A contribution to the knowledge of exine ultrastructure in subtribe Anisopappinae (Athroismeae, Asteraceae)

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A contribution to the knowledge of exine ultrastructure in subtribe *Anisopappinae* (*Athroismeae*, *Asteraceae*)

Abstract

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To better understand the taxonomy and phylogeny of the *Anisopappinae* and *Athroismeae*, the exine ultrastructure of two species of the two genera of that subtribe – *Anisopappus chinensis* Hook. & Arn. and *Cardosoa athanasioides* (Paiva & S. Ortiz) S. Ortiz & Paiva (both representing the types of the generic names) – were studied with transmission electron microscopy and scanning electron microscopy. As for other *Asteroideae* and *Asteraceae*, the existence of a mesoaperture intersecting the foot layer and the upper part of the endexine was established. The exines of both taxa have a Helianthoid pattern of structure, which supports the approaching of the *Athroismeae* to the *Heliantheae* s.l. but not to the *Inuleae*. There were no significant differences between the qualitative and quantitative characters of the two taxa, which support the inclusion of *Cardosoa* S. Ortiz & Paiva in the *Anisopappinae* and the consistency of the subtribe.

Additional key words: *Anisopappus*, *Cardosoa*, TEM, SEM, pollen morphology, taxonomy

Introduction

The *Anisopappinae*, one of the three subtribes of the small (five to six genera) and newly described tribe *Athroismeae* (Panero & Funk 2002) comprises two (Panero 2007; Anderberg 2009) to three (Ortiz 2010) genera and approximately 21 species (Panero 2007). They include annual to perennial herbs and their main diversity is found in tropical Africa and Madagascar, with one species (*Anisopappus chinensis* Hook. & Arn., representing the type of the generic name) also present in SE Asia (Eldenäs & Anderberg 1996; Panero 2007; Anderberg 2009; Chen & Anderberg 2011). In his morphology-based phylogenetic work, Eriksson (1991) placed some genera of the *Athroismeae* (e.g. *Athroisma* DC. and *Blepharispermum* DC.) in the *Heliantheae*. As a result of a phylogenetic analysis using morphological, anatomical and karyological data, Anderberg (1991) considered *Anisopappus* Hook. & Arn. to be sister to the *Inuleae*. Three years later, Anderberg (1994) included this genus in the *Inuleae*. Based on their analysis of *ndhF* sequences, Kim and Jansen (1995) considered that the *Blepharispermum* group should be placed in the *Heliantheae*, as a sister group to all the *Heliantheae* s.l. Employing morphological characters, Ortiz & Paiva (1995) described *Anisopappus athanasioides* Paiva & S. Ortiz. In the same year, Ortiz & al. (1996) and Eldenäs and Anderberg (1996) respectively published a taxonomic outline of *Anisopappus* and a morphology-based cladistic analysis of this genus. On the sequence of a molecular phylogenetic analysis using the

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Table 1. Assignments of the genera of *Anisopappinae* based on major treatments.

Treatment	Method	Genera	Results
Merxmüller & al. 1977	Morphology + pollen + chromosome number	*Anisopappus*	*Anisoppappus* was included in the *Inuleae–Inulinae*
Anderberg 1991	Phylogenetic analysis (morphology, karyology, anatomy)	*Anisopappus*	*Anisopappus* is sister to the *Inuleae*
Anderberg 1994	Phylogenetic analysis (morphology, karyology, anatomy)	*Anisopappus*	*Anisopappus* is sister to the *Inuleae* and was included in that tribe
Eldenäs & Anderberg 1996	Phylogenetic analysis (morphology)	*Anisopappus*	*Anisopappus latifolius* (S. Moore) B. L. Burtt is sister to the other species of the genus *Anisopappus*
Ortiz & al. 1996	Morphology	*Anisopappus*	Taxonomic and phylogenetic treatment of *Anisopappus*
Eldenäs & al. 1999	Phylogenetic analysis (*ndhF*)	*Anisopappus*	*Anisopappus* and *Blepharispermum* group were included in the *Heliantheae* s.l.
Wagstaff & Breitwieser 2002	Phylogenetic analysis (ITS)	*Anisopappus*	*Centipeda cunninghamii* is sister to a clade including the *Athroisma* group (incl. *Anisopappus smutsii* Hutch.) + *Heliantheae* s.l.
Panero 2005	Phylogenetic analysis (cpDNA: several coding regions)	*Anisopappus*, *Welwitschiella*	The *Anisopappinae* were described
Panero 2007	cpDNA: several coding regions + morphology	*Anisopappus*, *Welwitschiella*	*Anisopappus* and *Welwitschiella* were included in the *Anisopappinae*
Anderberg 2009	Morphology + chromosome number + molecular data	*Anisopappus*, *Welwitschiella*	*Anisopappus* was included in the *Anisopappinae*; *Welwitschiella* was included in the *Astereae*
Brouillet & al. 2009a	Phylogenetic analysis (*ndhF*, ITS)	*Welwitschiella*	*Welwitschiella* was included in the *Astereae*
Brouillet & al. 2009b	Phylogenetic analysis (ITS)	*Welwitschiella*	*Welwitschiella* was included in the *Astereae*
Ortiz 2010	Morphology	*Anisopappus*, *Cardosoa*	*Cardosoa* was recognized at generic rank

