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Pollen morphology of *Waltheria* L. (Malvaceae-Byttnerioideae) from Bahia, Brazil

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ABSTRACT

Waltheria encompasses about 60 species distributed in Brazil and Mexico. To improve the palynology of the genus, we analyzed 14 species occurring in the Brazilian State of Bahia. Pollen grains were acetolyzed and examined using light microscopy and scanning electron microscopy. Non-acetolyzed pollen grains were viewed by transmission electron microscopy. The results revealed two general pollen types based on exine ornamentation, such as reticulate and echinate, which are distinct with regard to (1) size and shape, (2) apertural type and number, (3) relative length of ectoapertures, and (4) thickening of the nexine compared to the sexine. An ultrastructural analysis of the pollen wall revealed a continuous foot layer and a compact and discontinuous endexine in most cases. The sexine consisted of heterogeneous columellae, which support a perforated semitectum (echinate type) or the semitectum was composed of perforations and muri-shaped thickening (reticulate type). These results confirm a high degree of pollen dimorphism in *Waltheria* associated with heterostyly.

Keywords: heterostyly, palynology, ultrastructure, taxonomy, *Waltheria*

Introduction

Waltheria (Malvaceae – Byttnerioideae) is a primarily neotropical genus, composed of mostly shrubs and subshrubs that bloom for several months during the year. It is distributed in two major centers of diversity, such as Brazil and Mexico, and encompasses about 60 species, of which 21 occur in Brazil, including 13 endemic species (Saunders 1993; Esteves 2015). *Waltheria* is characterized morphologically by a unicarpellate gynoecium (Schumann 1886), and the genus is divided into three subgenera (*Waltheria*, *Pringley* and *Virgata*) and two sections *Waltheria* and *Stegowaltheria*, based on the type of fruit dehiscence, stipule and seed testa morphology, and type of trichome (Schumann 1886; Saunders 1995).

According to Saunders (1993), approximately 50 *Waltheria* species are distylous, a type of heterostyly in which two mating types in a species differ in floral morphology, with regards to stamen and style length. Stamens are shorter than styles in longistylous morphs, whereas brevistylous morph styles are shorter than stamens. This floral heteromorphism is genetically controlled, and the reciprocal length of the gynoecium and androecium in longistylous and brevistylous flowers favors cross-fertilization between them, considering that most species are self-incompatible (Ganders 1979).

An extraordinary pollen dimorphism associated with this floral heteromorphism has evolved within the genus, as longistylous and brevistylous morphs produce reticulate and echinate pollen grains, respectively. The differences go beyond the features of the exine and include the size and shape of the pollen and the number and shape of the

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apertures (Köhler 1971; 1973; 1976; Melhem *et al*. 1976; Saunders 1993; 1995; 2005; Saba & Santos 2000; 2003; 2015; Saba *et al*. 2004). This degree of heteromorphism in Malvaceae is also observed in the genus *Melochia*, reflecting the close relationship of these taxa in the family (Silveira-Júnior *et al*. 2015).

Although heterostyly was recognized in early studies by Darwin (1877) and was observed in the genus *Waltheria* in the same century by Schumann (1886), the existing pollen dimorphism has not been studied by other authors. Only isolated species have been analyzed, and the pollen grains of brevistylous and longistylous morphs have not been compared (e.g. Erdtman 1952; Sharma 1970; Melhem *et al*. 1976; Palacios-Chávez *et al*. 1990; Roubik & Moreno 1991; Miranda *et al*. 1992; Saba & Santos 2003).

The most important palynological studies of *Waltheria*, which have provided detailed views of pollen grains under scanning electron microscopy (SEM) include Köhler (1971; 1976), Saunders (1993; 1995), Saba *et al*. (2004) and Saba & Santos (2015). These are the only authors who observed stratification of the pollen exine in *Waltheria* under transmission electron microscopy (TEM).

This study characterized the pollen morphology of heterostylous *Waltheria* species known in the flora of the Brazilian State of Bahia and verified the quantitative and qualitative differences in their pollen grains to provide taxonomic data of the genus.

Materials and methods

We selected 14 *Waltheria* species listed as occurring in the State of Bahia (Saunders 1995; Esteves 2015) from the CEPEC, HUEFS, SP, and SPF herbaria (acronyms according to Thiers, 2014). When possible, the specimens used were those that had been determined by experts as belonging to the genus. The pollen grains were collected from three specimens of each taxon that contained the floral morphs previously examined by the authors. Due to a lack of material, some material from other states was examined.

Examined material

Waltheria ackermanniana K. Schum: Brazil. Bahia: Rio de Contas, 20/I/1984, *Hatschbach G*. 47381 (CEPEC); Morro do Chapéu, 15/III/1990, *Saunders J*. 3105 (CEPEC); Morro do Chapéu, 15/III/1990, *Saunders J*. & *Carvalho A. M*. 3107 (CEPEC). *W. albicans* Turcz.: Brazil. Bahia: Brumado, 21/III/1990, *Saunders J. & Carvalho A. M*. 3149 (CEPEC); Bahia: Casa Nova, 16/VI/2001, *Nunes T. S. et al*. 520 (HUEFS); Bahia: Morro do Chapéu, 16/III/1990, *Saunders J. & Carvalho A. M*. 3117 (CEPEC). *W. brachypetala* Turcz: Brasil. Bahia: Morro do Chapéu, 16/III/1990, *Saunders J. & Carvalho A. M*. 3116 (CEPEC); Bahia: Lençóis, 17/III/1990, *Saunders J. & Carvalho* A. M. 3119 (CEPEC); Bahia: Canudos, 24/X/2001, *Queiroz L. P*. 7010 (HUEFS). *W. bracteosa* A. St.-Hil. & Naudin: Brazil. Bahia: Mucugê, 20/III/1990, *Saunders J. & Carvalho A. M*. 3143 (CEPEC); Bahia: Gentio do Ouro, 26/II/1977, *Harley R. M*. 19119 (CEPEC). *W. cinerascens* A. St.-Hil.: Brazil. Bahia: Canavieiras, 11/III/1990, *Saunders J. & Carvalho A. M*. 3222-B (CEPEC); Bahia: Valença, 11/XII/1980, *Matos-Silva L. A. et al*. 1252 (CEPEC); Bahia: Andaraí, 20/V/1989, *Matos-Silva L. A. et al*. 2819 (CEPEC). *W. communis* A. St.-Hil.: Brazil. Bahia: Porto Seguro, 01/V/1990, *Saunders J. & Carvalho A. M*. 3227-A (CEPEC); Bahia: Porto Seguro, 01/V/1990, *Saunders J. & Carvalho A. M*. 3229 (CEPEC); Bahia: Senhor do Bonfim, 27/III/2004, *Giulietti A. M*. 2455 (HUEFS). *W. coriacea* J. G. Saunders: Brazil, Bahia: Formosa do Rio Preto, 28/III/2000, *Harley R. M*. 53757 (CEPEC); Bahia: Formosa do Rio Preto, 03/IV/2000, *Oliveira R. P*. 467 (HUEFS). *W. excelsa* Turcz.: Brazil, Bahia: Maracajú, 17/III/1990, *Saunders J. & Carvalho* A. M. 3122 (CEPEC). *W. macropoda* Turcz.: Brazil. Bahia: Xique-xique, 17/III/1990, *Saunders J. & Carvalho A. M*. 3123 (CEPEC); Bahia: Oliveira dos Brejinhos, 16/IV/1999, *Sant'Ana S. C. et al*. 778 (CEPEC); Pernambuco: Orocó, 27/IV/2001, *Harley R. M. et al*. 54313 (CEPEC) *W. operculata* Rose: Brazil. Bahia: São Inácio, 25/II/1977, *Harley R. M*. 19024 (SPF); *W. rotundifolia* C. Presl.: Brazil. Bahia: Maracajú, 18/III/1990, *Saunders J. & Carvalho* A. M. 3126 (CEPEC); Bahia: Paulo Afonso, 18/V/1981, *Gonçalves L. M. C*. 76 (CEPEC). *W. selloana* K. Schum.: Brazil. Bahia: Rio de Contas, 13/VII/1985, *Kral R. et al*. 72731 (CEPEC); Bahia: Rio de Contas, 21/VII/1979, *Mori S. A. et al*. 12379 (CEPEC). *W. vernonioides* R. E. Fr.: Brazil. Mato Grosso: Cuiabá, no date, *Hoehne E. C*. 2976 (SP); Goiás: Monte Alegre de Goiás, 22/II/1990, *Saunders J. et al*. 3060 (CEPEC). *W. viscosissima* A. St.-Hil.: Brazil. Bahia: Santa Luzia, 01/V/1990, *Saunders J*. & *Carvalho* A. M. 3234 (CEPEC); Bahia: Lençóis, 02/VII/2001, *Roque N. et al*. 533 (CEPEC); Bahia: Feira de Santana, *Noblick L. R. et al*. 2359 (CEPEC).

Pollen grains for light microscopy (LM) were taken from two or three flowers per sample and were prepared using the acetolysis protocol (Erdtman 1960). The slides were mounted and deposited in the Palynology Laboratory of the Universidade do Estado da Bahia, *Campus* VII.

The major morphometric parameters (equatorial and polar diameters) were measured on 25 pollen grains, and the aperture diameters and exine, sexine and nexine thicknesses were measured in ten randomly chosen pollen grains. The arithmetic average, standard deviation, standard deviation of the average, coefficient of variation, and 95 % confidence intervals were calculated for the statistical analysis.

