilsenbeck (1990b) was observed in the test species of this study. In Hilsenbeck's progression, the first form is one in which the external sculpturing is uniform over the entire surface of the grain. He sees this progressing to a division of the surface into mesocolpium and margocolpus regions, with the margocolpus comprising solid bands of exine. In this study, *J. aconitiflora* (Fig. 4.3A), *J. betonica* (Fig. 4.5A), *J. campylostemon* (Fig. 4.5C), *J. guerkeana* (Fig. 4.6A), *D. leistneri* (Fig. 4.7A), *M. bella* (Fig. 4.7C), *P. cernua* (Fig. 4.7E), *H. forskaolii* (Fig. 4.8A) and *R. gracilis* var. *latilabiatus* (Fig. 4.8C), fit into this category. The next hypothetical step involves the subdivision of the margocolpus into insulae. This sculpturing was particularly noticeable in *J. brandegeeana* (Fig. 4.4E) and *M. longistrobus* (Fig. 4.8E) where the margocolpus remains a solid band in the polar region but forms individual insulae in the equatorial region. Hilsenbeck's final stage in the progression consists of a further breakdown of the periphery of the mesocolpium into rows of insulae in addition to those insulae of the margocolpus. Two test species, *J. protracta* (Fig. 4.3E) and *M. longistrobus* (Fig. 4.8E), exhibit this arrangement. The results obtained from this study show that when the mesocolpium and margocolpus bands are found together on grains, the surface sculpturing on both those regions for any species remains identical. If Hilsenbeck's progression is accurate, it suggests that the margocolpus is derived from the mesocolpium.

One can trace a progression from a 3-aperturate, 3-sided, externally and internally complex grain, to a bilaterally-flattened-elliptical, 2-aperturate, internally more simple grain (Balkwill and Getliffe Norris, 1988; Hilsenbeck, 1990b). If this reasoning is followed, the assumption could be made that a wall structure of internal chambers in the 3-aperturate, 3-sided species, *J. brandegeeana* may be more 'advanced' than the columellae arrangement seen in the 3-aperturate, 3-sided species *P. cernua* and *D. leistneri*. The 2-aperturate, flattened grains of *J. protracta* and *M. longistrobus* with insulae and peninsulae and internal wall chambers would be more advanced than the 3-aperturate grains of *H. forskaolii* and *R. gracilis* var. *latilabiatus* with an internal wall structure of individual columellae. The 2-aperturate grains where the margocolpus comprises many insulae and the mesocolpium is constructed from a thinner exine with internal chambers, as in *J. capensis* and *J. odora,* would be the most advanced.

Morphometric analyses were conducted to reveal how the pollen wall features would group species and to determine if the internal wall features could assist in clarifying relationships between species. At first, the number of apertures appeared to be a very important external characteristic for grouping species. Although this is a useful character when investigating relationships between species within the same genus, the number of apertures is however not advisable as a character when comparing species of different genera, as it is known that the number of apertures can vary within a single

genus (Baden, 1981). This is why a second cluster analysis was conducted where this character was excluded (to place more emphasis on the similarity between species shown by internal characters). In these analyses the internal wall characteristics played a major role in grouping species of *Justicia* (Fig. 4.10, Fig. 4.11 and Fig. 4.12).

There is much variation in the thickness of the exine and the ratio between the ectexine and endexine in various species. It has previously been suggested that the ratio of ectexine to endexine is phylogenetically important (Walker, 1974). This character did play a role in linking species, but not always validly, e.g., *J. brandegeeana* and *M. bella* (Fig. 4.11, Fig. 4.12 and Fig. 4.13) are only distantly related, but *J. betonica* and *J. campylostemon* (Figs 4.9–4.13) are closely related.

The results appear to be primarily based on cross-section shape and internal structure and show the sister taxa (as determined by McDade et al., 2000) grouped together (Fig. 4.9, Fig. 4.10 and Fig. 4.11). This is especially true in the analyses where the taxa with distinct, individual columellae form a cluster (Fig. 4.10 and Fig. 4.11), agreeing with molecular findings (McDade et al., 2000). The *Justicia* species were split into three or four groups, suggesting polyphyletic origins. In the first analyses, the New World species, *J. brandegeeana* and *J. carnea*, are grouped together (Fig. 4.9 and Fig. 4.10). The tree from the CA where the number of apertures was excluded (Fig. 4.10) more closely fits the molecular findings of McDade et al. (2000); with a definite split between Old and New World species. The sculpturing in *J. carnea* is different from the other species in this study as it does not have reticulate sculpturing. Sculpturing of the trema area external to the tectum consists of verrucae instead of insulae. The results of the morphometric analyses also consistently place *J. carnea* as an outlying group (Fig. 4.11, Fig. 4.12 and Fig. 4.13), consistent with findings from molecular (McDade et al., 2000) and morphological (Graham, 1988) studies.

Justicia aconitiflora was always placed on its own branch adjacent to similar species (Figs 4.9–4.13), perhaps unsurprisingly as there was disagreement as to its inclusion in *Justicia*. It was previously classified as *Duvernoia aconitiflora* and has since been placed in *Justicia* (Scotland and Vollesen, 2000). Although there is disagreement, there is some evidence that *Duvernoia* is reliably distinguished from *Justicia* in southern Africa (Manning and Getliffe Norris, 1985), although Manning has now placed the species in *Justicia* (pers. comm.).

Metarungia longistrobus, with its flattened-elliptical shape in cross-section and the presence of insulae, considered to be characteristic of *Justicia* (Lindau, 1895; Raj, 1961), was positioned separately from the other sister groups to *Justicia* in this study (Figs 4.9–4.13). It was always grouped together with *J. protracta* and with other examples that are flattened-elliptical and have an internal structure composed of walled chambers. The molecular study of McDade et al. (2000) also grouped *Metarungia* with

old world taxa such as *J. aconitiflora* (*Duvernoia*), similar to the arrangement found here (Figs 4.9–4.13).

The findings of McDade et al. (2000), based on phylogenetic analyses of molecular and morphological data, suggest that *Justicia* is not monophyletic. The morphometric analyses using pollen ultrastructure characters identified in this study appear to support this conclusion.

Pollen evolution from the perspective of internal wall structure

Examining the observations from this study, one is able to envisage the internal wall structure of the pollen grains comprising individual wall structural units. For the purposes of this discussion the units are represented by a hypothetical pentagon shape (Fig. 4.14). Several basic unit arrangements are observable:

A A solid-walled chamber containing a central columella, supporting surface sculpturing of a central plug capping the columella, producing fine rays of ectexine which extend out to meet the corners of the lumina and join the muri. This pattern of surface sculpturing makes up the reticulate sculpturing of the tectum and remains present in all of the basic unit types. *Justicia anagalloides* and *J. brandegeeana* fall into this category. *Justicia betonica, J. capensis, J. odora* and the related species *Metarungia longistrobus* are intermediate between this form and the next one.

B A solid-walled chamber, lacking a central columella, but still having the surface sculpturing – *J. aconitiflora, J. protracta, J. petiolaris, J. guerkeana, J. carnea* and also the sister species *Mackaya bella* have this arrangement. *Justicia flava* may also fit in here with the added breakdown of wall material forming porate structured walls. Consequently *J. flava* could also be seen to be intermediate between this type and type D.

C A chamber with a central columella, but with a loss of some wall material forming connecting cavities between chambers, or if walls are absent forming open chambers with supporting corner columns. This form is seen in *J. campylostemon* and possibly *Rhinacanthus gracilis* var. *latilabiatus*.

D A chamber where the central columella and the surrounding walls are absent, but supporting corner columns remain, forming an open chamber. *Justicia campylostemon* could possibly have this form or an intermediate form between C and D.

E A central columella remains supporting the surface sculpturing, forming an open cavity – *Hypoestes forskaolii, Dicliptera leistneri, Peristrophe cernua* show this pattern and possibly *R. gracilis* var. *latilabiatus*, although this species may also have supporting corners.

Hilsenbeck's (1990b) hypothetical scheme of pollen evolution – discussed earlier – has, as its most advanced stage, a 2-porate grain with the periphery of the mesocolpium broken into rows of insulae, in addition to those found in the margocolpus. The structural wall unit which is found in species with Hilsenbeck's most advanced stage is

type A or B. Using the structural wall units described above, it is easy to intuitively imagine a progression of internal pollen wall evolution beginning with type A and continuing by progressive loss of material to types D and E via types B and C (Fig. 4.14). This progression is obviously the reverse of that described by Hilsenbeck (1990b) using external sculpturing. If the progression suggested by the structural wall units is followed, the initial state would have a relatively unsculptured tectum; verrucae on the surface would fuse to form insulae which have an internal structure identical to the mesocolpium. These would then merge to build up the margocolpus and mesocolpium regions.

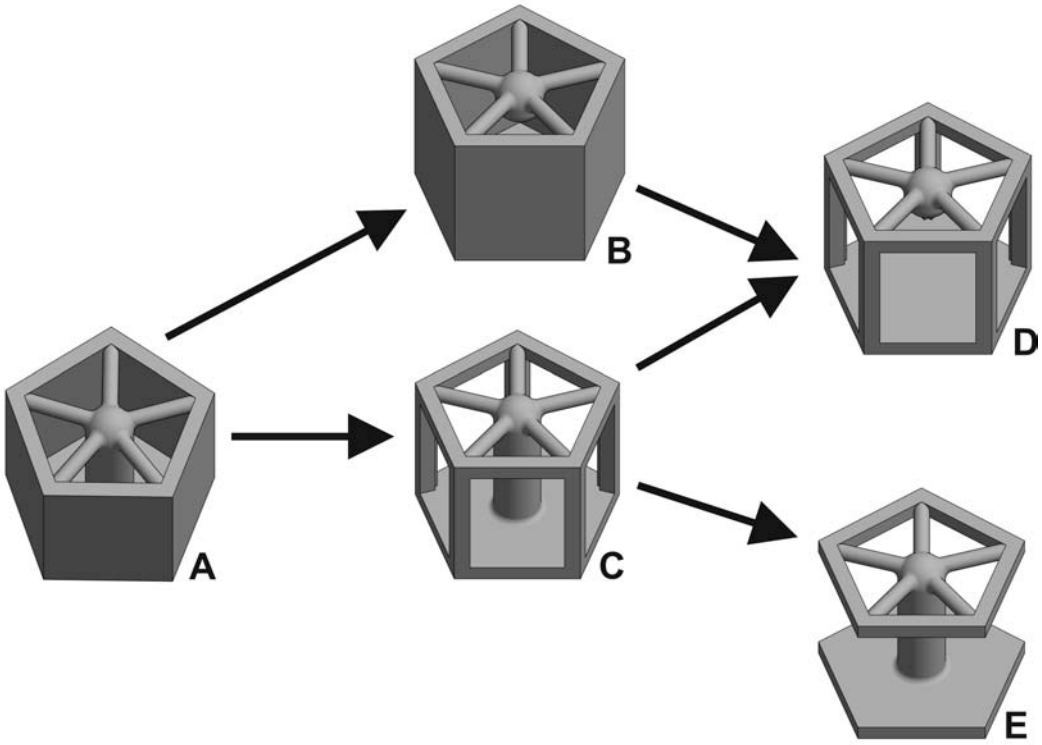

Figure 4.14 Schematic diagrams of pollen wall structural units showing a hypothetical progression of internal pollen wall structural evolution

Whilst external morphology is useful for classifying genera, the internal structure appears to be useful for determining relationships between species within genera or groups. In this way, if one is able to identify synapomorphic characters, it may be possible to identify closely related species. The results show distinct differences and variation in the internal pollen structures and features, which indicate that data obtained from the FIB-SEM technique, should be useful for taxonomic purposes and for showing relationships between species.

It is important to note that the sample size of twelve species is too small to make any conclusive taxonomic or phylogenetic findings, but this study has identified distinct,

internal structures within selected *Justicia* species which suggests that internal pollen wall features as revealed by the FIB-SEM could be useful from a taxonomic and phylogenetic perspective.

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APPENDIX

Table 4.A

Taxa		Shape P/E					Margocolpus			X-section Shape				Internal Structure			Ekt/Endexine Ratio		
	Type	2-aperturate	3-aperturate	1.1-1.5	1.6-2	>2	2-raised bands	2-rows insulae	>2-rows insulae	Round	Triangular	Flattened	Columellae	Chambers	Tunnels	1.1-2.0	2.1-3.0	3.1-4.0	> 4.1
J. betonica	1		x	x			x			x				x		x			
J. campylostemon	1		x		x		x			x				x		x			
J. flava	2		x		x			x			x				x				x
J. petiolaris	2		x		x			x			x				x				x
J. brandegeeana	3		x		x			x			x			x			x			
J. aconitiflora	4	x		x			x					x		x					x
J. guerkeana	4	x		x			x			x					x	x			
J. odora	5	x		x				x		x				x		x			
J. capensis	5	x		x				x		x				x			x		
J. anagalloides	5	x			x			x				x		x				x	
J. protracta	6/7	x			x				x			x		x				x	
J. carnea	8	x				x			x			x			x					x
Hypoestes	0		x		x		x			x			x					x	
Peristrophe	0		x		x		x				x		x						x
Rhinacanthus	0		x	x			x			x			x					x	
Dicliptera	0		x			x	x				x		x						x
Mackaya	0		x		x		x				x			x			x		
Metarungia	0	x			x				x			x		x			x		

Table 4.B

Taxa	Type	# apertures	Shape P/E	Raised bands	# rows insulae	Mesoc/ Margoc	X-section Shape Round	Triangular	Flattened	Mesocolpus Columellae	Int Structure Chambers	Tunnels	Mesocolpus Ektex/Endex
J. betonica	1	3	1.41	2	0	3.21	x				x		1.25
J. campylostemon	1	3	1.615	2	0	2.68	x				x		1.25
J. flava	2	3	1.657	0	2	4.547		x				x	4.58
J. petiolaris	2	3	1.66	0	2	3.843		x				x	5.61
J. brandegeeana	3	3	1.95	0	2	3.508		x			x		2.53
J. aconitiflora	4	2	1.418	2	0	3.01			x		x		4.24
J. guerkeana	4	2	1.19	2	0	3.445	x					x	1.13
J. odora	5	2	1.46	0	2	7.372	x				x		1.85
J. capensis	5	2	1.395	0	2	7.066	x				x		2.54
J. anagalloides	5	2	1.609	0	2	3.784			x		x		3.55
J. protracta	6 or 7	2	1.641	0	4	5.828			x		x		3.64
J. carnea	8	2	2.15	0	6	14.086			x			x	4.34
Hypoestes	0	3	1.65	2	0	3.375	x			x			3.33
Peristrophe	0	3	2.034	2	0	4.25		x		x			4.12
Rhinacanthus	0	3	1.22	2	0	3.988	x			x			3.74
Dicliptera	0	3	2.45	2	0	4.01		x		x			5.04
Mackaya	0	3	1.63	2	0	3.14		x			x		2.63
Metarungia	0	2	1.75	0	4	5.21			x		x		2.47

CHAPTER FIVE – DEVELOPMENT OF THE POLLEN WALL (EXINE) IN *BARLERIA OBTUSA* NEES (ACANTHACEAE)

Abstract
Anthers containing pollen grains of *Barleria obtusa* at various stages of development were examined to investigate pollen wall ontogeny, specifically with regard to the deposition of the exine, including the development of the reticulate sculpturing and the tapetum. Whole anthers containing pollen grains were viewed with a light microscope, firstly, to identify stages of pollen grain development and secondly, to assess the condition of the tapetum. Developing pollen grains were sectioned with the FIB-SEM in order to determine the suitability of this method to an investigation of this nature. The internal pollen wall development and in particular the development of the external elaborate, reticulate sculpturing was examined. The exine was found to be deposited on the immature pollen grains during the tetrad phase of development with the sculpturing - although somewhat condensed - already apparent at this early stage. The subsequent stages of development show the sculpturing advancing towards its final expanded structure as the grain increases in size. The tapetum was unusual in that it not only lined the anther locule but also surrounded each tetrad group without invaginating the tetrad arrangement, thus defining it as a secretory type.

INTRODUCTION

The wall of the pollen grain functions to protect the male gametophyte as it is transferred from the parent anther to the stigma of another flower. It is well adapted to perform this important function, being constructed of an extremely strong and chemically resistant substance, sporopollenin (Walker and Doyle, 1975). The wall is formed around a haploid cell, but includes material derived from the tapetum, a diploid cell layer lining the anther. Walls are often distinctively sculptured and patterned prompting palynologists to question how these sometimes intricately sculptured pollen walls are produced and copied so precisely in such large numbers in each anther of every species. This distinctive sculpturing is usually specific at the generic level and thus has formed the basis for pollen taxonomy and consequently palynology.

The role of the tapetum in pollen development has also been a subject of investigation (Pacini et al., 1985; Pacini, 1997). The major functions of the tapetum include:

- The supply of metabolic substances required for growth and development, essentially a nutritive function. Developing pollen grains grow to several times their original size whilst surrounded by the tapetum. This suggests a trophic role for the tapetum as all the material for developing pollen must pass through, or be metabolised by the tapetum.
- The provision of sporopollenin precursors for the formation of the exine. The relationship between the tapetum and exine development suggests that sporopollenin

precursors are synthesised by the tapetum (Zavada, 1984; Heslop-Harrison, 1968).

- The deposition of pollenkitt (an adhesive material) and tryphine (which protects pollen against water loss), on pollen grain surfaces. The tapetum degenerates as the pollen approaches maturity and leaves these substances as deposits on the grains. In addition to being adhesive, pollenkitt ensures that proteins which play a role in pollen-stigma recognition are maintained inside the exine cavities (Pacini and Hesse, 2005).

The type of tapetum is also considered to be taxonomically important (Pacini, 1997; Furness and Rudall, 1998). There are two main types of tapetum: (1) A multinucleate, plasmodium or amoeboid type tapetum formed from the fusion of the tapetal protoplasts, which completely encloses the individual microspores by intruding between them as they develop (Fernando and Cass, 1994); (2) A secretory tapetum maintains its parietal position surrounding the anther locule housing the tetrads (Murgia et al., 1991). Pacini (1997) regarded the secretory type as more primitive, as it is more characteristic of primitive Dicotyledons. Both types of tapetum are found in Monocotyledons. A third type of tapetum has been described in *Canna* L, an invasive type where there is a breakdown of cell walls and the tapetal protoplasts invade the locule, but do not fuse to form a plasmodium (Tiwari and Gunning, 1986).

The basic process of pollen wall development was first clarified through transmission electron microscope (TEM) investigations (e.g., Heslop-Harrison 1968; Owen and Makaroff 1995) and twelve stages of pollen wall development from premeiosis to anthesis were identified from these studies (Owen and Makaroff, 1995; Blackmore et al., 2007):

(1) Premeiosis I and (2) Premeiosis II - microsporocyte walls are thickened with callose, and protoplasmic strands develop between the cells forming a 'syncytium' in the anther locule (Heslop-Harrison 1968). This is thought to enable the synchronisation of nuclear divisions between cells.

(3) Meiosis of microspore nuclei resulting in four haploid nuclei in each microspore. At this stage organizational symmetry or polarity is established. With the onset of meiosis microspore mother cells (MMC) are invested in thick callose walls as additional callose is deposited around pollen cells.

(4) Cytokinesis occurs to produce four microspores, arranged in a tetrad. Exine development takes place, first by the deposition of the primexine forming the exine template, and then by the accumulation of sporopollenin.

(5)Tetrad phase - microspores are held together by the surrounding callose walls and the position of the apertures is determined.

(6) Released Microspores I and (7) II- the callose walls degenerate freeing individual microspores in the locule. Differentiation of the exine and incorporation of sporopollenin from haploid microspores and tapetum takes place.

(8) to (12) Development of the male gametophyte.

A new technique was developed by Blackmore and Barnes (1985, 1986 and 1987), utilizing freeze fracturing and cytoplasmic maceration of pollen grains. This afforded internal, 3D SEM views of developing pollen grains, enhancing understanding of the spatial relationships between the pollen wall and the cellular organelles involved in pollen wall ontogeny. Takahashi (1995) used this method to investigate pollen development in *Lilium longiflorum*, and found that the plasma membrane facilitates the formation of the exine pattern.

Barleria L. is a pantropical genus with approximately 300 species worldwide. It is mainly an Old World genus with the greatest centre of diversity in tropical east Africa, about 70 species in southern Africa and a single species in the tropical New World (Balkwill and Balkwill, 1998). It was named by Carl Linnaeus in 1753 to honour Jaques Barrellier (1606–1673) a Dominion monk, physician, botanist and plant collector (Gledhill, 2008). This genus was chosen as a candidate for investigating pollen wall development, as the pollen grains are large and exhibit complex, deep, elaborate, reticulate sculpturing. *Barleria obtusa* Nees, from Section *Barleria*, common name bush violet, was chosen as a test species. It is a multi-stemmed shrub, widely distributed in the subtropical, summer rainfall regions of South Africa and exhibits great horticultural potential. Clusters of flowers are borne in cymes at the end of branches. The flowers are a vivid violet colour and each has a style and two fertile stamens extending from the 5-lobed corolla (Fig. 5.1). The pollen grains are large (50–90 µm across), globose, 3-lobed and 3-aperturate.

The aim of this study was to prepare pollen grains at various stages of development - from pollen mother cells to fully mature grains - for examination and sectioning with the FIB-SEM, in order to observe pollen wall ontogeny.

MATERIALS AND METHODS

Barleria obtusa was selected as a test species due to the elaborate pollen wall structure, conspicuous, exserted, extrorse anthers and cymal arrangement of buds, making it easy to identify different stages of pollen development in a single inflorescence. A voucher specimen of was collected (J96503) and deposited in the C.E. Moss Herbarium (J), at the University of the Witwatersrand, Johannesburg. Inflorescences of the test species, containing flower buds at various stages of maturity, were collected in the field. A series of eight buds was removed from the compound cyme, from the youngest (outermost) to open mature flowers (innermost) (Fig. 5.1), representing a range of developmental stages spanning approximately two weeks (14–16 days). Bud 1 - housing very immature anthers with no visible pollen to Bud 8 - an open flower housing ripe anthers with mature released pollen, were selected. The buds were sliced open with a needle and the two anthers were excised from each bud. They were stored in herbarium envelopes in silica gel crystals.



Figure 5.1 Inflorescence of *B. obtusa* showing the arrangement of buds in the cyme and an open flower with two protruding anthers Scale bar =1 cm

The anthers were split open under a dissecting microscope and the pollen grains were removed with insect pins and placed on a SEM stub. The stubs were coated with 5 nm of gold/palladium alloy to stabilise the grains. The pollen sample was viewed with the SEM in order to identify an individual pollen grain of average size and external morphology, typical of the sample of grains at that stage of development. Suitable pollen grains from each stage of development were selected, photographed, orientated for sectioning with the FIB, and cross-sectioned once at a selected site with the FEI Nova NanoLab FIB-SEM at the Microscopy and Microanalysis Unit (MMU) at the University of the Witwatersrand, Johannesburg. FIB was operated at 30 kV. The sectioning site was determined by the size and shape of the grain. For a detailed discussion of the method refer to Chapter Two.

Whole anthers from the developing buds were also prepared for examination with the light microscope as a basis or foundation for comparison with FIB-SEM observations. The anthers with the remaining pollen grains were placed on glass slides, with Fuchsin jelly and cover slips, compressing the material. These slides were examined with an Olympus BX 63 OM/FM at the MMU, University of the Witwatersrand, Johannesburg. These samples afforded a light microscope comparison to the view observed with the FIB-SEM and were also used to assess the condition of the tapetum.

RESULTS

LM observations

The tissue squashes of anthers revealed various stages of pollen wall development which were distinguishable by the arrangement of microspores or developing pollen grains and the condition of the tapetum.

Bud 1 (youngest): primordial anthers containing meiotic and post-meiotic microspore mother cells (MMC) or pollen mother cells (Fig. 5.2A). The pollen mother cells are separated by walls irregularly thickened with callose. Tapetal cells appear to have dense cytoplasm and surround the MMCs. The tapetum is parietal. This corresponds to stage 3 of Owen and Makaroff (1995), ‘meiosis of microspore nuclei’.

