

## POLLEN EVOLUTION IN THE GENUS *ECHINOPS* (CARDUEAE, ASTERACEAE): DECIPHERING THE ORIGIN OF GIANT POLLEN GRAINS

Oriane Hidalgo,<sup>1,\*</sup>† Ismael Sánchez-Jiménez,<sup>\*</sup> Luis Palazzesi,<sup>‡</sup>§ Benoît Loeuille,<sup>†</sup> and Teresa Garnatje<sup>\*</sup>

<sup>\*</sup>Institut Botànic de Barcelona (IBB), Consejo Superior de Investigaciones Científicas (CSIC) Ajuntament de Barcelona, Passeig del Migdia sn, 08038 Barcelona, Catalonia, Spain; <sup>†</sup>Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AB, United Kingdom; <sup>‡</sup>Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), C1405DJR Buenos Aires, Argentina; and <sup>§</sup>Sección Paleopalinoología, Museo Argentino de Ciencias Naturales “Bernardino Rivadavia,” C1405DJR Buenos Aires, Argentina

Editor: Jennifer Mandel

**Premise of research.** The genus *Echinops* is unique among the Cardueae tribe of Asteraceae for presenting two distinctive features, both related to reproductive structures: a syncephalium (=secondary capitulum) and an impressively large pollen grain with a triangular section and probably the thickest of all plant cell walls. While the syncephalium constitutes a synapomorphy for the genus, recent evidence suggests that some *Echinops* species have pollen similar to that of other Cardueae. This study therefore seeks to contribute insights into the spatiotemporal frame of pollen evolution within the genus.

**Methodology.** Micromorphological characterization was provided for 35 specimens from 28 *Echinops* species using light and scanning electron microscopy. Pollen counts were carried out for two *Echinops* species and *Cardopatum corymbosum*. Pollen data are discussed in the context of a dated *Echinops* phylogeny. For comparison purposes, new and published pollen data of 622 Cardueae species and 303 taxa of Vernonieae, another tribe where syncephaly has evolved, were collated.

**Pivotal results.** The “Perennial” *Echinops* pollen type of huge size and triangular section likely derived from the “Annual” *Echinops* pollen type, more similar in shape, size, and exine structure to that of other Cardueae. Pollen type transition took place in the genus long after syncephaly evolved. Pollen size increase did not occur at the expense of pollen quantity and could respond to warmer environmental conditions and increased male competition.

**Conclusions.** This study of *Echinops* pollen evidenced the evolutionary exploration of novel phenotypic space in the genus, most certainly in response to the climatic context in which the species have diversified.

**Keywords:** Compositae, exine, light microscopy (LM), scanning electron microscopy (SEM), syncephalium.

**Online enhancements:** tables.

### Introduction

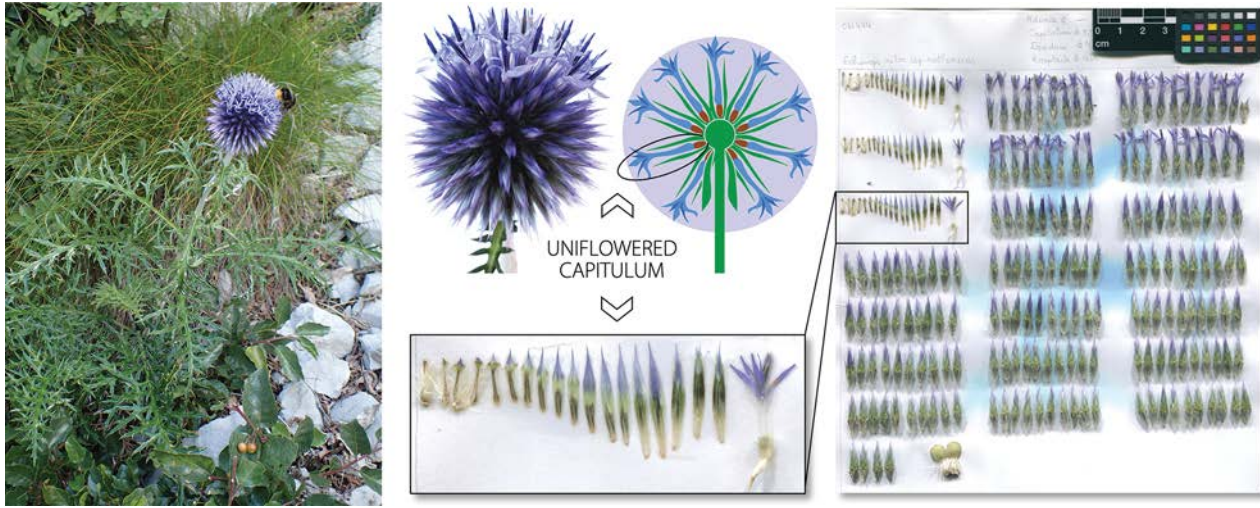
*Echinops* L. is a genus of ca. 120 species from Eurasia and Africa, classified in the monotypic subtribe Echinopsinae (Cardueae, Asteraceae; Susanna et al. 2020). The genus is notable among Cardueae in that it is the only one to have evolved a syncephalium (=secondary capitulum), which represents a synapomorphy for the taxon. The spherical shape of this syncephalium, its basipetal flowering sequence, and the fact that it is constituted by uniflorous capitula (often with blue flowers, hence the common name “blue thistle”), make *Echinops* species easily recognizable (fig. 1). The genus also stands out for producing impressively large pollen grains, triangular prisms rounded

at the poles with slightly sunken lateral sides (Mohl 1835), and probably the thickest of all plant cell walls (Gabarayeva et al. 2018). It is likely that both traits (i.e., syncephalium and giant pollen grains) have had a considerable impact on the reproductive biology of *Echinops* species, which are mainly pollinated by insects (Grant 1950), although cases of bird-pollinated species are also reported (Vogel 2015).

Of the published classifications of Asteraceae and Cardueae pollen grains, most consider that *Echinops* pollen should constitute a separate pollen type (Mohl 1835; Stix 1960; Pla Dalmau 1961; Díez 1987; Tormo and Uberta 1988, 1995; Perveen 1999; Jafari and Ghanbarian 2007; Punt and Hoen 2009). However, while *Echinops* has long been model for the study of pollen morphogenesis (e.g., Stix 1964, 1970; Blackmore 1990; Gabarayeva et al. 2018), little attention has been paid to describing and interpreting pollen diversity within the genus. To our knowledge, pollen characterization data are limited to about 20 *Echinops* species (i.e., only ca. 17% of the species; Tormo and Uberta 1988;

<sup>1</sup> Author for correspondence; email: oriane.hidalgo@ibb.csic.es.

Manuscript received November 2022; revised manuscript received January 2023; electronically published May 1, 2023.



**Fig. 1** *Echinops ritro* subsp. *ruthenicus*. Photo of an individual in the field (left) and dissection of its syncephalium (right), showing 184 uniflowered capitula and the secondary receptacle (protocol described in Fu et al. 2023). Photograph credit: Oriane Hidalgo.

Garnatje and Martín 2007; Osman 2009; Punt and Hoen 2009; Vural et al. 2010; Kallajxhiu et al. 2014; Tahmasebi et al. 2022). Nevertheless, the few data available revealed a particularly wide range of pollen size, from polar axis (P) of 14.50 to  $>100 \mu\text{m}$  (Erdtman 1952; Tormo and Uberta 1988; Garnatje and Martín 2007; Tahmasebi et al. 2022). Garnatje and Martín (2007) consider that pollen grains of the lower size range, corresponding to annual *Echinops* species, should constitute a different type, which they call the “Annual” *Echinops* pollen type. This pollen type characterized by a  $P < 50 \mu\text{m}$  and a circular section in polar view shows affinities with other pollen types in the tribe Cardueae (e.g., the crupina type; Wagenitz 1955). The pollen of  $P > 50 \mu\text{m}$  with triangular section and prominent bridge-shaped intercolpia, exclusive to *Echinops* and in particular its perennial species, constitutes the “Perennial” *Echinops* pollen type (Garnatje and Martín 2007).

Whether the arising of giant pollen grains could be related to syncephaly is unknown, although one would certainly expect the evolution of these two highly distinctive features of *Echinops*, both related to reproductive structures, to be linked to some extent. A temporal frame of pollen type transitions within the genus is lacking; however, it was suggested that the Annual *Echinops* type could have evolved in the most recent common ancestor of *Echinops* species, while the Perennial *Echinops* type could have evolved when perennial species diverged (Sánchez-Jiménez et al. 2010). If this were the case, this would mean that the pollen of huge size and triangular section evolved much after the syncephalium. Nevertheless, the possibility that the Annual *Echinops* type derived from the Perennial type cannot be totally excluded, and its similarities with other Cardueae pollen would then be convergences due to sharing a short growth cycle (e.g., as found in some annual/biennial, high-mountain, or invasive species; Hidalgo et al. 2008b). Regarding the possible impact of the inflorescence context on pollen evolution, the examination of pollen traits in other syncephalous Asteraceae could shed some light on the subject. The tribe Vernoniae is particularly interesting in this respect, as it includes representatives with syncephalia resem-

bling those of *Echinops* (e.g., *Chresta* Vell. ex DC.; Siniscalchi et al. 2017).

