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FIB-SEM enhances the potential taxonomic significance of internal pollen wall structure at the generic level

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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 five closely related genera was investigated using a focused ion beam-scanning electron microscope (FIB-SEM). 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 in the mesocolpium region. This internal wall layer contains elongate, arched, walled cavities or polygonal, walled chambers, each with or without a central columella. A morphometric analysis using a combination of documented external characters and the internal wall structure characters obtained in this study was conducted. The resulting tree clustered the taxa (the 18 selected species) into separate groups broadly in line with existing classifications. This verifies that the characters identified by means of FIB-SEM have the potential to be applicable in taxonomic studies at genus level.

1. Introduction

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 with the interpretation of the phylogeny of the family. 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). 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 three dimensions (3D) with the possibility of identifying internal features or characters of potential taxonomic value. A new technique involving precise cross-sectioning or slicing of pollen grains at a preselected position using a focused ion beam-scanning electron microscope (FIB-SEM), has proved most useful for examining the internal structure of pollen grains in 3D (House and Balkwill 2013, 2016). The FIB-SEM is an instrument with a FIB column and a SEM column integrated in the same vacuum or specimen chamber. The FIB system is operated at high beam currents and uses a finely focused beam of ions (Ga^+) to mill or cross-section the specimen at a chosen position. Directly thereafter the SEM is operated at low beam currents and uses an electron beam to image the cut specimen in 3D. Results obtained with this technique allow one to identify distinct features which otherwise appear similar when viewed as thin cross-sections or surface scans and these features have the potential to assist in making taxonomic decisions at tribal level (House and Balkwill 2016).

The members of the large and contentious genus *Justicia* L. are interesting from a palynological perspective in that they encompass a wide range of morphological pollen structures. Consequently, it was felt that *Justicia* could afford a pertinent opportunity to test the taxonomic and systematic merits of internal pollen wall structures for use at generic level.

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

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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 2004); in Paraguay and Argentina (Ezcurra 2002); in tropical Africa (Hedrén, 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 von Esenbeck (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 (1989) 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.

The aim of this paper is to determine whether FIB-SEM technology will provide characters of internal structure of pollen walls in *Justicia* that may be useful in determining the relationships among the species of the genus. If this is the case, it will suggest that FIB-SEM is a practical technique for obtaining characters to use for making taxonomic decisions at generic level.

2. Materials and methods

2.1. Terminology

The main external and internal pollen wall terminology used in this paper is explained in Fig. 1, p. 1536 of House and Balkwill (2013), and in Fig. 1, p. 227 of House and Balkwill (2016). For the sake of clarity and convenience the terminology used to describe the pollen grains in this study is further highlighted in labelled micrographs (Fig. 1), with the external features indicated in Fig. 1a and the internal wall layers in Fig. 1b. The special morphological terms used: margocolpus (Immelman, 1989), mesocolpium and trema region (Raj, 1961) are identified.

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 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, Subsection 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 in 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 five other genera of Justiciinae was also investigated. These are:

Hypoestes forskaolii (Vahl)R.Br.

Rhinacanthus latilabiatus (K.Balkwill) I.Darbysh.

Dicliptera cernua (Nees) J.C.Manning & Goldblatt (previously *Peristrophe cernua* Nees)

Dicliptera leistneri K.Balkwill

Mackaya bella Harv.

Metarungia longistrobus (C.B.Clarke) C.Baden

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

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. Sample preparation was in line with that for standard SEM investigation. In order to stabilise the specimens and prevent charging samples were lightly coated (5 nm) with Au-Pd alloy or with carbon if Au-Pd alloy was not available (for *J. campylostemon*). Samples were viewed with a FIB-SEM (Auriga FIB FE SEM, Carl Zeiss) with a Cobra FIB column and Gemini FE SEM column. Suitable pollen grains were selected, photographed, orientated for sectioning and each pollen grain was sectioned once with the FIB at a selected site. The coarse milling current used for cross-sectioning was 4 nA and each cut took approximately 45 min. The cut surface was then polished at a lower beam current of 600 pA to remove deposits and reveal more detail. Directly thereafter the pollen grains were imaged with the SEM portion of the microscope, all within the microscope vacuum chamber. Analysis of this kind identifies areas of different chemical composition and electron density making it possible to distinguish the various layers of the pollen wall which are represented in different shades of grey. It was therefore possible to measure the width of the ectexine and endexine using a line measuring tool. The FIB was operated at 30 kV and imaging with the SEM was conducted at 15 kV. This work was conducted at the CSIR, Brummeria, Pretoria. A detailed explanation of the method and rational is given in House and Balkwill (2013).