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RESEARCH ARTICLE

COMPARATIVE LEAF MICROMORPHOLOGY AND PALYNOLOGY OF SELECTED *Ruellia* L. AND *Justicia* L. SPECIES FROM ACANTHACEAE FAMILY AND ITS TAXONOMIC SIGNIFICANCES

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Abstract. A leaf micromorphology and palynological study was conducted on the selected Acanthaceae species namely *Justicia betonica* L, *Justicia carnea* Lindl., *Justicia procumbens* (L.) Lam., *Ruellia repens* L., *Ruellia simplex* C. Wright and *Ruellia tuberosa* L. Taxonomists often faced difficulties in identifying and classifying species within the Acanthaceae family, especially when plant specimens obtained from the field samplings are incomplete such as the absence of flowers and fruits. Thus, the objective of this study is to identify and list the characters of leaf micromorphology and palynology that are useful in identification of species in Acanthaceae. The procedures involved such as dehydration, critical point drying, gold coated and examination under scanning electron microscope Zeiss Supra 55VP and were analyses using SmartSEM software. Results revealed the common and variations characteristics of leaf micromorphology that can be useful in identification of species and genera studied such as type of epicuticular waxes, cuticular ornamentations, stomata characteristics and the presence of trichomes. Findings in this study also have shown some variations in the pollen morphology that can be used in species identification and classification. In conclusion, the results have demonstrated that leaf micromorphology and pollen morphology characteristics have taxonomic significance and can be used as an additional data especially in identification and classification of species as well as genera of Acanthaceae.

Keywords: Acanthaceae, leaf micromorphology, palynology

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1. INTRODUCTION

The family Acanthaceae has been placed under the order Lamiales along with the families Verbenaceae, Bignoniaceae, Schorphulariaceae, Lamiaceae, Gesneriaceae and Plantaginaceae [1]. This family has more than 3000 species in the area tropical and subtropical [2] meanwhile [3] recorded as many as 29 genera and 129 species which has been identified in Peninsular Malaysia. The Acanthaceae family consists of mainly herbaceous plants and shrubs, rarely climbing plants (subfamily Thunbergioideae) and trees [4]. This family can be characterized through certain characteristics such as the presence of cystolith on the surface of the leaf epidemic, flowering characteristics, and calyx structures. In addition, one of the main characteristics of the Acanthaceae family is the presence of retinacula on the seed surface, which are hook-shaped structures that serve to eject the seeds (except in the subfamilies Thunbergioideae and Nelsonioideae).

Justicia species include herbs and shrubs with opposite leaves, which can be sessile or petiolate with smooth, wavy, or fibrous margins. Cystoliths are clearly visible, measuring 0.1–0.5 mm. The inflorescence consists of simple or stalked spikes with dicasium or spicate subunits, and occasionally single flowers. Bracts are triangular, lanceolate, spatulate, ovate, or rounded, and can be smaller, equal to, or larger than the calyx; they are green or variegated, with papery edges [4]. The genus *Ruellia* comprises herbaceous and shrub species, exhibiting creeping to upright growth forms, with cystoliths present. Leaves are either sessile or have petioles, with smooth, scalloped, or toothed edges. The bracts are opposite, green, with smooth edges and may have two small bracteoles or none. Flowers are sessile, semi-sessile, or on short stalks. The calyx has five lobes, which may be equal or slightly varied. The corolla is funnel-shaped, with five lobes that vary in shape and size. Four stamens are present in pairs, enclosed within the corolla tube and positioned similarly at the base, with one sterile stamen or none [4].

Recent research on several *Justicia* species highlights their potential in both ethnomedicine and ornamental horticulture. For instance, *Justicia* species is cultivated in Indian gardens due to its unique floral morphology and attractive foliage [5]. Many Acanthaceae species possess medicinal properties and have been widely used in traditional medicine and horticulture. For instance, *Ruellia repens* has been used in folk remedies, meanwhile various parts of *Ruellia* species being utilized to treat conditions such as bladder stones and bronchitis [6].