cpDNA *ndhF*, Eldenäs & al. (1999) placed *Anisopappus* in the same clade as the genera of the *Blepharispermum* group and suggested that they should provisionally be considered as members of the *Heliantheae* s.l. Wagstaff and Breitwieser (2002), in their study of nuclear ribosomal ITS sequences, included the genera of *Athroismeae* in a clade together with the genera of the *Heliantheae* complex of tribes. Based on a molecular phylogenetic analysis of an extensive sampling of *Asteraceae* using many cpDNA markers, Panero & Funk (2002) suggested that the *Blepharispermum* group should be considered a tribe (*Athroismeae*) and situated this taxon between a clade including the *Inuleae* and the *Heliantheae* s.l. In 2008, Panero & Funk stated these same conclusions in their new phylogenetic analysis. Panero (2005) described for the first time the *Anisopappinae*, included in that subtribe the genus *Anisopappus*, and proposed to include *Welwitschiella* O. Hoffm. Ortiz (2010), based on anatomical and macro- and micromorphological characters, treated *Anisopappus athanasioides* in a new monospecific genus: *Cardosoa* S. Ortiz & Paiva.

Despite Panero's (2005) proposal to include *Welwitschiella* in *Athroismeae*, supported by morphological features and followed by Panero (2007) and Ortiz (2010), the molecular phylogenetic analysis (ITS, *ndhF*) performed by Brouillet & al. (2009a–b) clearly concluded that this genus belongs to the *Astereae* (subtribe *Grangeinae*). Anderberg (2009) reached the same conclusion. The *Astereae* form a monophyletic group with *Anthemideae*, *Calenduleae* and *Gnaphalieae* (Funk & al. 2009), strongly supported (Panero & Funk 2008), while *Athroismeae* is part of another well-supported monophyletic group (Panero & Funk 2008) together with *Inuleae* and the different tribes of the "*Heliantheae* alliance" (Funk & al. 2009). So we decided to exclude *Welwitschiella* from *Athroismeae*, a decision also recently taken by, e.g., Pruski (2014).

The assignments of the genera of *Anisopappinae* based on major treatments are summarized in Table 1.

Leins (1971) investigated, with light microscopy (LM), the pollen morphology of 20 species of *Anisopappus*, which he included in three pollen types. Pornpongrungrueng & Chantaratothai (2002) studied, with LM and scanning electron microscopy (SEM), the pollen morphology of one single specimen of *A. chinensis*. No works on the exine ultrastructure of *Anisopappinae* or, with the sole exception of *Centipeda* Lour. (Skvarla & al. 1977), of other *Athroismeae* have been published until now, either with transmission electron microscopy (TEM) or SEM. For these reasons, the exine ultrastructure of *Anisopappinae* has been very insufficiently known.

Many authors (e.g. Wagenitz 1976; Skvarla & al. 1977, 2005; Bolick 1978; Wortley & al. 2008, 2012; Pereira Coutinho & Dinis 2009, 2012; Blackmore & al. 2010) stressed the importance of the pollen morphology data in understanding the taxonomy, ecology and evolution of the *Asteraceae*. For that reason, we decided to study, with TEM and SEM, the ultrastructure of the exines of *Anisopappus chinensis* and *Cardosoa athanasioides* (Paiva & S. Ortiz) S. Ortiz & Paiva (both representing the types of the generic names). Our main aim was to contribute to the knowledge of the pollen morphology of the *Anisopappinae* and *Athroismeae* in a taxonomic and phylogenetic context and to use the palynological data to verify the taxonomic consistency of these taxa. Secondarily, we wanted to contribute to the question of the existence of a mesoaperture in the pollen of the *Asteraceae*.