The pollen surface was analyzed by SEM (JEOL 6390LV; Tokyo Japan) at the Electron Microscopy Platform of Centro de Pesquisas Gonçalo Moniz-FIOCRUZ. The pollen grains were acetolyzed, washed, and dehydrated in an ascending hydroethanol series (50, 70, 90, and 100 %) for about 10 min in each phase. Absolute alcohol containing the pollen grains was dripped directly over SEM stubs (in a specimen holder), which were metallized by gold evaporation in a high vacuum after they dried completely.

The TEM analysis was carried out at FIOCRUZ using a Zeiss EM 109 microscope (Zena, Germany). The chemical treatment of the samples followed the protocol used by Silveira-Júnior *et al*. (2015). Briefly, non-acetolyzed pollen grains were fixed in glutaraldehyde (2.5 %) in 0.1 M sodium cacodylate buffer solution, washed in a sodium cacodylate buffer solution and post-fixed in osmium tetroxide (1 %), potassium ferricyanide (0.8 %), and 5 mM calcium chloride in sodium cacodylate buffer. The pollen grains were washed again and dehydrated in an ascending acetone series (30, 50, 70, 90, and 100 %). The samples were embedded in Epon resin, the blocks were cut at 70 nm on an ultramicrotome (Ultracut E), and contrasted in uranyl acetate (7 %) and lead citrate.

All material was illustrated by photomicrographs and scanning and transmission electromicrographs. We adopted the palynological terminology used by Punt *et al*. (2007) and Hesse *et al*. (2009) to describe the pollen characters.

Results

The pollen grains are shown in the Figures 1–8. The morphological characteristics are summarized in Tables 1 and 2, and the morphometric data are shown in Tables 3 and 4. The species was grouped into reticulate and echinate types based on exine ornamentation. The apertural type, number, and distribution allowed grouping the species into three (reticulate pollen grains) and four (echinate) categories (Fig. 8).

Reticulate type

The longistylous and homostylous (*W. operculata*) specimens characterized the reticulate type of pollen grain: mostly medium to large; isopolar or apolar only in *W. communis* and *W. rotundifolia*; oblate spheroidal, prolate spheroidal and spherical (apolar pollen grains); amb circular, subcircular, rarely quadrangular (*W. coriacea,* Fig. 2 I) and polygonal; zonocolporate and pantocolporate (*W. communis* and *W. rotundifolia*).

The ectoapertures are long to short, often narrow, with a generally regular outline, without a margin, with tapered ends less often rounded (Figs. 1F, J, M, 2B, F, J, 3B, F, M). The ectoapertures are difficult to identify and measure in *W. communis* and *W. rotundifolia*, and they barely extend to the limits of the endoapertures.

The endoapertures are lolongate in *Waltheria bracteosa* (Fig. 1M), circular in *W. communis* (Fig. 2E) and *W. rotundifolia* (Fig. 3G), or lalongate in the other species, showing variations in relation to height and width between species. The inferior and superior margins of the endoapertures are frequently concave and elliptical in shape. A slight constriction in the middle of both margins may occur in *W. albicans* (Fig. 1F) and the lateral margins, when visible, are rounded or elliptical. A costa and fastigium are present in almost all species, except in *W. communis* and *W. rotundifolia*.

The sexine is often thicker than the nexine (Tabs. 3, 4); a thinner sexine occurs in *W. brachypetala* (Fig. 1J) and is of equal thickness in *W. bracteosa*. Under ML, the reticula have irregular lumina and are usually heterogeneous with respect to size (Figs. 2F, 3E). Homogeneous lumina occurred in pollen grains of *W. ackermanniana* and *W. bracteosa* and a circular form occurred only in *W. ackermanniana*. The lumina are typically ≥ 1 µm in diameter. The walls are simplicolumellate, continuous, and straight.

The columellae are distinct in optically sectioned and SEM images, alternating between larger and smaller, maintaining an undulating or slight undulating semitectum (Figs. 1F, 3F-G, 4E', M'); however, no undulation was observed in *W. coriacea* (Fig. 2I). SEM also revealed perforations on the muri of *W. ackermanniana*, *W. albicans*, *W. brachypetala, W. communis*, and *W. rotundifolia* pollen grains (Figs. 4B, E, G, 5B, M). Furthermore, the lumina possessed perforations in most species.

The ultrastructure of the pollen grain wall revealed a distinct nexine in a continuous foot layer and a discontinuous and compact endexine (Fig. 7A-H). The foot layer is generally thinner than the endexine; however, both have the same thickness in *W. operculata* (Fig. 7G). The sexine is formed by thin and heterogeneous columellae with respect to size. The columellae in *W. ackermanniana* is short at the apertural region where there is a thickening of the nexine (costa, Fig. 7B). The columellae are uniformly distributed in *W. ackermanniana* (Fig. 7A), although they are further apart in *W. operculata*. The columellae in *W. ackermanniana* and *W. operculata* support a relatively thick semitectum with internal foramina and sparse or densely distributed perforations in *W. macropoda* (Fig. 7E). Muri shaped thickening on the semitectum was observed in all samples. A thick and continuous intine was observed only in *W. macropoda*.

Echinate type

The echinate type characterized pollen grains of the brevistylous specimens and homostylous specimens of *W. selloana* and *W. vernonioides*: medium or mostly large pollen grains; isopolar, but apolar in *W. communis*, *W. rotundifolia*, and *W. vernonioides*; suboblate, oblate spheroidal (most species), and spherical (apolar pollen grains); amb circular and polygonal; 5–8 zonocolporate or less often 10–14 panto-brevicolporate (*W. rotundifolia*) and 10–16 pantopororate (*W. communis* and *W. vernonioides*).

The ectoapertures are long or short, slightly wide, and circular in *W. communis* (Fig. 2H) and *W. vernonioides* (Fig. 3K). The outline of the ectoaperture is often irregular or not clearly visible in *W. ackermanniana* (Fig. 1D) and *W. albicans* (Fig. 1H). The ectoapertures in *W. rotundifolia* are difficult to identify and measure due to the slightly stretched limits of the endoaperture (brevicolporus). The apertural membrane may present with ornamented echinae, as in *W. coriacea* (Fig. 2L) and *W. selloana* (Fig. 3I). The ectoapertures have