This study is essential to uncover the latest information and identify the unique characteristics of each species analyzed, contributing to a deeper understanding, and aiding in resolving plant taxonomy challenges. Leaf micromorphological and palynological traits can significantly aid in species identification, even when the specimens lack flowers and fruits. Previous studies on Acanthaceae have primarily emphasized macromorphological traits, molecular phylogenetics, or phytochemical properties, but detailed micromorphological and palynological analyses remain limited. Pollen morphology has been underutilized in resolving taxonomic complexities especially in *Ruellia* and *Justicia* species. Therefore, the aim of this study is to develop a comprehensive database of leaf micromorphological and palynological features for *Justicia* and *Ruellia* species, serving as a valuable resource for future studies and references. The results also can be useful to support taxonomic revisions by validating or redefining species boundaries using micromorphological traits such as trichomes, stomatal types, and pollen features.

2. MATERIALS AND METHODS

A scanning electron microscope (SEM) is an instrument that used a focused high- energy beam of electrons to produce micromorphological images. This research involved six plants species of Acanthaceae with total of three to five replications for each species studied were collected from various localities in Peninsular Malaysia such as in Selangor, Perak and Pahang. For the leaf micromorphological method, the selected plant materials were taken from the dried sample of the

herbarium. The samples were cut at the lamina area in 1 cm^2 measurement and mounted on a mounting holder. The samples were coated with gold by using a sputter-coated machine. The samples were observed under a scanning electron microscope Zeiss Model Evo 50. Key features, including wax structure, cuticular sculpturing, types of trichomes and the presence of stomata, were observed at magnifications of 100x, 500x, 1000x, and 2000x.

For pollen analysis, the pollen from six species studied were observed under scanning electron microscopy (SEM). The pollen was then dehydrated through a graded alcohol series (50%, 70%, 95%, and 100%) before undergoing critical point drying (CPD) to preserve structural integrity. Then, the dried pollen grains were mounted on aluminum stubs using double-sided carbon tape and coated with a gold layer (10–15 nm thickness) using a sputter coater. Pollens were observed under Scanning Electron Microscope Zeiss Supra 55VP with various magnification. The morphological terminology for pollen description followed [7] and [8] to ensure consistency and accuracy.

3. RESULTS AND DISCUSSION

3.1 Leaf Micromorphology

The results and observations in this study revealed several common and variation characteristics in the leaf micromorphological of selected Acanthaceae species. Shared traits, such as stomatal shape and the presence of granule and crustose waxes across all studied species, serve as valuable supporting data for species identification and classification. Meanwhile, the variation characteristics of leaf micromorphology can be used to differentiate the Acanthaceae species such as cuticular sculpturing, type of stomata and trichomes.

3.1.1 Cuticular Sculpturing

Previous research by [9] opined that epidermal sculpturing has provided little systematic information yet has considerable diagnostic value in plants. The features of cuticular sculpturing somehow may characterize some species and could serve as a criterion to distinguish some species in plants. The results of this study manage to describe five patterns of cuticular sculpturing which are Pattern 1 (Epidermal cells outlines are distinguishable, anticlinal walls raised into ridges and periclinal walls sunken), Pattern 2 (Epidermal cells outlines are slightly distinguishable, anticlinal walls raised into ridges and periclinal walls sunken, Pattern 3 (Epidermal cells outlines are indistinguishable, anticlinal and periclinal walls cannot be differentiated), Pattern 4 (Epidermal cells outlines are distinguishable, periclinal walls raised into ridges and anticlinal walls sunken) and Pattern 5 (Epidermal cells outlines are slightly distinguishable, periclinal walls raised into ridges and anticlinal walls sunken). Study by [9] stated that cuticular sculpturing features can be used in identification species and very useful in taxonomic studies as well as plant phylogeny studies in *Thunbergia* species. The findings of this study align with the research by [10], which reported variations in cuticular sculpturing patterns on the adaxial and abaxial epidermal surfaces of *Schoutenia* leaves. In addition, results of this study recorded the presence of epidermal folds and cuticular striations present on the adaxial and abaxial of epidermal surface in all species studied. Therefore, the character has proven to be a common character of Acanthaceae especially on the occurrence of epidermal folds and cuticular striations since this character is useful to recognize the *Justicia* and *Ruellia* species. According to [11], the presence of striae on epidermal surfaces of plants is related to the environmental condition, in which the loss of structure perhaps of deposition of foreign particles on the surfaces.