Bud 2: Pollen grains are arranged in tetrads (Figs 5.2B–5.2E). The tapetum is parietal, surrounding tetrads (Fig. 5.2C). Intercellular air spaces or thick callose walls separate pollen grains (Fig. 5.2D). Cytoplasmic connections are observable between tapetal cells and developing pollen cells (Fig. 5.2B and Fig. 5.2E). These seem to persist longer than reported in other studies (e.g., Blackmore and Barnes, 1985; Takahashi and Skvarla, 1991; Owen and Makaroff, 1995). Although they may be confused with exine sculpturing, the cytoplasmic strands observed here are too long to be wall material. A thin layer of primexine – the earliest stage of pollen wall deposition – is observable (Fig. 5.2D and Fig. 5.2E). This corresponds to stage 5 of Owen and Makaroff (1995), the ‘tetrad phase’.

Bud 3: Individual pollen grains are found, and the primexine is thickened (Fig. 5.2F and Fig. 5.4A). The tapetum is degenerating or senescing (Fig. 5.2F). Future sites of apertures can be identified by thinner areas of exine (Fig. 5.4A). Exine sculpturing is advanced.

Bud 4: The pollen grains continue to develop and the tapetum breaks down further. Some of the lytic products of the senescing tapetum may be utilised by the developing pollen grains. (Fig. 5.2G). These buds correspond to stages 6 and 7 of Owen and Makaroff (1995), ‘release of microspores’.

Bud 5: The anther locules are still intact (Fig. 5.3A). Pollen grains continue to develop and enlarge. The exine is stretched over the grain appearing thinner (Fig. 5.4B and Fig. 5.4C).

Bud 6: The developing grains pack the anther locule, arranged in parallel rows (Fig. 5.3B).

Bud 7: The tapetum is completely broken down, forming a saturating film of pollenkitt over the surface of the mature pollen grains (Fig. 5.3C).

Bud 8: The anther ruptures longitudinally at the stomium (an indentation between the locules of the theca) and individual pollen grains are released (Fig. 5.3D).

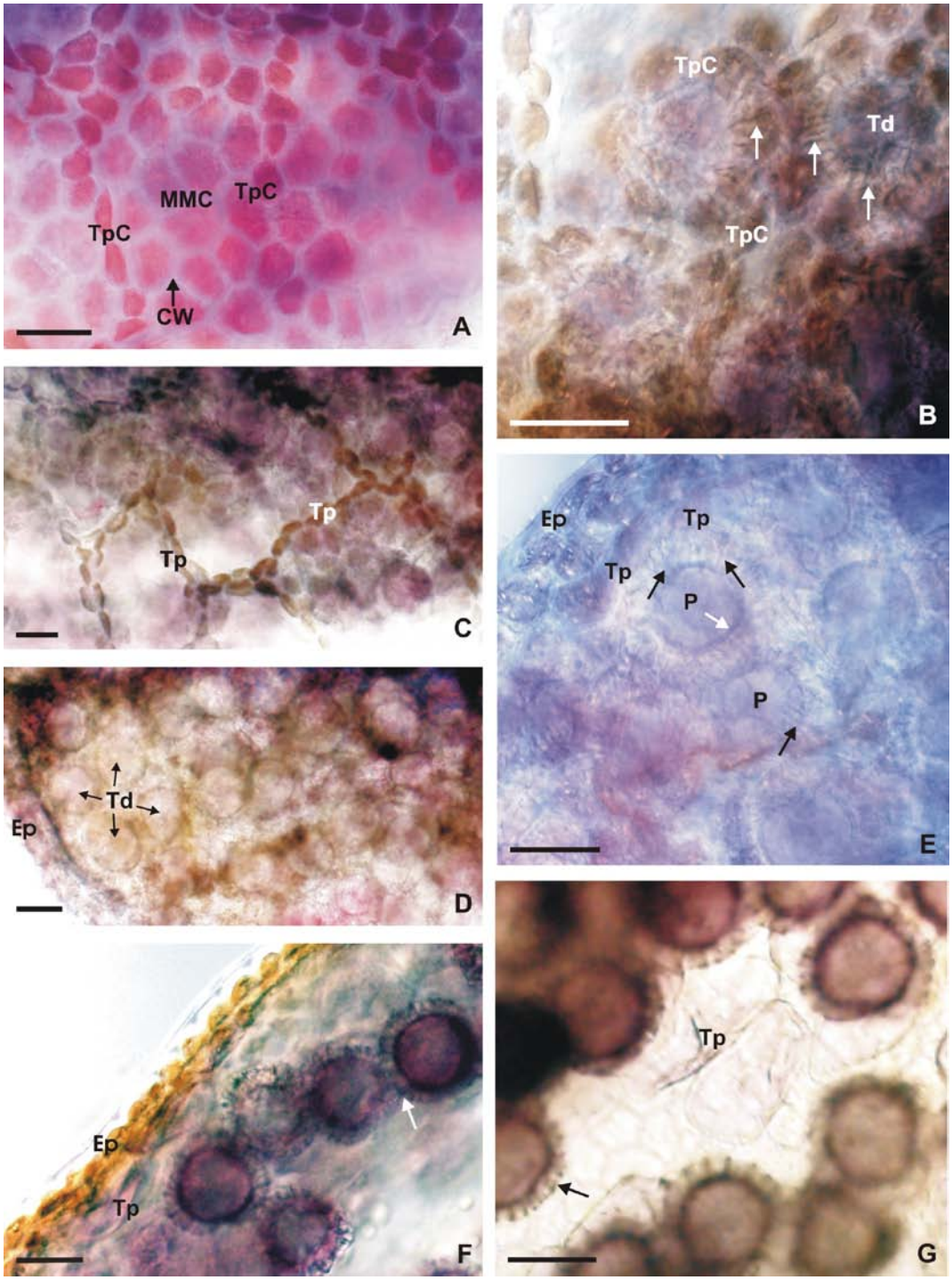

Figure 5.2 LM examination of immature anthers ***Bud 1*** (youngest): **A** megaspore mother cells (MMC) are enclosed in thick callose walls (CW) and surrounded by tapetal cells (TpC) ***Bud 2***: **B** tapetal cells are thickened, cytoplasm condensed (TpC) enclose tetrads (Td) and cytoplasmic channels (arrows) are seen between tapetum and developing pollen grains **C** arrangement of tapetum (Tp) **D** arrangement of tetrads (Td) within anther epidermis (Ep) **E** developing pollen grains (P) surrounded by tapetum (Tp), within anther epidermis (Ep). Few cytoplasmic connections seen (black arrows) and primexine deposition begins (white arrow) ***Bud 3***: **F** tapetum (Tp) starts to degenerate, exine well developed (white arrow) ***Bud 4***: **G** tapetum (Tp) continues to senesce, pollen grains packed together, exine sculpturing well developed (arrow) Scale bars = 50 µm

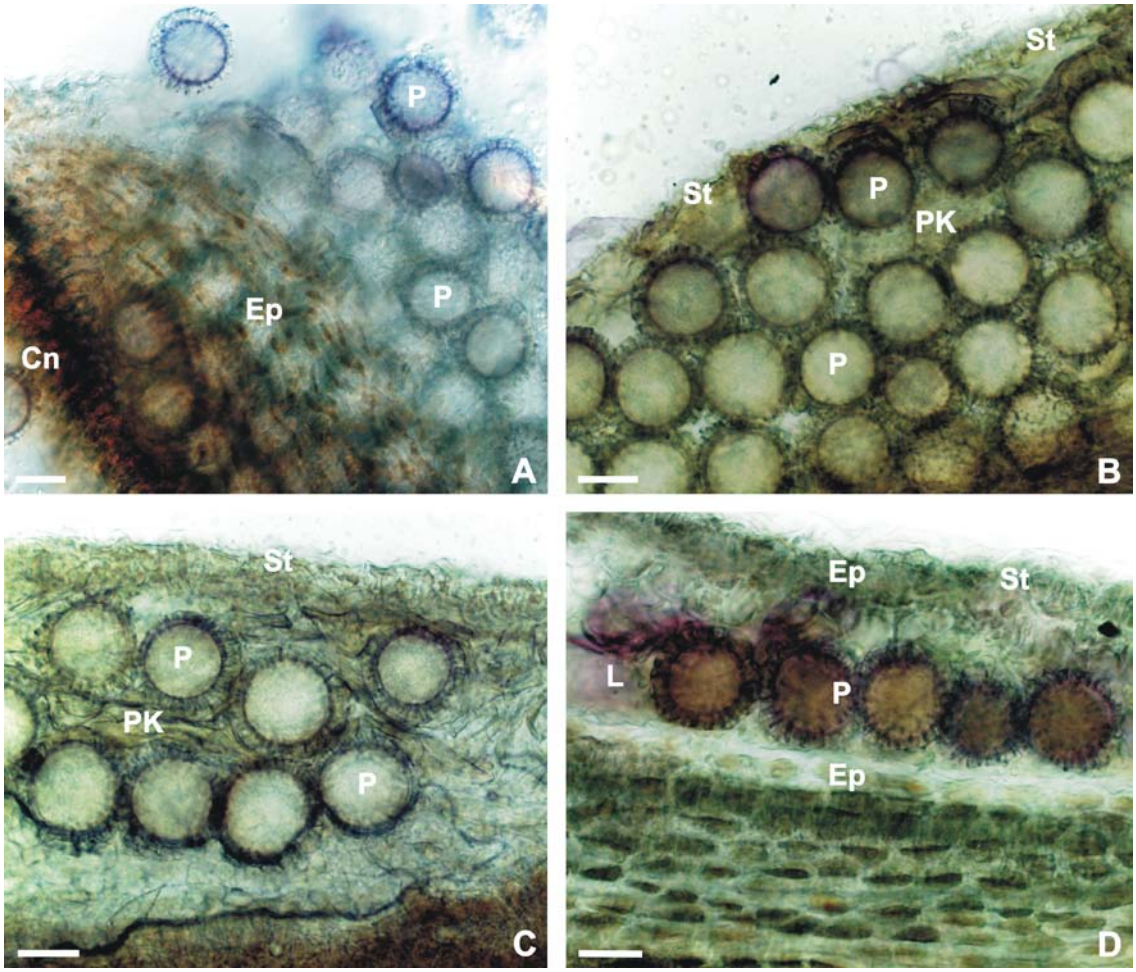

Figure 5.3 LM observations ***Bud 5***: **A** epidermis (Ep) of locule wall remains intact attached to connective (Cn), grains (P) tightly packed ***Bud 6***: **B** pollen grains (P) with developed cell walls packed closely in parallel rows within the anther locule. Pollenkitt (PK) surrounds grains, anther will dehisce at stomium (St) ***Bud 7***: **C** anther stomium (St) dehiscing and tapetum degenerates and deposits pollenkitt (PK) on pollen grains (P) ***Bud 8***: **D** individual pollen grains (P) within the locule (L), will be released through the ruptured stomium (St). Epidermis (Ep) on each side of stomium intact. Scale bars = 50 µm

FIB-SEM observations

Internal pollen wall structure was not easily discernible and the individual wall layers could not be clearly identified. This could be a result of the FIB sectioning technique being too harsh for the early primexine wall of the immature grain as the formation of this wall precedes sporopollenin deposition.

Externally, development of pollen walls can be seen to progress from a more condensed, reticulate pattern with thick muri, surrounding lumina of a reduced size, with no clear internal wall structures visible (Fig. 5.5aC), to an expanded, open, reticulate pattern, with refined muri and a few pila in the centre of each lumina (Fig. 5.5bN).

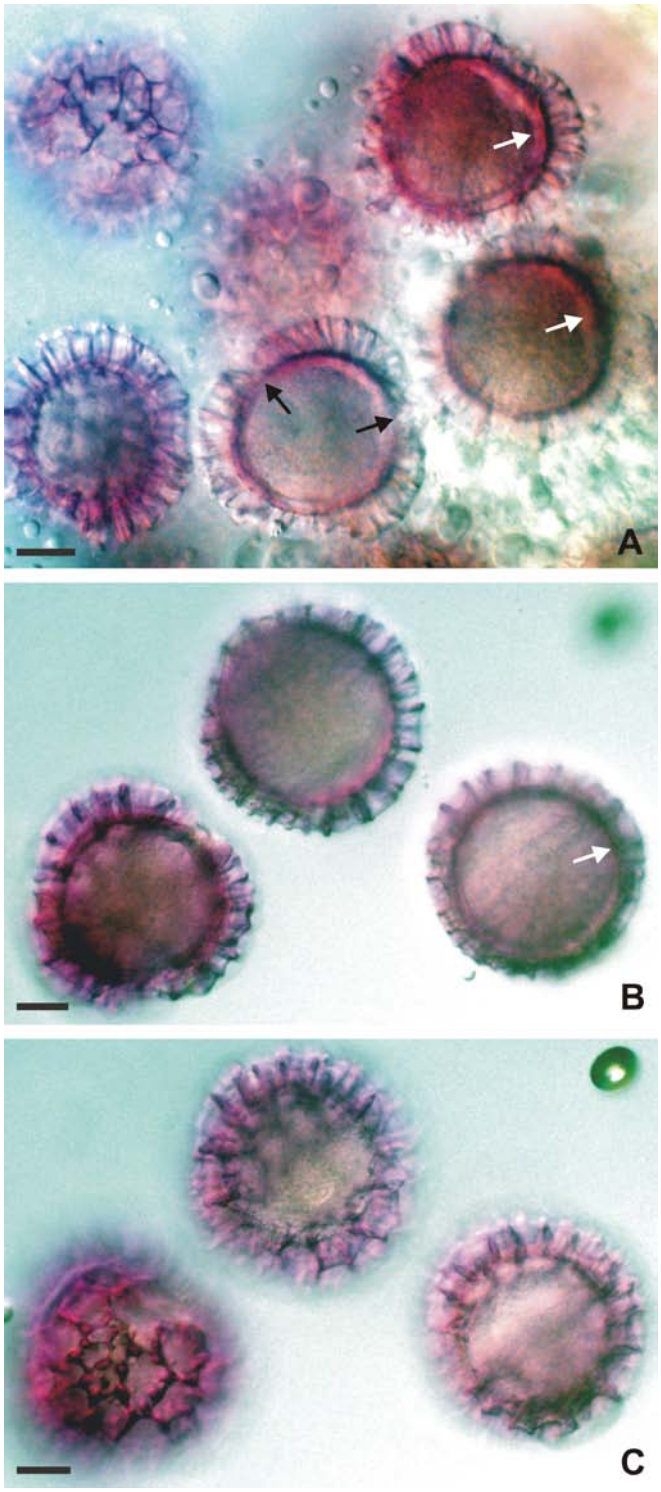

Figure 5.4 Developing pollen grains **A** Pollen grains from Bud 3 showing thickened primexine deposition (white arrow) and position of apertures (black arrows) **B** and **C** A through focus of pollen grains from Bud 5. Grains have expanded to stretch exine over surface of grain
Scale bars = 20 µm

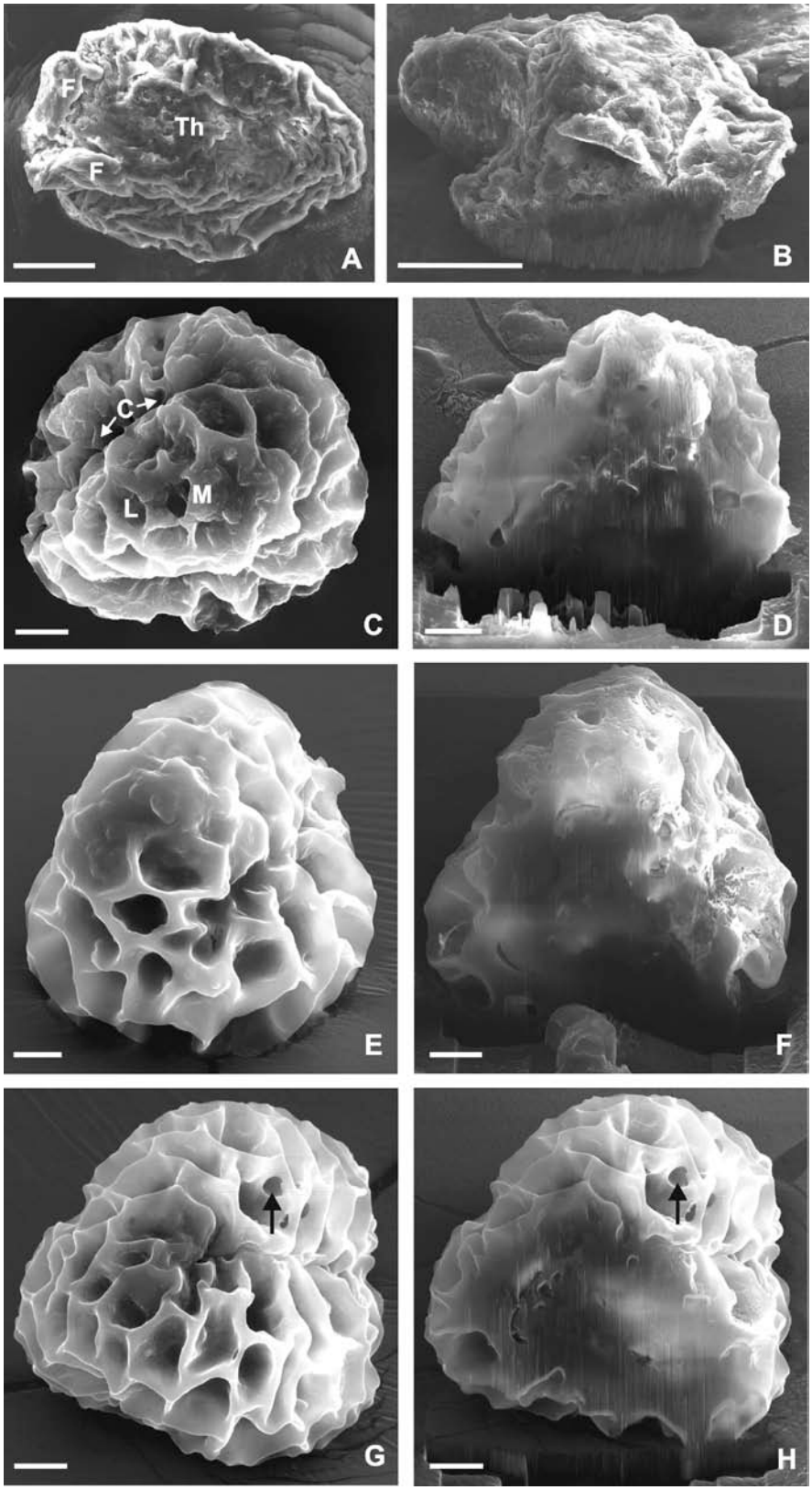

Figure 5.5a FIB-SEM micrographs of developing pollen grains ***Bud 1*** (youngest): **A** whole anther **B** cross-section through whole anther. No pollen grains are visible ***Bud 2– 4***: **C – H** whole developing pollen grains and corresponding cross-sections through the grains. C-colpus containing the aperture; F-filament; L-lumina; M-muri; Th-theca; arrows show developing cavities in muri
Scale bars: A and B = 100 µm; C–H = 10 µm

Bud 1 (Fig. 5.5aA and Fig. 5.5aB): No individual pollen grains were visible within the anthers; therefore the whole anther was sectioned. The anthers contain MMC or pollen mother cells at this stage (Fig. 5.2A), not visible with the SEM.
Bud 2 (Fig. 5.5aC and Fig. 5.5aD): Pollen grains in the tetrad phase- the immature pollen wall already has a clear reticulate pattern (Fig. 5.5aC). The muri are solid, thickened and shallow, the lumina are reduced in size. The positions of the colpi with corresponding apertures are clearly demarcated and the grain is 3-lobed. The average grain size is 40–50 µm across. There are no internal wall layers discernible (This stage corresponds to Fig. 5.2B –Fig. 5.2E).
Buds 3 and 4 (Fig. 5.5aE, 5.5aF, Fig. 5.5aG and 5.5aH): After release from the thick callose wall surrounding the tetrads, the grains mature and increase in size to around 60 µm across. The muri become deeper and begin to develop cavities in the solid walls. The internal area (volume of cytoplasm) of the pollen grain increases. The internal structure of the pollen wall is not clear (Corresponds to Fig.5.2F and Fig. 5.2G and 5.4A).
Buds 5 to 6 (Fig. 5.5bI, 5.5bJ, Fig. 5.5bK and 5.5bL): grains increase in size to about 80 µm across. The reticulate pattern appears to be stretched out across the expanding grains, refining into the typical elaborate arching muri pattern of the mature specimens. It does not appear as though more wall material is being added during the development, rather that the immature grains already contain the basis for the end result, which is refined and expanded as the developing pollen grains increase in volume. The endexine is deposited at this stage. The observed pattern of development suggests that the endexine and intine are derived from the pollen cell itself and not from the tapetum (Corresponds to Fig. 5.3A and Fig. 5.3B; Fig. 5.4B and Fig. 5.4C).
Bud 7 (Fig. 5.5bM) and Bud 8 (Fig. 5.5bN): The grains increase in size to about 90 µm across. The external sculpturing is complete. Individual, mature pollen grains are released from the anthers (Corresponds to Fig. 5.3C and Fig. 5.3D).

DISCUSSION

The technique of sectioning developing pollen grains with the FIB-SEM is useful for investigating pollen ontogeny, as varying stages of the developing grains are clearly identifiable. It is possible that the materials forming the outer wall of the pollen grains are at this stage, not sufficiently rigid to completely withstand the milling of the focused ion beam (Fig. 5.5aD, Fig. 5.5aF and Fig. 5.5aH). In contrast to the walls of the fully mature grains (Fig. 5.5b M and 5.5bN), they appear to collapse somewhat, although details such as the exine thickness and some of the initial sculpturing can still be discerned. Internal pollen wall layers, however, were not easily discernible in the immature pollen grains. It may be the case that these features are obscured by the ion beam process, but it is also possible that they may not be well formed at this stage and are still in the process of developing, or being laid down.

It is clear that the deposition of the rudimentary pollen wall happens very early on in the

process and is complete before the end of the tetrad phase. The sculpturing then appears to refine, almost as a consequence of being stretched out across an increasing area, as the pollen grains expand to their mature size. Although not quantified in this study, the thickness of the exine did not appear to increase as pollen grains matured, implying that the full complement of material was already present from very early on and that no further deposition of exine materials took place. This is in contrast to other studies where the exine is observed to be deposited in stages (e.g., Takahashi, 1995; Paxon-Sowders et al., 1997). Any activity of the genes, which have recently been proposed as having a role in influencing the patterns of the reticulate sculpturing (Paxson-Sowders et al., 2001; Ariizumi et al., 2008), would presumably also occur early on in the developmental stages, as the pattern appears to be in place early in the tetrad phase. The final internal wall layers (the endexine and intine) appear to only be deposited by almost mature pollen grains, just prior to release (Fig. 5.5bL).

The condition of the tapetum was also observed, as it has been shown to play a major role in the development of the pollen grains (Pacini, 1997). The light microscope data revealed the tapetum to be secretory, with some unusual features. The tapetal cells were noticeably aligned to surround each tetrad (Fig 5.2C), with no breakdown of individual cell walls or invasion of the tetrad groups, as observed in *Canna* (Tiwari and Gunning, 1986) and definitely did not form a plasmodium. Cytoplasmic strands were clearly observable linking the tapetal cells and the developing pollen grains in the tetrad phase (Fig 5.2B and Fig. 5.2E), possibly even extending through the primexine walls of the developing grains (Fig. 5.2E). In the later stages of development, the tapetum breaks down after the degeneration of the callose walls surrounding the tetrads, enveloping the maturing grains in pollenkitt (Fig. 5.3C). Thereafter, individual grains appear to be arranged in tightly packed rows within the anther locules (Fig. 5.3).

The process of pollen ontogeny in angiosperms has been well established and documented (Blackmore et al., 2007), but this study has highlighted several new features. Most notably, development of the exine sculpturing appears to be established early on in development and is refined as a consequence of the expanding volume of the grain, rather than by progressive deposition of additional material. The arrangement of the tapetal cells around the tetrads in this species is unusual, as are the long cytoplasmic strands extending from the taptum, through the primexine, into the grains themselves.

Finally, the results of this exploratory study confirm the partial efficacy of the microscopy techniques used for investigations of this nature. Similar studies of additional Acanthaceae species would establish whether these findings are typical of this group or if pollen grain ontogeny in this family is as variable and diverse as the elaborate sculpturing of the grains themselves.