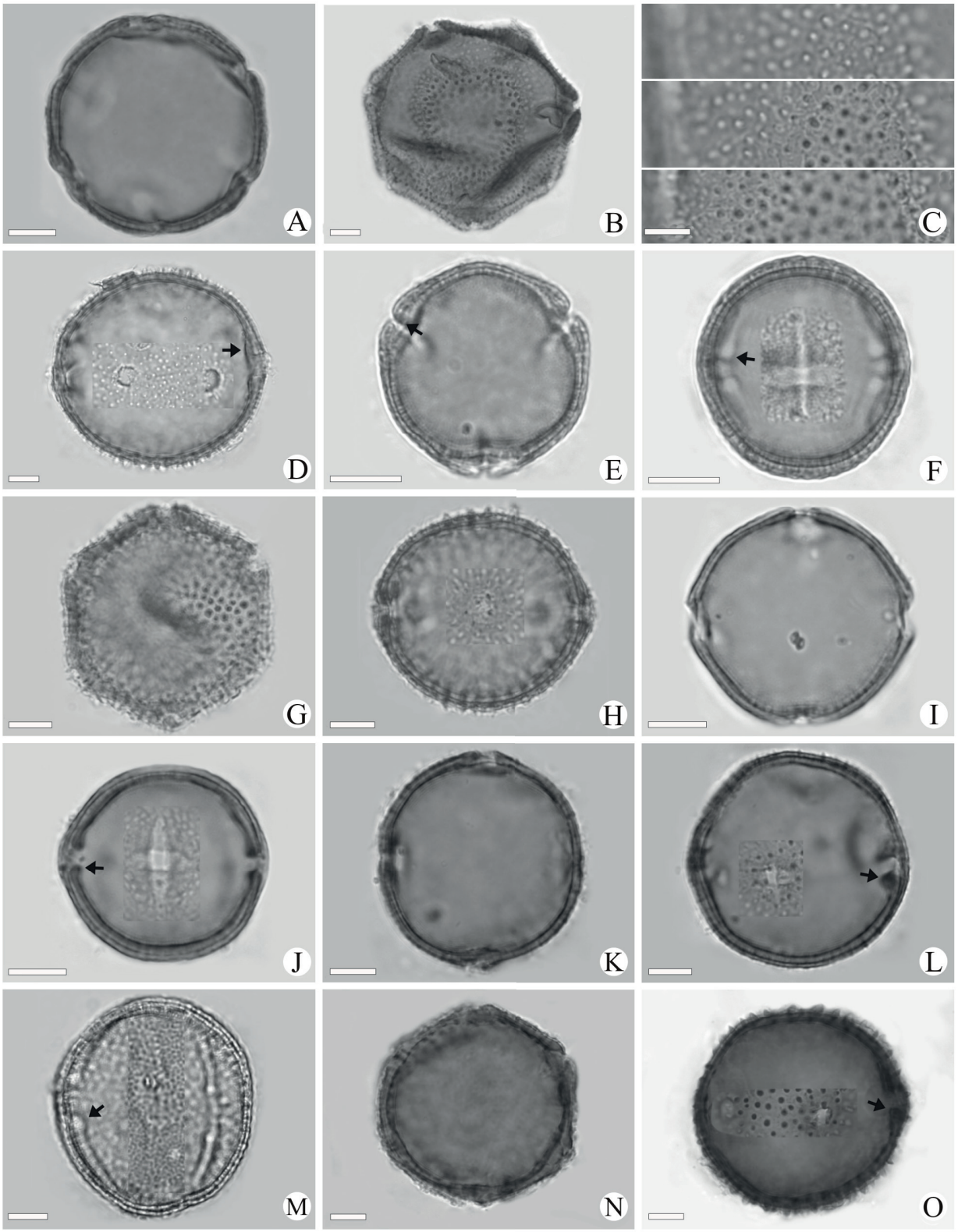

Figure 1. Light micrographs of *Waltheria* L. pollen grains. *Waltheria ackermanniana* (Reticulate): **A.** Polar view; *W. ackermanniana* (Echinate): **B.** Polar view; **C.** LO Analysis; **D.** Equatoial view, costa detail (arrow). *W. albicans* (Reticulate): **E.** Polar view, fastigium detail (arrow); **F.** equatorial view, costa detail (arrow); *W. albicans* (Echinate): **G.** Polar view; **H.** Equatorial view. *W. brachypetala* (Reticulate): **I.** Polar view; **J.** Equatorial view, costa detail (arrow); *W. brachypetala* (Echinate): **K.** Polar view; **L.** Equatorial view, costa detail (arrow). *W. bracteosa* (Reticulate): **M.** Equatorial view, costa detail (arrow); *W. bracteosa* (Echinate): **N.** Polar view; **O.** Equatorial view, costa detail (arrow). Scales bar = 10 *μm*.

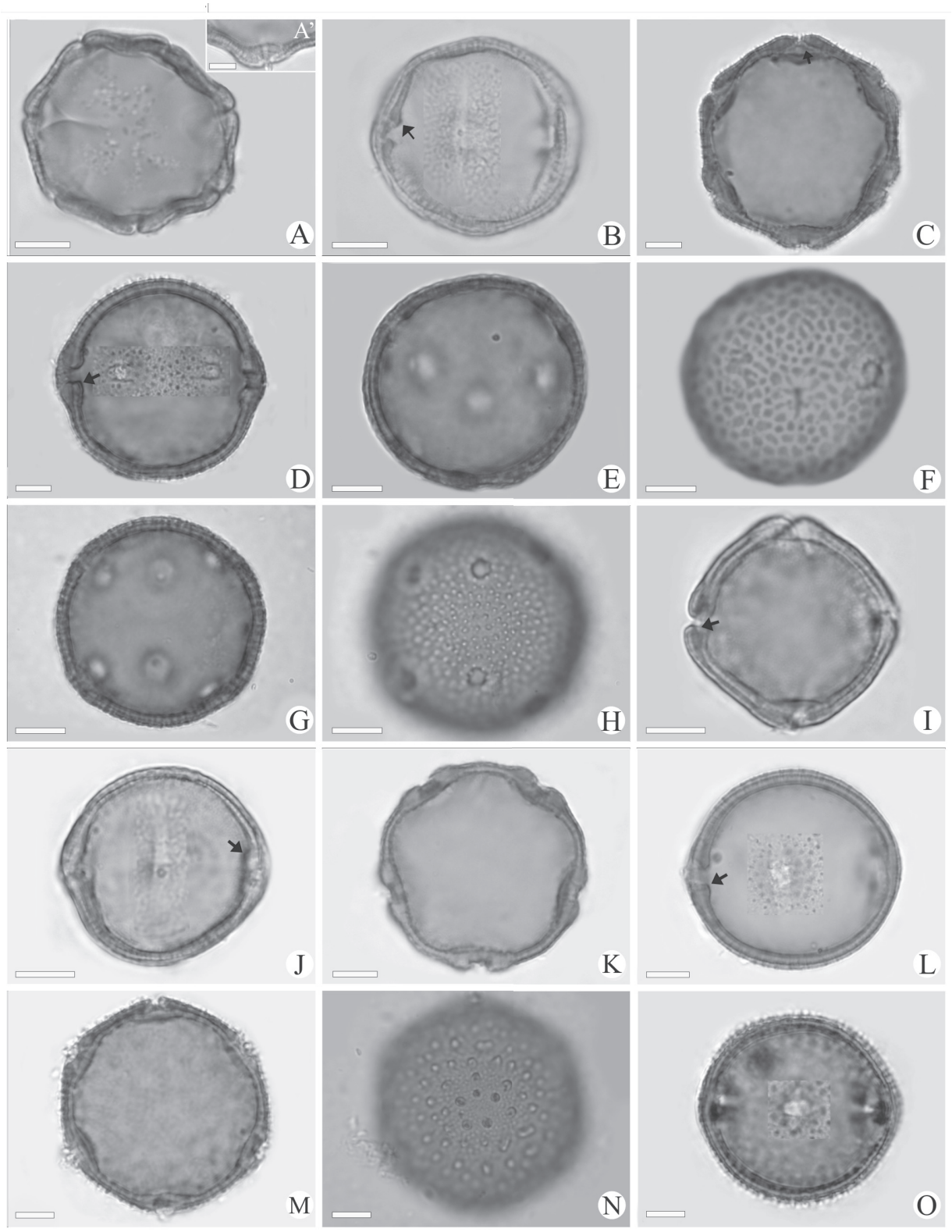

Figure 2. Light micrographs of *Waltheria* L. pollen grains. *W. cinerascens* (Reticulate): **A.** Polar view; A'. Fastigium detail; **B.** Equatorial view, costa detail (arrow). *W. cinerascens* (Echinate): **C.** Polar view, fastigium detail (arrow); **D.** Equatorial view, costa detail (polar view). *W. communis* (Reticulate): **E.** Polar view, optical section; **F.** Polar view, surface. *W. communis* (Echinate): **G.** Polar view, optical section; **H.** Polar view, surface. *W. coriacea* (Reticulate): **I.** Polar view, fastigium detail (arrow); **J.** Equatorial view, costa detail (arrow). *W. coriacea* (Echinate): **K.** Polar view; **L.** Equatorial view, costa detail. *W. excelsa* (Echinate): **M.** Polar view, optical section; **N.** Polar view, surface; **O.** Equatorial view. Scales bar: A'= 5 *µm; other figures=10 µm*.

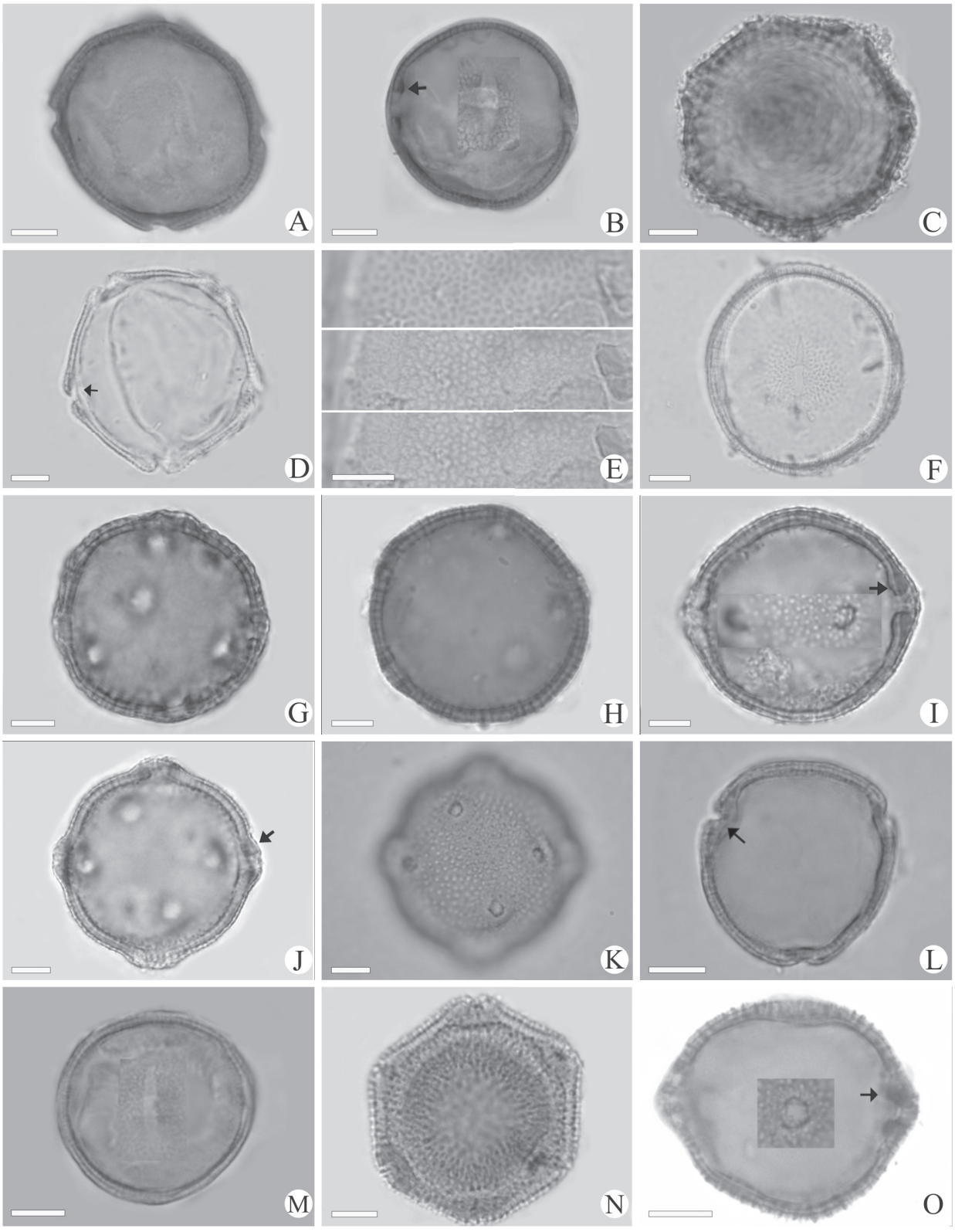

Figure 3. Light micrographs of *Waltheria* L. pollen grains. *W. macropoda* (Reticulate): **A.** Polar view; **B.** Equatorial view, costa detail (arrow). *W. macropoda* (Echinate): **C.** Polar view. *W. operculata* (Reticulate): **D.** Polar view, fastigium detail (arrow); **E.** LO Analysis; **F.** Equatorial view. *W. rotundifolia* (Reticulate): **G**. General view; *W. rotundifolia* (Echinate): **H.** General view. *W. selloana* (Echinate): **I.** Equatorial view, costa detail (arrow). *W. vernonioides* (Echinate): **J.** General view, aspis detail (arrow); **K.** General view, surface. *W. viscosissima* (Reticulate): **L.** Polar view, fastigium detail (arrow); **M.** Equatorial view; *W. viscosissima* (Echinate): **N.** Polar view; O. Equatorial view, costa detail (arrow). Scales bar = 10 μm.