The aims of this study were to (i) assess the extent of pollen diversity in *Echinops*, (ii) provide insights into pollen type transitions, and (iii) address the evolutionary and functional significance of pollen traits in the genus.

## Material and Methods

### Plants

Floral buds were obtained from herbarium specimens (table 1) and preserved in paper envelopes until processed for pollen examination by light microscope (LM) and scanning electron microscope (SEM). Sampling covers the nine *Echinops* sections whose circumscription was verified and redefined by Sánchez-Jiménez et al. (2010; i.e., sects. *Acantholepis* (Less.) Jaub. & Spach, *Chamaechinops* Bunge, *Echinops*, *Hamolepis* R.E.Fr., *Hololeuce* Rech.f., *Oligolepis* Bunge, *Phaeochaete* Bunge, *Psectra* Endl., and *Ritropsis* Greuter & Rech.f.), along with one representative of sect. *Cenchrolepis* Hochst. (*E. hispidus* Fresen) and two tropical African species, *E. eryngiifolius* O.Hoffm. and *E. gracilis* O.Hoffm., that are not attributed to any section. For pollen counting, fresh plant material was collected from cultivated plants at the Botanical Garden of Barcelona and the Royal Botanic Gardens, Kew. The five mature, undehisced anthers of a floral bud were collected and stored in an open PCR tube in a box with silica gel and left to dry for at least 1 wk.

### Light Microscopy

Pollen was acetolyzed following the micromethod described by Avetissian (1950), mounted on glycerogelatine, and sealed with nail polish. Pollen plates were photographed with a Zeiss AxioCam HRm digital camera mounted on a Zeiss Axioplan microscope, and images were analyzed with AxioVision LE version 4.8.2. Depending on the specimen, 7–50 fully developed

Table 1

Pollen Size, Shape, and Type of <i>Echinops</i> Accessions, with Indication of Collection Data							
Taxon	P $\pm$ SD ( $\mu$ m)	E $\pm$ SD ( $\mu$ m)	P/E	Shape	Type	<i>n</i>	Accession data
<i>E. albicaulis</i>	88.80 $\pm$ 7.3	72.99 $\pm$ 6.1	1.22	SP	EA	15	Kazakhstan; Goloskokov s.n. (GB)
<i>E. amplexicaulis</i>	80.21 $\pm$ 6.3	51.95 $\pm$ 4.7	1.55	P	Pe	13	Cameroon; Jacques-Felix 9083 (YA)
<i>E. bannaticus</i>	72.12 $\pm$ 4.8	55.22 $\pm$ 4.0	1.31	SP	Pe	23	Montenegro; Garnatje & Sánchez-Jiménez 14 (BC)
<i>E. cephalotes</i>	78.77 $\pm$ 3.5	63.63 $\pm$ 4.6	1.24	SP	Pe	50	Iran; Babakanlou & Amin s.n., 13.VII.1973 (W 1973-05105)
<i>E. cornigerus</i>	99.62 $\pm$ 5.8	71.50 $\pm$ 5.2	1.40	P	Pe	39	Afghanistan; Grey-Wilson & Hewer 1656 (GB)
<i>E. davuricus</i>	64.01 $\pm$ 3.7	46.10 $\pm$ 3.2	1.39	P	Pe	47	China; Liu, Cao & Vallès s.n., 29.VIII.2007 (BC)
<i>E. elbursensis</i>	...	...	...	...	EA	...	Iran, Tehran; 25.VIII.1975 (W)
<i>E. echinatus</i>	76.45 $\pm$ 4.5	61.43 $\pm$ 3.7	1.25	SP	Pe	46	Pakistan; (W 1979-05252)
<i>E. emiliae</i>	61.21 $\pm$ 4.3	57.07 $\pm$ 4.5	1.07	Sph	EA	44	Turkey; Ertugrul, Garcia-Jacas & Susanna 2262 (BC)
<i>E. eryngiifolius</i>	103.80 $\pm$ 5.9	64.28 $\pm$ 5.6	1.62	P	Pe	35	Rwanda; Raynal 20690 (B 10 0024097)
<i>E. exaltatus</i>	66.89 $\pm$ 5.4	55.75 $\pm$ 5.6	1.20	SP	Pe	50	Slovenia; Garnatje & Sánchez-Jiménez 6 (BC)
<i>E. fastigiatus</i>	21.84 $\pm$ .96	20.19 $\pm$ .90	1.08	Sph	An	31	Kazakhstan; Goloskokov s.n. (LE)
<i>E. fontqueri</i>	103.70 $\pm$ 3.5	74.98 $\pm$ 2.8	1.38	P	Pe	15	Morocco; Gómiz s.n., 19.V.2002 (BC)
<i>E. freitagii</i>	55.14 $\pm$ 2.1	44.13 $\pm$ 3.5	1.26	SP	Pe	40	Iran, Khorasan; 1.VIII.1975 (W 1979-07328)
<i>E. gmelinii</i>	41.91 $\pm$ 3.7	36.36 $\pm$ 2.9	1.15	SP	An	7	China; Vallès, Yan & Zhao s.n., 2.IX.2007 (BC)
<i>E. gracilis</i>	67.69 $\pm$ 6.1	50.71 $\pm$ 5.9	1.34	P	Pe	48	Cameroon; Letouzey 8784 (YA)
<i>E. graecus</i>	67.01 $\pm$ 3.7	61.77 $\pm$ 3.6	1.09	Sph	Pe	27	Greece; Garnatje & Sánchez-Jiménez 35 (BC)
<i>E. griffithianus</i>	103.3 $\pm$ 3.6	76.70 $\pm$ 4.3	1.35	P	Pe	42	Afghanistan; Jørgensen 569 (GB)
<i>E. hoehnelii</i>	80.65 $\pm$ 4.1	51.19 $\pm$ 4.1	1.58	P	Pe	45	Kenya; Galbany & Arrabal s.n., 10.IX.2006 (BC)
<i>E. hololeucus</i>	58.14 $\pm$ 3.7	46.49 $\pm$ 3.2	1.25	SP	Ho	37	Afghanistan; 34°00'N, 64°45'E, 27.VII.1962 (W 1972-19421)
<i>E. humilis</i>	33.67 $\pm$ 1.1	37.49 $\pm$ 1.0	.90	Sph	An	47	Russia; Tipokhina & Danilyuk s.n., 10.VIII.1973 (LE)
<i>E. hystrioides</i>	99.22 $\pm$ 3.2	70.24 $\pm$ 4.3	1.41	P	Pe	29	Saudi Arabia; Collenette 1570 (E00276929)
<i>E. hystrioides</i>	101.10 $\pm$ 7.3	70.84 $\pm$ 6.1	1.43	P	Pe	21	Yemen; Hein 3806 (BC)
<i>E. hystrioides</i>	135.02 $\pm$ 14.9	94.33 $\pm$ 7.0	1.43	P	Pe	35	Yemen; Hein 3806 (B 10 0220615)
<i>E. hystrioides</i>	96.95 $\pm$ 5.4	71.02 $\pm$ 7.0	1.37	P	Pe	35	Yemen; Hein 3806 (B 10 0220613)
<i>E. hystrioides</i>	122.34 $\pm$ 6.9	97.23 $\pm$ 11.4	1.26	SP	Pe	16	Yemen; Hein 3942 (B 10 0220608)
<i>E. integrifolius</i>	26.34 $\pm$ 1.60	24.05 $\pm$ 1.07	1.09	Sph	An	15	China; (PE 00554105)
<i>E. longifolius</i>	78.15 $\pm$ 4.7	48.23 $\pm$ 6.9	1.62	P	Pe	16	Togo; Hein & Pircher 772 (B 10 0293577)
<i>E. przewalskyi</i>	68.82 $\pm$ 2.7	51.21 $\pm$ 3.5	1.35	P	Pe	43	China; Vallès & Zhao s.n., 5.IX.2007 (BC)
<i>E. spinosissimus</i>	125.14 $\pm$ 6.2	88.22 $\pm$ 6.7	1.42	P	Pe	7	Somalia; Bally 9941 (G00160590)
<i>E. spinosissimus</i>	94.64 $\pm$ 6.0	66.36 $\pm$ 2.8	1.42	P	Pe	16	Yemen; Hein 6936 (B 10 0220616)
<i>E. spinosissimus</i>	95.85 $\pm$ 4.9	74.00 $\pm$ 5.9	1.30	SP	Pe	32	Chad; Pappi 131 (G00160589)
<i>E. spinosissimus</i> subsp. <i>spinosissimus</i>	145.50 $\pm$ 4.1	106.30 $\pm$ 4.1	1.37	P	Pe	39	Greece; Rechanger12702 (W)
<i>E. talassicus</i>	76.24 $\pm$ 3.7	58.64 $\pm$ 4.3	1.30	SP	Pe	32	Kazakhstan; Ivaschenko, Susanna 2180 & Vallès (BC)
<i>E. transcaucasicus</i>	...	...	...	...	Pe	...	Armenia; Avetisian, Arevschatian & Pogolian s.n., 30.VIII.1979 (W)

Note. P = polar axis; E = equatorial axis. Shape: Sph = spheroidal P/E [0.88–1.14]; SP = subprolate P/E [1.14–1.33]; P = prolate P/E [1.33–2). Pollen type: An = annual; EA = Echinata-Annual; Ho = Hololeucus; Pe = Perennial. *n* = number of pollen grains measured by LM.

grains were measured. The parameters considered were polar and equatorial (E) diameters and shape (P/E ratio). For pollen grains with a triangular cross section, we consider that the diameter E corresponds to the length of the side.