Material and methods

Pollen grains were collected from a total of six herbarium specimens of *Anisopappus chinensis* and two of *Cardosoa athanasioides* from the following institutions: COI, SANT (herbarium codes follow Thiers 2014+). After collection, the grains were acetolised according to Erdtman (1960). In order to increase the number of exine fractures, the acetolysis was prolonged to ten minutes (100 °C). The terminology for exine description follows Punt & al. (2007) and Hesse & al. (2009).

TEM — Pollen grains of *Anisopappus chinensis* and *Cardosoa athanasioides* were fixed in 2 % osmium tetroxide in 0.1 M sodium cacodilate buffer, pH 7.2 for 24 h, dehydrated in a graded ethanol series (70 %–100 %) and embedded in Spurr's resin. Thin sections were obtained with a LKB Ultrotome Nova ultramicrotome equipped with a diamond knife, conventionally stained with uranyl acetate and lead citrate, and observed with a JEOL JEM-100 SX at 80 kV. Using micrographs of the exine of the species studied, 15 measurements of the following characters were taken: sexine, nexine, endexine, foot layer, tectum and connected columellar bases thickness, length and width of the inter-spinular columellae. We also calculated the ratio of inter-spinular columellae length to width.

SEM — After being dehydrated in a graded acetone series, the pollen grains of both species were mounted on aluminium stubs, coated with gold-palladium with an ion-sputter and observed with a Hitachi SU-70 (4 kV) scanning electron microscope. Fifteen measurements of the maximum diameter of spinular perforations were taken.

Results

General description of exine structure

Ectoapertures intersecting the sexine (Fig. 1C–D), mesoapertures intersecting the foot layer and the upper part of the endexine (Fig. 1C–D), endoapertures intersecting the inner part of the endexine (Fig. 1C–D), colpal membrane formed by the lower part of the endexine (Fig. 1C–D). Exine with a Helianthoid pattern, i.e. caveate (Fig. 1A–F), foraminate (Fig. C–F), with a single layer of columellae (Fig. 1A–F); sexine composed by a tectum and an infratectum, the infratectum formed by a columellar layer and the connected columellar bases (Fig. 1A, C–F); nexine thinner than the sexine (Fig. 1A–F); inter-apertural endexine with an irregular inner surface (Fig. 1A–F), apertural endexine thicker (Fig. 1C–D); foot layer quite smooth (Fig. 1C–F); connected columellar bases perforate (Fig. 1C–F); inter-spinular columellae originating, by lower and upper branching, respectively the connected columellar bases and the tectum (Fig. 1A, C, E, F); tectum perforate (Fig. 1A–F). Sculpture echinate-perforate (Fig. 1); spines conic, with concave (Fig. 1A, C) or ± straight (Fig. 1E) sides, attenuate, apex generally acute (Fig. 1A, C) sometimes ± obtuse, with a sub-apical cavity (Fig. 1C, E), spinular columellae reaching c. ⅔ of the height of the spine (Fig. 1C, E), longer than the inter-spinular, ± branched (Fig. 1C–F), sometimes originating various columellae levels (Fig. 1E); spinular perforations reaching c. ⅔ of the height of the spine (Fig. 1, A, C, E), variable in shape (Fig. 1A) and size (Fig. 1A, E), larger nearer the apex (Fig. 1A, C, D, E), inter-spinular perforations smaller, variable in size and shape (Fig. 1A, C, E, F).

Anisopappus chinensis

Exine thickness = 2.50–3.70 (2.87 ± 0.86) µm; sexine thickness = 0.78–1.32 (0.91 ± 0.09) µm; nexine thickness = 0.44–0.80 (0.65 ± 0.12) µm; connected columellar bases thickness = 0.35–0.55 (0.45 ± 0.10) µm; inter-spinular columellae length = 0.30–0.80 (0.50 ± 0.12) µm, width = 0.15–0.35 (0.24 ± 0.05) µm, length / width = 2.06–3.50 (2.16 ± 0.67); tectum thickness = 0.24–0.40 (0.31 ± 0.05) µm; maximum diameter of the spinular perforations = 0.35–0.57 (0.44 ± 0.08) µm.