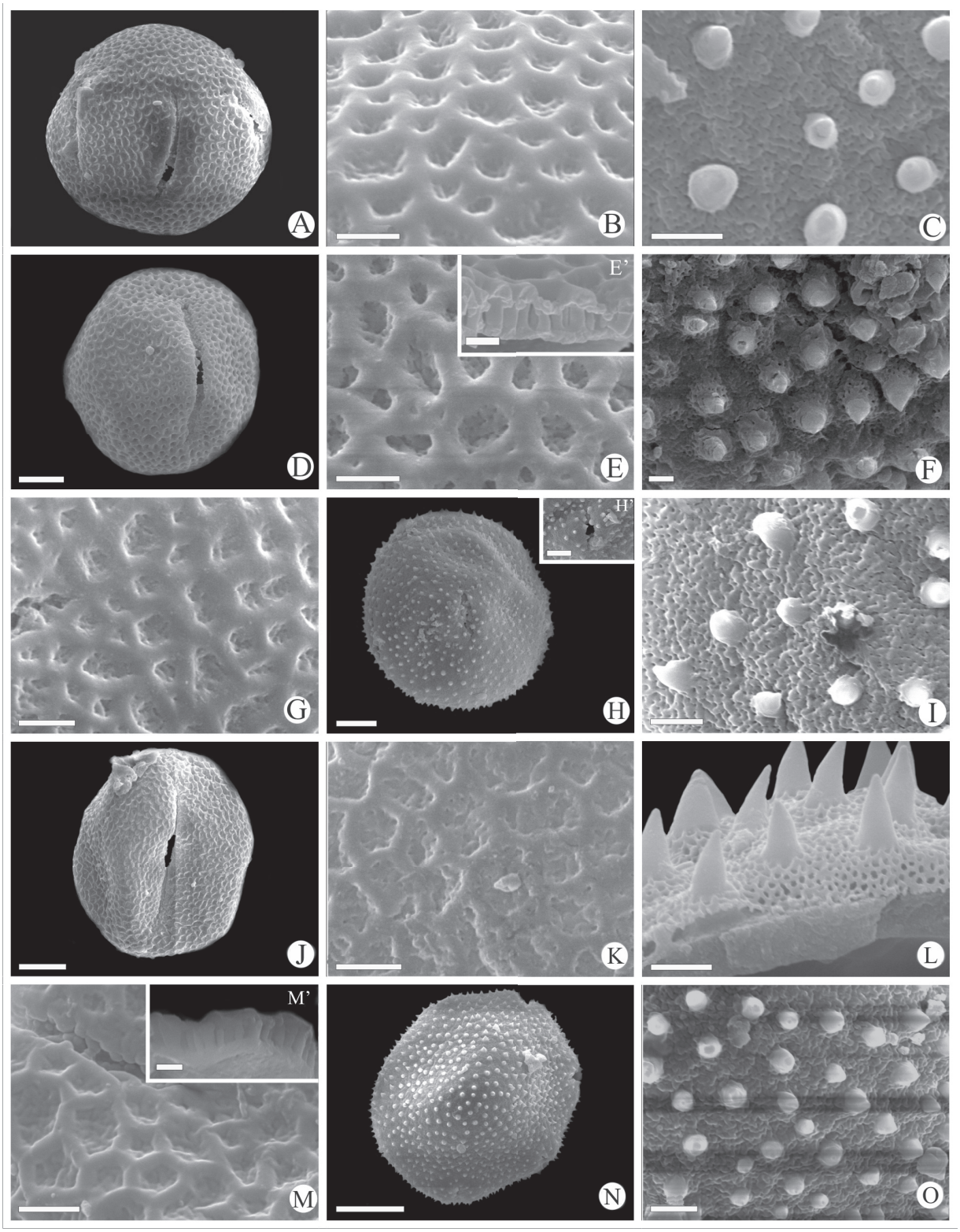

Figure 4. Scanning electron micrographs of *Waltheria* L. pollen grains. *W. ackermanniana* (Reticulate): **A**. Equatorial view; **B.** Exine ornamentation; *W. ackermanniana* (Echinate): **C.** Exine ornamentation. *W. albicans* (Reticulate): **D.** Equatorial view; **E.** Exine ornamentation; **E'.** Detail of the exine in a fragmented pollen grain. *W. albicans* (Echinate): **F.** Exine ornametation. *W. brachypetala* (Reticulate): **G.** Exine ornamentation. *W. brachypetala* (Echinate): **H.** Equatorial view; **H'.** Aperture detail; **I.** Exine ornamentation. *W. bracteosa* (Reticulate): **J.** Equatorial view; **K.** exine ornamentation. *W. bracteosa* (Echinate): **L.** Exine ornamentation. *W. cinerascens* (Reticulate): **M.** Exine ornamentation; **M'.** Detail of the exine in a fragmented pollen grain; *W. cinerascens* (Echinate): **N.** Polar view; **O.** Exine ornamentation. Scales bar: E', M' = 1 *μm; B, C, E, F, G, I, K, L, M, O = 2 μm. H' = 5 μm A, D, H, J, N = 10 μ*m.