#### Scanning Electron Microscopy

We followed the procedure of Halbritter (1998) with slight modifications. Pollen grains from herbarium vouchers were rehydrated by placing the anthers into an Eppendorf tube filled with pure water and further dehydrated overnight in 30 mL of 2,2-dimethoxypropane (DMP) acidified with a drop of 0.2 M HCl. Anther tissues were subsequently removed, and the tube was centrifuged before eliminating the DMP. Pollen grains were critical-point dried in CO<sub>2</sub>, using ethanol as intermediate fluid, further mounted on stubs, sputter-coated with graphite, and observed with an ESEM Quanta 200 FEI XTE 325/D8395 at high-vacuum conditions at the Centres Científics i Tecnològics of the Univer-

sitat de Barcelona. In the case of *E. elbursensis* Rech.f. and *E. transcaucasicus* Iljin, pollen grains were acetolyzed as described above, gold-coated with diode sputtering, and observed under a Hitachi 52300. Pollen description followed the nomenclature recommendations of Punt et al. (2007).

#### Pollen Counts by Flow Cytometry

We used lycopod spore tablets as a standard for pollen counting (9666  $\pm$  671 spores per capsule; batch 3862; Department of Quaternary Geology, Lund University, Lund, Sweden) following the protocol of J. M. de Vos, Y. Woudstra, I. J. Leitch, and O. Hidalgo (personal communication). Samples were analyzed using a CyFlow SL3 flow cytometer (Partec, Münster, Germany) fitted with a 100-mW green solid-state laser (Cobolt Samba, Solna, Sweden). For each species, we analyzed two flowers per individual, with three technical replicates each.

### Divergence Time Estimation

Sequences of nr-ITS and cp-*trnL*F regions were gathered from GenBank and edited with BioEdit (ver. 7.2.3; Hall 1999) to constitute a data set of 97 taxa, including 85 *Echinops* and 12 outgroups species. Alignment was carried out on GUIDANCE web server (ver. 1.5; Penn et al. 2010) using MAFFT multiple alignment algorithm (ver. 7; Katoh and Standley 2013), with 100 bootstrap repeats, a max iterate of 1000, and a local pair refinement strategy. Divergence times were estimated with BEAST (ver. 2.1.3; Bouckaert et al. 2014) using the combined ITS and *trnL*F data set. A minimum age constraint of 47.5 mya was applied based on the fossil pollen grain *Mutisiapollis telleriae* for calibration (Palazzesi et al. 2022b and references therein). Each gene was analyzed under separate substitution models selected using the Akaike information criterion implemented in jModelTest (ver. 2.1.4; Darriba et al. 2012)—namely, SYM+I+G for ITS and GTR+G for *trnL*F—while assuming the clock and tree were linked. Uncorrelated, lognormal relaxed clock model and birth-death tree prior model were specified.

### Tribe-Level Surveys

New and published P and E length data were collated for 997 accessions of Cardueae (see raw data in table A1). Plant names were updated primarily using the Plants of the World Online and Euro+Med Plantbase databases (Greuter 2006; POWO 2022), leading to a total of 622 species represented. P and E values were then averaged per species. Subtribe assignment follows the circumscriptions of Herrando-Moraira et al. (2019, 2020). For comparative purposes, we also compiled data for another tribe, the Vernonieae, that includes representatives with a syncephalium of globular shape and a small number of flowers per capitulum resembling that of *Echinops* (e.g., *Chresta*; Siniscalchi et al. 2017) as well as other types of syncephalium (e.g., *Eremanthus* Less [Loeuille et al. 2012]; *Paralychnophora* MacLeish [Souza-Souza et al. 2016]). P and E length data were gathered from the literature for 303 taxa of Vernonieae (table A2), together with information on whether these taxa were syncephalous.

### Data Visualization and Analysis

Data visualization, processing, and analysis were carried out in R (ver. 3.2.2; R Core Team 2019) using the package ggplot2 (Wickham 2016) and the function ace in phytools (Revell 2012) for ancestral state reconstruction.

## Results

This study contributed pollen measurements (images not shown; table 1) and SEM views (figs. 2, 3) for 35 specimens from 28 *Echinops* species. Our results further extend the already remarkable pollen size diversity known in the genus, to a range of P length spanning 1 order of magnitude, up to 145.5  $\mu\text{m}$  in *E. spinosissimus* Freyn subsp. *spinosissimus* (table 1). The study provides the first pollen count data for the genera *Cardopatium* Juss. and *Echinops*, with mean values of  $2669.84 \pm 754.01$  for *C. corymbosum* (L.) Pers.,  $4716.34 \pm 651$  for *E. pappii* Chiov., and  $4142.95 \pm 321.90$  for *E. sphaerocephalus* L. Details of pollen count data are provided in table A3.

We collated new and previously published pollen data for about 50 *Echinops* species, which were then used to assign pollen type—either Annual or Perennial—following the description of Garnatje and Martín (2007). However, the pollen of some species did not correspond to either type, which led us to describe two new pollen types: the Echinete-Annual and the Hololeucus pollen types (description provided in table 2). It is worth noting that the affinities of Annual, Echinete-Annual, and Hololeucus pollen types, while differing in size (fig. 4) and exine ornamentation (figs. 2, 3), are much closer to each other than to the Perennial type (table 2). The latter is clearly distinguished by its peculiar shape and its exceptionally complex exine (Gabarayeva et al. 2018; table 2).

The mapping of pollen types onto the phylogeny shows an almost complete correspondence to *Echinops* sections (fig. 4). Indeed, excluding two species, each section is characterized by a single pollen type: Annual for sections *Acantholepis* and *Chamaechinops*, Echinete-Annual for sect. *Psectra*, Hololeucus for sect. *Hololeuce*, and Perennial for the remaining sections (table 2; fig. 4). The two exceptions are *E. strigosus* in sect. *Psectra*, with Annual type, and *E. albicaulis* Kar. & Kir. in sect. *Echinops*, with Echinete-Annual type. This attests to the low evolutionary lability of pollen type despite the great diversity of pollen size and shape displayed by the genus.