Cardosoa athanasioides

Exine thickness = 2–4 (3.07 ± 0.44) µm; sexine thickness = 0.66–1.10 (0.88 ± 0.09) µm; nexine thickness = 0.69–1.37 (1.05 ± 0.27) µm; connected columellar bases thickness = 0.22–0.45 (0.31 ± 0.06) µm; inter-spinular columellae length = 0.33–0.66 (0.45 ± 0.07) µm, width = 0.12–0.27 (0.18 ± 0.04) µm, inter-spinular columellae length / width = 1.83–3.15 (2.22 ± 0.39); tectum thickness = 0.11–0.40 (0.21 ± 0.10) µm; maximum diameter of the spinular perforations = 0.31–0.67 (0.42 ± 0.09) µm.

Discussion

Bolick (1991) stated that caveate pollen grains correlate with a generally thinner exine. Our own results (exine thickness mean = 2.87 µm in *Anisopappus chinensis*

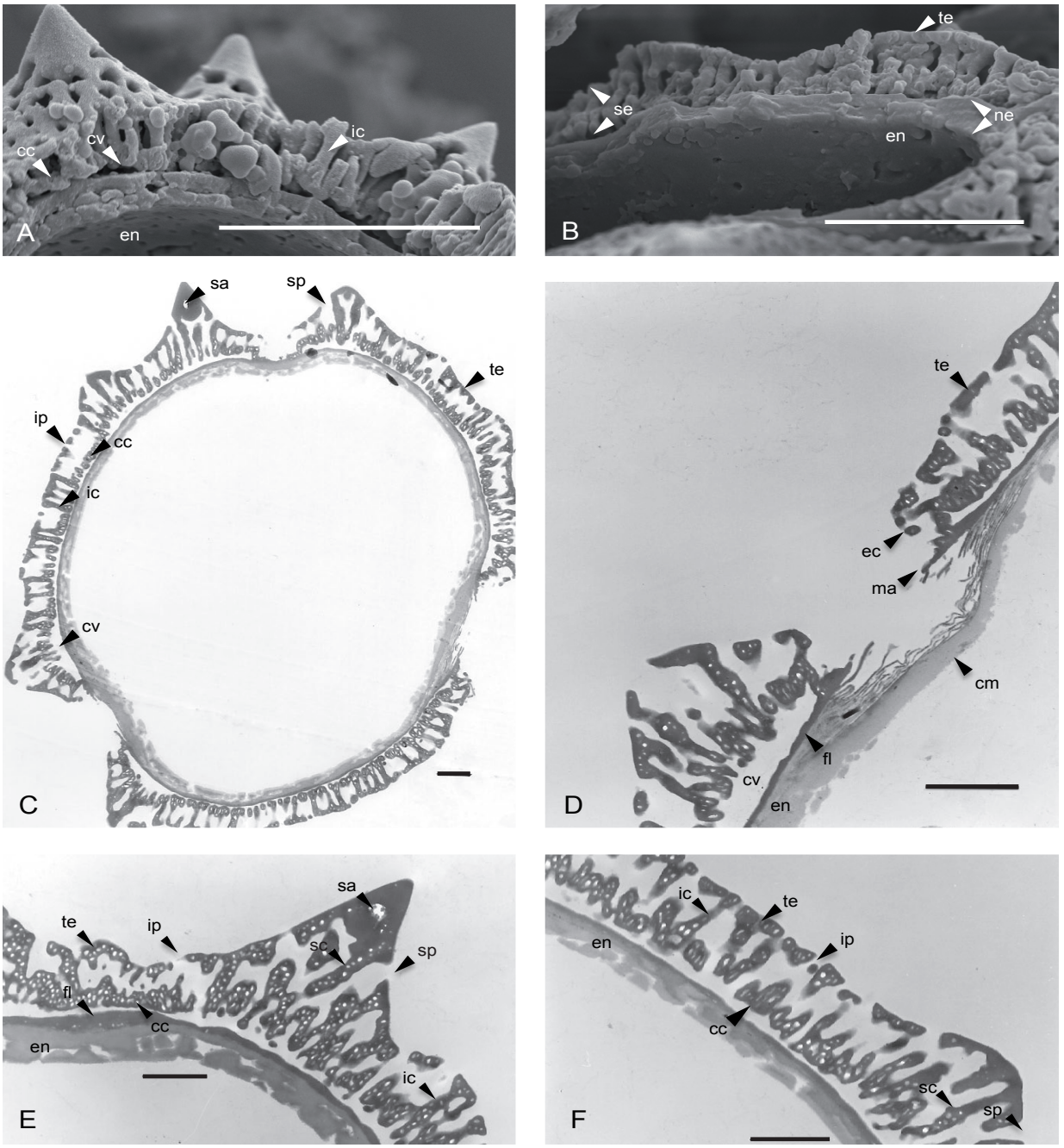

Fig. 1. SEM (A & B) and TEM (C–F) micrographs of pollen grains of *Anisopappus chinensis* and *Cardosoa athanasioides* – A: *A. chinensis*, fractured exine; B: *C. athanasioides*, fractured exine; C: *C. athanasioides*, section at equator; D: *C. athanasioides*, section of exine through an aperture; E & F: *A. chinensis*, details of sections of exine: *cc* connected columellar bases, *cm* colpal membrane, *cv* cavea, *ec* ectoaperture, *en* endexine, *fl* foot layer, *ic* inter-spinular columellae, *ip* inter-spinular perforations, *it* internal tectum, *ma* mesoaperture, *ne* nexine, *sa* sub-apical cavity, *sc* spinular columellae, *se* sexine, *sp* spinular perforations, *te* tectum. – Scale bars: A = 4 µm; B = 3 µm; C–F = 1 µm.

and and 3.07 µm in *Cardosoa athanasioides*) agree with those of Bolick (exine thickness mean of caveate pollen grains = 2.3–3.7 µm).

The presence of a sub-apical cavity in the pollen spines of *Anisopappus chinensis* (Chen & Anderberg 2011), confirmed by this study, is a very common feature in the *Asteraceae* (see e.g. Skvarla & al. 1977; Salgado-Labouriau 1982; Pereira Coutinho 2002; Pereira Coutinho & Paiva 2003).

As far as data obtained using light microscopy and TEM/SEM may be compared, the qualitative and quantitative features of the exine structure obtained by us are generally close to those pointed out by Leins (1971) for the pollen type *Anisopappus lastii* (O. Hoffm.) Wild (which includes *A. chinensis*). For example, that author described the existence of thickenings at the base of the spinular columellae, a characteristic that is visible in our micrographs and corresponds to the connected columellar