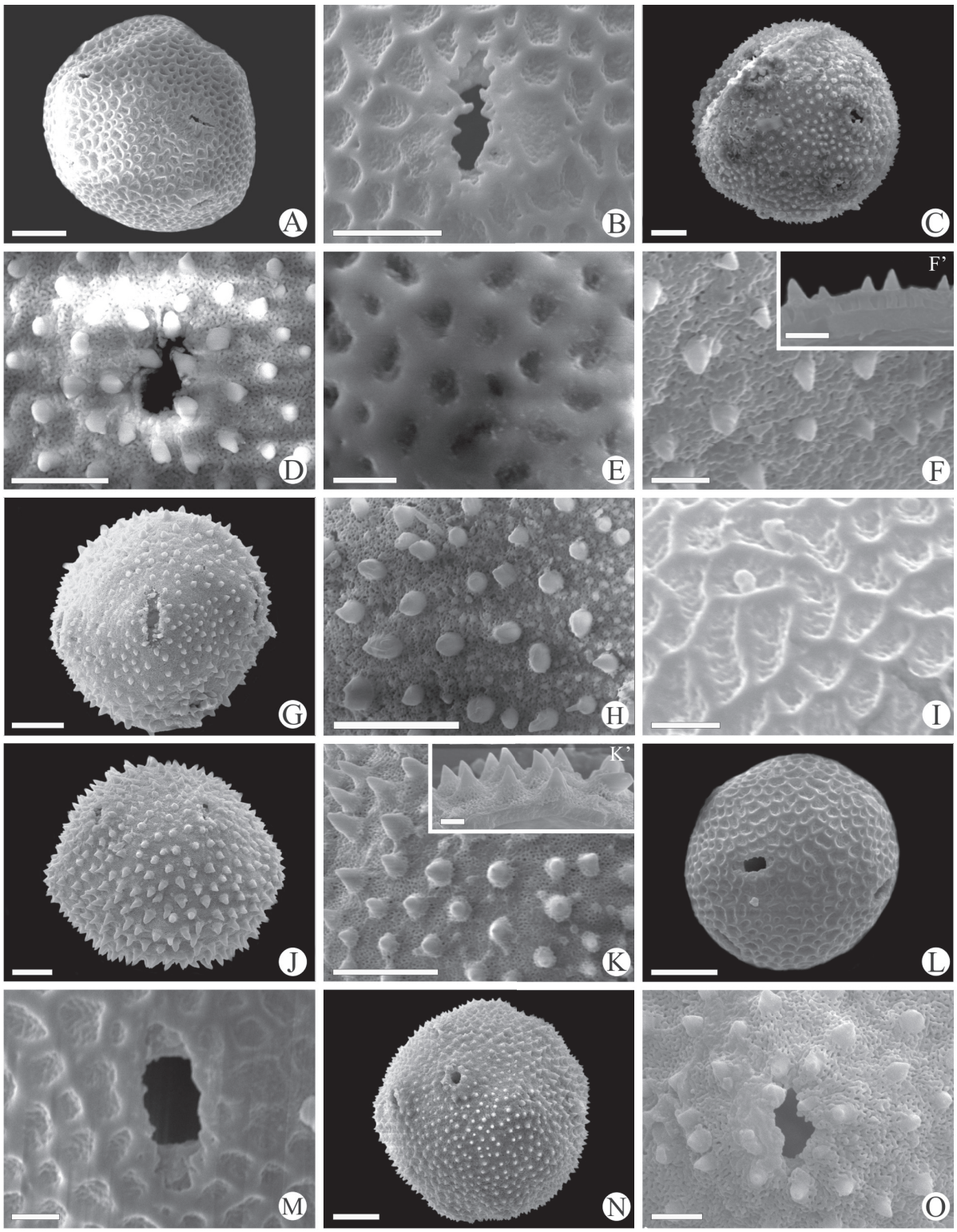

Figure 5. Scanning electron micrographs of *Waltheria* L. pollen grains. *W. communis* (Reticulate): **A.** General view; **B.** Exine ornamentation, aperture detail. *W. communis* (Echinate): **C.** General view; **D.** Exine ornamentation, aperture detail. *W. coriacea* (Reticulate): **E.** Exine ornamentation. *W. coriacea* (Echinate): **F.** Exine ornamentation; **F'.** Detail of the exine in a fragmented pollen grain. *W. excelsa* (Echinate): **G.** Equatorial view; **H.** Exine ornamentation. *W. macropoda* (Reticulate): **I.** Exine ornamentation; *W. macropoda* (Echinate): **J.** Equatorial view; **K.** Exine ornamentation; **K'.** Detail of the exine in a fragmented pollen grain. *W. rotundifolia* (Reticulate): **L.** General view; **M.** Exine ornamentation, aperture detail. *W. rotundifolia* (Echinate): **N.** General view; **O.** Exine ornamentation, aperture detail. Scales bar: E, F, F', I, K', M, O = 2 *μm; B, D, H = 5 μm; A, C, G, J, K, L, N = 10* μm.

tapered ends in almost all species, except they are rounded in *W. excelsa* (Fig. 2O).

The endoapertures are circular, lolongate in *W. macropoda* and *W. rotundifolia* and lalongate in most species. The latter may present superior and inferior concave (elliptical) or parallel (rectangular) margins (Figs. 1L, 2D, L, O, 3O). Both patterns occur in *W. excelsa*. *W. communis* and *W. vernonioides* have pollen grains with circular endoapertures (Figs. 2G-H, 3J-K), which are difficult to visualize, precluding their measurement and illustration under LM. Costae were observed in almost all species, whereas fastigia were only present in *W. cinerascens* (Fig. 2C). *W. vernonioides* was the only species to present pollen grains with aspides (Fig. 3J).

The nexine was thicker than the sexine in most cases; a thicker sexine was observed only in *W. brachypetala*, *W. rotundifolia,* and *W. selloana*. The supratectal elements are small and acute echinae, with a broad base, densely distributed throughout the surface of the pollen grain (Figs. 1C, 4H, L, N, 5C, F', G, H, J, K', N, 6A, B', D). The form of the echinae distribution varied slightly among species: they were located more distant from each other in *W. brachypetala* (Fig. 4H-I) and *W. excelsa* (Fig. 5G-H), while interspinal distance was about 1 µm in *W. rotundifolia*, *W. selloana*, and *W. vernonioides*. Smaller echinae were observed in *W. cinerascens* (Fig. 4N) and the largest in *W. macropoda* (Fig. 5J-K').

The L.O. analysis revealed elevations in the tectum, similar to granules, smaller than echinae but also distributed over the surface of the pollen grain (Figs. 1C, 2N, 3K). The SEM analysis showed that these elevations are, in most species, the highest undulations on the semitectum wall. The presence of granules was confirmed only in *W. excelsa* pollen grains (Fig. 5H).

The semitectum was one more element that differentiated some species under MEV, which may be either perforate in *W. albicans* (Fig. 4F), *W. communis* (Fig. 5D), *W. selloana* (Fig. 6B), and *W. vernonioides* (Fig. 6E); granulate-perforate in *W. excelsa* (Fig. 5H); microreticulate in *W. bracteosa* (Fig. 4L), *W. macropoda* (Fig. 5K), and *W. viscosissima* (Fig. 6F), or rugulate-perforate in *W. ackermanniana* (Fig. 4C), *W. brachypetala* (Fig. 4I), *W. cinerascens* (Fig. 4O), *W. coriacea* (Fig. 5F), and *W. rotundifolia* (Fig. 5O).

The walls of the echinate pollen grains were differentiated by a foot layer thicker than the compact and discontinuous endexine under TEM. The foot layer in *W. macropoda* is continuous and spongy in appearance (Fig. 7F). Furthermore, the wall has short and heterogeneous columellae, which are wider at the base of the echinae and have a densely perforated semitectum (Figs. 7D, F, H). *W. brachypetala* had a thick intine at the apertural region (Fig. 7D).

Discussion

The data presented here corroborate those found in the literature regarding pollen dimorphism among floral morphs of *Waltheria* L. Two distinct types of pollen grains have been observed in a single taxa (Köhler 1973; 1976;

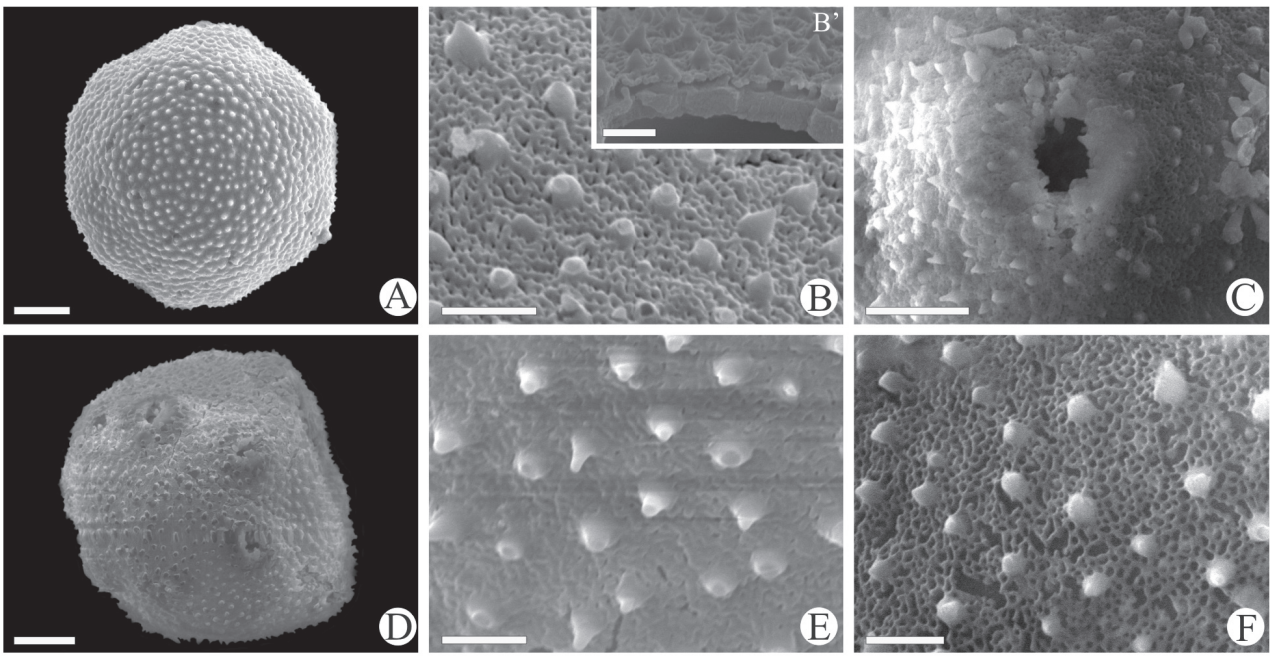

Figure 6. Scanning electron micrographs of *Waltheria* L. pollen grains. *W. selloana* (Echinate): **A.** Polar view; **B.** Exine ornamentation; **B'.** Detail of the exine in a fragmented pollen grain; **C.** Aperture detail. *W. vernonioides* (Echinate): **D.** General view; **E.** exine ornamentation. *W. viscosissima* (Echinate): **F.** Exine ornamentation. Scales bar: B, B', E, F = 2 *µm; C = 5 µm; A, D = 10 µm.*