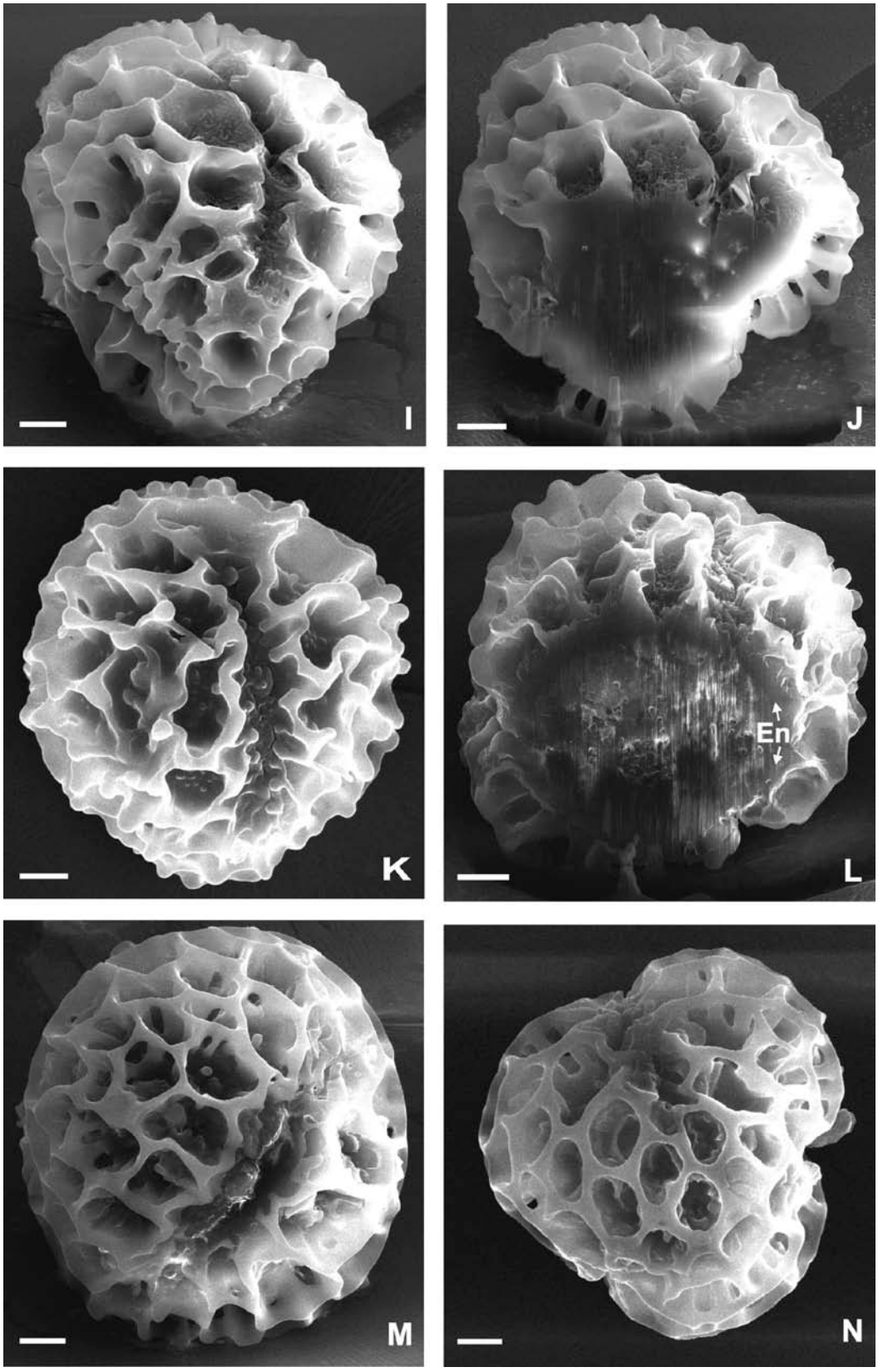

Figure 5.5b FIB-SEM micrographs of developing pollen grains ***Bud 5 – 6***: **I, J, K, L** external sculpturing almost complete. Internal structure becomes visible, endexine (En) formation visible ***Bud 7***: **M** mature pollen grain ready for release ***Bud 8***: **N** mature pollen grain
Scale bars: I–N = 10 µm

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CHAPTER SIX–DISCUSSION

The major thrust of this study was to investigate internal structure of pollen grain walls to identify features or characters of potential taxonomic value. The family Acanthaceae was selected for this purpose due to the great morphological diversity of its pollen and the historic use of external pollen features in formulating its classification systems. In order to conduct this study a new microscopy technique, focused ion beam-scanning electron microscopy (FIB-SEM), was utilised and evaluated. FIB-SEM proved to be an extremely effective tool for investigating internal pollen wall structures in the Acanthaceae (Chapter Two and Chapter Three). In addition to sample preparation being fairly simple, the results obtained afford an excellent three-dimensional view of the interior pollen wall structure. Data obtained with this technique allow one to distinguish between distinct features which could otherwise appear similar when viewed as thin cross-sections or surface scans. As a result, five different internal wall structures were identified from a wide range of genera within the Acanthaceae (Chapter Three). This included being able to determine whether certain walls comprised actual chambers or tunnels, or whether they contained an open cavity interspersed with free-standing columns or columellae. In standard two-dimensional cross-sections, the walls between adjacent chambers or tunnels often give the impression of columellae, but the three-dimensional view afforded by FIB-SEM allows one to accurately distinguish between chamber walls and discrete columellae. The objective of establishing a methodology for sample preparation which produced useful data was therefore most successfully met, with the FIB-SEM more than proving its effectiveness in this regard (Chapters Two and Three). The appropriateness of using FIB-SEM on softer material, such as developing pollen grains within anthers was also tested (Chapter Five). The strength and stability of sporopollenin makes mature pollen grains excellent candidates for this technique and immature, developing grains are also able to withstand exposure to the ion beam (but to a lesser degree than mature grains), making this a useful method for investigating pollen wall development as well.

Internal wall characteristics and features identified in this research (Chapter Three) were used in morphometric analyses, which largely support existing classifications based on molecular data and external morphological characters (Chapter Four). Within the subtribe Justiciinae, it was noted that in all the species of *Justicia* examined, the typical internal wall arrangement included chambers and elongate, arched cavities or tunnels, but the structure was never restricted to distinct, individual columellae alone. This was in contrast to the specimens of the closely related genera *Hypoestes, Rhinacanthus Peristrophe* and *Dicliptera* that were investigated. In spite of the similar, reticulate external sculpturing shared by *Justicia* and these species, the studied examples of the latter genera had internal walls structured with definite, free-standing, individual columellae, showing that external features are not necessarily indicative of similar internal wall structures (Chapter Three and Chapter Four). Within *Justicia* itself, it was

also found that different or similar external sculpturing of grains from various species, could not be used to infer similar or different internal wall structures. *Justicia carnea*, *J. flava* and *J. petiolaris* have similar internal wall arrangements, with arched tunnels (Chapter Four), but their external grain sculpturing differs widely. Conversely, for the subtribe as a whole, *J. carnea* and *Isoglossa ovata* have similar external sculpturing and share the same internal wall structure of tunnels and chambers (Chapter Three). It can therefore be surmised, that the taxonomic and phylogenetic importance of the internal wall features identified in this study, may lie in suggesting infra-generic relationships, rather than making inferences at any higher taxonomic levels. The importance of these features from a phylogenetic perspective within genera must still be assessed.

The data obtained in this study were insufficient to point towards any evolutionary sequence of wall arrangements, but this possibility is worth exploring in future studies. Prior work has suggested that an alveolate ectexine may be a basal state (Walker, 1974). In this study, *Justicia flava* and *Thunbergia atriplicifolia*, from different tribes and subfamilies, both exhibit this arrangement (Chapter Three). In fact *J. flava* exhibits a combination of internal wall structures, with a porous, alveolate ectexine containing elongate, arched chambers or tunnels. Within *Justicia*, other species examined were found to have different internal wall structures, so the potential for determining a phylogenetic sequence certainly seems possible and could prove useful for identifying relationships within closely related species (Chapter Four). This in turn allows for a more accurate assessment of homologies and better use of character states in phylogenetic analyses.

When reviewing the external and internal pollen grain morphology data obtained from this study, it is possible to propose a hypothetical progression of pollen wall structural evolution (Fig. 6.1). For the purposes of this explanation, the pollen wall is envisaged as comprising closely packed individual pentagonal shaped pollen wall units. The tectal sculpturing of muri surrounding radiating arms was chosen, but this may also vary according to the species.

In this scenario, an internal solid walled chamber containing a central columella supporting a sculptured tectum within surrounding muri which form an overall reticulate pattern (**A**) progresses to an open reticulate pattern (**G**), by the successive loss of wall material and tectal sculpturing.

From a solid walled chamber with a central columella (**A**) (e.g., *Justicia*) the first step is the loss of the surrounding walls, leaving only corner supports (**B**) (e.g., *Rhinacanthus*), which are subsequently lost, leaving an open cavity with discrete columellae supporting the tectum (**C**) (e.g., *Hypoestes*, *Dicliptera*, *Peristrophe*). Alternatively, the central columella is lost, and either the solid walls remain (**D**) (e.g., *Acanthus*, *Hygrophila*, *Justicia*, *Asystasia*, *Isoglossa*) or are also lost leaving an open chamber with corner

supports for the tectum (**E**) (e.g., *Sclerochiton*, *Petalidium*). With the consequent loss of the tectal sculpturing, muri roughly in the shape of pentagrams remain (**G**), forming the deep reticulate pattern found in species of *Barleria* and *Ruellia*. In some instances solid walls remain (**F**), such as the sculpturing seen in *Crabbea*.

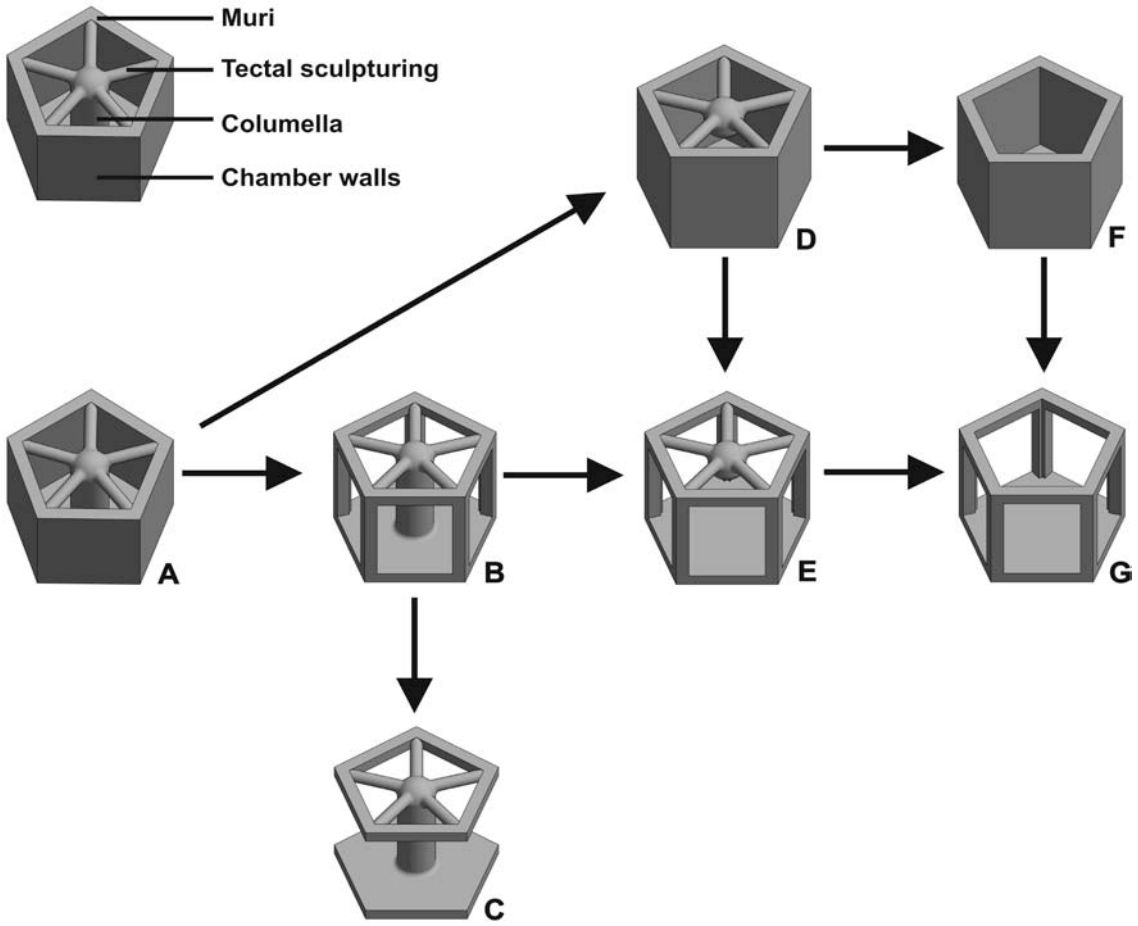

Figure 6.1 A schematic diagram of pollen wall structural units showing a hypothetical progression of internal pollen wall structural evolution in the Acanthaceae

The stated objectives of the study, to

- Evaluate the use of FIB-SEM for investigating the detailed structure of the pollen walls in species of Acanthaceae.
- Develop a methodology for utilising FIB-SEM to investigate internal pollen wall features (Chapter Two).
- Accumulate pollen wall data from selected genera from across the family using FIB-SEM (Chapter Three).
- Identify internal pollen wall characteristics from the collected data and analyse the characters for taxonomic potential (or phylogenetic signal) in the family (Chapter Four).

- To determine whether a selected species shows a similar pollen wall development pattern to documented examples, in order to examine phylogenetic signal in ontogeny (Chapter Five).
- Discuss the relationships suggested by the internal wall structures from this study with respect to existing classifications and phylogeny based on external morphology and molecular studies (Chapter Six).

were therefore all successfully met.

Discussion of Results

A number of key questions were posed, and the results that answer them are presented below:

- *Do species currently accepted as being closely related share any internal pollen wall characteristics?*

Of the wide range of species that were investigated, it was noted that many species within the same tribe, while sharing characteristic external pollen sculpturing, also shared a similar internal wall structure. This was the case for *Acanthus* and *Blepharis* (Tribe Acantheae); *Hygrophila* and *Dyschoriste* (Tribe Ruellieae, Sub-tribe Ruelliinae); *Hypoestes* and *Rhinacanthus* (Tribe Ruellieae, Sub-tribe Justiciinae); *Peristrophe* and *Dicliptera* (Tribe Ruellieae, Sub-tribe Justiciinae). These data correspond to associations made from molecular studies (McDade and Moody, 1999; McDade et al., 2000; McDade et al., 2005; McDade et al., 2008; Tripp et al., 2013). Furthermore, the species examined from the genus *Justicia*, exhibited a wide range of varying external pollen sculpturing, but were found to have walled chambers, structurally never restricted to individual columellae alone, within their internal walls. Species of *Isoglossa* have the same external wall sculpturing as *Justicia carnea* (although they differ with respect to other external pollen morphological features), but consistently show the same internal wall structure of large chambers which decrease in size towards the poles. All these closely related taxa share internal pollen wall characteristics.

- *Do species currently accepted as being closely related but with very different external appearance share any internal pollen wall characteristics?*

The genus *Justicia* is a prime example of this. As noted in Chapter Four, the genus displays a wide range of external sculpturing, but the internal wall characteristic, namely walled chambers, remained consistent. This is an extremely useful finding, as it supports the existing placement of species within this genus, in spite of the varying external appearance of their pollen grains, particularly between Old and New World species. The Old World species of *J. betonica*, *J. capensis* and *J. protracta*, have distinctly different external pollen sculpturing, but have a similar internal wall structure of walled chambers. The same applies to the Old World species, *J. anagalloides*, and the

New World species, *J. brandegeeana*. The New World species, *J. carnea*, has very different external sculpturing to the other *Justicia* species described in this study, but shares a similar internal structure to the Old World species, *J. guerkeana*, *J. flava* and *J. petiolaris*, of arched, elongate chambers.

- *Do species with very similar external morphology, but not accepted as being closely related (divergent), have distinct or similar pollen wall structures?*

  This point is elegantly illustrated when examining the wall structure and sculpturing in species of *Barleria*, *Crabbea* and *Ruellia* investigated in this study. *Barleria* and *Crabbea* in Sub-tribe Barleriinae and *Ruellia* in Sub-tribe Ruelliinae have similar external pollen wall sculpturing. All are semitectate with the tectum restricted to the muri which outline roundish-hexagonal lumina forming a very open reticulate pattern. In *Barleria* and *Ruellia* individual columellae support this open reticulum, while in *Crabbea* solid wavy walls are found. The supporting structures of columellae or walls extend from the rudimentary tectum down to the foot layer which is pilate to verrucate. FIB-SEM results revealed a simple internal wall structure with well defined, solid endexine and a thin intine in all cases. *Barleria* is unique in that firstly, the muri appear hollow internally with a thin, double wall and the endexine appears to be separated into two layers by a fine granular layer. Interestingly, *Crabbea,* in the same tribe as *Barleria,* exhibits more external and internal pollen characters in common with *Ruellia* than it does with other *Barleria* species. This is in line with molecular data (McDade et al., 2008) that separates *Barleria* from other genera, including *Crabbea*, in the Barleriinae. This result fits with the general conclusions of this study, that making inferences as to phylogenetic relationships on the basis of internal wall characters, is perhaps most suited to infra-generic relationships.

- *Do species from different tribes share any internal pollen wall characteristics or are their wall structures distinct?*

  While certain tribes definitely appear to have similar external pollen sculpturing, the limited data across tribes in this study did not identify any specific internal wall feature to be characteristic for a tribe. While walled chambers were characteristic of *Justicia* in the sub-tribe Justiciinae, fellow sub-tribe members from *Rhinacanthus*, *Hypoestes*, *Dicliptera* and *Peristrophe* had distinct columellae present in their walls. Justiciineae is the largest sub-tribe with widely divergent external pollen morphology, and the variability of the internal structure appears to follow this pattern as well.
  Justicia flava has an interesting internal wall structure which appears to be a combination of the alveolate form and arching, elongate chambers. The only other example of an alveolate wall structure was in *Thunbergia*, a genus in a completely different sub-family from the Justiciinae.
  It seems as though all internal structures are found across the family and are not

restricted to a particular tribe [e.g., columellae are found in Acantheae (*Sclerochiton*), Ruelliinae (*Petalidium*), Justiciinae (*Hypoestes*), Barleriinae (*Barleria*)].

- *Do internal pollen wall characteristics support existing classifications of the Acanthaceae or are these characteristics not indicative of phylogeny?*

  The internal pollen wall characteristics identified in this study are useful for clarifying similarities within taxa (as discussed in Chapter Four) and are supportive of existing classifications when considered together with external pollen and other plant morphological characteristics. Morphometric analyses suggest internal pollen features may be useful in inferring phylogeny within lower taxonomic units, such as genera.

- *Does any discernible relationship exist between external sculpturing and internal pollen wall structure, or are they unrelated?*

  From the data collected in this study, it can be concluded that the internal wall structures cannot be predicted from external sculpturing. This idea has been proposed before (Moore et al., 1991) and has been confirmed for Acanthaceae by this study. Within the Justiciinae, the ratio between the ectexine and endexine within the margocolpus region was measured. It was noted that in species with a well developed (high) margocolpus region, some had corresponding thick ectexine and endexine layers. In these species the internal structure was constructed from elongate, arched chambers. This pattern was seen in *J. flava, J. petiolaris, J. carnea* and in *Asystasia* Blume. *Isoglossa* presented a similar structure, but the chambers had a flat ceiling and were not arched. It has been suggested that the ratio of ectexine to endexine is phylogenetically important (Walker, 1974), and the observations from this study support this notion (e.g., *Justicia flava, J. petiolaris* and *J. brandegeeana* all have very similar external pollen wall morphology typical of the genus. *J. flava* and *J. petiolaris* are Old World species and both have a much wider (about five times wider) ectexine than an endexine layer, however *J. brandegeeana* is a New World species and has a narrow ectexine, only twice as wide as the endexine. As a result *J. brandegeeana* has a different internal wall structure from the other two species).

- *Does the pattern of development of the pollen wall within anthers reveal any phylogenetic trends?*

  The ontogeny of pollen grains has not been investigated in the Acanthaceae, but a number of studies have been conducted within the Asteraceae. The pattern of development of the pollen in *Barleria obtusa* follows a similar pattern to that of the Asteraceae, with the pollen wall material laid down in the early stages of development. As the grain increases in volume the exine is stretched out into its final, mature pattern. The tapetum is of a secretory nature, and single pollen grains are released from a mature

anther.

- *Is it possible to use internal pollen wall characteristics for classification purposes?*

  This investigation suggests that internal pollen wall characteristics are a valuable asset for fine tuning existing classifications. There are a few unplaced genera within the Acanthaceae and pollen data would be helpful in placing these genera in the family. Classifications which utilize many levels of data are the most accurate and the FIB-SEM allows us a currently unique 3D perspective and has yielded new insights affording a more comprehensive understanding of pollen grain morphology.

  In conclusion, this study has successfully introduced FIB-SEM methodologies for gathering previously difficult to obtain characters of potential taxonomic significance in the interior of pollen walls. New characters have been identified, and the results suggest intriguing new possibilities for clarifying infra-genetic relationships in the Acanthaceae and beyond.

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APPENDIX

I. QUICK GUIDE – FIB-SEM MICROGRAPHS OF ALL SPECIES (page 7.2–7.9)

II. QUICK GUIDE – HIGH MAGNIFICATION FIB-SEM MICROGRAPHS OF ALL SPECIES (page 7.10–7.17)

These quick guides are intended to serve as a comprehensive reference to all the species investigated and sectioned with the FIB-SEM method in the course of this study.

In all cases the micrographs are labelled as follows:

(a) External view of a whole pollen grain
(b) Detail of the external sculpturing
(c) (I) FIB-SEM cross-section of the grain
Or (II) High magnification cross-section through the pollen wall

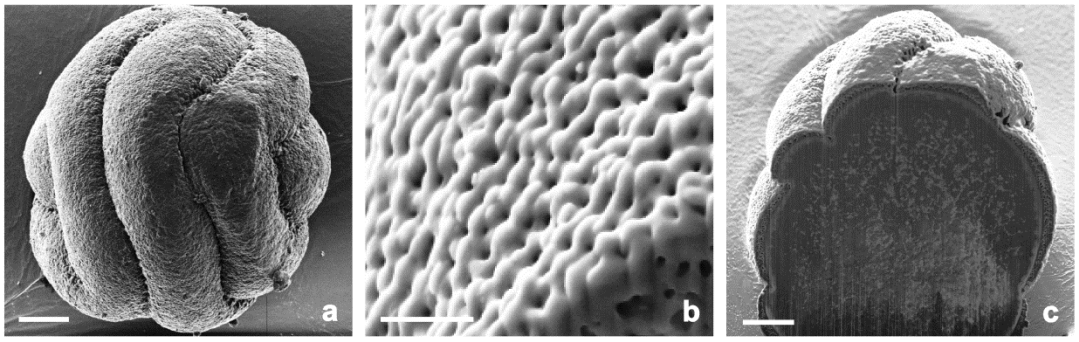
The micrographs are organised according to the classification of Scotland and Vollesen (2000) as depicted in Chapter One, Fig. 1.1: pg. 1.4. The species are listed in alphabetical order within these groupings.