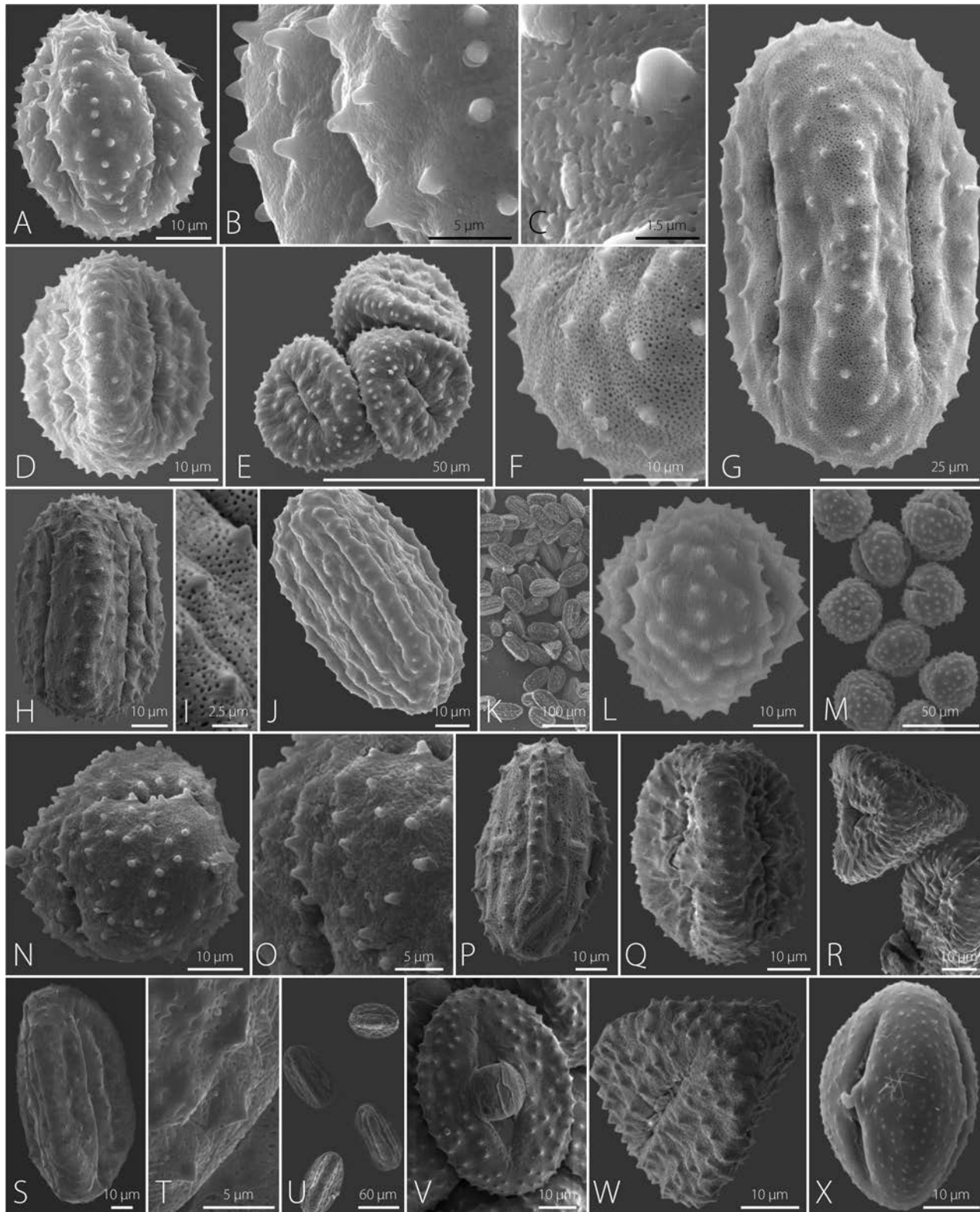
The ancestral state reconstruction suggests that *Echinops* Annual pollen type characterizes the backbone of the genus, while *Echinops* Perennial pollen type evolved in the common ancestor of the clade comprising sects. *Echinops*, *Hamolepis*, *Oligolepis*, *Phaeochaete*, and *Ritropsis*, 6.10 mya (node marked with a B in the chronogram of fig. 4).

A general trend in pollen evolution in *Echinops* is an increase in size, especially of the P-axis (fig. 4) that varies over a 10.03-fold range and reaches in sect. *Ritropsis* and *Oligolepis* the largest values for the Cardueae (fig. 5A, 5B). Changes in size are also accompanied by changes in shape, the smallest grains being spheroidal or subprolate and the largest ones being prolate (fig. 5A). In comparison, such size and shape changes are not observed in the tribe Vernonieae (fig. 5C, 5D). Indeed, the range of P-axis values in this tribe is only 4.30-fold, and all of the 303 taxa studied so far have spheroidal or suboblate pollen (fig. 5C).

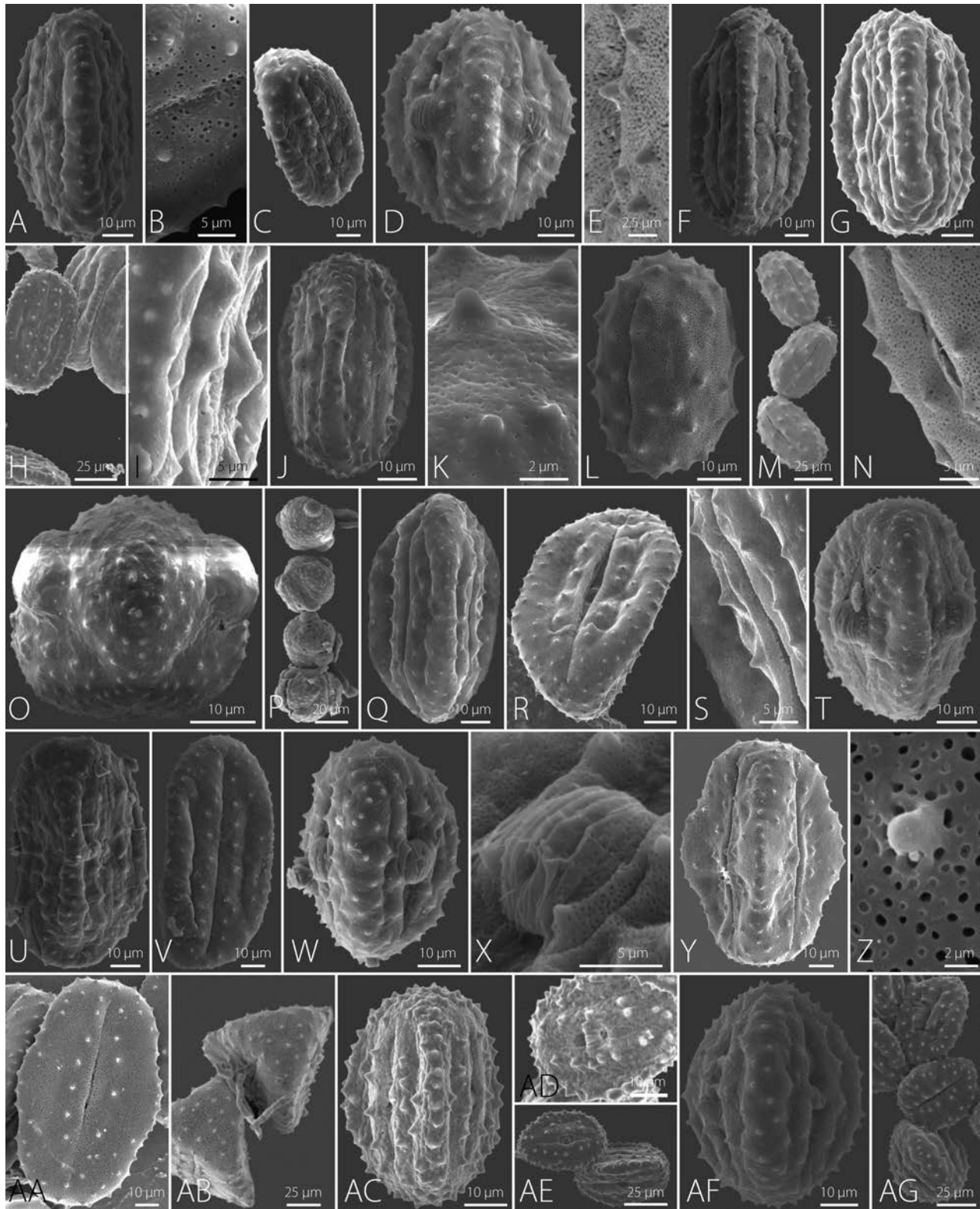
## Discussion

### Evolution of *Echinops* within Cardueae: Climatic Context

The phylogenomics hypothesis of Mandel et al. (2019) places *Echinops* as a sister group to the remaining Cardueae, with the exception of *Carlina* L. and *Cardopatium*; these last two taxa form a clade sister to the remaining Cardueae. The tribe Cardueae may have diverged from the rest of Asteraceae about 40 mya in western Asia according to dating analyses and ancestral area reconstructions (Barres et al. 2013; Mandel et al. 2019). We inferred here that the origin of Cardueae occurred during a global warming event (i.e., Mid-Eocene Climatic Optimum [MECO]), which has been recorded throughout the world (Bijl et al. 2010). Aridification appears to have intensified during and after the MECO, particularly in places today occupied by massive deserts such as the Tibetan Plateau (Bosboom et al. 2014). The crown Cardueae—that is, the most recent common ancestor of the tribe—evolved about 36 mya, during the Late Eocene, when



**Fig. 2** Scanning electron microscopy views of pollen grains for *Echinops* species. A–C, *Echinops albicaulis*. D–G, *Echinops bannaticus*. H, I, *Echinops cephalotes*. J, K, *Echinops cornigerus*. L, M, *Echinops elbursensis*. N, O, *Echinops emiliae*. P, *Echinops eryngiifolius*. Q, R, *Echinops exaltatus*. S–U, *Echinops fontqueri*. V, W, *Echinops freitagii*. X, *Echinops gmelini*.