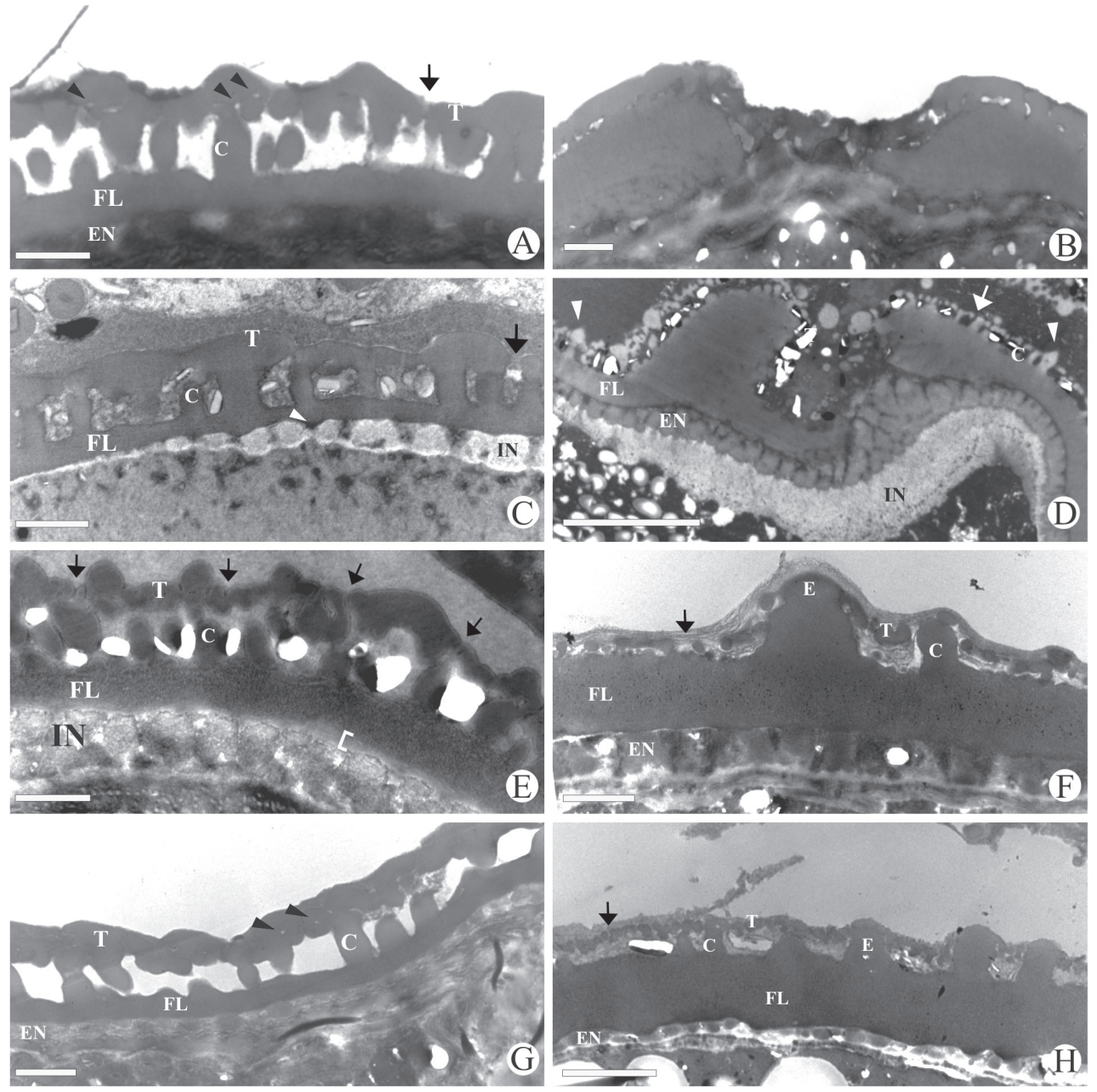

Figure 7. Trasmission electron micrographs of *Waltheria* L. pollen grains. *W. ackermanniana* (Reticulate): **A.** Wall structure showing internal foramina (arrowheads) and a perforation (arrow); **B.** Wall structure, apertural region. *W. brachypetala* (Reticulate): **C.** Wall structure showing endexine (arrowhead) and a perforation (arrow). *W. brachypetala* (Echinate): **D.** Wall structure on apertural region, showing perforation (arrow) and echinae (arrowhead). *W. macropoda* (Reticulate): **E.** Wall structure showing endexine (bracket) and a perforation (arrows). *W. macropoda* (Echinate): **F.** Wall structure showing perforation (arrow). *W. operculata* (Reticulate): **G.** Wall structure showing internal foramina (arrowheads). *W. rotundifolia* (Echinate): **H.** Wall structure showing a perforation (arrow). (C= Columella; EN= Endexine; E= Echinus; FL=Foot Layer; IN= Intine; T= Tectum). Scales bar: D = 5 μm; other images = 1 μm.

Miranda & Andrade 1989; Saunders 1993; 1995; Saba & Santos 2000; 2003; Saba *et al*. 2004; Saunders 2005; Saba & Santos 2015).

Brevistylous morphs always presented with larger pollen grains than those of the longistylous, which corroborated other studies (Köhler 1973; 1976; Miranda & Andrade 1989; Saunders 1993; 1995). Medium pollen grains prevailed among the reticulate type, while large ones prevailed among the echinate type. Saba *et al.* (2004) reported that medium-sized pollen grains are dominant among longistylous specimens, but they did not observe the prevalence of large pollen on the brevistylous morphs.

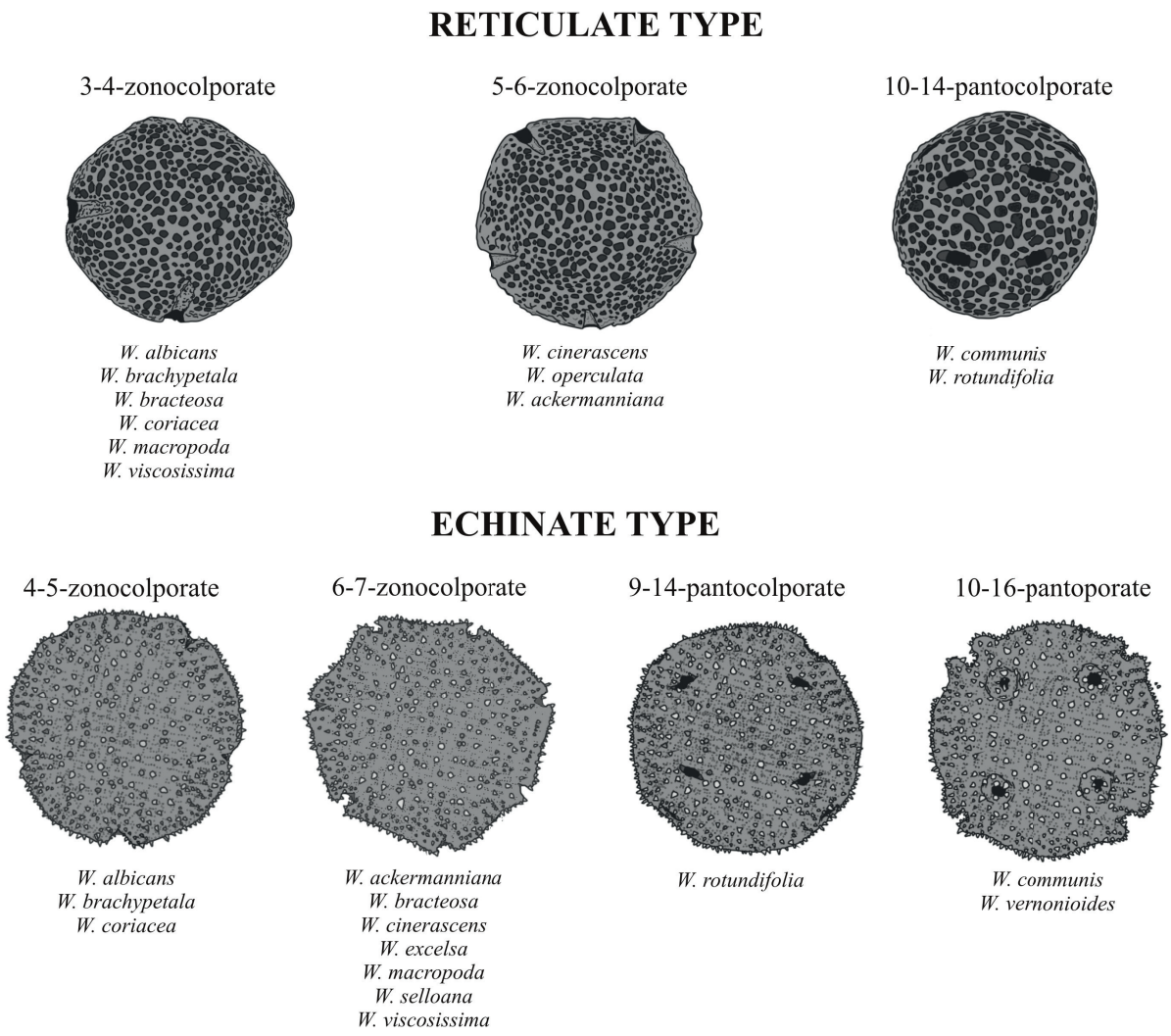

Figure 8. Grouping of the species relative to the apertural characteristics.

Echinate and reticulate pollen grains of *Waltheria* have a tendency to have prolate and oblate spheroidal shapes, respectively (Köhler 1971; 1973; Saba *et al.* 2004; Saba & Santos 2015). According to Köhler (1976), this tendency is caused by the increase in the number of apertures in the echinate pollen grains.

The apolar pollen grains presented apertures arranged in a loop-like pattern, similar to the line on a tennis ball (Figs. 2E-H, 3G-H, J-K), a feature that can also be observed in Rubiaceae and Eriocaulaceae (Dessein *et al*. 2002; Dessein *et al*. 2005; Borges 2008). According to Dessein *et al*. (2005), these apertural types are intermediate forms between zono and genuine pantoaperturate grains.

Overall, echinate pollen grains had smaller ectoapertures than those of the reticulate type (Tabs 4, 5). This has also been observed by other authors who have studied *Waltheria* (Köhler 1971; 1976; Miranda & Andrade 1989; Saba & Santos 2000; 2015; Saba *et al*. 2004).