3.1.2 Type of Stomata

According to [12], stomatal types not only have diagnostic significance but can also serve as indicators of natural taxonomic affinity. Additionally, the results of the study by [13] show that *C. vespertilionis* green leaves, with stomata are present on both of adaxial and abaxial epidermis

(amphistomatic). The findings of this study also highlight variations in stomatal distribution, with some species exhibiting a hypostomatic condition, while others display an amphistomatic arrangement. A study conducted by [12] on 22 species from the Acanthaceae family, including those in the genus *Justicia*, identified as hypostomatic features. The present study revealed two types of stomata which are hypostomatic which can be used to identify *Justicia* species (Figures 1(e) and 1(h)) except in *J. betonica*, whereas amphistomatic stomata are recorded in all *Ruellia* species studied (Figure 1i,1m &1o) as well as in *J. betonica* (Figure 1(a).

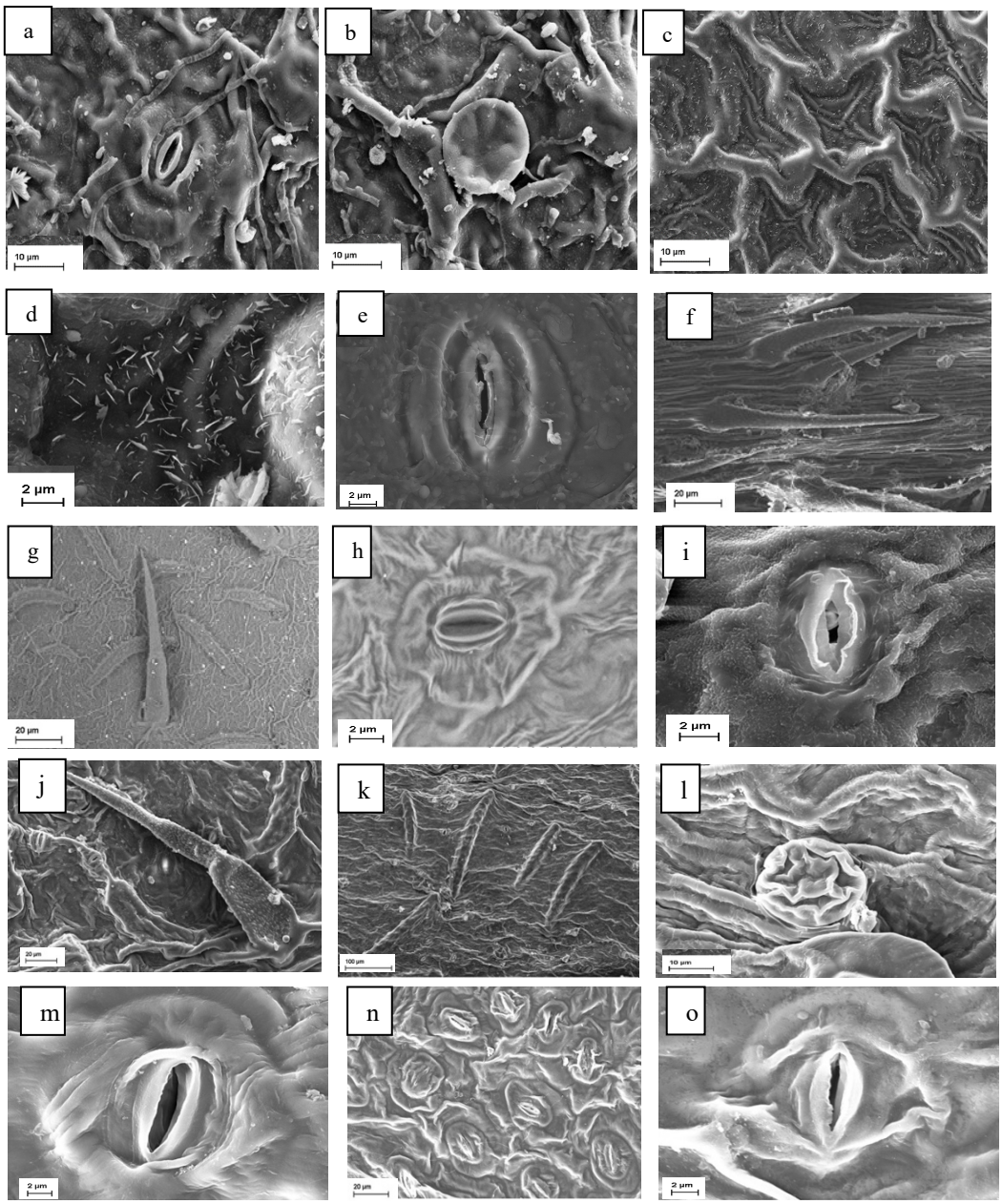

Figure 1: FESEM micrographs of *Justicia* species (a) stomata of *J. betonica*, (b) glandular trichome of *J. betonica*, (c) cuticular sculpturing of *J. carnea*, (d) wax of *J. carnea*, (e) stomata of *J. carnea*, (f) non glandular tricomes of *J. carnea*, (g) cystoliths and trichome of *J. procumbens*, (h) stomata of *J. procumbens.* FESEM micrographs of *Ruellia* species (i) stomata *R. repens*, (j) non glandular trichome of *R. repens*, (k) cystoliths of *R. repens*, (l) glandular trichome of *R. simplex*, (m) stomata of *R. simplex*, (n) cuticular sculpturing of *R. tuberosa* and (o) stomata of *R. tuberosa*