**Fig. 3** Scanning electron microscopy views of pollen grains for *Echinops* species. A–C, *Echinops gracilis*. D–F, *Echinops graecus*. G–I, *Echinops griffithianus*. J, K, *Echinops hoehmellii*. L–N, *Echinops hololeucus*. O, P, *Echinops humilis*. Q–S, *Echinops hystricoides*. T, *Echinops latifolius*. U, V, *Echinops longifolius*. W, X, *Echinops przewalskyi*. Y–AB, *Echinops spinosissimus* subsp. *spinosissimus*. AC–AE, *Echinops talassicus*. AF, AG, *Echinops transcaucasicus*.

**Table 2**  
**Pollen Types in the Genus *Echinops***

<i>Echinops</i> pollen type	Description	Taxa included
Annual	Pollen grains small or medium, $P < 50 \mu\text{m}$ , spheroidal to subprolate; exine ornamentation microechinated with suprategal pointed elements from 0.10 to 0.15 $\mu\text{m}$ , which are regularly distributed; <sup>2</sup> circular section in polar view	Sect. <i>Acantholepis</i> : <i>E. acantholepis</i> , <sup>1</sup> <i>E. gmelinii</i> , <i>E. namus</i> ; <sup>1</sup> sect. <i>Chamaechinops</i> : <i>E. fastigiatus</i> , <i>E. humilis</i> , <i>E. integrifolius</i> ; sect. <i>Psectra</i> : <i>E. strigosus</i> <sup>1</sup>
Echinate-Annual	Pollen grains medium or large, $20 < P < 100 \mu\text{m}$ , spheroidal to subprolate, resembling the Annual type but with ornamentation echinated consisting of spines with rounded tips; circular section in polar view	Sect. <i>Echinops</i> clade VIII: <i>E. albicaulis</i> ; sect. <sup>1</sup> <i>Psectra</i> : <i>E. elbursensis</i> , <i>E. emiliae</i>
Hololeucus	Pollen grains of medium size, $P$ around 60 $\mu\text{m}$ , subprolate in shape; exine ornamentation consisting of sparsely distributed spines with rounded tips; circular section in polar view	Sect. <i>Hololeuce</i> : <i>E. hololeucus</i>
Perennial	Pollen grains large or very large, $P > 50 \mu\text{m}$ , subprolate to prolate shape, in few cases spheroidal (e.g., <i>E. graecus</i> , <i>E. transcaasicus</i> ); exine ornamentation microechinated/echinated-verrucated; intercolpal zone raised, producing a pronounced bridge; triangular section in polar view <sup>2</sup>	Sect. <i>Cenchrolepis</i> : <i>E. giganteus</i> , <sup>1</sup> <i>E. hispidus</i> ; <sup>1</sup> sect. <i>Echinops</i> clade VIII: <i>E. bannaticus</i> , <i>E. davuricus</i> , <i>E. exaltatus</i> , <i>E. freitagii</i> , <i>E. graecus</i> , <i>E. microcephalus</i> , <sup>3</sup> <i>E. obliquilobus</i> , <sup>1</sup> <i>E. ossicus</i> , <sup>1</sup> <i>E. przewalskyi</i> , <i>E. ritro</i> , <sup>1</sup> <i>E. sphaerocephalus</i> , <sup>1</sup> <i>E. talassicus</i> ; sect. <i>Echinops</i> clade IX: <i>E. transcaasicus</i> ; sect. <i>Hamolepis</i> : <i>E. hoehnelii</i> ; sect. <i>Oligolepis</i> : <i>E. cornigerus</i> , <i>E. dumanii</i> , <sup>2</sup> <i>E. echinatus</i> , <i>E. griffithianus</i> , <i>E. phaeocephalus</i> , <sup>2</sup> <i>E. polygamus</i> ; <sup>3</sup> sect. <i>Phaeochaete</i> : <i>E. amplexicaulis</i> , <i>E. cephalotes</i> , <i>E. longifolius</i> , <i>E. mildbraedii</i> ; <sup>1</sup> sect. <i>Ritropsis</i> : <i>E. fontqueri</i> , <sup>1</sup> <i>E. glaberrimus</i> , <sup>4</sup> <i>E. hystrichoides</i> , <i>E. macrochaetus</i> , <sup>4</sup> <i>E. macrophyllus</i> , <sup>5</sup> <i>E. spinosissimus</i> , <i>E. tenuisectus</i> ; <sup>3</sup> unknown or doubtful sect.: <i>E. chlorophyllus</i> , <sup>6,a</sup> <i>E. eryngiifolius</i> , <i>E. galalensis</i> , <sup>4</sup> <i>E. gracilis</i> , <i>E. hussonii</i> , <sup>4</sup> <i>E. robustus</i> , <sup>6,a</sup> <i>E. saissanicus</i> <sup>1</sup>

Sources. 1 = Garnatje and Martín (2007); 2 = Vural et al. (2010); 3 = Tahmasebi et al. (2022); 4 = Osman (2009); 5 = Jafari and Ghanbarian (2007); 6 = Ikuse (1962).

Note. In one case, for *E. taeckholmianus* (Osman 2009), we were unable to assign a pollen type. P = polar axis.

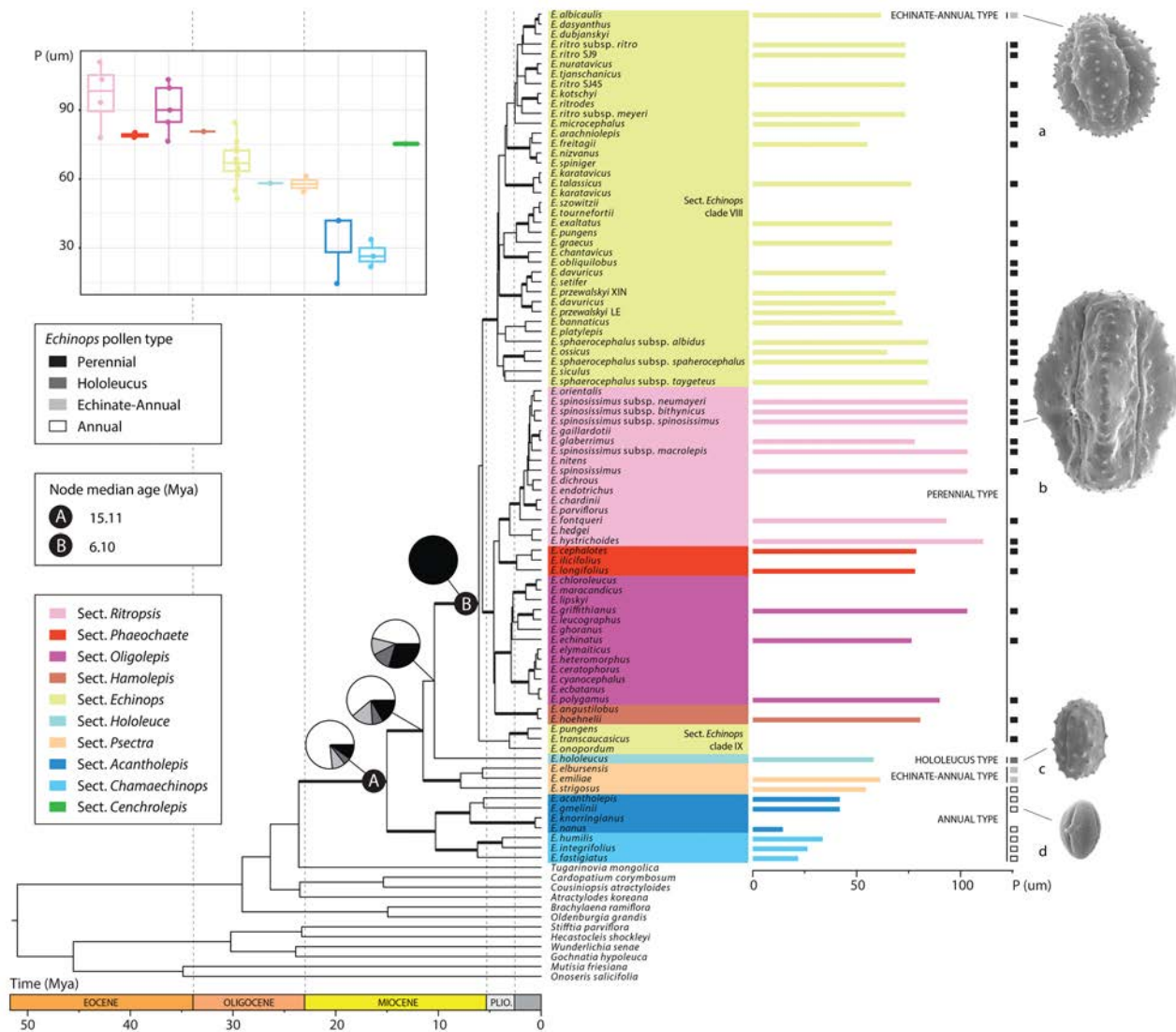
<sup>a</sup> *Echinops chlorophyllus* Rech.f. and *E. robustus* Bunge have been traditionally ranged into sect. *Oligolepis*. However, a very similar species, *E. cephalotes*, was removed from this section and placed in sect. *Phaeochaete* (Sánchez-Jiménez et al. 2010). These species should likely be reassigned to sect. *Phaeochaete*; however, pending molecular phylogenetic data, we chose to let them be unassigned.