III. FIB-SEM: A new technique for investigating pollen walls

A chapter in: Microscopy: advances in scientific research and education.
(A. Méndez-Vilas, Ed). Volume 1, Chapter 30, pages 54–58.
© FORMATEX

I. Acanthaceae

Thunbergioideae

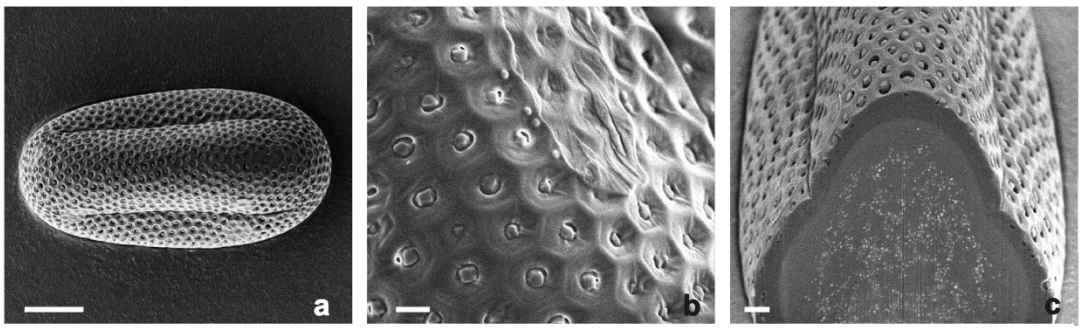


Thunbergia atriplicifolia

Scale bar: a 10 µm b 1 µm c 10 µm

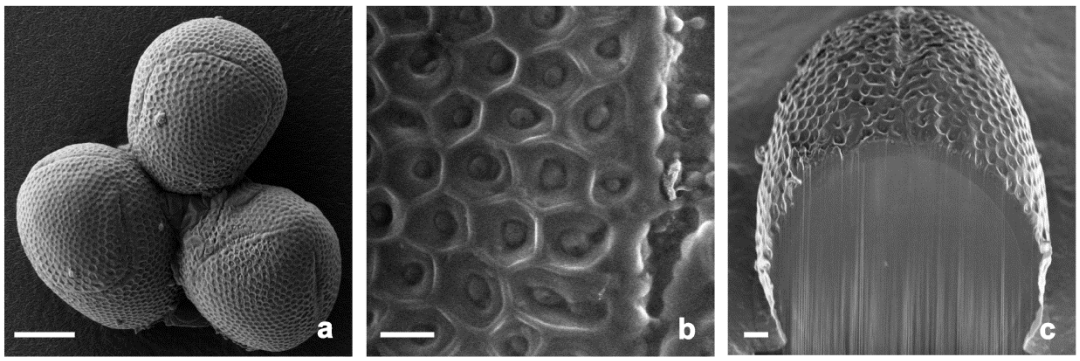
Acanthoideae

Acantheae



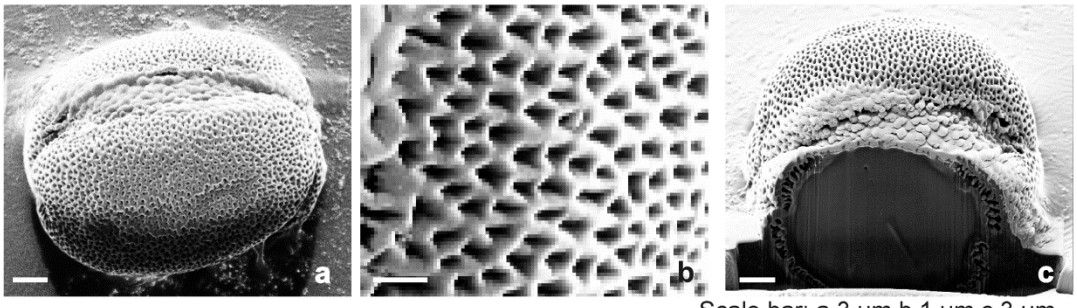
Acanthus mollis

Scale bar: a 10 µm b 1 µm c 2 µm



Blepharis stainbankiae

Scale bar: a 10 µm b 1 µm c 2 µm

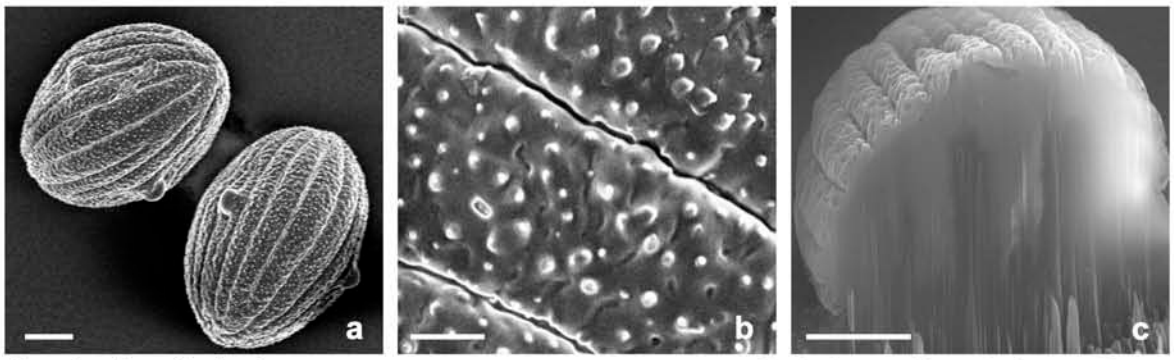


Sclerochiton harveyanus

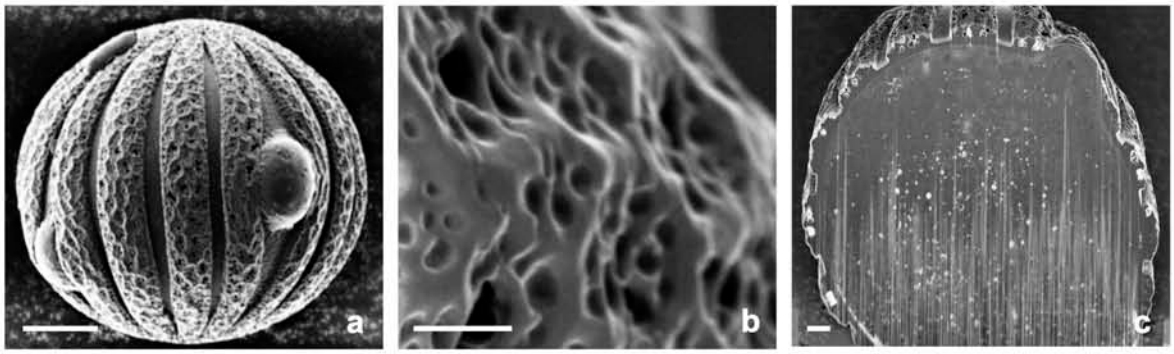
Scale bar: a 3 µm b 1 µm c 3 µm

Ruellieae

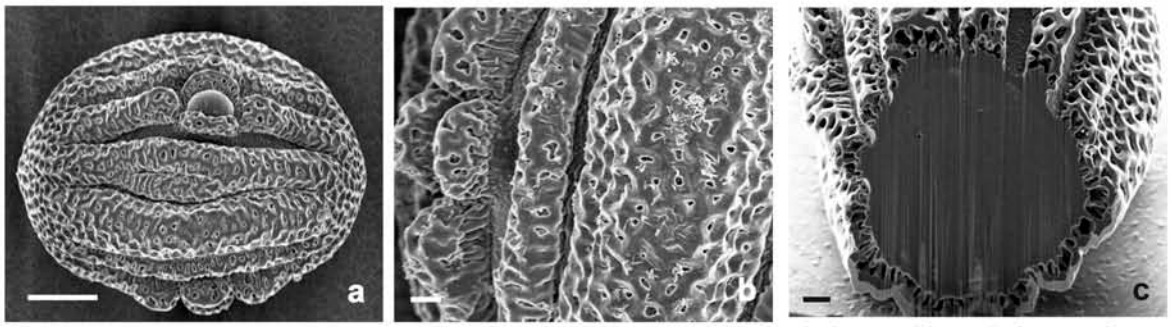
Ruelliinae



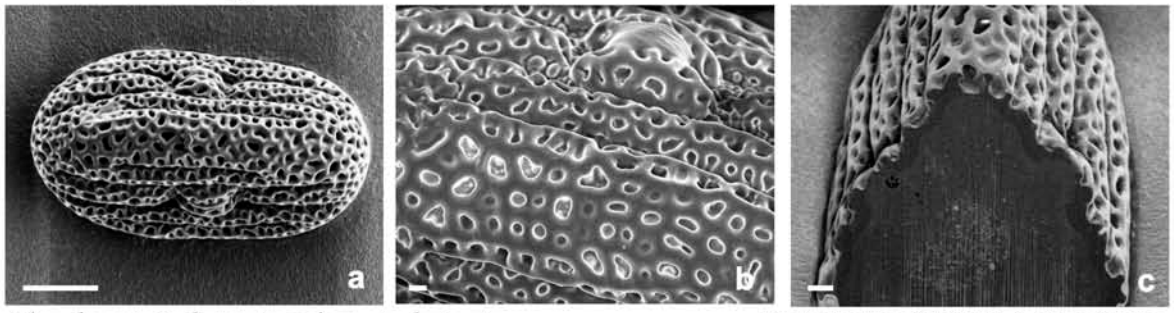
Dyschoriste fischeri Scale bar: a 10 µm b 2 µm c 5 µm



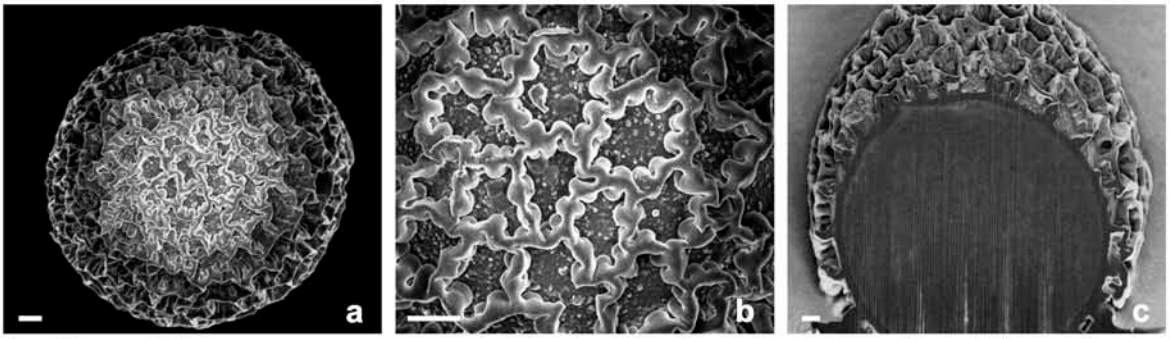
Hygrophila auriculata Scale bar: a 10 µm b 1 µm c 3 µm



Petalidium oblongifolium Scale bar: a 10 µm b 2 µm c 3 µm



Phaulopsis imbricata subsp. *imbricata* Scale bar: a 10 µm b 1 µm c 2 µm

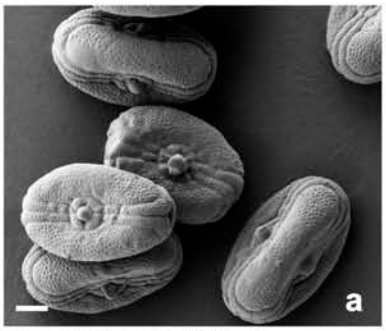


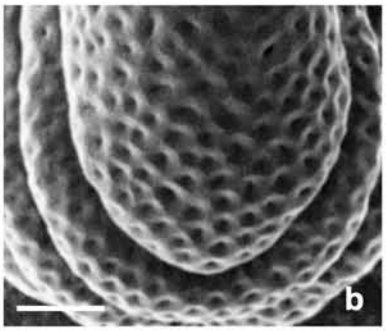
Ruellia cordata Scale bar: a 3 µm b 3 µm c 2 µm

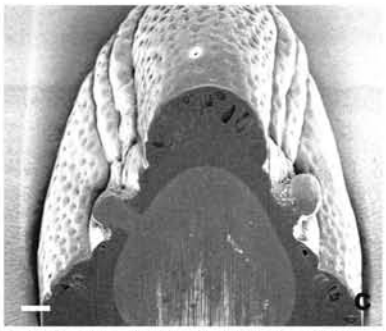
Justiciinae



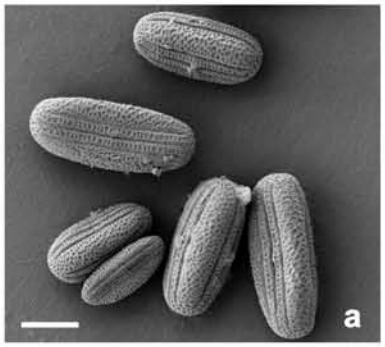
Anisotes formosissimus Scale bar: a 20µm

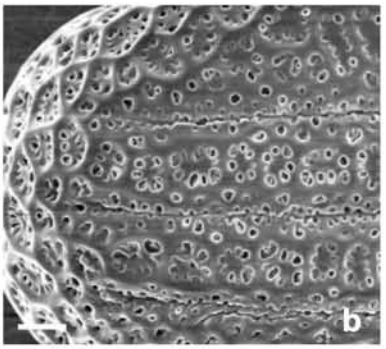


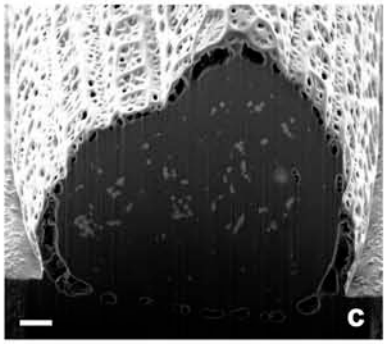




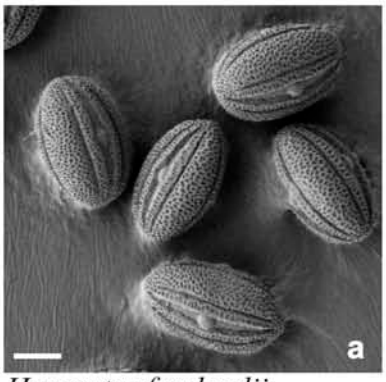
Asystasia subbiflora Scale bar: a 3 µm b 5µm c 3µm

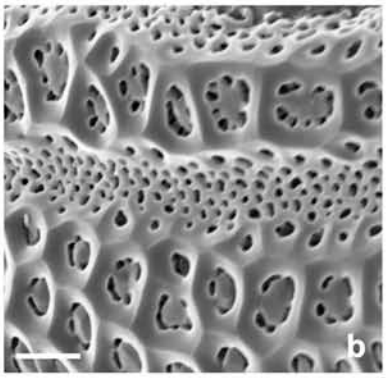


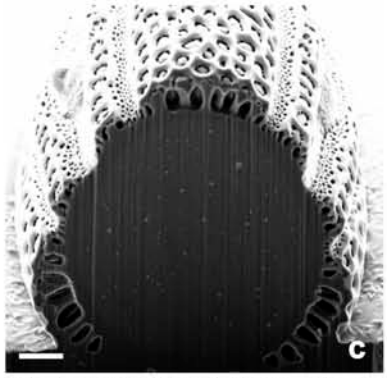




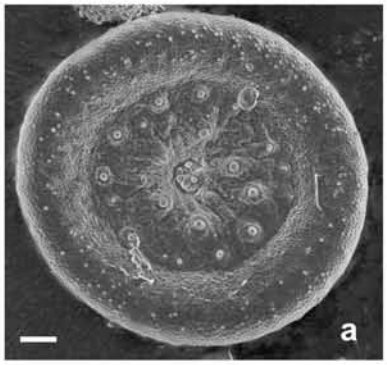
Dicliptera leisterni Scale bar: a 20 µm b 2µm c 2 µm

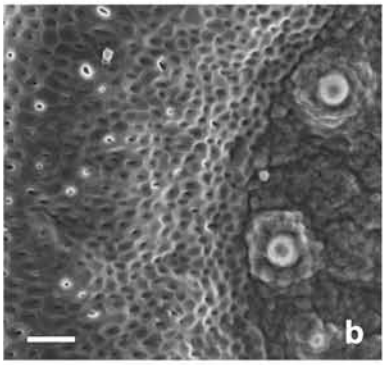


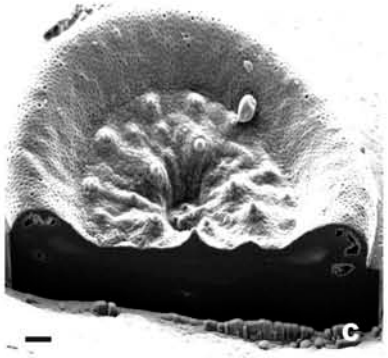




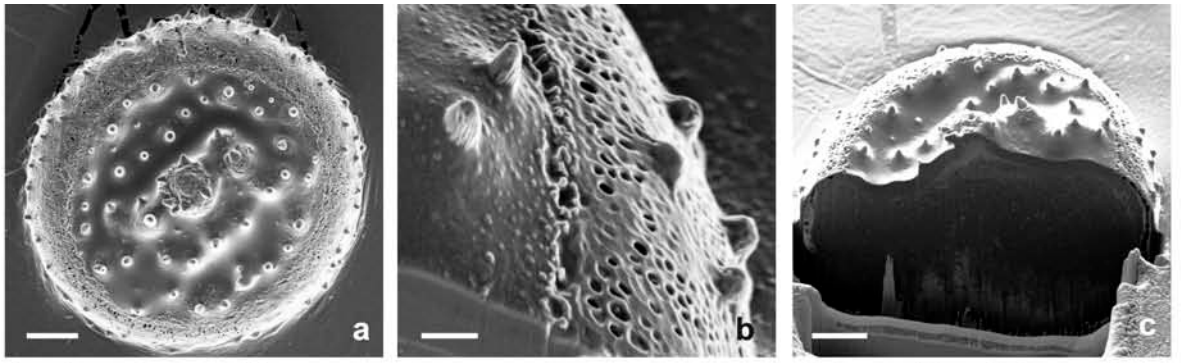
Hypoestes forskaolii Scale bar: a 20 µm b 2µm c 2 µm