Previous research by [14], the existence of hypostomatic leaves in Acanthaceae indicates that the stomata trait is significant and could be a useful criterion for plant identification. Other than that, the epidermal and subsidiary cells around stomata for all species studied cannot be differentiated due to the presence of epidermal folds and the abundance of waxes at the epidermis surface. Other than that, the elliptic stomata also have been recorded in all species studied. The summaries of the results have been showed in Table 1.

Table 1: Description of stomata for species studied

Species /characters	Type of stomata	Epidermal and subsidiary cells	Shape of stomata	Stomata size (average)
J. betonica	Amphistomatic	Epidermal and subsidiary cells cannot be differentiated	Elliptic	(24.79 – 27.70 µm) x (11.82 – 20.70 µm)
J. carnea	Hypostomatic	Epidermal and subsidiary cells cannot be differentiated	Elliptic	(17.91 – 25.23 µm) x (9.30 – 13.25 µm)
J. procumbens	Hypostomatic	Epidermal and subsidiary cells can be differentiated	Elliptic	13.60 – 15.20 µm) x (9.53 – 11.40 µm
R. repens	Amphistomatic	Epidermal and subsidiary cells cannot be differentiated	Elliptic	(12.74 – 19.17 µm) x (6.11 – 9.72 µm)
R. simplex	Amphistomatic	Epidermal and subsidiary cells cannot be differentiated	Elliptic	15.49 – 22.77 µm) x (13.39 – 19.02 µm)
R. tuberosa	Amphistomatic	Epidermal and subsidiary cells cannot be differentiated	Elliptic	(17.27 – 19.70 µm) x (13.76 – 18.35 µm)

3.1.3 Type of Wax

Earliest studies on the leaf micromorphological study of adaxial and abaxial epidermal surfaces have explained that the type of wax on the epidermal surface has taxonomic value and can be used in species identification. The findings of this study recorded three types of wax present on the adaxial and abaxial epidermal surfaces. Two types of wax which are crustose, and granules can be found in all species studied (Table 2).

