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

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Source: Willdenowia, 44(3) : 431-437

Published By: Botanic Garden and Botanical Museum Berlin (BGBM)

URL: https://doi.org/10.3372/wi.44.44314
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Abstract

DOI: http://dx.doi.org/10.3372/wi.44.44314

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.

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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 Athroismae 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 (Athroismae) 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 athanasioideae in a new monospecific genus: Cardosoa S. Ortiz & Paiva.

Despite Panero’s (2005) proposal to include Welwitschiella in Athroismae, 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 Grangeineae), 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 Athroismae 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 Athroismae, 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. Pornpong-rungrueng & 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 Athroismae 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. Secondly, 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 aceticised according to Erdtman (1960). In order to increase the number of exine fractures, the acetylosis 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 Heliandroid 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...
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...

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**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.
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 approximatively 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 (Eriksen 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, Seneciod (Skvarla & al. 1977; Pereira Coutinho & Dinis 2007). The sub-Gnaphalioi 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 Gnaphalioi 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 _Gnaphalioeae_, 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 _Polygonoaceae_ (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_

Cardosoa athanasioides

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

Acknowledgements

The authors are grateful to the late curator of the Coimbra Herbarium, Dr Maria Teresa Almeida, for having allowed the study of the vouchers of Anisopappus chinensis and Cardosoa athanasioides and to three anonymous reviewers for their comments on an earlier draft of this paper.

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