The endoapertures of echinate pollen grains in most species were taller and smaller when compared with the reticulate type. These data corroborate, in part, the results reported by Köhler (1976), who observed only a smaller width of the endoapertures. Miranda & Andrade (1989) described the same characteristics as the present study in specimens of *W. viscosissima*, which were also reported by Saunders (1993) for other species in the genus. Saba *et al*. (2004) found this same pattern in *W. cinerascens* and *W. selloana*, but only in the height of the endoapertures.

The number of apertures ranged from three to 14 on the reticulate pollen grains and from four to 16 on the echinate pollen. *W. cinerascens* and *W. rotundifolia* presented with the same number of apertures between the two floral morphs. The same results were found by Miranda & Andrade (1989) and Saba *et al.* (2004) for *W. brachypetala*, *W. cinerascens*, and *W. viscosissima*.

The echinate pollen grains often had a thicker exine, but only *W. rotundifolia* had a thicker exine on its reticulate type pollen. Köhler (1973; 1976) and Saunders (2005) reported

Table 1. Morphological characteristics of *Waltheria* L. pollen grains – Reticulate type

Species	Size	Shape	Polarity	Apertures						Exine ornamentation (LM and SEM)
				nº	type	endoaperture	ectoaperture	costa	fastigium	
W. ackermanniana	M	OS	isopolar	(5)-6	Colporus	lalongate: elliptical	long, narrow, apices acute	-	-	Reticulate-perforate; homogeneous and circular lumina; semitectum slightly undulating
W. albicans	M	PS	isopolar	3-(4)	Colporus	lalongate: elliptical	short, narrow, apices rounded	+	+	Reticulate-perforate; heterogeneous and irregular lumina, semitectum slightly undulating
W. brachypetala	M	OS	isopolar	(3)-4-(5)	Colporus	lalongate: elliptical	long, broad, apices rounded	+	-	Reticulate-perforate; homogeneous and irregular lumina; semitectum slightly undulating
W. bracteosa	L	PS	isopolar	3	Colporus	lolongate	narrow, apices acute	+	-	Reticulate-perforate; homogeneous and irregular lumina; semitectum undulating
W. cinerascens	M	OS	isopolar	(5)-6-(7)	Colporus	lalongate: elliptical	long, narrow, apices acute	+	+	Reticulate; heterogeneous and irregular lumina; semitectum undulating
W. communis	M to L	S	apolar	9-10-11-12-(13-14)	Colporus	circular	broad, apices rounded	-	-	Reticulate-perforate; heterogeneous and irregular lumina; semitectum undulating
W. coriacea	M	OS	isopolar	4	Colporus	lalongate: elliptical	short, narrow, apices acute	+	+	Reticulate-perforate; heterogeneous and circular lumina; semitectum non-undulating
W. macropoda	M to L	PS	isopolar	(3)-4	Colporus	lalongate: elliptical	short, broad, apices acute	+	-	Reticulate-perforate; heterogeneous and irregular lumina; semitectum slightly undulating
W. operculata	M to L	OS-PS	isopolar	(3-4)-5-(6)	Colporus	lalongate: rectangular a elliptical	short to long, narrow to broad; apices acute	-	+	Reticulate; heterogeneous and irregular lumina; semitectum undulating
W. rotundifolia	M	S	apolar	(10)-12-13-(14)	Colporus	circular	narrow, apices rounded	-	-	Reticulate-perforate; heterogeneous and irregular lumina; semitectum undulating
W. viscosissima	M	OS	isopolar	3	Colporus	Lalongate: elliptical	short, narrow, apices rounded	-	+	Reticulate; heterogeneous and irregular lumina; semitectum undulating

Note: L=large; M=medium; OS=oblate spheroidal; PS=prolate spheroidal; S=spheroidal; [+] present; [-] absent

Table 2. Morphological characteristics of *Waltheria* L. pollen grains – Echinate type

Species	Size	Shape	Polarity	Apertures						Exine ornamentation (LM and SEM)
				nº	type	endoaperture	ectoaperture	costa	fastigium	
W. ackermanniana	L	OS	isopolar	(5)-6-7	Colporus	circular	long, narrow, apices acute	+	-	Echinate-rugulate-perforate; echinae densely distributed;
W. albicans	M	OS	isopolar	(4)-5-(6)	Colporus	circular	short, narrow, apices acute	-	-	Echinate-perforate; echinae densely distributed;
W. brachypetala	M to L	OS	isopolar	4-5-(6)	Colporus	lalongate: elliptical	short, narrow, apices acute	+	-	Echinate-rugulate-perforate; echinae sparsely distributed;
W. bracteosa	L	OS	isopolar	6-(7)	Colporus	circular	long, broad, apices acute	+	-	Echinate-microreticulate; echinae densely distributed;
W. cinerascens	L	SO-OS	isopolar	(5)-6-7	Colporus	lalongate: rectangular	long, narrow, apices acute	+	+	Echinate-rugulate-perforate; echinae densely distributed;
W. communis	L	S	apolar	10-(11)-12-13-14	Porus	circular	circular	-	-	Echinate-perforate; echinae densely distributed;
W. coriacea	L	OS	isopolar	4-5-(6)	Colporus	lalongate, elliptical to circular	very short, broad, apices acute	+	-	Echinate-rugulate-perforate; echinae densely distributed;
W. excelsa	L	SO	isopolar	(5)-6	Colporus	lalongate: rectangular to elliptical	long, broad; apices rounded	-	-	Echinate-perforate-granulate; echinae sparsely distributed;
W. macropoda	L	OS	isopolar	(5)-6-7-(8)	Colporus	lolongate	long; apices acute	-	-	Echinate-micoreticulate; echinae densely distributed;
W. rotundifolia	L	S	apolar	(10)-12-13-(14)	Colporus	lolongate	narrow; apices acute	-	-	Echinate-rugulate-perforate; echinae densely distributed;
W. selloana	M to L	OS	isopolar	(5)-6-(7)	Colporus	circular	short to long, narrow; apices acute	+	-	Echinate-perforate; echinae densely distributed;
W. vernonioides	L	S	apolar	12-13-14-15-(16)	Porus	circular	broad; apices rouded	-	-	Echinate-perforate; echinae densely distributed;
W. viscosissima	L	OS	isopolar	(5)-6	Colporus	lalongate: elliptical	short; apices acute	+	-	Echinate-microreticulate; echinae densely distributed;

Note: L=large; M=medium; OS=oblate spheroidal; SO=suboblate; S=spheroidal; [+] present; [-] absent

similar results, whereas Saba *et al.* (2004) and Saba (2007) found no such correlation. In this study, no significant difference in thickness was observed between the floral morphs of *W. bracteosa* and *W. coriacea*.

Among the reticulate pollen grains, the sexine type was almost always thicker than the nexine, while the reverse was true among the echinate type. However, most authors noted this "rule" only among longistylous morphs (Erdtman 1952; Melhem *et al.* 1976; Saba & Santos 2003; 2015; Saba *et al.* 2004).

This study was the second time that the ultrastructure of *Waltheria* pollen grains has been investigated. Saba & Santos (2015) observed four levels of sexine stratification: Sexine 1 corresponding to the compact continuous foot layer, sexine 2 formed by thicker columellae, sexine 3 is defined as the tectum, which is thinner, and sexine 4 was formed by muriform thickening on sexine 3, which gives

Table 3. Morphometric data of *Waltheria* L. pollen grains (isopolar)