Table 2: Leaf micromorphology of species studied

Species /characters	Type of wax	Cuticular sculpturing (adaxial)	Cuticular sculpturing (abaxial)	Trichomes
J. betonica	Granules, crustose, flakes	Pattern 4	Pattern 4	-Peltate glandular trichome
J. carnea	Granules, crustose, flakes	Pattern 1	Pattern 2	-Simple unicellular (short, pointed end)
J. procumbens	Granules, crustose	Pattern 5	Pattern 5	-Simple multicellular (long, pointed end, echinate ornamentation)
R. repens	Granules, crustose	Pattern 3	Pattern 3	-Peltate glandular trichome - Simple multicellular (long, pointed end, echinate ornamentation)
R. simplex	Granules, crustose	Pattern 3	Pattern 5	-Peltate glandular trichome
R. tuberosa	Granules, crustose	Pattern 5	Pattern 5	-Peltate glandular trichome -Simple multicellular (short, blunted end, echinate ornamentation)

However, the presence of flake wax was recorded only in *J. betonica* and *J. carnea* (Figures 1(c) and 1(d). Therefore, the variations of waxes in species studied can be used as additional data for the identification of certain species. [14] also demonstrated variations of waxes in *Justicia* species, therefore could be taxonomic important in the identification process.

3.1.4 Type of Trichomes

Previous research conducted by [15] stated that trichomes may be among the most significant anatomical features and importance in taxonomic delimitations due to their accessibility. [16] explained that the variations of trichomes present in selected Asteraceae species can serve as additional data for species identification. Furthermore, [10] also mentioned about the importance of trichomes that can be useful in analyzing hybrid variation of certain species in *Schoutenia* species. The finding of this study manages to record four types of trichomes in six species studied as state in Table 2.

Glandular trichome specifically glandular peltate trichomes are the most common and abundant trichomes in Acanthaceae species [17]. Simple multicellular (long, pointed end, echinate ornamentation) present in all species studied except in *J. betonica, J. carnea, R. simplex* and *R. tuberosa*. Meanwhile, glandular trichomes were observed in all species studied except in *J. carnea* and *J. procumbens*. Additionally, the findings of this study revealed that non-glandular trichomes sometimes exhibit echinate ornamentation which is characterized by spiny or bristle-like projections on the trichome surface, as shown in Figure 1(j). This unique structural character may enhance protection against herbivores, reduce water loss, and influence interactions with environmental factors. Therefore, the presence and variation of echinate ornamentation in trichomes can be an important distinguishing characteristic for species identification and taxonomic classification.

3.2 Palynological Analysis

Furthermore, the results of this study on six species from the Acanthaceae family recorded the similarities and variations in pollen characteristics. Similarities or general characteristics shared by all species can used as a feature that supports the grouping of the genus in the family meanwhile the variation in pollen morphological characteristics such as the exine ornamentations and the pollen shape can be used for identification and differentiation at the taxon level.

3.2.1 Pollen Characteristics

Research by [18] stated that the ratio of the length of the polar axis (P) to the length equatorial axis or pollen diameter (E) can be used as an index for determine the shape of a pollen. This index can be obtained through a range of value ratios the length P is divided by the value of the diameter E (P/E). [19] argues that this index is very important in taxonomic studies because of the determination of the shape of the pollen depends on the value of the P/E index. Table 3 shows the variation the pollen characteristics that been recorded in species studied. Two type of pollen shapes have been identified in this study namely prolate which reported in *Justicia* species (Figures 2(a) to (c)) and prolate-spheroidal in *Ruellia* species (Figures 2(d) and (e)). A study by [20] on the genus *Ruellia, Ecbolium, Asystasia, Blepharis* and *Dicliptera* from Yemen also explained the importance of pollen shape variation characteristics which are useful in genus classification in the Acanthaceae family.