ephemeral continental-scale glaciations occurred in Antarctica (Van Breedam et al. 2022), anticipating a more widespread glaciation event at the Eocene-Oligocene transition (34.44–33.65 mya). This period of a large temperature drop across the Eocene-Oligocene transition marks the onset of the modern “ice-house” world. In this context, the family Asteraceae as well as other grasslands’ components (e.g., Poaceae) started to rise in diversification linked to a global decrease in CO<sub>2</sub> (Palazzesi et al. 2022a). Also, *Echinops* may have split from its sister group during this climatic scenario, although previously published data suggested a later origin of the genus, during the Miocene (Montazerolghaem et al. 2017). The lack of fossil records assigned to *Echinops* prevents any substantial interpretation about the origin and diversification of the genus. In line with this, Partridge (1978) recorded some fossil pollen grains that he claimed to belong to *Echinops* or *Mutisia* L.f. from Neogene offshore sediments in western Africa. However, the overall morphology of the pollen grains—for example, the size and distribution of the spines—appears not to be related to either *Echinops* or *Mutisia* but to other Cardueae. The unquestionable Quaternary record of *Echinops*, however, allows us to better interpret the climatic context in which the genus grew and expanded. For example, dispersed pollen grains recorded by Jiang et al. (2014) in China and deposited during the Last Glacial Maximum, about 20,000 years

ago, lead them to suggest that *Echinops*, associated with other arid-adapted shrubs and herbs (e.g., *Taraxacum* F.H.Wigg. and Chenopodiaceae), bloomed during very dry conditions at the expense of humid-demanding taxa (e.g., grasses). Further interpretations of the climatic context during the early evolution of *Echinops* (late Paleogene–early Neogene) await new fossil discoveries.

#### *Evolution of Pollen in Echinops: Decoupled from That of the Syncephalium and from Any Polyploidization Event*

Our results show that the Perennial pollen type appeared 6.10 mya at the earliest, well after the syncephalium, which evolved in the most recent common ancestor of all *Echinops* 15.11 mya (fig. 4). Previous studies suggest that one or more polyploidization events may have accompanied the evolution of these characters in *Echinops* (Sánchez-Jiménez et al. 2009). Polyploidy and subsequent genome diploidization mechanisms are widely recognized as key players promoting evolutionary diversification (e.g., Dodsworth et al. 2016; Landis et al. 2018). However, mixed evidence has been found for their effect as drivers of pollen morphological evolution (Jardine et al. 2022), including pollen size (reviewed in Sanders 2021).



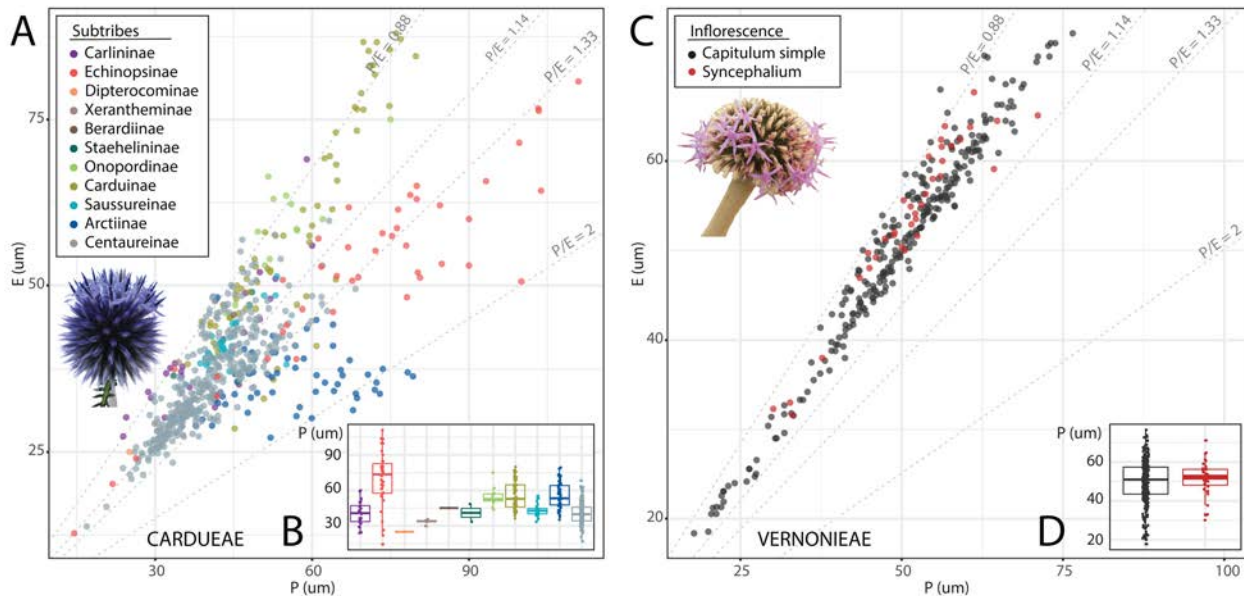
**Fig. 4** Distribution of mean polar axis (P) values per species and pollen types across the different sections of *Echinops* (following the taxonomic circumscription of Sánchez-Jiménez et al. 2010). Ancestral state reconstruction results of pollen type are presented as pie charts at selected nodes of the dated phylogeny. Branch support is provided for *Echinops*, with bold branches indicating a posterior probability  $\geq 95\%$ . Photos (at the same scale for a better comparison) illustrate the different types of pollen in the genus: *Echinops albicaulis* for Echinata-Annual type (a), *E. spinosissimus* subsp. *spinosissimus* for Perennial type (b), *E. hololeucus* for Hololeucus type (c), and *E. gmelinii* for Annual type (d).

The most frequent chromosome numbers in *Echinops* are superior or equal to 30 and considered to correspond to diploidized polyploids (Garnatje et al. 2004). Nevertheless, some annual species have much lower chromosome numbers, the most extreme case being *E. acantholepis* Jaub. & Spach with  $2n = 14$ . Consequently, two possible configurations were proposed (Sánchez-Jiménez et al. 2009). One situates a polyploidization event at the base of the genus with subsequent drastic dysploidy in the clade of annual species. The other involves several independent polyploidization events (at least four) that affected all but a few annual species. Unexpectedly, the most recent developments in transcriptomics and phylogenomics support another configuration, where the last polyploidization event in the evolutionary

history of *Echinops* would be the one common to all Asteraceae except Barnadesieae (Zhang et al. 2021; Palazzesi et al. 2022b).

The pollen size increase observed in *Echinops* Perennial type is then probably not related to polyploidization; however, its decrease in the annual species may well be associated with other genomic processes that impact on genome size. Indeed, genome size correlates with cell cycle duration, and annual species that are generally fast growing therefore tend to have smaller genomes than their perennial relatives (in *Echinops*; Garnatje et al. 2004; Sánchez-Jiménez et al. 2009). This downsizing is achieved through genomic restructuring, which often also results in chromosome number changes. Since genome size also correlates with cell size (Beaulieu et al. 2008), genome downsizing would then result





**Fig. 5** Diversity of pollen shape and size in 622 species of Cardueae (A, B) and 303 species and subspecies of Vernoniae (C, D). P indicates polar axis; E indicates equatorial axis. Dashed lines indicate P/E ratio of 0.88, 1.14, 1.33, and 2, which delimitate the suboblate P/E [0.75–0.88], spheroidal P/E [0.88–1.14], subprolate P/E [1.14–1.33], prolate P/E [1.33–2], and perprolate P/E  $\geq 2$  shape in the morphospace. B, D, Boxplots with individual jitter values depicting the distribution of P size. Colors correspond to subtribes for Cardueae (all presenting a simple capitulum except for the Echinopsinae and possibly the Dipterocominae, with a syncephalum) and to the inflorescence type (simple capitulum vs. syncephalum) for Vernoniae. A picture of a syncephalous representative is provided for each tribe: *Echinops* (Cardueae) and *Chresta* (Vernoniae). Photograph credits: Oriane Hidalgo (*Echinops ritro*), Carolina M. Siniscalchi (*Chresta sphaerocephala*).

in smaller cells, including pollen. *Echinops acantholepis* and *E. gmelinii* Turcz. illustrate this trend, where annual taxa show both genome downsizing—here accompanied by dysploidy—and reduced pollen size, as previously found in other Asteraceae such as in *Callicephalus* C.A.Mey., *Centaurea* L., and *Oligochaeta* K.Koch (Hidalgo et al. 2008a, 2008b) within Cardueae and in *Reichardia* Roth (Siljak-Yakovlev et al. 2017).