Species/Specimen	PD		ED		EDp		P/E	Ecto	Endo	PAI	Sex	Nex	Lum	E.H	I.D
	$\bar{x}$ ±S$\bar{x}$	Fv	$\bar{x}$ ±S$\bar{x}$	Fv	$\bar{x}$ ±S$\bar{x}$	Fv									
Waltheria ackermanniana															
Saunders J. 3105 (CEPEC) (B)	53.4*	50.0-60.0	58.8*	55.0-60.0	57.2*	55.0-60.0	0.90	10.3x5.0	5.6x8.0	0.48	1.0	2.0	-	2.2	2.0
Hatschbach G. 47381 (CEPEC) (B)	57.2*	50.0-65.0	62.0*	55.0-72.5	59.5*	47.5-72.5	0.92	12.2x4.0	5.5x5.0	0.45	1.4	1.8	-	1.8	1.8
Saunders J. 3107 (CEPEC) (L)	44.7±0.7	40.0-47.5	48.8±1.0	45.0-53.7	48.8±1.0	45.0-52.2	0.91	23.8x3.4	4.9x9.6	0.39	1.8	1.1	2.1	-	-
W. albicans															
Saunders J. 3149 (CEPEC) (B)	44.8±1.2	40.0-50.0	49.5±1.1	45.0-52.5	48.1±1.2	42.5-52.5	0.90	8.2x2.7	4.7x4.6	0.57	1.2	1.6	-	1.9	2.0
Nunes T. S. et al. 520 (HUEFS) (L)	38.8±0.8	35.0-42.5	37.4±0.7	32.5-40.0	37.3±0.7	35.0-40.0	1.03	22.1x2.1	5.0x4.5	0.56	1.9	1.0	1.2	-	-
Saunders J. 3117 (CEPEC) (L)	34.1±0.6	30.0-37.5	30.7±0.5	27.5-32.5	28.9*	30.0-35.0	1.11	21.2x1.1	4.5x7.2	0.58	1.8	1.0	1.2	-	-
W. brachypetala															
Saunders J. 3116 (CEPEC) (B)	45.1*	40.0-50.0	47.1*	42.5-52.5	45.8*	42.5-50.0	0.95	9.8x2.4	5.2x6.8	0.56	1.1	1.4	-	1.8	2.2
Saunders J. 3119 (CEPEC) (B)	44.7±0.9	40.0-47.5	47.0±1.3	37.5-52.5	47.0±1.6	37.5-52.5	0.95	12.6x2.7	3.4x6.8	0.55	1.0	1.3	-	1.5	2.0
Queiroz. L. P. 7010 (HUEFS) (L)	34.8±0.9	32.5-40.0	37.9±0.8	32.5-42.5	37.4±1.0	32.5-42.5	0.91	20.0x4.4	4.9x8.4	0.43	1.0	1.3	1.0	-	-
W. bracteosa															
Harley R. M. 19119 (CEPEC) (B)	56.0±0.9	50.0-60.0	60.2±1.0	55.0-65.0	51.5±3.7	40.0-70.0	0.93	8.0x3.2	6.0x5.9	0.47	1.0	1.1	-	2.6	2.6
Saunders J. 3143 (CEPEC) (L)	52.0±1.0	47.5-57.5	46.4±1.2	42.5-57.5	37.5*	37.5-37.5	1.12	24.6x1.7	8.6x4.4	-	1.0	1.0	1.8	-	-
W. cinerascens															
Saunders J. 3222 (CEPEC) (B)	49.0*	47.5-50.0	57.5*	55.0-60.0	55.2*	50.0-60.0	0.85	9.0x3.3	2.6x6.0	0.40	1.1	1.8	-	1.1	2.0
Silva L. A. M. 2819 (CEPEC) (B)	58.6±2.1	45.0-65.0	65.3±2.3	62.5-72.5	59.4±2.7	50.0-60.0	0.89	13.4x5.1	6.6x10.6	0.44	1.0	2.3	-	1.8	2.9
Silva L. A. M. 1252 (CEPEC) (L)	38.3±0.7	35.0-41.2	39.2±0.7	37.5-43.7	42.5±1.2	37.5-47.5	0.97	23.2x1.4	4.4x4.2	0.45	2.0	1.0	1.2	-	-
W. coriacea															
Harley R. M. 53757 (CEPEC) (B)	48.7±1.2	41.2-52.5	51.2±1.1	42.5-57.5	50.4±1.1	43.7-57.5	0.95	16.2x4.9	5.3x6.6	0.54	1.1	1.4	-	1.7	2.0
Oliveira R. P. 467 (HUEFS) (L)	34.7*	30.0-37.5	36.2*	32.5-40.0	35.6*	32.5-40.0	0.95	18.8x2.3	3.4x7.8	0.52	1.5	0.9	1.2	-	-
W. excelsa															
Saunders J. 3122 (CEPEC) (B)	47.2±0.7	45.0-50.0	53.2±0.8	47.5-57.5	51.5±1.0	47.5-57.5	0.88	7.8x3.3	4.7x5.7	0.48	1.2	2.0	-	2.8	3.4
W. macropoda															
Sant'Ana S. C. 778 (CEPEC) (B)	59.5±0.9	57.5-65.0	64.0±1.1	60.0-70.0	63.5±1.0	60.0-67.5	0.92	__	7.5x6.2	0.44	1.0	2.0	-	3.7	2.5
Saunders J. 3123 (CEPEC) (L)	50.1±1.1	45.0-57.5	46.3±1.2	42.5-52.5	45.7*	40.0-50.0	1.08	23.8x1.7	6.6x8.1	0.65	1.1	1.0	2.0	-	-
Harley R. M. 54313 (CEPEC) (L)	44.0±1.2	37.5-50.0	43.0±1.1	37.5-50.0	44.7±0.8	40.0-50.0	1.02	22.2x3.8	5.8x9.5	0.53	1.4	1.0	1.4	-	-
W. operculata															
Harley R. M. 19024 (SPF) (H)	52.5±1.4	45.0-60.0	51.3±1.2	45.0-57.5	53.9±1.6	45.0-62.5	1.02	23.0x4.9	5.9x7.8	0.60	1.5	1.0	<1.0	-	-
W. selloana															
Kral R. 72731 (CEPEC) (H)	47.6±0.9	42.5-52.5	51.4±1.0	47.5-57.5	52.2±1.5	45.0-57.5	0.92	8.8x2.6	5.2x5.7	0.43	1.0	1.6	-	2.0	1.0
Mori S. A. 12379 (CEPEC) (H)	47.0*	42.5-47.5	48.3*	45.0-53.5	47.1±1.4	37.5-53.7	0.97	___	5.0x5.0	0.52	1.8	0.9	-	1.7	1.3
W. viscosissima															
Saunders J. 3234 (CEPEC) (B)	46.0±0.8	42.5-50.0	50.0±1.2	45.0-57.5	49.3±1.2	42.5-55.0	0.92	8.4x--	5.3x4.5	0.52	1.0	2.1	-	1.5	1.1
Roque N. 533 (CEPEC) (L)	43.4±1.2	35.0-47.5	40.7±1.0	37.5-45.0	39.3±0.9	35.0-45.0	1.06	20.4x1.7	5.6x9.8	0.70	1.9	0.8	1.0	-	-
Noblick L. R. 2359 (CEPEC) (L)	37.6±0.7	35.0-40.0	36.7±0.6	35.0-40.0	37.5±1.0	32.5-40.0	1.02	18.4x1.5	4.6x8.7	0.69	1.5	0.5	1.0	-	-

Note: (B)=Brevistylous morph; (H)= Homostylous morph; (L)= Longistylous morph; PD=Polar diameter; ED=Equatorial diameter; EDp= Equatorial diameter in polar view; Ecto=Length x width of the ectoaperture; Endo=Height x width of the endoaperture; PAI= polar area index; Sex=Sexine; Nex=Nexine; Lum=Lumina diameter; E.H.=Echinus height; I.D.=Interespinal distance; *n<25; measures in µm and indexes in absolute numbers.

Table 4. Morphometric data of *Waltheria* L. pollen grains (apolar)

Species/Specimen	D		P/E	Ecto	Endo	Sex	Nex	Lum	E.H.	I.D
	$\bar{x}\pm S_{\bar{x}}$	Fv								
W. communis										
Saunders J. 3227 (CEPEC) (B)	58.5±1.0	52.5-62.5	1.00	7.5x4.0	4.7x4.9	1.0	2.0	-	1.8	2.5
Giulietti. A. M. 2455 (HUEFS) (B)	55.7±1.2	50.0-62.5	1.00	__	5.3x4.9	1.3	2.3	-	2.1	1.9
Saunders J. 3229 (CEPEC) (L)	52.7±1.8	45.0-65.0	1.00	6.1x3.0	5.6x5.2	1.5	1.1	4.2	-	-
W. rotundifolia										
Gonçalves L. M. C. 76 (CEPEC) (B)	55.9±1.0	50.0-60.0	1.00	5.5x1.7	5.1x4.4	1.0	1.8	-	2.1	1.7
Saunders J. 3126 (CEPEC) (L)	49.8±0.7	47.5-52.5	1.00	6.4x1.5	4.7x4.6	2.8	0.9	3.8	-	-
W. vernonioides										
Saunders J. 3060 (CEPEC) (H)	55.4±1.4	47.5-60.0	1.00	5.7x3.0	4.7x4.5	1.0	2.3	-	1.5	1.0
Hoehne E. C. 2976 (SP) (H)	54.8±1.2	50.0-60.0	1.00	7.0x4.3	4.6x4.6	2.8	2.8	-	1.4	1.0

Note: (B)=Brevistylous morph; (H)= Homostylous morph; (L)= Longistylous morph; D=Polen grains diameter; Ecto= Length x width of the ectoaperture; Endo= Height x width of the endoaperture; Sex= sexine; Nex= nexine; Lum= Lumina diameter; E.H.= Echinus heigh; D.I= Interespinal distance; *n<25; measures in μm and indexes in absolute numbers.

the undulating appearance to the tectum. Of the species studied here, only *W. operculata* did not have the fourth layer, despite its undulating appearance under LM.

Most authors who have studied *Waltheria* pollen grains determined that the exine ornamentation is microreticulate, reticulate, or suprareticulate among longistylous morphs (Erdtman 1952; Köhler 1971; Melhem *et al.* 1976; Miranda & Andrade 1989; Saunders 1993; Saba & Santos 2000; Saunders 2005). Here we clearly observed perforations located on the lumina under SEM. This type of ornamentation was interpreted as reticulate-perforated in this study.

As in the present study, Köhler (1971) was unable to clearly observe perforations on the tectum of brevistylous pollen grains under LM. Köhler (1976) confirmed the existence of a semitectum using SEM and reported undulations and perforations distributed densely over the surface. While the specimens studied by Saba *et al.* (2004) showed pollen grains with microreticulate semitectum, demonstrating that these perforations are difficult to detect under LM.

Conclusion

A high degree of pollen heteromorphism due to heterostyly was confirmed in *Waltheria*, which is frequently detected by palynologists (Saba *et al.* 2004; Saba & Santos 2015).

The apertural type and number and their distribution throughout the pollen grains were important factors for grouping the species, and exine ornamentation could not be used to differentiate species, although it was useful to precisely distinguish the pollen grains of brevistylous and longistylous morphs.

A better understanding of the pollen ultrastructure of *Waltheria* was achieved through this study, as the data obtained here are presented for the first time for most of these species.

The palynological characterization of the species studied here provide essential data for future taxonomic and/or phylogenetic studies on the genus *Waltheria*, as well as other applied research among its species. However, further studies are necessary for a complete palynological characterization and, consequently, a more concise definition of the taxa, which will require that a larger sample size be investigated under SEM and TEM.

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