The term ambitus or better known as amb is an external form of polar or polar surface. A study by [19] uses the term ‘amb’ for pollen characters on the genus of the mangrove swamp. Findings have shown two types of ambitus (amb) in this study which are horizontal oblong that can be found in *Justicia* species (Figures 2(a) to (c)) and round in *Ruellia* species studied (Figures 2(d) and (e)). Therefore, type of ambitus can be one of the characters in classification of genus in Acanthaceae family.

According to [8], the pollen wall consists of two layers sporoderm which is the inner layer called intin while the outer layer is outside is known as exine. The characteristic variation present in the pollen wall is very important in the identification of a plant species [19]. [20] concluded that the results

of the observations below scanning electron microscopy on pollen morphological characteristics has a high impact in plant taxonomy. The results of this study managed to record three types of exine ornamentations which are identified as bireticulate (Figure 2(f)), micro reticulate (Figure 2(b)) and coarsely reticulate (Figure 2(h)). In *Justicia betonica* and *Justicia procumbens*, two type of reticulate have been reported which are reticulate in ectexine and micro reticulate in endexine (Figure 2(f)) as compared in *Justicia carnea* which recorded only one type of exine ornamentation only. The results of this study also manage to record coarsely reticulate for the exine ornamentation in all *Ruellia* species. Thus, this could be useful in identifying the genus in Acanthaceae family.

Table 3: Pollen characteristics of species studied

Species /characters	Apertures	Exine ornamentation	Ambitus (amb)	Shape
J. betonica	Planaaperturate	Bireticulate	Horizontal oblong	Prolate
J. carnea	Planaaperturate	Micro-reticulate	Horizontal oblong	Prolate
J. procumbens	Planaaperturate	Bireticulate	Horizontal oblong	Prolate
R. repens	Triporate	Coarsely reticulate	Round	Prolate-spheroidal
R. simplex	Triporate	Coarsely reticulate	Round	Prolate-spheroidal
R. tuberosa	Triporate	Coarsely reticulate	Round	Prolate-spheroidal

Two dichotomous keys of the selected genus in Acanthaceae species were constructed based on the leaf micromorphology and palynological characteristics as per below.

Dichotomous key of *Justicia*

1 Granules, crustose and flakes wax present at the epidermis of abaxial and adaxial surface..……..……...2

1 Granules and crustose wax present at the epidermis of abaxial and adaxial surface……..*J. procumbens*

2 Amphistomatic stomata present at epidermis surface; peltate glandular trichome present; exine ornamentation bireticulate present at pollen surface…….*J. betonica*

2 Hypostomatic stomata present at epidermis surface; simple unicellular (short, pointed end) present; exine ornamentation micro-reticulate present at pollen surface…….....*J. carnea*

Dichotomous key of *Ruellia*

1 Epidermal cell outline for abaxial surface are slightly distinguishable, periclinal walls raised into ridges and anticlinal walls sunken (pattern 5)……...2

1 Epidermal cells outline are indistinguishable, anticlinal and periclinal walls cannot be differentiated (pattern 3)……..*R. repens*
.

2 Two type of trichomes: Peltate glandular trichome and simple multicellular (short, blunted end, echinate ornamentation) present at the epidermis surface……*R. tuberosa*