#### Increase of Pollen Size in Echinops Perennial Pollen Type Did Not Occur at the Expense of Pollen Quantity

Because of the existence of a trade-off between the number and size of pollen grains (Cruden and Miller-Ward 1981; Vohhof and Harder 1995), it is important to also consider the quantitative aspects of *Echinops* pollen evolution. We carried out pollen counts to find out whether *Echinops* species with Perennial pollen type have a particularly low amount of pollen per flower. Furthermore, reproductive systems are reflected in male and female investments through the pollen-to-ovule ratio (Cruden 1977; Cruden 2000), and the number of pollen grains in *Echinops* species could therefore provide information on where they fall on the spectrum of self-compatibility.

*Echinops sphaerocephalus* presented a mean of 4142.95 pollen grains per flower, suggesting that the pollen-to-ovule ratio is maintained at a level corresponding to xenogamy (Cruden 1977) despite a huge pollen size. This fits our expectations, given our unsuccessful attempts to reproduce *Echinops* species in the past. Unfortunately, there is little data to compare these results with, as the breeding system of *Echinops*—and even more gener-

ally that of Cardueae—has so far been poorly studied (East 1940). Previous reports of pollen counts in the tribe show mean values per flower of 5895 for *Berardia lanuginosa* (Lam.) Fiori & Paol. (facultative xenogamy; Guerrina et al. 2016), ca. 850 for *Carduus baeocephalus* Webb subsp. *baeocephalus* (facultative xenogamy), ca. 190 for *Carduus baeocephalus* subsp. *microstigma* Gaisberg & Wagenitz (facultative autogamy; Gaisberg 2002), 3650.01–6500.01 for *Carthamus tinctorius* L. (facultative xenogamy; Shao et al. 2012), and 107.17–138.92 and 926.58–2308.38, respectively, for cleistogamous and chasmogamous flowers of *Centaurea melitensis* L. (Porras and Muñoz Álvarez 2000). In this sense, the values for *Echinops* and *Cardopatum* are at the high end of the range for Cardueae. Taken together, rather than a rebalancing of male reproductive investment to favor pollen grain size over number, the pattern observed in *Echinops* species with Perennial pollen type more likely suggests an increase of the overall resources devoted to male function.

#### Functional Significance of the Evolution of the Echinops Perennial Pollen Type

Among the many factors that contribute to determining pollen size, postpollination processes, rather than pollen transport, appear to be prevalent in animal-pollinated species (Harder 1998; Sarkissian and Harder 2001). In particular, a significant relationship exists between pollen volume and style length (Torres 2000). Indeed, pollen tube length is predetermined by the nutrient reserves of the pollen and must adjust to the pistil length (Cruden 2009). In addition, pollen tube growth rate also depends

on nutrient reserves, which gives the large pollen grains an advantage in the competition for fertilization. Pollen size increase in *Echinops* could therefore respond to an evolutionary trend toward producing larger flowers and syncephalia and/or a selection pressure for improved male fitness. Flower length varies considerably in *Echinops*, up to 4–4.5 cm in the gigantic Ethiopian species *E. ellenbeckii* O.Hoffm. and *E. longisetus* A.Rich. (Tadesse 2004), with enormous syncephalia up to 25 cm in diameter and pollinated by birds (Vogel 2015). Certainly, these *Echinops* species, for which unfortunately no palynological or phylogenetic data are available, should constitute a target for future studies on *Echinops* pollen evolution. Indeed, in *Chresta* (a syncephalous representative of the Vernoniaeae tribe), the largest pollen grains are found in *C. curumbensis* (Philipson) H.Rob. and *C. speciosa* Gardner, which also have the largest flowers (up to 3.3 cm) and are bird pollinated (Siniscalchi et al. 2017).

There is growing evidence that environmental factors also participate in determining pollen size and shape (Ejmond et al. 2011, 2015). Pollen undergoes dehydration when released from the anther and rehydration once deposited on the stigma, processes that can alter pollen viability and whose intensity depends on the level of dryness of the environment. Accordingly, a trend toward larger pollen (i.e., with a lower surface-to-volume ratio and therefore a lower desiccation rate) is observed at high temperatures (Ejmond et al. 2015). This trend, however, is interpreted as more likely resulting from increased competition between pollen on the stigma than representing an adaptation to high desiccation stress (Ejmond et al. 2015). Larger pollen grains in *Echinops* may therefore reflect the adaptation to warmer environmental conditions.

In addition to the effect of water stress on pollen viability, dehydration and rehydration can also affect the structure of the exine and cause the pollen to break. The size and shape of pollen grains influence their harmomegathic functioning (i.e., the capacity to accommodate changes in volume through exine elasticity and harmomegathic structures), which is optimized in smaller and more elongated pollen (Wodehouse 1935). In this sense, the particular structure of *Echinops* Perennial pollen type, its exceptionally complex wall and elongated shape, could certainly have the function of allowing a considerable increase in size while maintaining sufficient harmomegathic capacity.

Although some taxa may share certain pollen characteristics with *Echinops*, such as an elongated shape in the case of Arctiinae (fig. 5A), the evolutionary trajectory of *Echinops* pollen remains quite exceptional. Actually, other strategies have been explored in Asteraceae lineages to cope with arid and hot climates, which do not involve the huge increase of pollen size and a dramatic shape change seen in *Echinops*. For example, in Centaureinae,

adaptation to such climates is achieved through the development of a harmomegathic structure, the cavea (Hidalgo et al. 2008a, 2008b and references therein). Similarly, in Vernoniaeae, type F pollen (sensu Keeley and Jones 1979) is described by a reduced amount of tectum, thick columellae around the lacunae and germinal pores, and a highly geometric form with patterned elevated ridges and lacunae regularly spaced. These characteristics probably confer improved tensile strength that may be related to volume changes and an increased resistance to desiccation (Bolick 1978; Keeley and Jones 1979). Type F pollen is found in very few Vernoniaeae species that grow in very harsh conditions (e.g., the syncephalous *Chresta martii* (DC.) H.Rob. over rock outcrops in the dry Brazilian caatinga and *Linzia* Sch.Bip. ex Walp. spp. in dry or recently burnt grasslands; Jeffrey and Beentje 2000; Siniscalchi et al. 2017) and in some syncephalous weedy species (e.g., *Elephantopus mollis* Kunth and *Rolandra fruticosa* (L.) Kuntze; Robinson 1992; Skvarla et al. 2005).

### Concluding Remarks

In this work, we provide insights into the evolution of pollen gigantism in *Echinops* and its potential relationships with adaptation to warmer environmental conditions. As evidence accumulates across plant lineages of a response of pollen morphology to climate, we can anticipate that current global changes might also influence pollen phenotypic spectrum as well as pollination success. In this sense, the present study contributes to increasing the body of knowledge on pollen traits that is still too limited and yet crucial for modeling the impact of global change.

### Acknowledgments

We thank herbaria listed in table 1 and the botanical gardens listed in table A3 for providing material; the personnel of the Centres Científics i Tecnològics of the Universitat de Barcelona for technical support; Joan Vallès, Joan Martín, and Maruxa Suárez for helpful comments; Carolina M. Siniscalchi for the picture of *Chresta sphaerocephala*; and two reviewers for comments on an earlier version of the manuscript. This work has been supported by projects CGL2010-22234-C02-01/BOS and CGL2010-22234-C02-02/BOS from DGICYT of the Spanish government and COMPOSITAE (PID2020-116480GB-I00) funded by MCIN/AEI/10.13039/501100011033. Support was also provided by Ajuts a Grups de Recerca Consolidats (2017/SGR/1116 and 2021/SGR/00315) from the Generalitat de Catalunya. I. Sánchez-Jiménez benefited from a FPU predoctoral grant.

### Literature Cited

- Avetissian EM 1950 Simplified method of pollen preparation by aceto-lysis. *Bot Z* 35:385.
- Barres L, I Sanmartín, CL Anderson, A Susanna, S Buerki, M Galbany-Casals, R Vilatersana 2013 Reconstructing the evolution and biogeographic history of tribe Cardueae (Compositae). *Am J Bot* 100:867–882.
- Beaulieu JM, IJ Leitch, S Patel, A Pendharkar, CA Knight 2008 Genome size is a strong predictor of cell size and stomatal density in angiosperms. *New Phytol* 179:975–986.
- Bijl PK, AJP Houben, S Schouten, SM Bohaty, A Sluijs, G-J Reichart, JS Sinninghe Damsté, H Brinkhuis 2010 Transient Middle Eocene atmospheric CO<sub>2</sub> and temperature variations. *Science* 330:819–821.
- Blackmore S 1990 Sporoderm homologies and morphogenesis in land plants, with a discussion of *Echinops sphaerocephala* (Compositae). Pages 1–12 in M Hesse, F Ehrendorfer, eds. *Systematics*. Vol 5. *Plant Systematics Evolution*. Springer, Vienna.