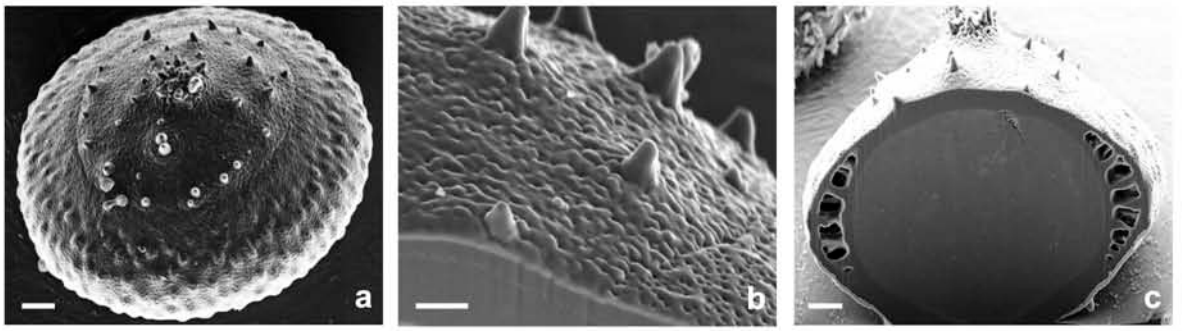


Isoglossa delicatula Scale bar: a 3 µm b 5 µm c 2 µm



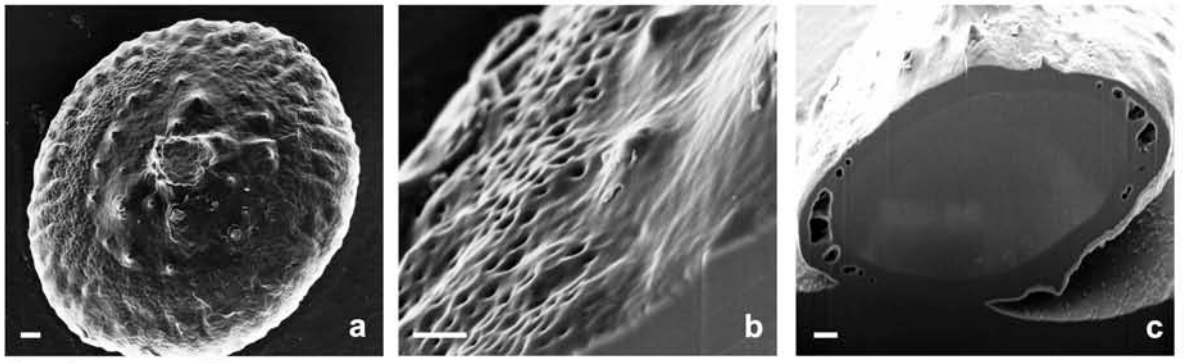
Isoglossa hypoestiflora

Scale bar: a 10 µm b 2 µm c 10 µm



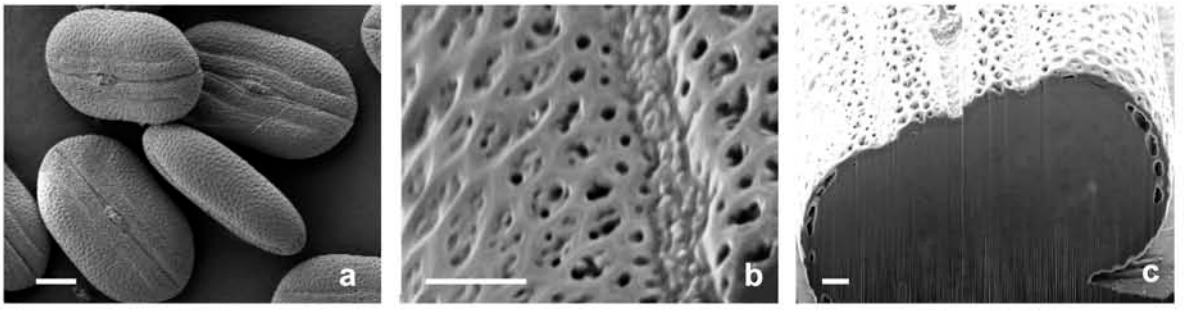
Isoglossa ovata

Scale bar: a 3 µm b 1 µm c 3 µm



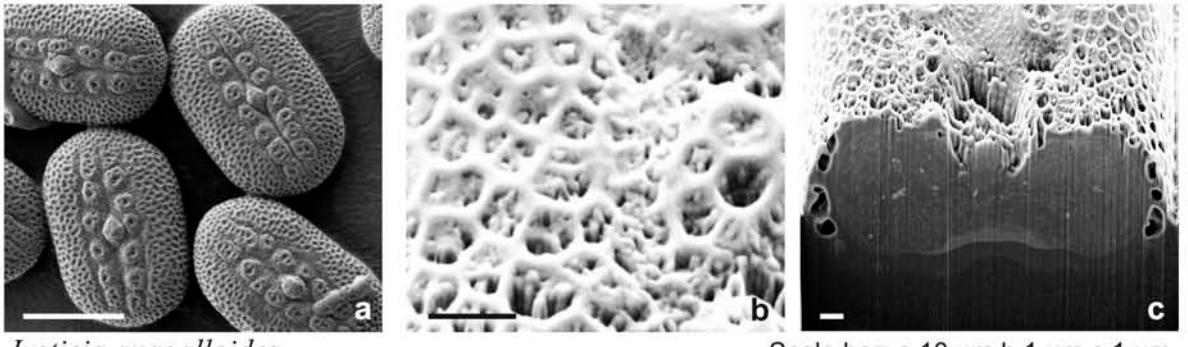
Isoglossa woodii

Scale bar: a 2 µm b 1 µm c 2 µm



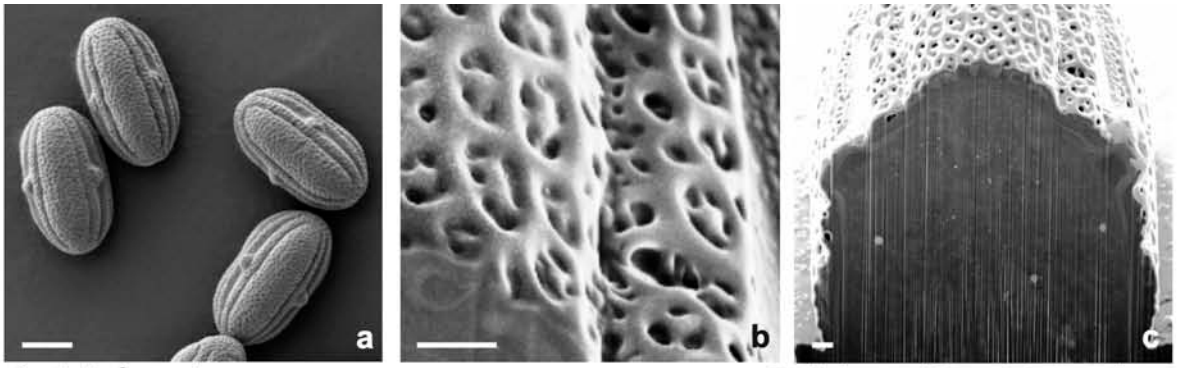
Justicia aconitiflora

Scale bar: a 10 µm b 2 µm c 2 µm



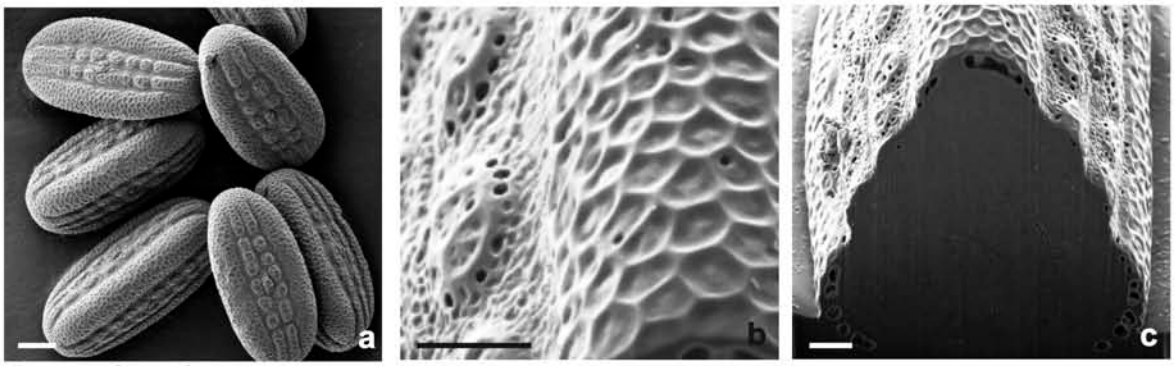
Justicia anagalloides

Scale bar: a 10 µm b 1 µm c 1 µm



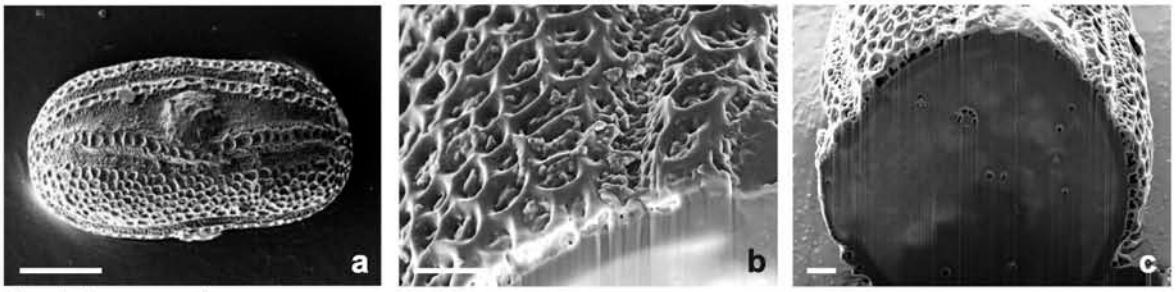
Justicia betonica

Scale bar: a 10 µm b 1 µm c 1 µm



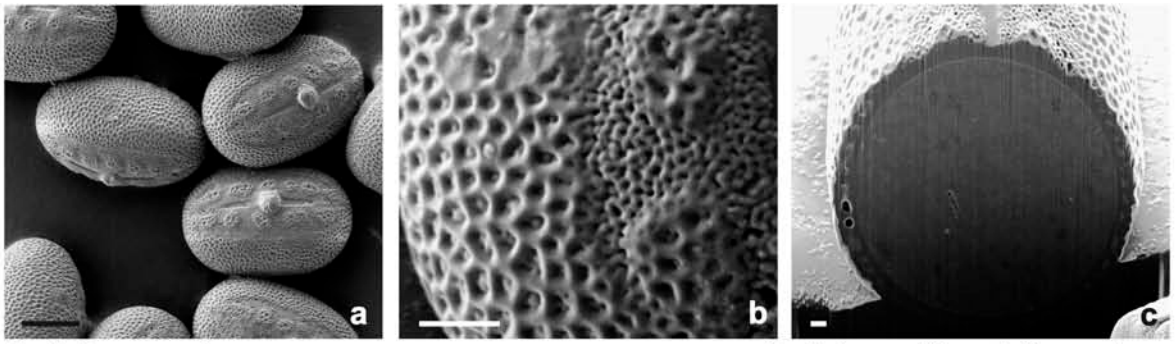
Justicia brandegeeana

Scale bar: a 10 µm b 3 µm c 3 µm



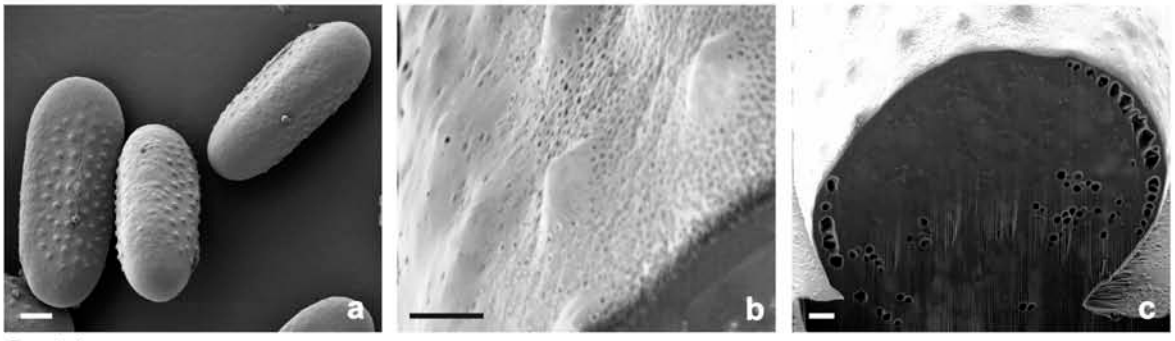
Justicia campylostemon

Scale bar: a 10 µm b 2 µm c 2 µm



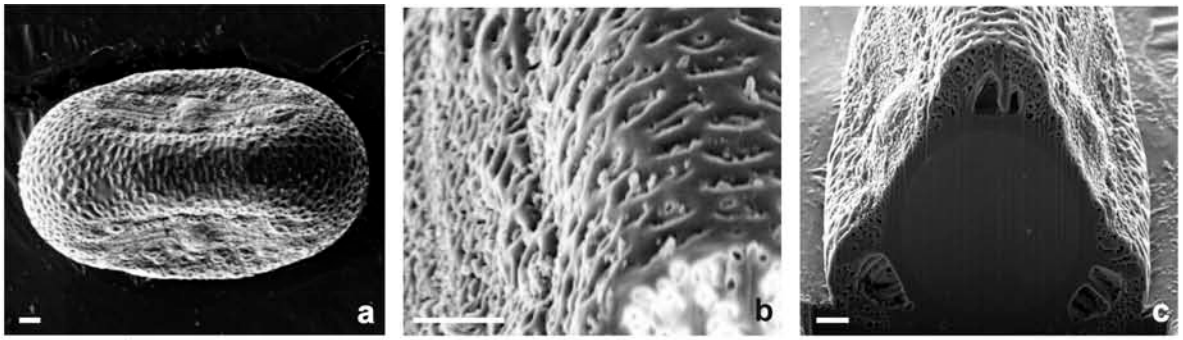
Justicia capensis

Scale bar: a 10 µm b 2 µm c 1 µm



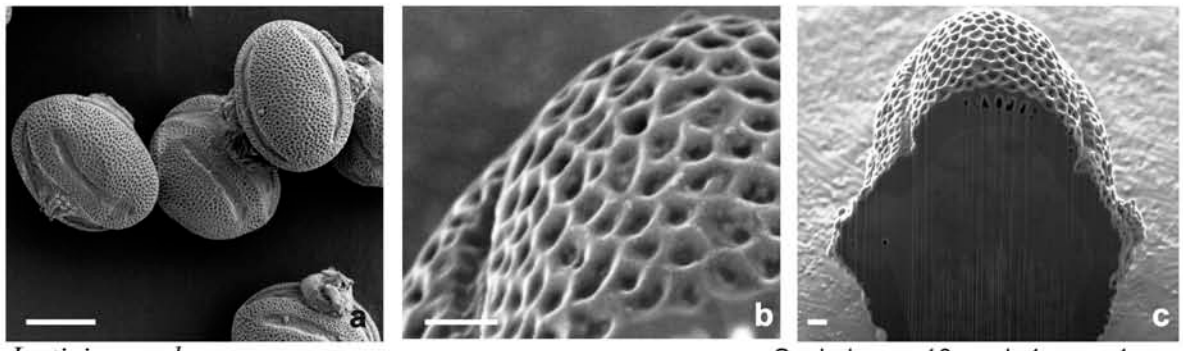
Justicia carnea

Scale bar: a 10 µm b 2 µm c 2 µm



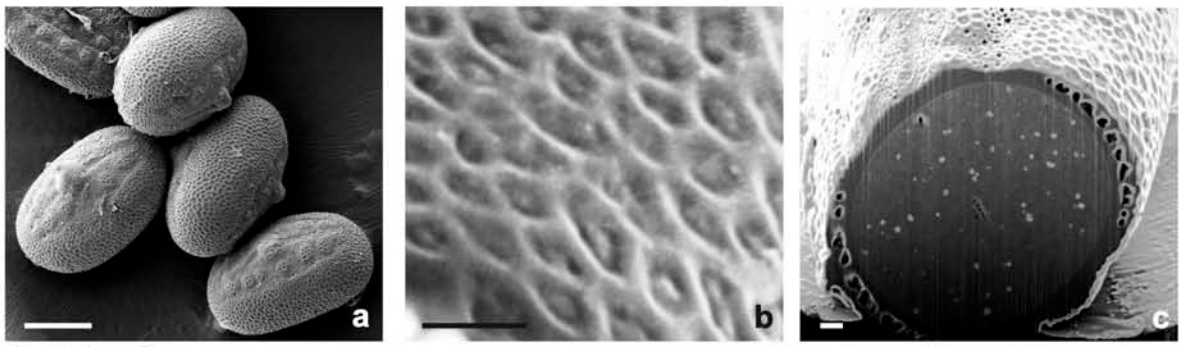
Justicia flava

Scale bar: a 2 µm b 2 µm c 2 µm



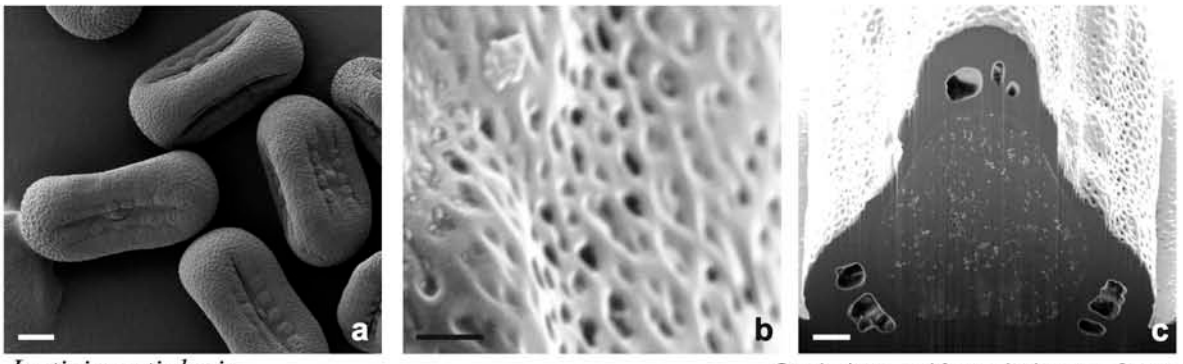
Justicia guerkeana

Scale bar: a 10 µm b 1 µm c 1 µm



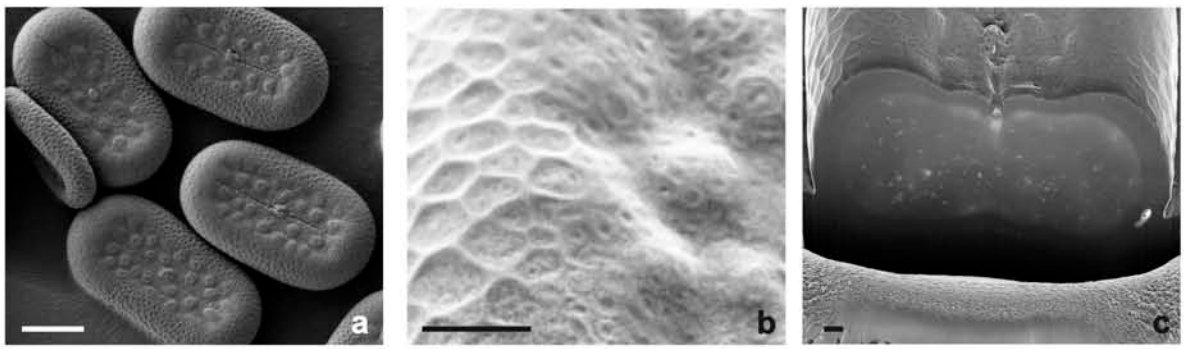
Justicia odora

Scale bar: a 10 µm b 1 µm c 1 µm



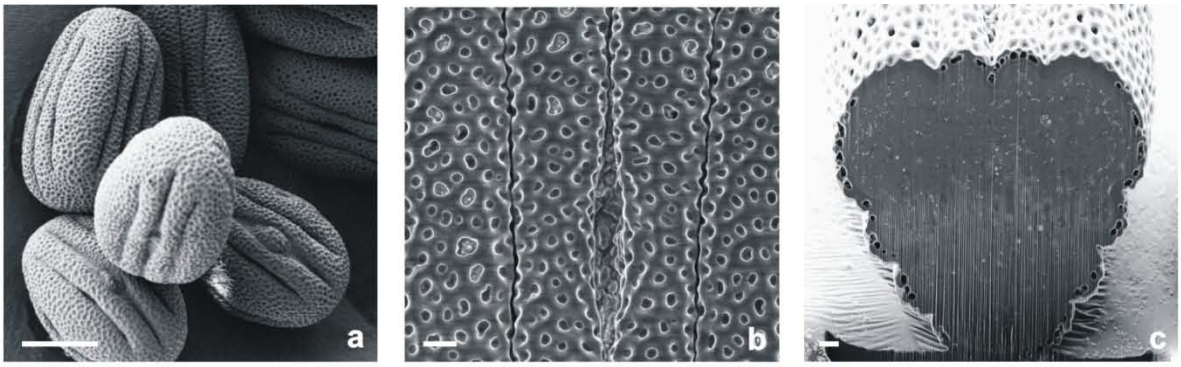
Justicia petiolaris

Scale bar: a 10 µm b 1 µm c 3 µm



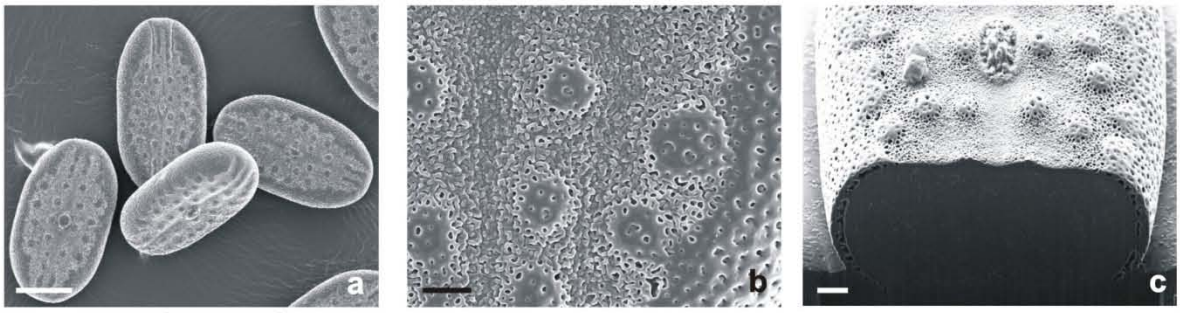
Justicia protracta

Scale bar: a 10 µm b 2 µm c 1 µm



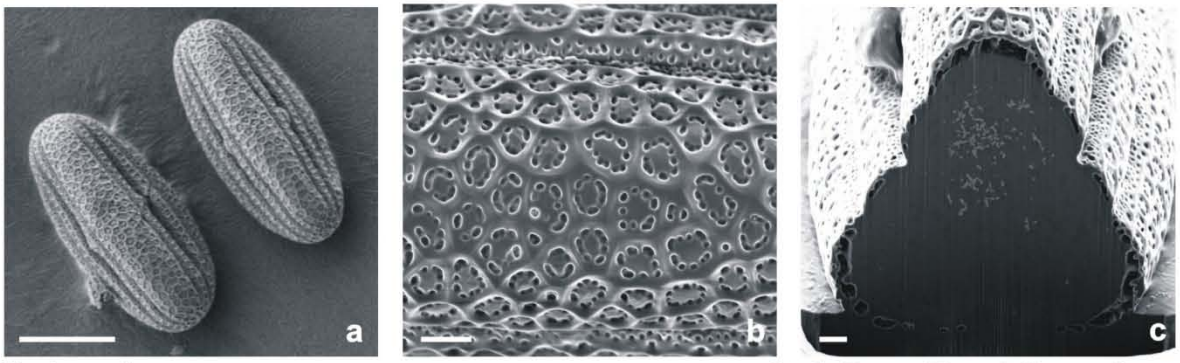
Mackaya bella

Scale Bar: a 20 µm b 2 µm c 2 µm



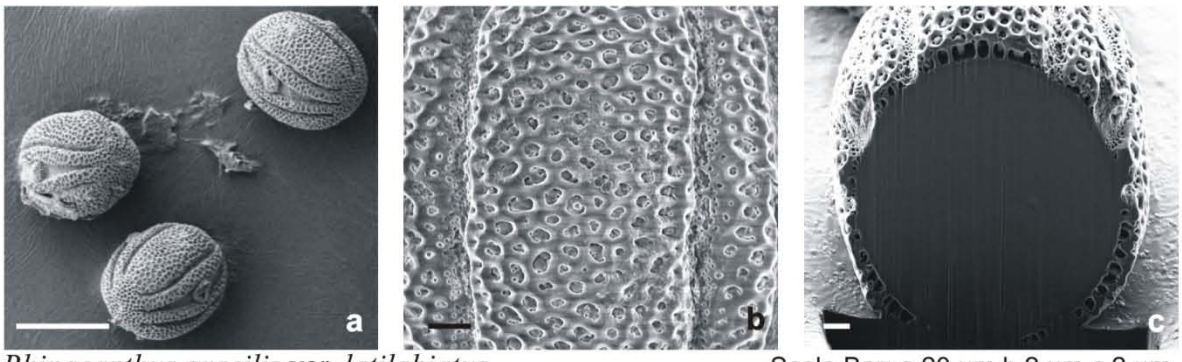
Metarungia longistrobus

Scale Bar: a 20 µm b 2 µm c 3 µm



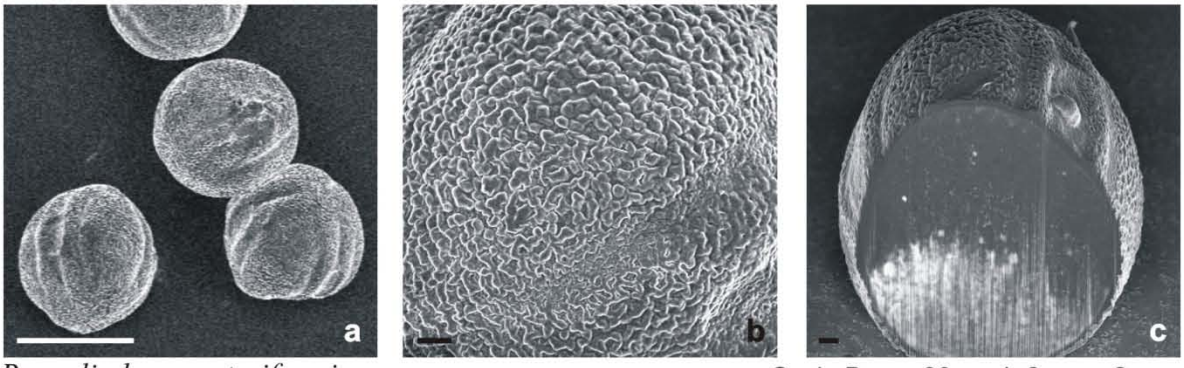
Peristrophe cernua

Scale Bar: a 20 µm b 2 µm c 2 µm



Rhinacanthus gracilis var. *latilabiatus*

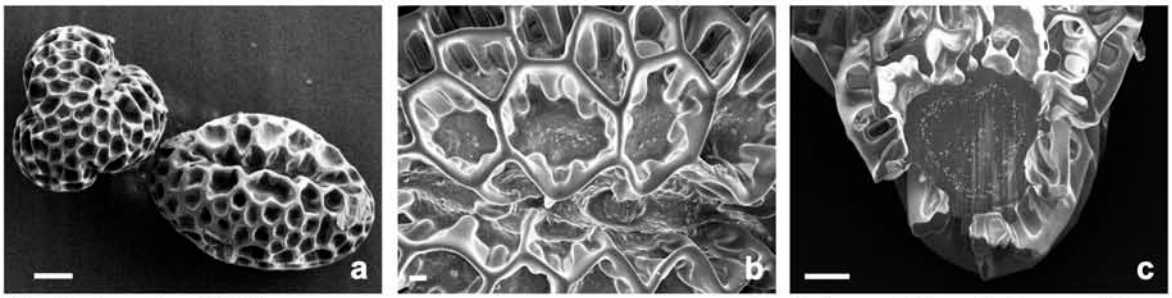
Scale Bar: a 20 µm b 2 µm c 2 µm



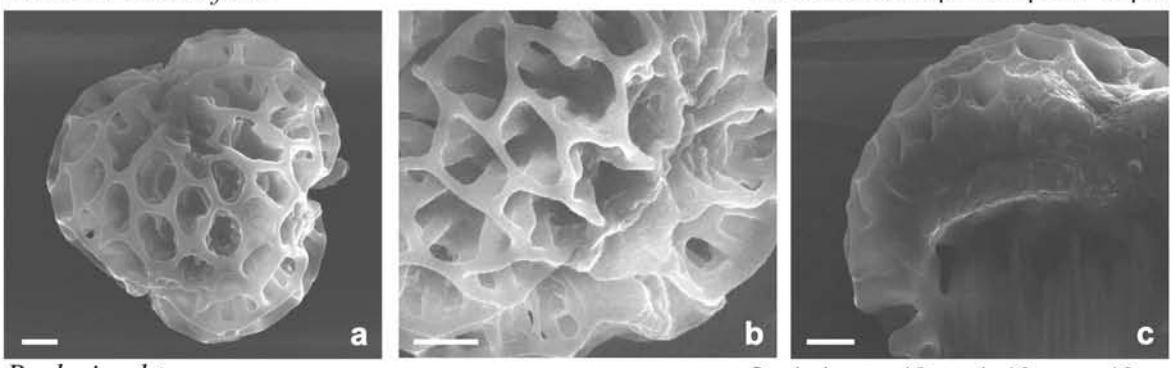
Ruspolia hypocrateriformis var. *nov*.