2 One type of trichome: Peltate glandular trichome only present at the epidermis surface ……..…..*R. simplex*

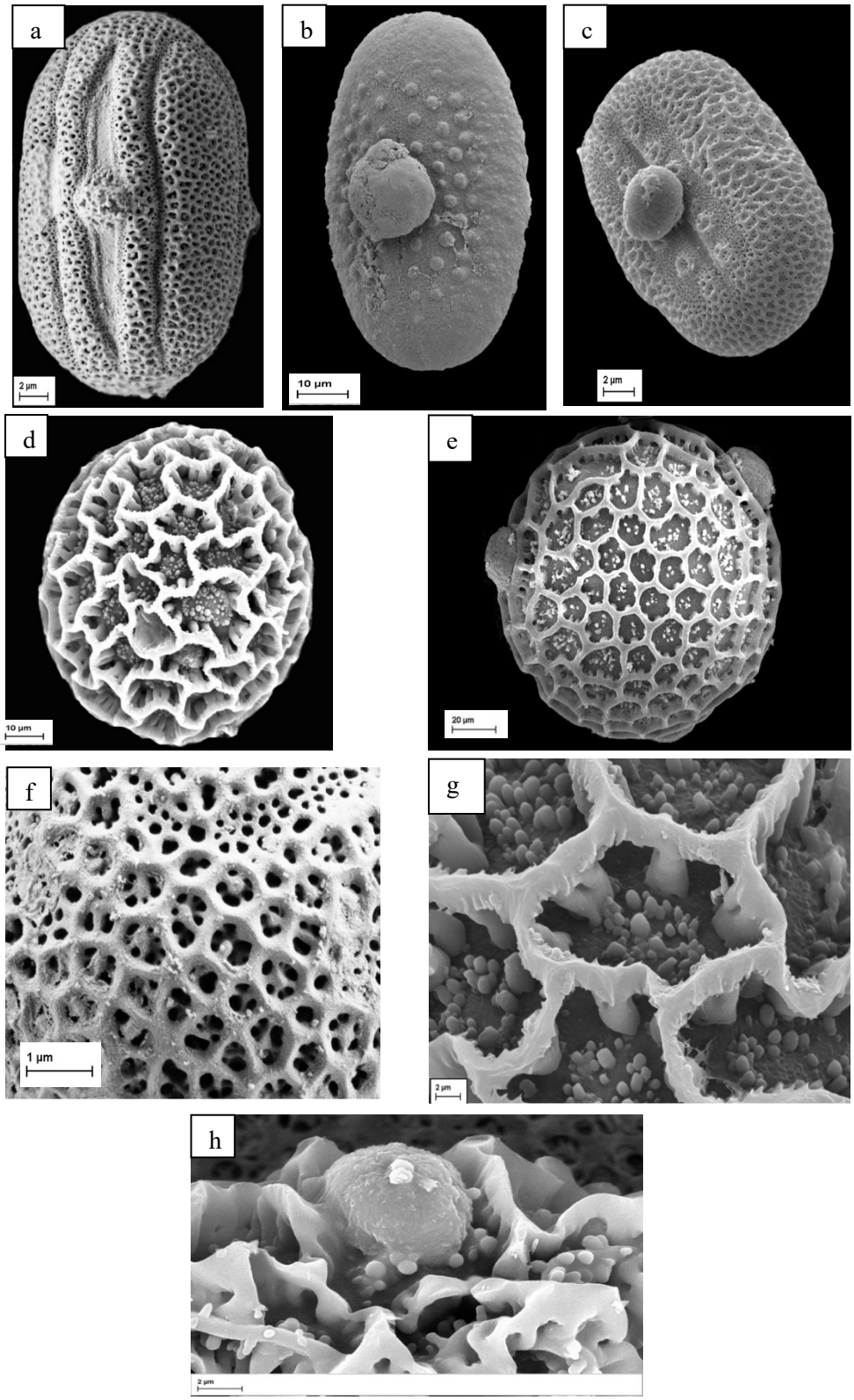

Figure 2: FESEM micrographs of pollen (a) *J. betonica*, (b) *J. carnea*, (c) *J. procumbens*, (d) *R. simplex*, (e) *R. tuberosa*, (f) exine ornamentation of *J. betonica*, (g) exine ornamentation of *R. simplex* and (h) exine ornamentation of *R. repens*

4. CONCLUSIONS

This study highlights the significance of leaf micromorphology and palynological characteristics variability among six species of Acanthaceae, particularly at the genus and taxon levels. Key diagnostic features, including cuticular sculpturing, wax type, stomatal presence, trichome diversity, pollen shape, ambitus, and exine ornamentation, contribute to a more refined taxonomic framework for the family. The integration of leaf and pollen traits enhances the resolution of species identification and provides valuable supplementary data for genus-level classification. These findings reinforce the importance of micromorphological and palynological analyses in plant systematics, offering a robust approach for distinguishing closely related taxa within Acanthaceae.

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Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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