bases. Also, the mean values we found for the thicknesses of, respectively, the inter-spinular sexine and the nexine of *A. chinensis* (0.91 µm, 0.65 µm) and *Cardosoa athanasioides* (0.88 µm, 1.05 µm) are quite close to those of Leins (1.2 µm, 0.7 µm). Nevertheless, in the figure he published, the connected columellar bases are represented with approximately the same thickness of the tectum, which is faulty (see descriptions).

The fact that we were not able to find any reliable qualitative or quantitative character to distinguish the exine of *Anisopappus chinensis* from that of *Cardosoa athanasioides* supports Ortiz (2010) statement that *Cardosoa* belongs to the *Anisopappinae* and the taxonomic consistency of this subtribe.

The TEM sections of the exine revealed the existence of a typical Helianthoid pattern for *Anisopappus chinensis* and *Cardosoa athanasioides*. The same pattern is recognizable in the fractured exines obtained, with SEM, for both taxa. In fact, it must be noted that many internal foramina are observable in the sexine of the two species. These conditions do not contradict the elevation of the *Athroismeae* to tribe and support its proximity to the *Heliantheae* s.l., as suggested by several authors (Eriksson 1991; Anderberg 1994; Eldenäs & Anderberg 1996; Eldenäs & al. 1999; Wagstaff & Breitwieser 2002; Panero & Funk 2002, 2008; Funk & al. 2009), but not to the *Inuleae*, as proposed by other botanists (Merxmüller & al. 1977; Anderberg 1989, 1991; Eldenäs & Anderberg 1996; Ortiz & al. 1996; Eldenäs & al. 1998), since the ultrastructural pattern of the exine of this last tribe is, always, Senecioid (Skvarla & al. 1977; Pereira Coutinho & Dinis 2007). The sub-Gnaphalioid pattern described by Skvarla & al. (1977) for *Centipeda cunninghamii* (DC.) A. Braun & Asch. differs from that of *Anisopappus* and *Cardosoa* mainly by the presence, within the sexine, of an interlaced layer. It must be noted, however, that the exines of some *Helenieae* (Skvarla & al. 1977; Pereira Coutinho 2002) and *Heliantheae* (Skvarla & al. 1977) present transitional forms of ultrastructure between the Helianthoid and the Gnaphalioid patterns. The foot layer of the exine of *A. chinensis* and *C. athanasioides* is quite smooth, a common condition in the *Heliantheae*, but not in the *Gnaphalieae*, which predominantly have a rather rough foot layer (Pereira Coutinho & Dinis 2009). Curiously, in the TEM micrograph published by Skvarla & al. (1977), the foot layer of *C. cunninghamii* seems to present an intermediate condition (i.e. foot layer somewhat rough).