Scale Bar: a 30 µm b 2 µm c 2 µm

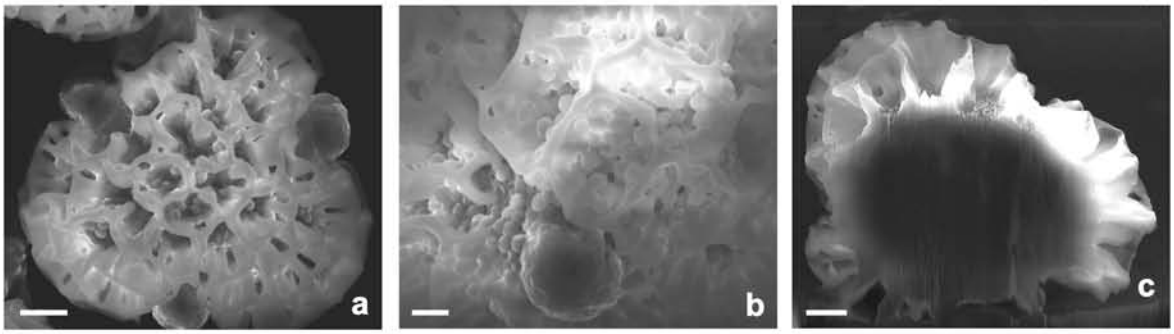
Barleriinae



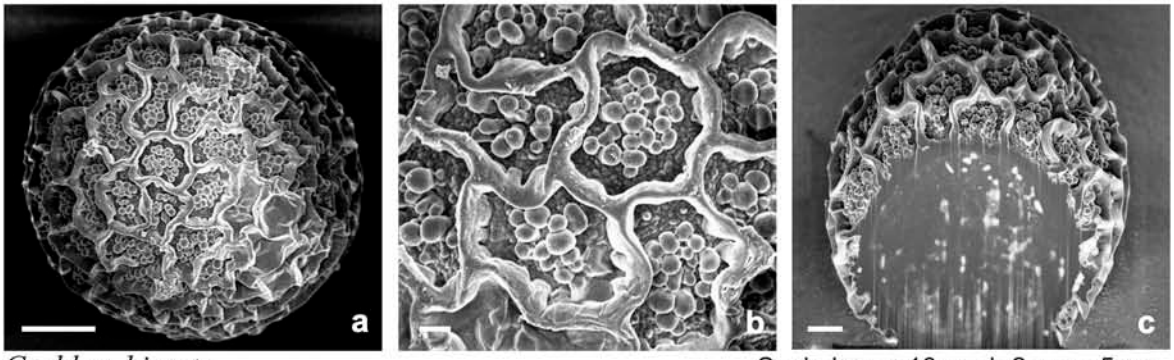
Barleria rotundifolia Scale bar: a 20 µm b 3 µm c 10 µm



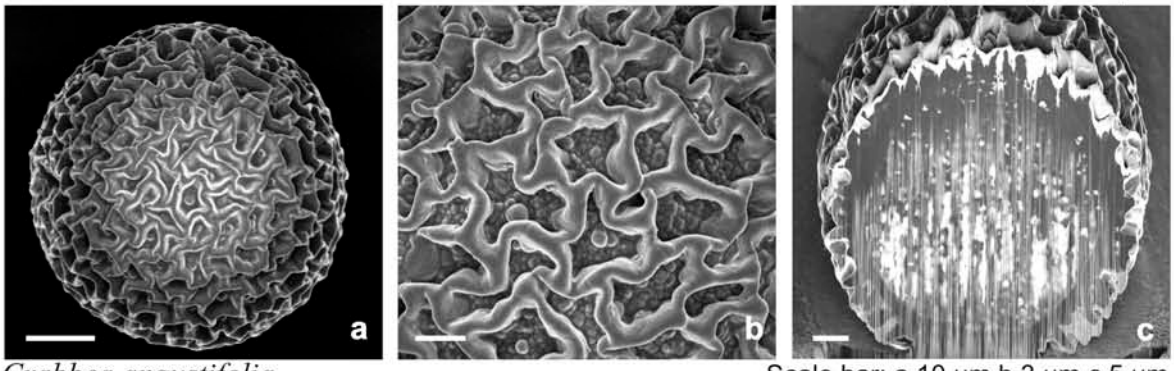
Barleria obtusa Scale bar: a 10 µm b 10 µm c 10 µm



Barleria greenii Scale bar: a 10 µm b 5 µm c 10 µm



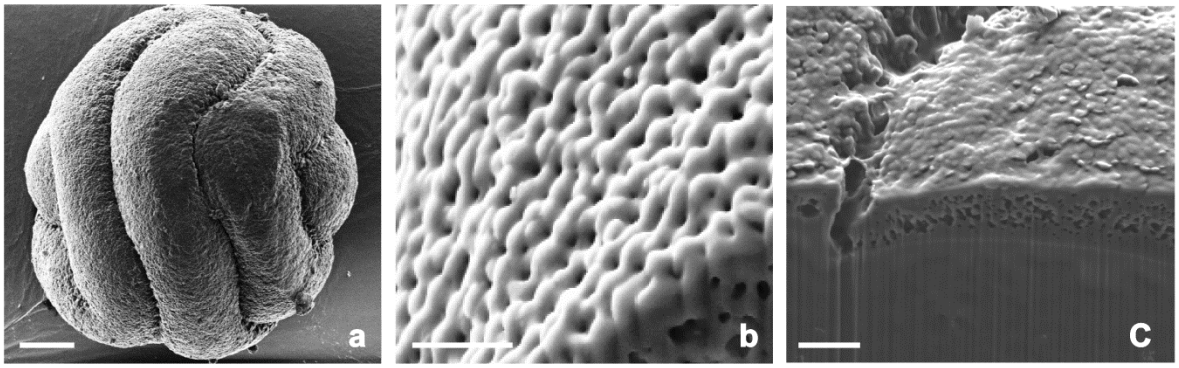
Crabbea hirsuta Scale bar: a 10 µm b 2 µm c 5 µm



Crabbea angustifolia Scale bar: a 10 µm b 3 µm c 5 µm

II. Acanthaceae

Thunbergioideae

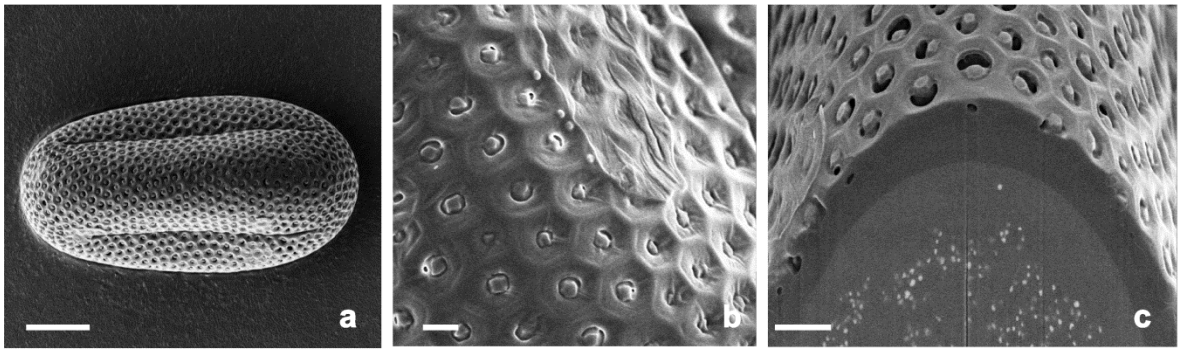


Thunbergia atriplicifolia

Scale bar: a 10 µm b 1 µm c 2 µm

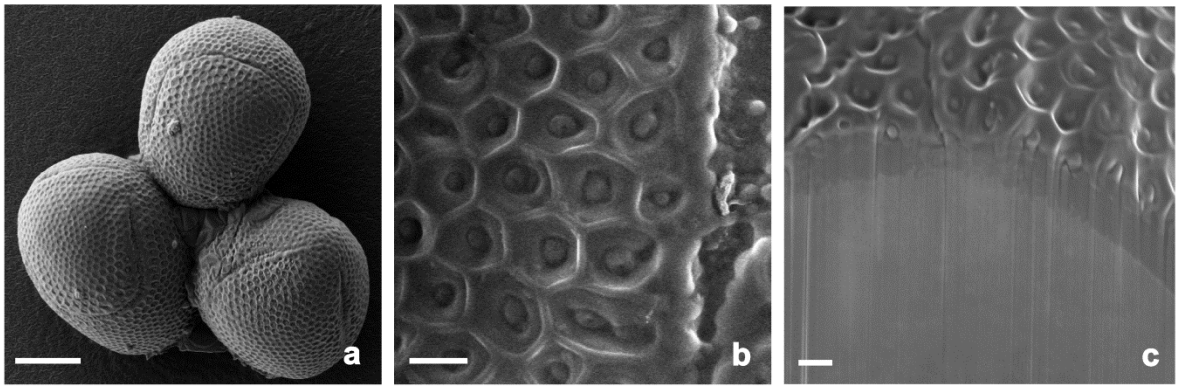
Acanthoideae

Acantheae



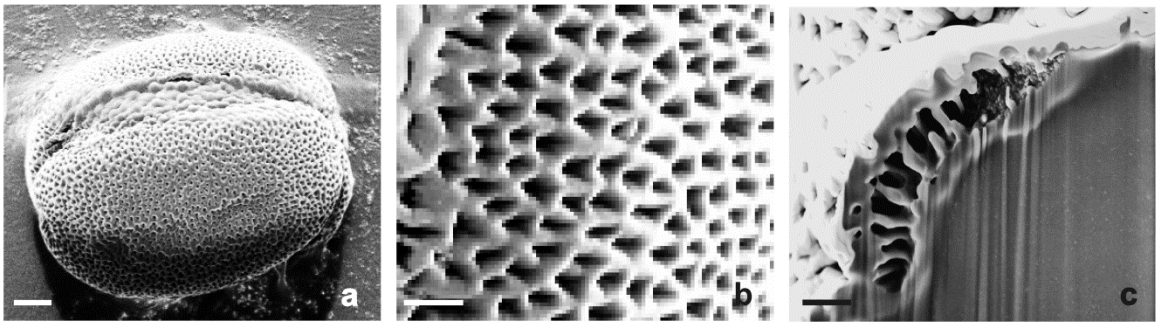
Acanthus mollis

Scale bar: a 10 µm b 1µm c 2 µm



Blepharis stainbankiae

Scale bar: a 10 µm b 1µm c 1 µm

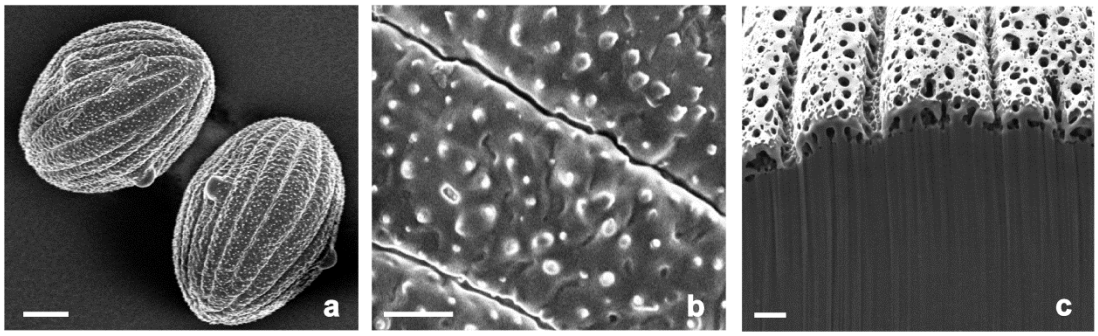


Sclerochiton harveyanus

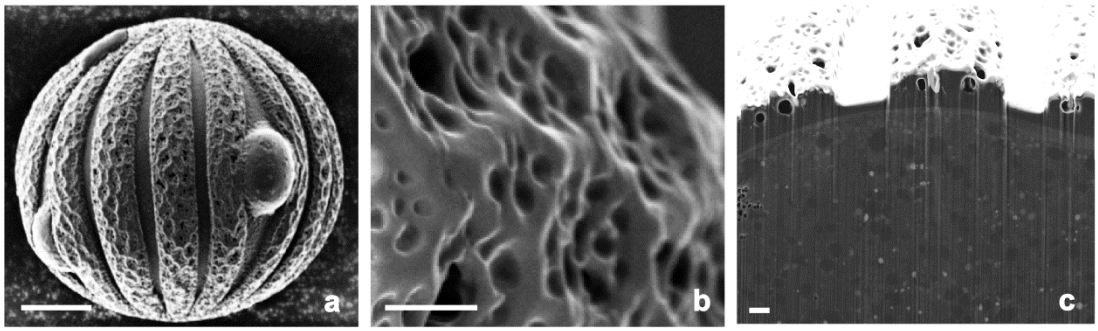
Scale bar: a 3 µm b 1µm c 1µm

Ruellieae

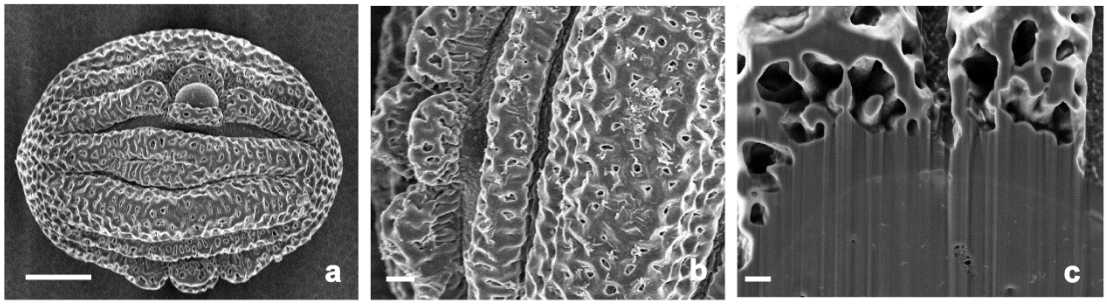
Ruelliinae



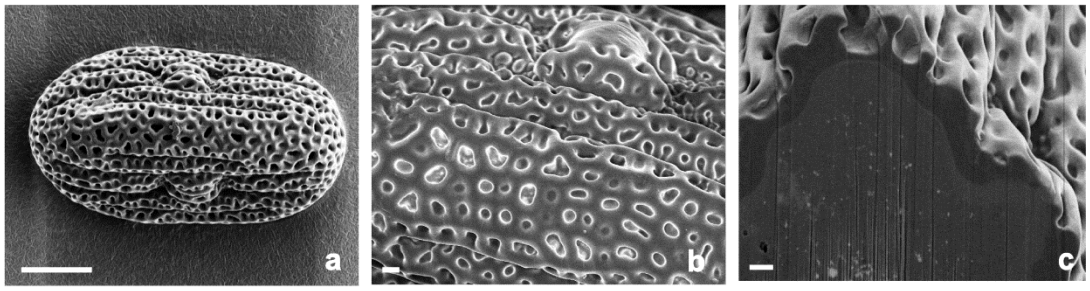
Dyschoriste fischeri Scale bar: a 10 µm b 2 µm c 1 µm



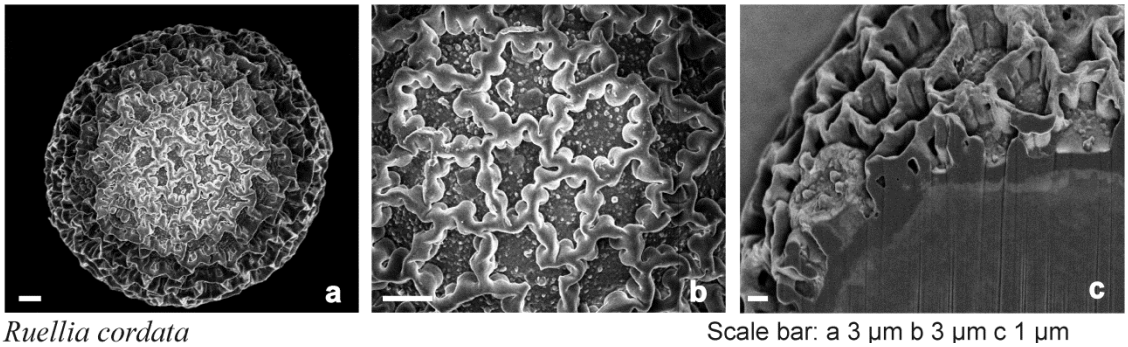
Hygrophila auriculata Scale bar: a 10 µm b 1 µm c 1 µm



Petalidium oblongifolium Scale bar: a 10 µm b 2 µm c 1 µm



Phaulopsis imbricata subsp. *imbricata* Scale bar: a 10 µm b 1 µm c 1 µm

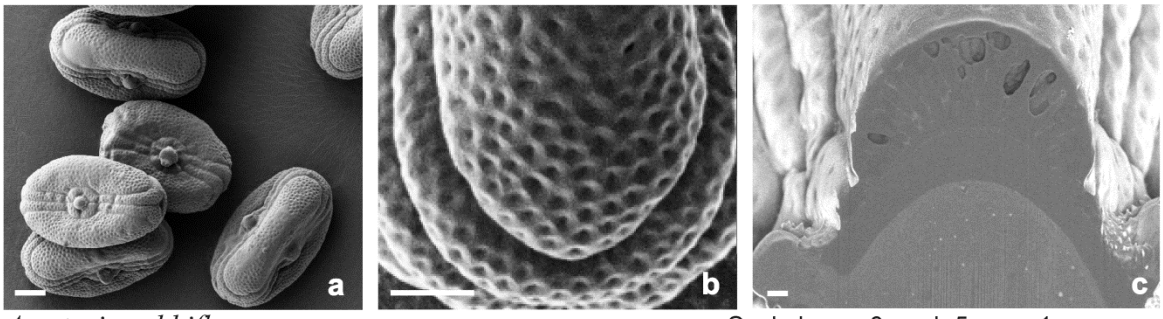


Ruellia cordata Scale bar: a 3 µm b 3 µm c 1 µm

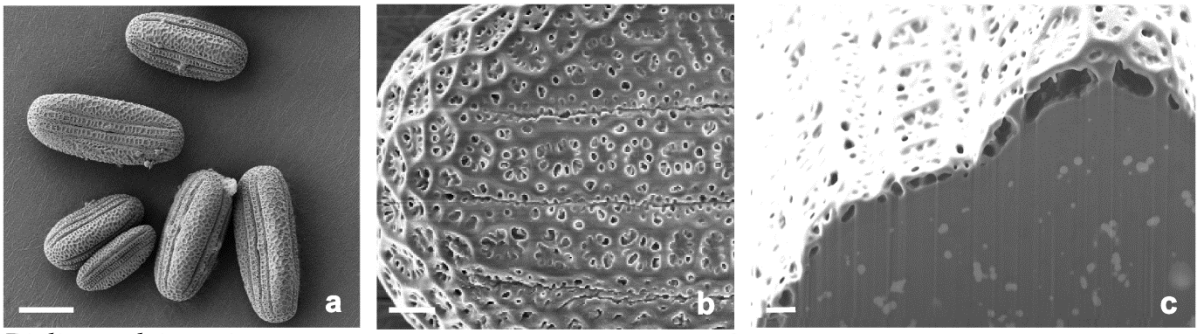
Justiciinae



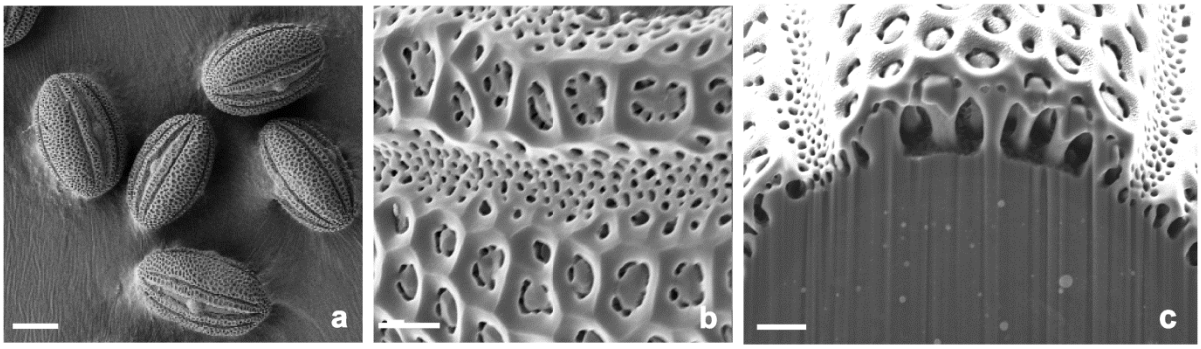
Anisotes formosissimus Scale bar: a 20µm



Asystasia subbiflora Scale bar: a 3 µm b 5 µm c 1 µm



Dicliptera leisterni Scale bar: a 20 µm b 2 µm c 1 µm



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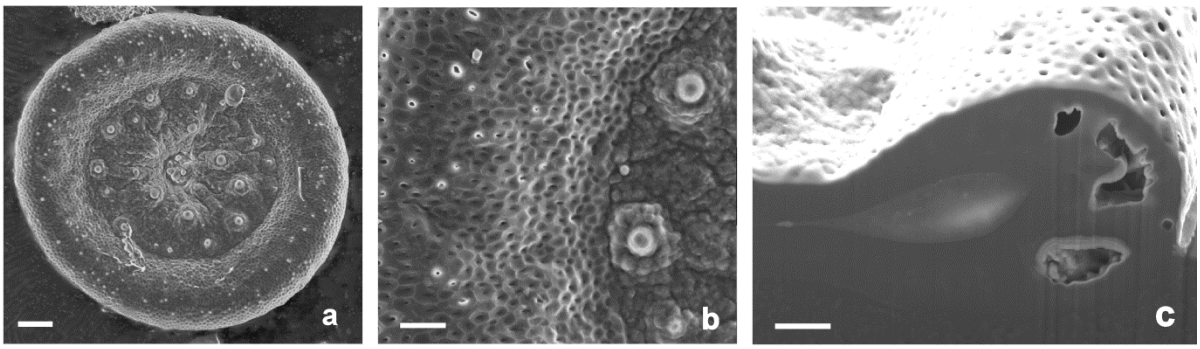

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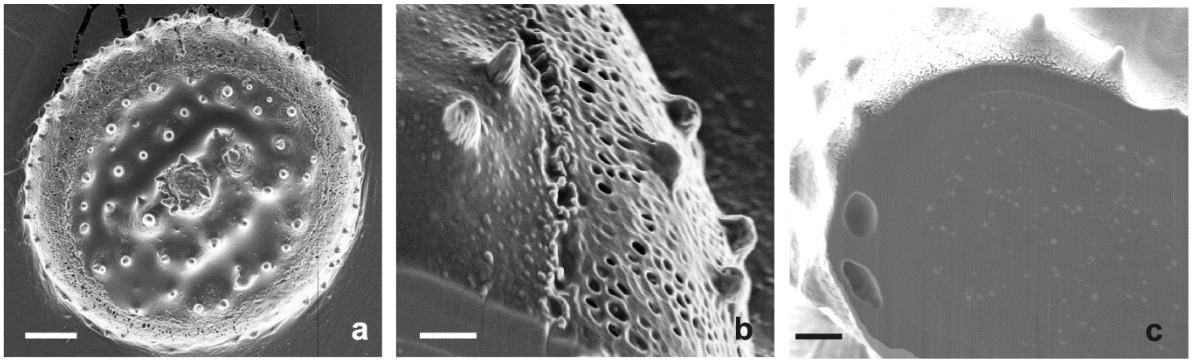

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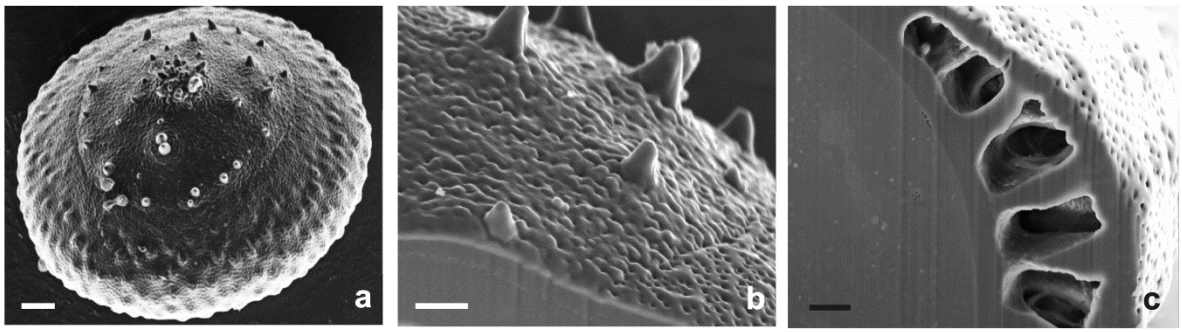

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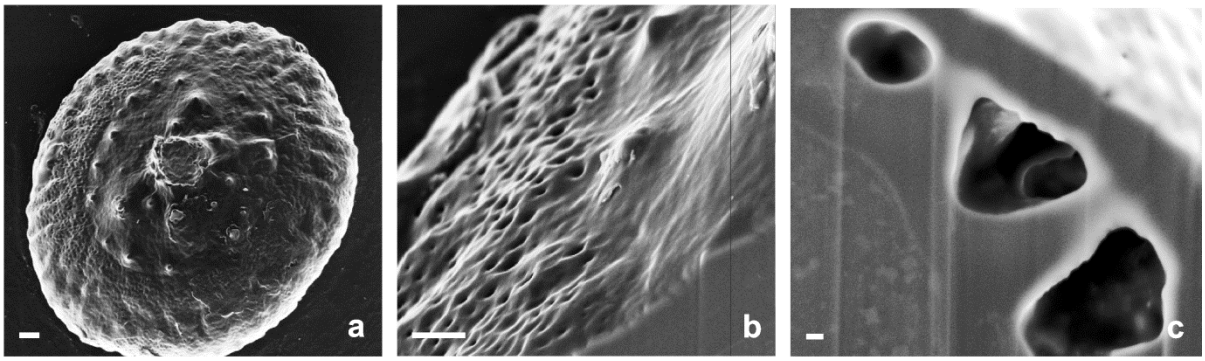
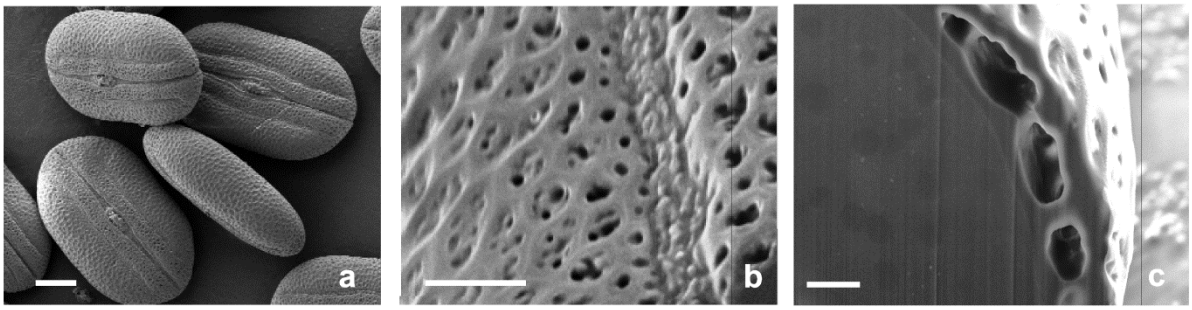


Isoglossa woodii Scale bar: a 2 µm b 1 µm c 0.2 µm



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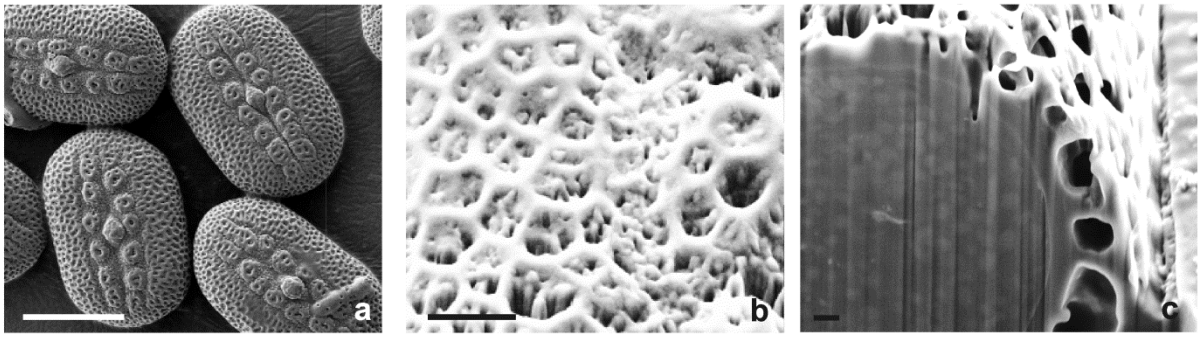

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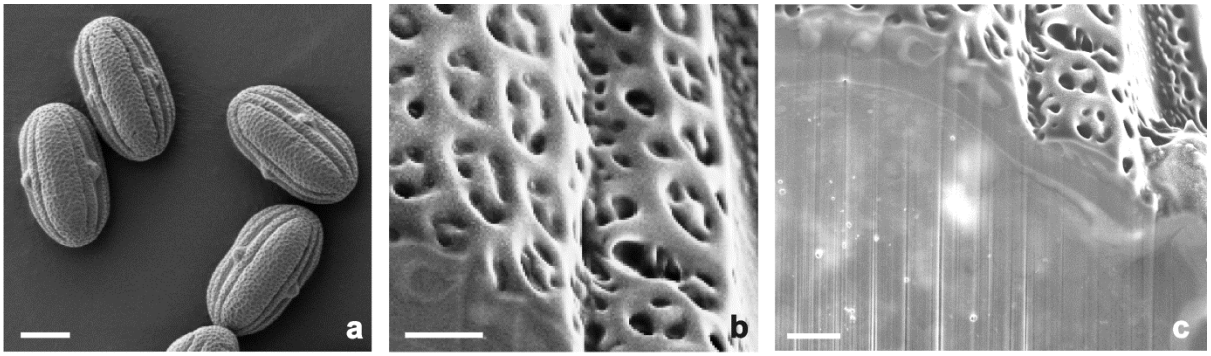

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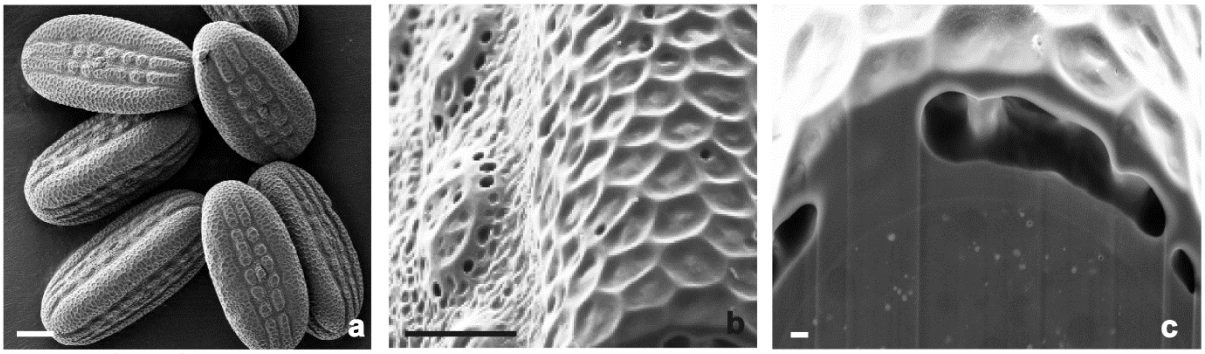

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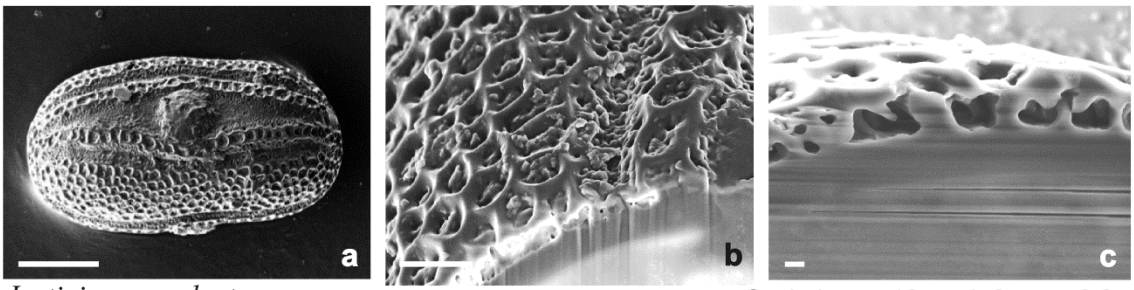
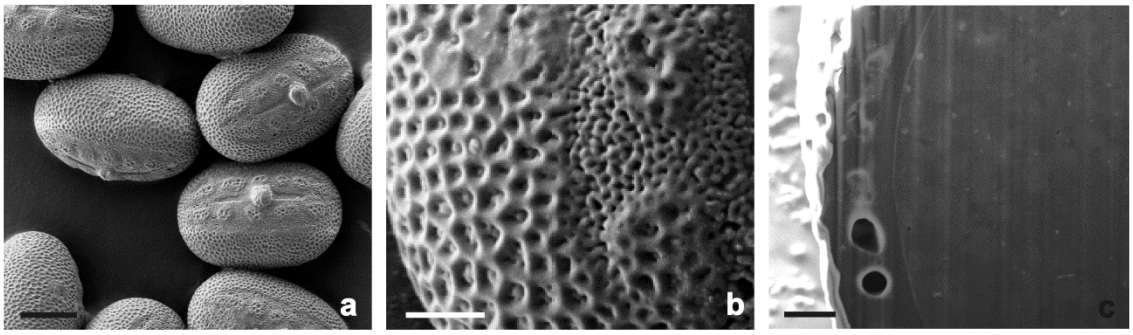


Justicia campylostemon Scale bar: a 10 µm b 2 µm c 0.3 µm



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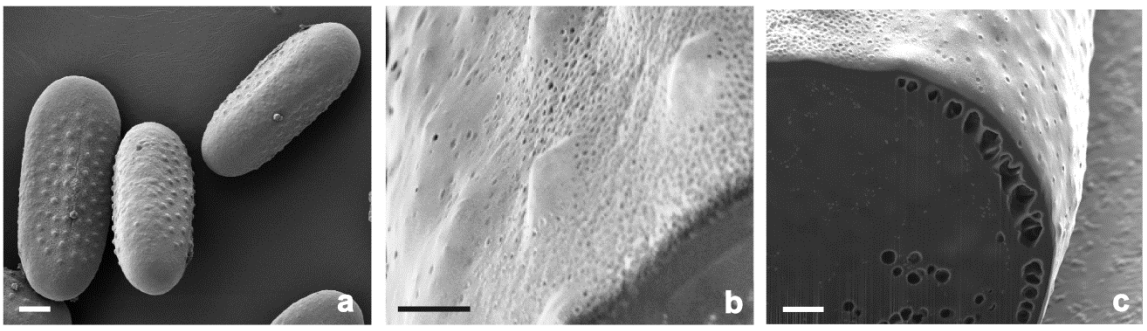
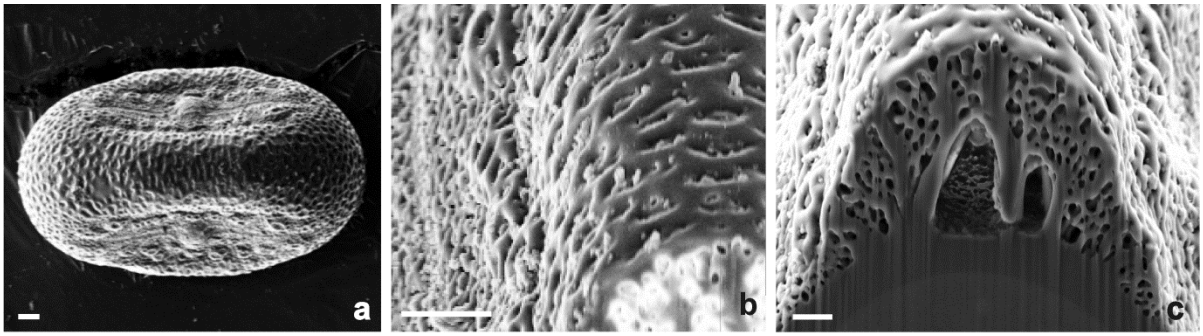


Justicia carnea Scale bar: a 10 µm b 2 µm c 2 µm



Justicia flava

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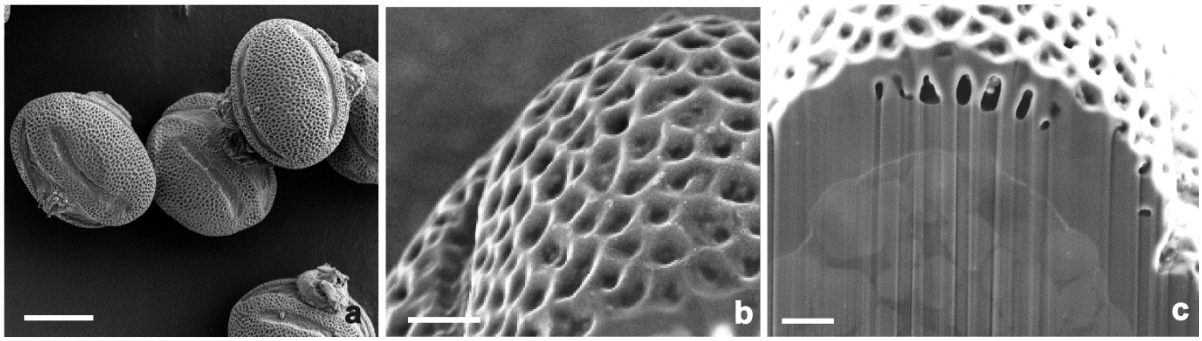

Justicia guerkeana

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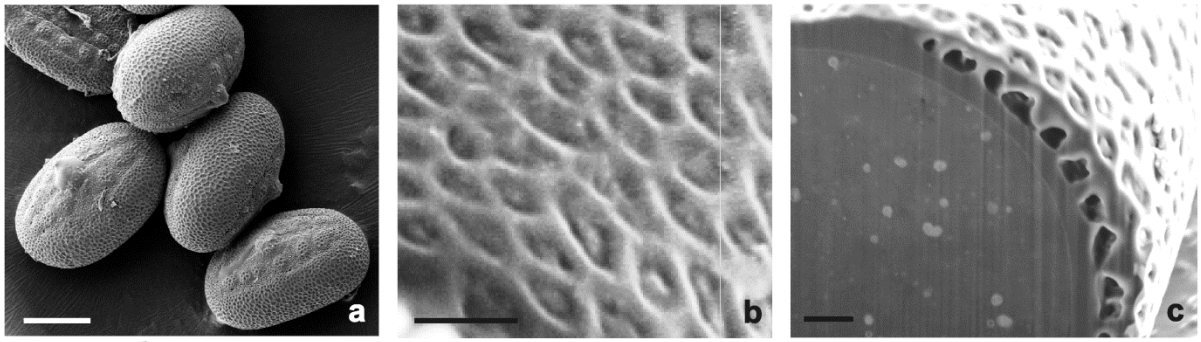

Justicia odora

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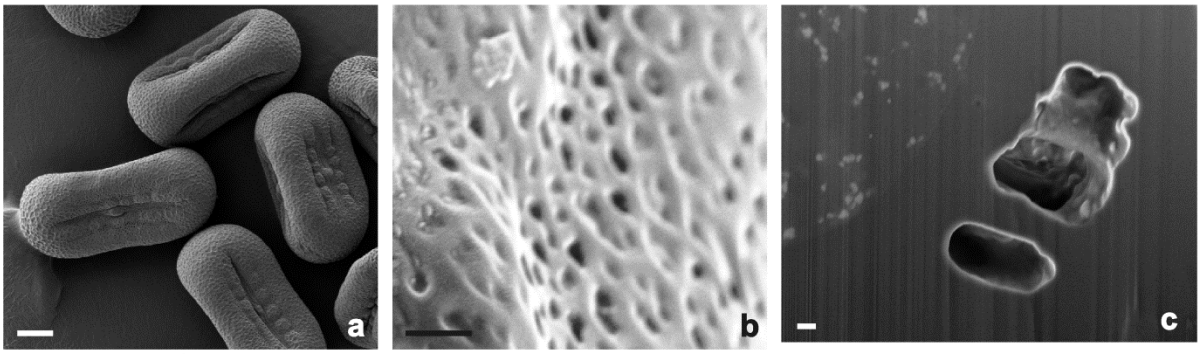

Justicia petiolaris

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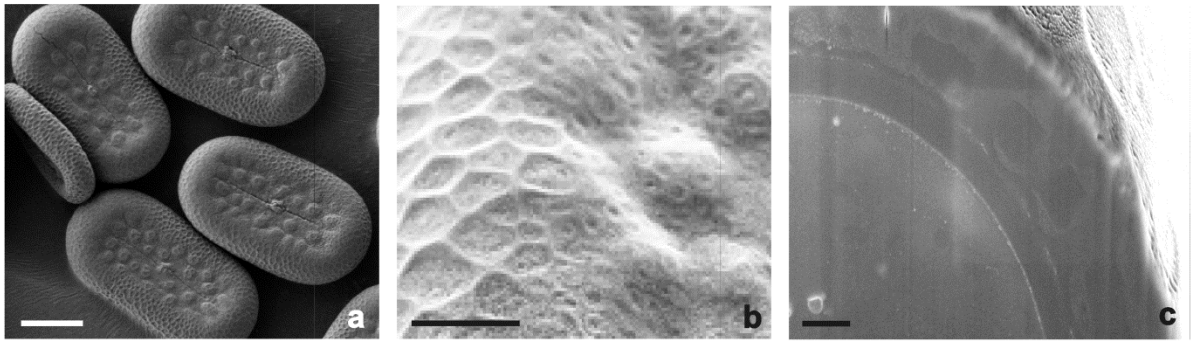

Justicia protracta

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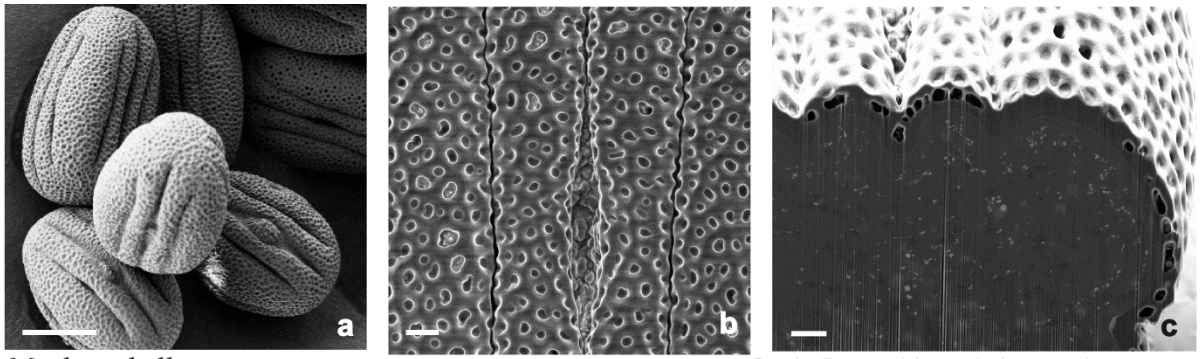

Mackaya bella

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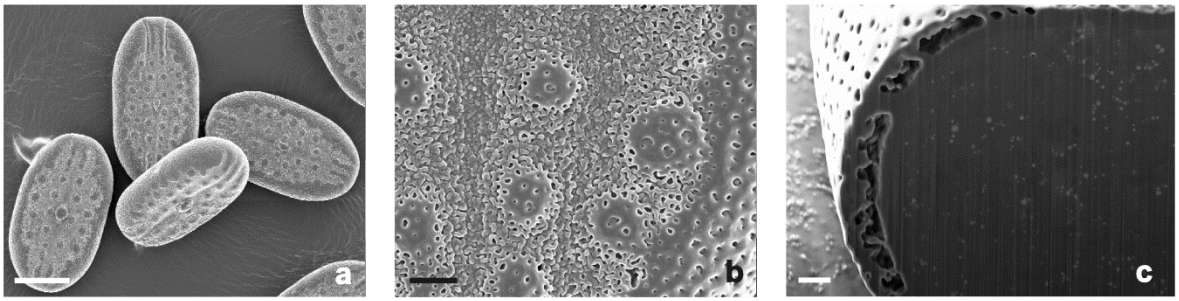

Metarungia longistrobus

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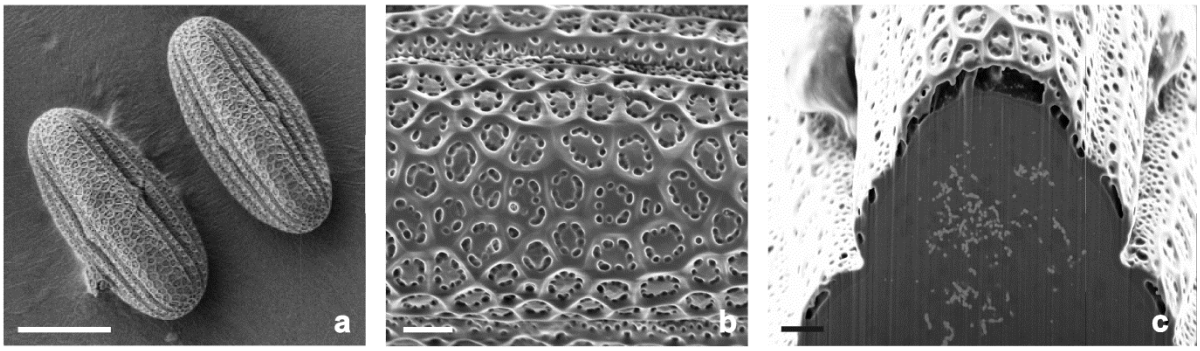

Peristrophe cernua

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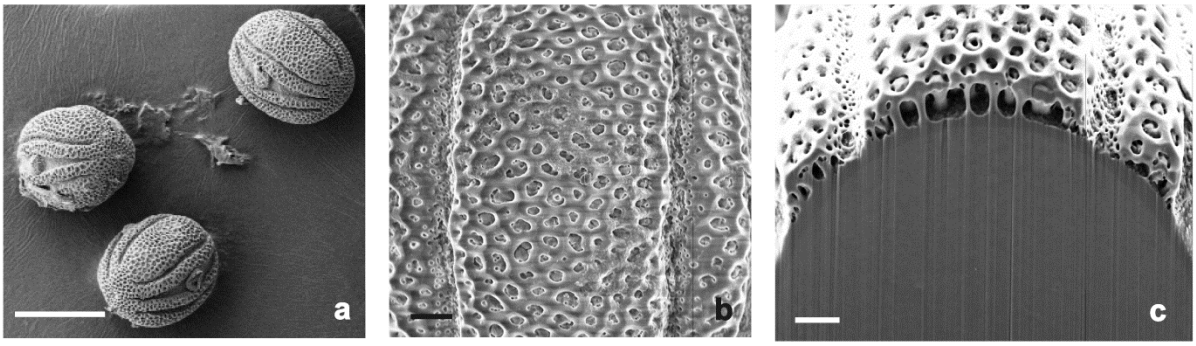
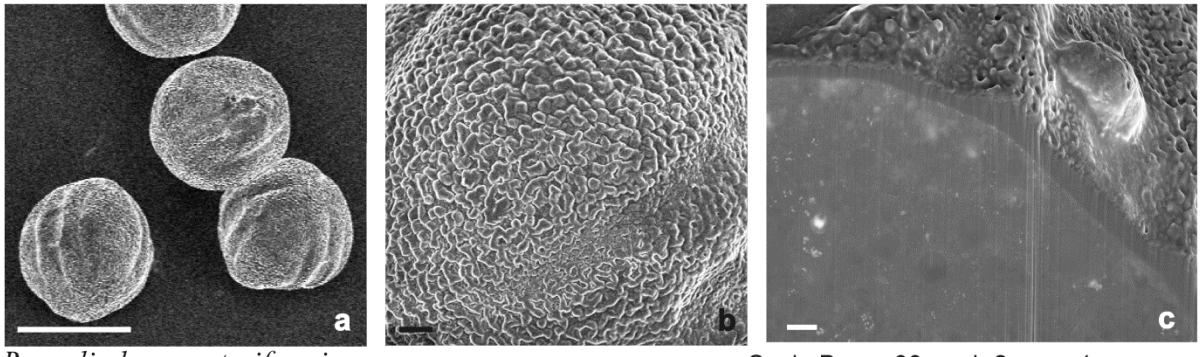