The existence of a mesoaperture in the apertural system of the pollen grains of the angiosperms is quite a rare feature, being limited only to some families like the *Boraginaceae* (Saad-Limam & al. 2002) and the *Polygonaceae* (Punt & al. 2008). In the case of *Asteraceae*, Dimon (1971) pointed out the existence of an intermediate aperture for all the taxa of the Mediterranean area she studied. According to that palynologist, the mesoapertures intersected the foot layer. In their study of the *Cardueae* exine, Tormo-Molina & Ubera-Jiménez (1990) indicated that the intermediate aperture concerned not only the foot layer, but also the upper part of the endexine. Some other authors pointed out its existence for several taxa of *Asteraceae* but did not indicate which layers of the exine it intersected (e.g. Diez 1987; Blackmore & al. 2009) or stated that their observation is frequently quite difficult (Diez 1987; Pereira Coutinho & Dinis 2009). This last condition is certainly correlated to the fact that the limits of the ectoapertures often cover those of the mesoapertures (Pereira Coutinho & Dinis 2009). For this reason, the employment of TEM is important to elucidate the questions of the existence or not of an intermediate aperture in the exine of the *Asteraceae* and which layers it concerns. In the case of the *Anisopappinae*, our own results agree with those of Tormo-Molina & Ubera-Jiménez (1990). In fact, as it happened with other taxonomic groups of *Asteraceae* (e.g. Pereira Coutinho & Paiva 2003; Pereira Coutinho & Dinis 2007, 2009; Pereira Coutinho & al. 2011), the existence of a mesoaperture intersecting the foot layer and the upper part of the endexine was well established. Apparently, this condition constitutes a synapomorphy for all the *Asteraceae* or, at least, for all the *Asteroideae*.

It is interesting to note that in the *Anisopappinae* as in other taxa of *Asteraceae* (e.g. *Inulinae* – Pereira Coutinho & Dinis 2007) the spinular columellae and perforations reach the same height, a condition that enhances the function of the spines as repositories and conductors of exine hold substances (like enzymes and recognition proteins) to the stigma surface (Bolick 1978; Blackmore 1982; Salgado-Labouriau 1982).

Conclusions

This study investigated, for the first time, the exine ultrastructure of the *Anisopappinae*. As in other *Asteroideae*, the presence of a well-defined mesoaperture concerning the foot layer and the upper part of the endexine was established. The presence of a Helianthoid pattern of the exine in the two taxa studied places *Athroismeae* close to the *Heliantheae* s.l., but not to the *Inuleae*. The qualitative and quantitative data support the inclusion of *Cardosoa* in the *Anisopappinae* and enhances the taxonomic consistency of this subtribe.

Specimens investigated

Anisopappus chinensis

Angola: Bié, Cassuango, Cuiriri, Mar 1906, *Gossweiler 3738* (COI); Cassuango, Manangue, vale do rio Cuiriri, 24 Mar 1906, *Gossweiler 3687* (COI). — Huambo: Caputo Ruilundo, 24 May 1962, *Teixeira & Andrade 8233* (COI). — Huíla: Kampuluvé, 6 Apr 1900, *H. Baum s.n.* (COI [isotypus]); Sá da Bandeira, entre o rio Nene e Huíla, 14 May 1966, *C. Henriques 975* (COI); Ungueria pr. rio Chacuto, 2 Jun 1937, *Gossweiler 11137* (COI).

Cardosoa athanasioides

Angola: Huambo: Cachihugo, 23 Jun 2007, *S. Ortiz, Paiva, Rodriguez-Oubiña, Carballal, Serrano & Soares 847* (SANT); Chianga, Nova Lisboa, 10 May 1965, *Hélder Cardoso s.n.* (COI [holotypus]).

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