Rhinacanthus gracilis var. *latilabiatus*

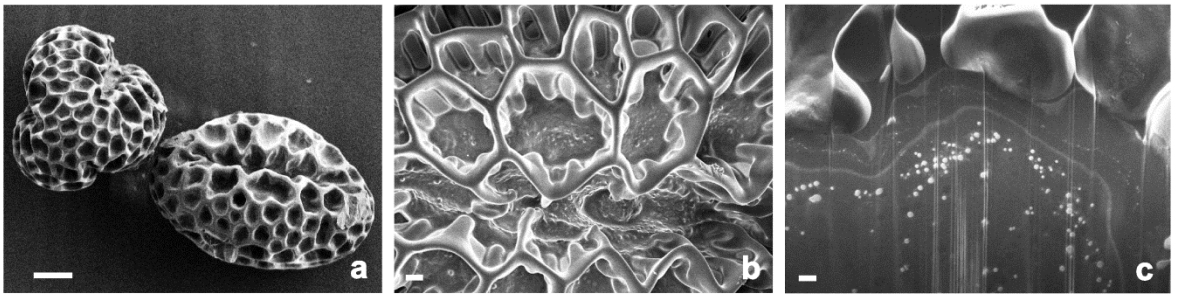
Scale Bar: a 20 µm b 2 µm c 2 µm



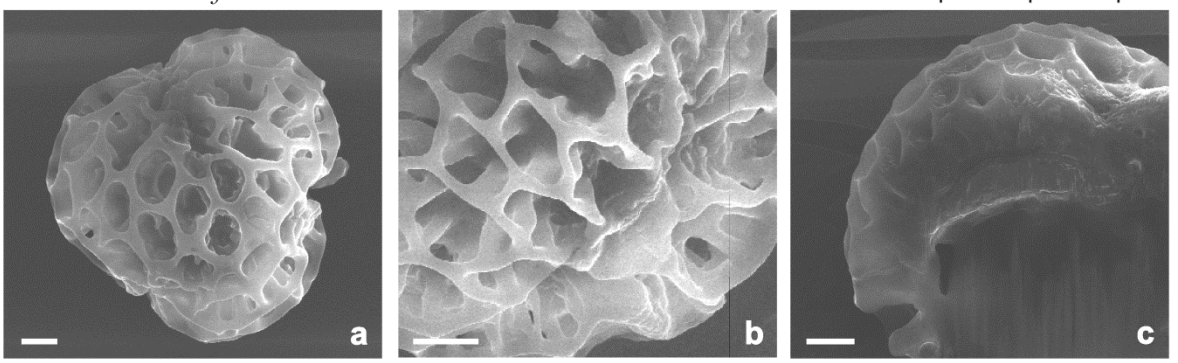
Ruspolia hypocrateriformis var. *nov.*

Scale Bar: a 30 µm b 2 µm c 1 µm

Barleriinae



Barleria rotundifolia Scale bar: a 20 µm b 3 µm c 1 µm



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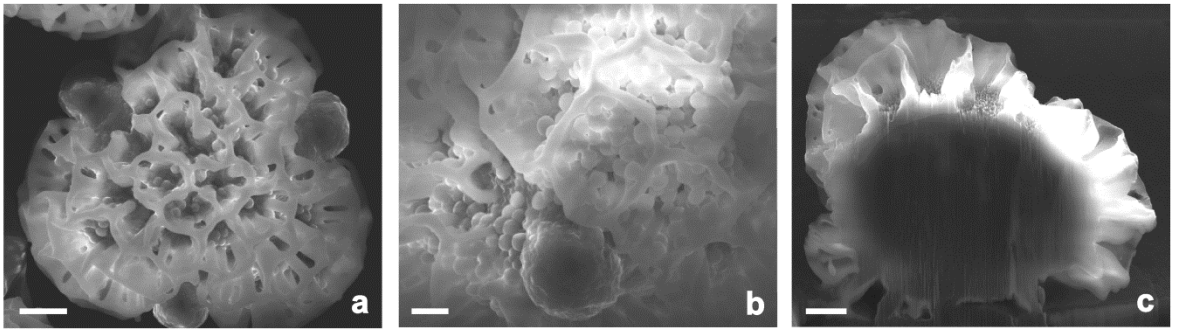
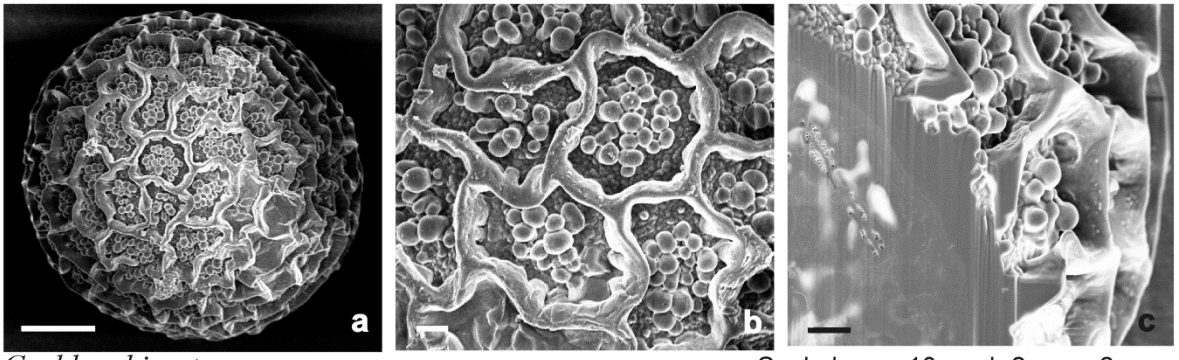
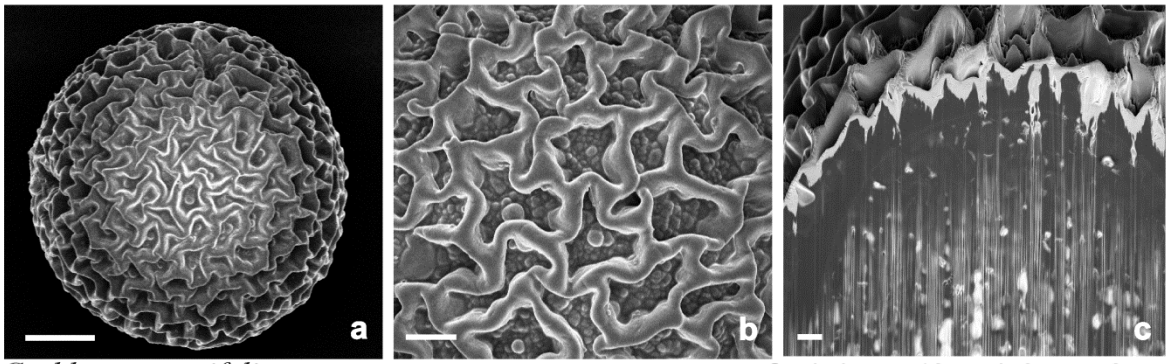


Barleria greenii Scale bar: a 10 µm b 5 µm c 10 µm



Crabbea hirsuta Scale bar: a 10 µm b 2 µm c 2 µm



Crabbea angustifolia Scale bar: a 10 µm b 3 µm c 2 µm

FIB-SEM: A new technique for investigating pollen walls

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The typically distinctive and elaborate external structures and sculpturing of pollen grains have been widely used in the taxonomy and systematics of the flowering plants. The family Acanthaceae is a striking example, where the widely diverse external pollen grain morphology has proved useful in determining relationships between taxa. Detailed information for external pollen wall morphology has traditionally been accumulated using light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) techniques. Internal pollen wall features from which the external sculpturing is derived, also exhibit complex and diverse characteristics, but lack investigation due to the difficult and cumbersome methods required to examine the underlying exine. Advancing technology in the field of microscopy has made it possible to view the internal structure of pollen grain walls in far greater detail and in 3D. A new technique involving precise cross sectioning or slicing of pollen grains at a selected position for examining pollen wall ultra-structure, using a focused ion beam-scanning electron microscope (FIB-SEM), has been explored and is yielding data which will prove to be of taxonomic importance. The FIB-SEM affords high resolution, 3D views for investigating internal pollen wall structure.

Keywords: Acanthaceae; exine; focused ion beam-scanning electron microscope; FIB-SEM; palynology; pollen wall ultra-structure

1. Introduction

The external pollen wall morphology has proved to be a reliable characteristic when formulating classification systems for flowering plants and this makes the study of pollen, or palynology, an attractive field for taxonomy and systematics, especially in families that show a wide variety of pollen surface features, such as the Acanthaceae [1, 2]. The members of this family are interesting from a palynological perspective in that they encompass a wide range of pollen morphological features, from supposedly primitive right through to more advanced types [2].

General pollen wall morphology is highlighted in figure 1. The terminology follows that of Walker and Doyle [3].

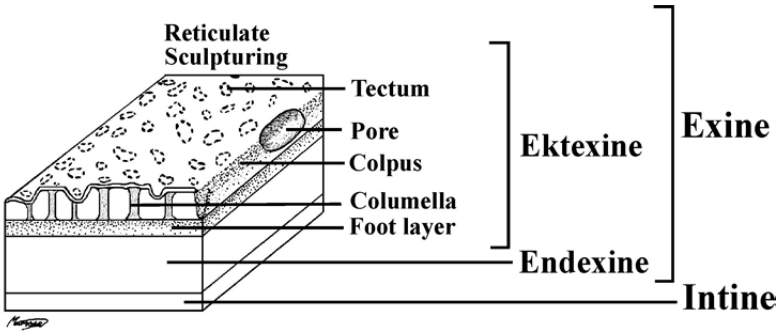

Fig. 1 A representation of a cross section through a pollen grain wall indicating the main external and internal wall characteristics.

A majority of the palynological data to date have been derived from external structures and sculpturing of pollen grains obtained from scanning electron microscopy (SEM) studies. Although light microscopy (LM) and transmission electron microscopy (TEM) have provided two dimensional (2D) images of the internal structure of the pollen grain wall, these data can be difficult to interpret. Currently, the internal structure of the pollen grain wall, which is suggested to be taxonomically important, is difficult to examine and hence lacks investigation. Although similarities can be drawn between certain external features, the possibility exists that pollen grains which have the same external appearance when viewed with the SEM may have distinctly different internal wall structures [4]. For example, species which have pollen grains with the surface sculpturing forming a network or reticulum, the most common sculpturing found in the Acanthaceae, could have very different underlying wall structures. In fact, focused ion beam-scanning electron microscopy (FIB-SEM) studies of internal pollen wall features in the Acanthaceae have so far identified five distinct, basic, internal pollen wall structures [5].

A new technique involving precise cross sectioning or slicing of pollen grains at a selected position for examining wall ultra-structure, using a focused ion beam-scanning electron microscope or FIB-SEM, has been explored [6]. The FIB-SEM is an instrument with a SEM column and a FIB column integrated in the same specimen chamber. The SEM uses a focused beam of electrons to image the sample in the chamber, and crucially, the FIB uses a focused beam of

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gallium ions to cut or mill a cross section while the sample remains in the chamber. Specimen material is milled away at a selected site to a chosen depth, with each pass of the beam, allowing for precise cross sectioning of the specimen. Although typically used for semiconductor research, the ultra-structure of fossilized pollen walls has previously been investigated using FIB techniques with promising results [7]. FIB techniques have also been used to characterize fragile and delicate biological specimens by cutting a window through the surface to observe subsurface layers [8], or by cutting an ultra-thin layer or lamella out of the specimen to view with a TEM [9], or by FIB tomography, which involves obtaining serial sections through a resin embedded specimen [10]. In these cases, the specimens are fixed and prepared in the same way as for TEM examination.

The outer layer of the pollen wall or exine is composed of an extremely strong and resistant substance known as sporopollenin, which due to its nature, requires complex sample preparation to obtain results from TEM. With FIB sectioning, however, a single pollen grain may be successfully sectioned without using the extensive sample preparation required for TEM work. Sectioning pollen grains for TEM examination is a rather haphazard process where sectioning and viewing of samples are carried out on separate machines. Whereas, with the FIB technique, the exact position of the region to be sectioned can be selected. In addition serial sections of an entire pollen grain may be obtained where the grain is viewed and imaged with the SEM portion of the microscope after each cut, to prevent further damage to the specimen. This allows for high resolution 3D investigation of the pollen grain wall and as the sectioning is achieved inside the microscope chamber, the results are instantaneous.

2. Methodology

2.1 Test species

Pollen from a test species *Isoglossa woodii* C.B.Clarke was examined with LM, SEM and FIB-SEM and compared to TEM cross sections of pollen grains of this species, obtained from a previous study conducted at the University of the Witwatersrand.

2.2 Collection and preparation of pollen grains

Fresh, whole flowers with ripe anthers were collected and placed in small herbarium paper envelopes, and stored in silica gel granules. Individual pollen grains were collected directly from the anthers under a dissecting microscope and transferred to a glass slide or a carbon coated disc using fine insect pins.

2.3 Light microscopy

A small clump of Fuchsin jelly was placed on a glass slide and pollen grains were transferred onto the jelly. This was melted and a cover slip placed on top of the liquid and then viewed and imaged using a Zeiss Axio Imager.M2 light microscope.

2.4 FIB-SEM microscopy

A carbon coated disc with pollen grains was attached to a SEM stub and allowed to dry for 24 h. Samples were coated with a gold-palladium alloy at 20 mA for 1 min in order to lend stability and minimize charging. Samples were viewed with a Zeiss Auriga microscope with a dual-crossbeam FIB workstation and Gemini FE SEM column at the Council for Scientific and Industrial Research (CSIR), Brummeria, Gauteng, South Africa. Selected pollen grains were sectioned once at a predetermined site.

A detailed method is explained in House and Balkwill [6].

3. Comparison of microscopy data

Light microscopy is very useful when identifying external and some internal features in the pollen grain wall. Extensive light microscope studies of pollen morphology in the Acanthaceae have been conducted [11, 12] in order to formulate a more comprehensive classification system for this diverse family. These studies have more recently prompted researchers to examine the external features of pollen grains in greater detail and in 3D with the aid of the SEM for the family as a whole [1] and for individual genera [e.g. 13, 14, 16]. External pollen morphological features have been used as characteristics for defining taxa and relationships between taxa. These studies not only highlighted the usefulness of pollen morphology in formulating classification systems, but also the shortfall of examining external features alone because the internal pollen wall characteristics have not, to date, been the focus of investigations. Transmission electron microscopy investigations have been conducted on a few individual genera [e.g. 13, 14, 15]. For the purposes of comparison in this study, pollen grains of *Isoglossa woodii* were examined.

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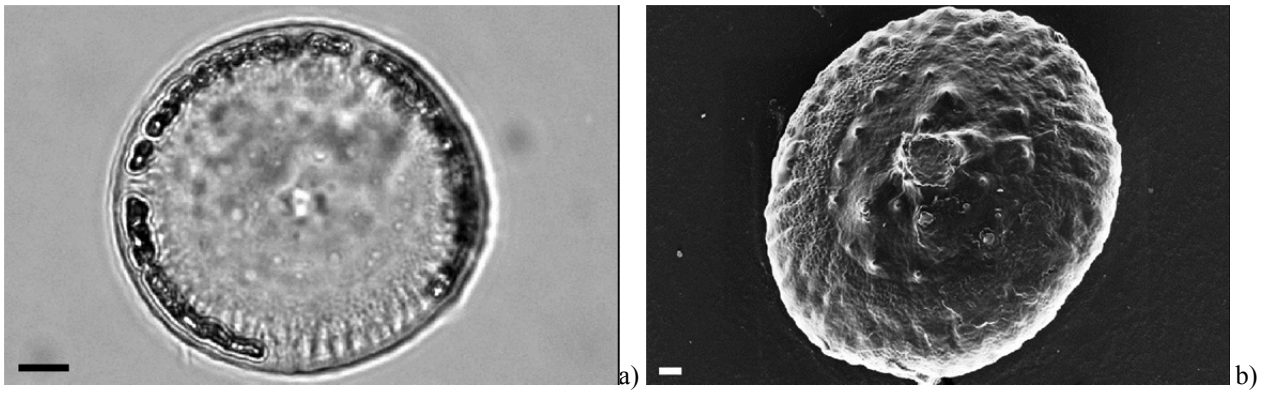

Fig. 2 a) LM of broad surface of *Isoglossa woodii*, showing external sculpturing detail and limited internal structure. Scale bar = 5 µm. b) SEM of *I. woodii* showing surface sculpturing in 3D detail. Scale bar = 2 µm.

The external reticulate sculpturing and scattered spines over the surface are clearly visible with LM (fig. 2a) and these features are also clearly discernible in 3D (fig. 2b). Some internal structures can be seen with the LM (fig. 2a), where a ring of dark cavities is visible just within the circumference of the grain, known as the girdle area. This structure is verified with the TEM (fig. 3a), where detail of the internal wall structure shows large cavities in the girdle area. The three dimensional structure of these cavities within the pollen wall becomes apparent in greater, unambiguous detail when viewed after FIB-SEM techniques are applied (fig. 3b). With LM and TEM, internal structure is seen in 2D. The images obtained with FIB verify this structure in 3D, with the obvious advantage of confirming the observed features within the wall as being empty cavities and not areas of different density or material. Furthermore, an impression of the extent to which each cavity extends through the girdle area is better obtained from the FIB section, as well as a view of how, if at all, the cavities are inter-connected. Overall, the FIB sections afford a far more comprehensive, clear and detailed view of the internal wall structure.

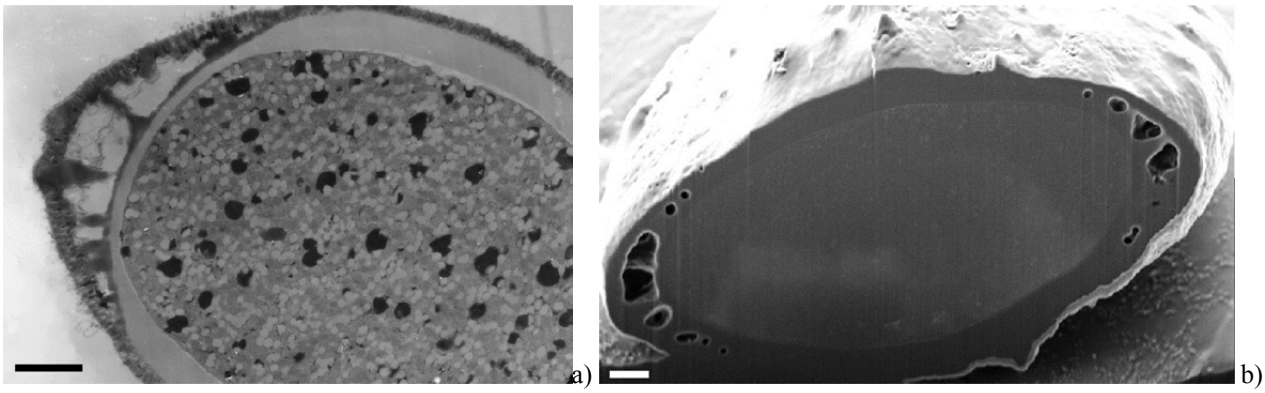

Fig. 3 a) TEM showing internal wall detail and cavities in the girdle area. Scale bar = 2 µm. b) FIB-SEM section showing 3D detail of external and internal pollen wall morphology. Scale bar = 2 µm.

FIB-SEM results obtained from pollen of various species of the Acanthaceae, affording detailed, 3D perspectives, have now resulted in the identification of at least five basic internal wall structures in this family, with further investigations likely to reveal more. This methodology has made it far easier to distinguish between cavities in the walls (fig. 4C) and tunnels running through the walls (fig. 4E). It is now also possible to determine if cavities or tunnels have distinct internal columellae (fig. 4D), or whether the walls of adjacent cavities or tunnels are merely giving the impression of columellae.

The five internal wall structures identified thus far in the Acanthaceae are depicted in fig. 4. They are described as:

1. Porate, sponge-like in appearance – e.g. *Thunbergia atriplicifolia* (fig. 4A).
2. Solid internal wall with no cavities, columellae, tunnels or other features – e.g. *Acanthus mollis* (fig. 4B).
3. Cavities or chambers irregularly dispersed throughout the wall – e.g. *Justicia petiolaris* (fig. 4C). These cavities are typically not an interconnected network of tunnels and occur discretely as they dwindle and disappear in successive sections.
4. Distinct, individual, regularly arranged columellae – e.g. *Rhinacanthus gracilis* (fig. 4D).
5. Labyrinthine interconnected tunnels – e.g. *Dicliptera leistneri* (fig. 4E).

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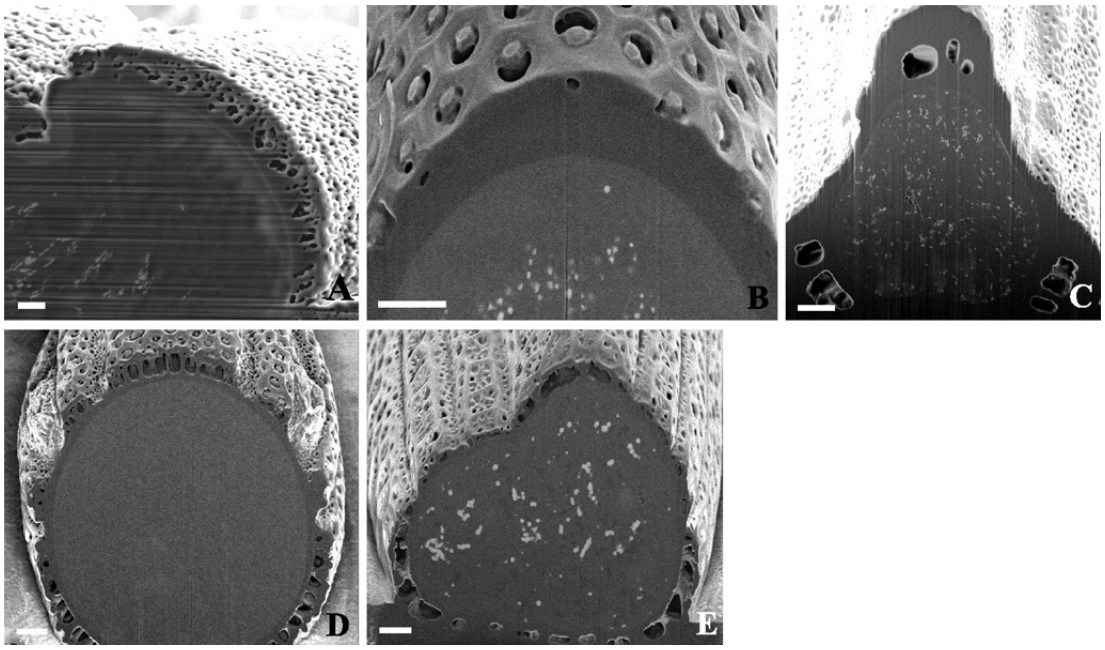

Fig. 4 FIB-SEM cross sections showing 3D detail of the internal pollen grain walls of five species of Acanthaceae:

A. *Thunbergia atriplicifolia*. Scale bar = 1 μm.
B. *Acanthus mollis*. Scale bar = 2 μm.
C. *Justicia petiolaris*. Scale bar = 3 μm.
D. *Rhinacanthus gracilis*. Scale bar = 2 μm.
E. *Dicliptera leistneri*. Scale bar = 2 μm.

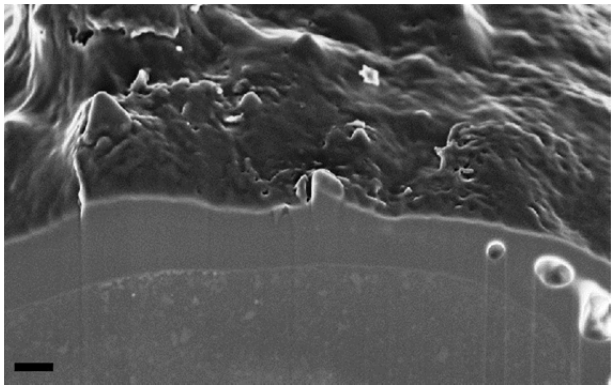

Fig. 5 FIB-SEM of the internal pollen wall showing the endexine layer protruding into an external spine. Scale bar = 1 μm.

Due to the perspective obtained from FIB-SEM work on pollen grains, it is now also possible to determine which internal layers give rise to the external features (fig. 5), or more accurately observe the relationship between the external features and the internal structures directly underlying them. In this example (fig. 5), a section of the endexine can be seen protruding upwards into the surface sculpturing on a pollen grain.

3. Conclusions

The FIB-SEM affords high resolution, 3D views for investigating internal pollen wall structure, which supplements the information gained from other methods of microscopy. These additional data contribute to a better understanding of which internal wall layers and components give rise to the external features of the pollen grain wall and the relative structure of the internal wall.

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