- Bolick MR 1978 Taxonomic, evolutionary, and functional considerations of Compositae pollen ultrastructure and sculpture. *Plant Syst Evol* 130:209–218.
- Bosboom RE, HA Abels, C Hoorn, BCJ van den Berg, Z Guo, G Dupont-Nivet 2014 Aridification in continental Asia after the Middle Eocene Climatic Optimum (MECO). *Earth Planet Sci Lett* 389:34–42.
- Bouckaert R, J Heled, D Kühnert, T Vaughan, C-H Wu, D Xie, MA Suchard, A Rambaut, AJ Drummond 2014 BEAST 2: a software platform for Bayesian evolutionary analysis. *PLoS Comput Biol* 10:e1003537.
- Cruden RW 1977 Pollen-ovule ratios: a conservative indicator of breeding systems in flowering plants. *Evolution* 31:32–46.
- 2000 Pollen grains: why so many? Pages 143–165 in A Dafni, M Hesse, E Pacini, eds. *Pollen and pollination*. Springer, Vienna.
- 2009 Pollen grain size, stigma depth, and style length: the relationships revisited. *Plant Syst Evol* 278:223–238.
- Cruden RW, S Miller-Ward 1981 Pollen-ovule ratio, pollen size, and the ratio of stigmatic area to the pollen-bearing area of the pollinator: an hypothesis. *Evolution* 35:964–974.
- Darriba D, GL Taboada, R Doallo, D Posada 2012 jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods* 9:772.
- Díez MJ 1987 Asteraceae (Compositae). Pages 332–357 in B Valdés, MJ Díez, I Fernández, eds. *Atlas Polínico de Andalucía Occidental*. Universidad de Sevilla, Spain.
- Dodsworth S, MW Chase, AR Leitch 2016 Is post-polyploidization diploidization the key to the evolutionary success of angiosperms? *Bot J Linn Soc* 180:1–5.
- East EM 1940 The distribution of self-sterility in the flowering plants. *Proc Am Philos Soc* 82:449–518.
- Ejsmond MJ, A Ejsmond, Ł Banasiak, M Karpińska-Kołaczek, J Kozłowski, P Kołaczek 2015 Large pollen at high temperature: an adaptation to increased competition on the stigma? *Plant Ecol* 216:1407–1417.
- Ejsmond MJ, D Wrońska-Pilarek, A Ejsmond, D Dragosz-Kluska, M Karpińska-Kołaczek, P Kołaczek, J Kozłowski 2011 Does climate affect pollen morphology? optimal size and shape of pollen grains under various desiccation intensity. *Ecosphere* 2:art117.
- Erdtman G 1952 Pollen morphology and plant taxonomy: angiosperms. *Almqvist & Wiksell*, Waltham, MA.
- Fu L, L Palazzesi, J Pellicer, M Balant, MJM Christenhusz, L Pegoraro, I Pérez-Lorenzo, IJ Leitch, O Hidalgo 2023 Let's pluck the daisy: dissection as a tool to explore the diversity of Asteraceae capitula. *Bot J Linn Soc* 201:391–399.
- Gabarayeva NI, SV Polevova, VV Grigorjeva, S Blackmore 2018 Assembling the thickest plant cell wall: exine development in *Echinops* (Asteraceae, Cynareae). *Planta* 248:323–346.
- Gaisberg MV 2002 Contribución al conocimiento de la taxonomía, la biología reproductiva y la distribución de *Carduus baeocephalus* Webb. *Rev Acad Canar Cienc* 14:253–261.
- Garnatje T, J Martín 2007 Pollen studies in the genus *Echinops* L. and *Xeranthemum* group (Asteraceae). *Bot J Linn Soc* 154:549–557.
- Garnatje T, J Vallès, S Garcia, O Hidalgo, M Sanz, MÁ Canela, S Siljak-Yakovlev 2004 Genome size in *Echinops* L. and related genera (Asteraceae, Cardueae): karyological, ecological and phylogenetic implications. *Biol Cell* 96:117–124.
- Grant V 1950 The protection of the ovules in flowering plants. *Evolution* 4:179–201.
- Greuter W 2006 Asteraceae (pro parte majore). In *Euro+Med Plantbase: the information resource for Euro-Mediterranean plant diversity*. <http://www.europlusmed.org>.
- Guerrina M, G Casazza, E Conti, C Macri, L Minuto 2016 Reproductive biology of an Alpic paleo-endemic in a changing climate. *J Plant Res* 129:477–485.
- Halbritter H 1998 Preparing living pollen material for scanning electron microscopy using 2,2-dimethoxypropane (DMP) and critical-point drying. *Biotech Histochem* 73:137–143.
- Hall TA 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95–98.
- Harder LD 1998 Pollen-size comparisons among animal-pollinated angiosperms with different pollination characteristics. *Biol J Linn Soc* 64:513–525.
- Herrando-Moraira S, JA Calleja, M Galbany-Casals, N Garcia-Jacas, J-Q Liu, J López-Alvarado, J López-Pujol, et al 2019 Nuclear and plastid DNA phylogeny of tribe Cardueae (Compositae) with Hyb-Seq data: a new subtribal classification and a temporal diversification framework. *Mol Phylogenet Evol* 137:313–332.
- Herrando-Moraira S, Cardueae Radiations Group, J-A Calleja, Y-S Chen, K Fujikawa, M Galbany-Casals, et al 2020 Generic boundaries in subtribe Saussureinae (Compositae: Cardueae): insights from Hyb-Seq data. *Taxon* 69:694–714.
- Hidalgo O, N Garcia-Jacas, T Garnatje, K Romashchenko, A Susanna, S Siljak-Yakovlev 2008a Extreme environmental conditions and phylogenetic inheritance: systematics of *Myopordon* and *Oligochaeta* (Asteraceae, Cardueae-Centaureinae). *Taxon* 57:769–778.
- Hidalgo O, A Susanna, N Garcia-Jacas, J Martín 2008b From acaveate to caveate: evolution of pollen types in the *Rhaponticum* group (Asteraceae, Centaureinae) related to extreme conditions. *Bot J Linn Soc* 158:499–510.
- Ikuse M 1962 On pollen grains of the Compositae collected by the Kyoto University scientific expedition to the Karakoram and Hindukush, 1955. *Acta Phytotaxon Geobot* 20:112–119.
- Jafari E, G Ghanbarian 2007 Pollen morphological studies on selected taxa of Asteraceae. *J Plant Sci* 2:195–201.
- Jardine PE, L Palazzesi, MC Tellería, VD Barreda 2022 Why does pollen morphology vary? evolutionary dynamics and morphospace occupation in the largest angiosperm order (Asterales). *New Phytol* 234:1075–1087.
- Jeffrey C, HJ Beentje 2000 Vernoniae. Pages 108–285 in HJ Beentje, SAL Smith, eds. *Flora of tropical East Africa, Compositae (part 1)*. Royal Botanic Gardens, Kew.
- Jiang W, X Yang, Y Cheng 2014 Spatial patterns of vegetation and climate on the Chinese Loess Plateau since the Last Glacial Maximum. *Quat Int* 334/335:52–60.
- Kallajxhiu N, P Naqellari, B Pupuleku, S Turku 2014 Palynological description of five plants of different families in the Albanian region. *Int J Bot Res* 4:31–38.
- Katoh K, DM Standley 2013 MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 30:772–780.
- Keeley SC, SB Jones 1979 Distribution of pollen types in *Vernonia* (Vernoniaeae: Compositae). *Syst Bot* 4:195–202.
- Landis JB, DE Soltis, Z Li, HE Marx, MS Barker, DC Tank, PS Soltis 2018 Impact of whole-genome duplication events on diversification rates in angiosperms. *Am J Bot* 105:348–363.
- Loeuille B, RMB Souza-Souza, VHR Abreu, CBF Mendonça, V Gonçalves-Esteves 2012 Pollen morphology of the genus *Eremanthus* Less. (Vernoniaeae, Asteraceae). *Acta Bot Bras* 26:46–57.
- Mandel JR, RB Dikow, CM Siniscalchi, R Thapa, LE Watson, VA Funk 2019 A fully resolved backbone phylogeny reveals numerous dispersals and explosive diversifications throughout the history of Asteraceae. *Proc Natl Acad Sci USA* 116:14083–14088.
- Mohl H 1835 Sur la structure et les formes des graines de pollen. *Ann Sci Nat Bot Ser* 2 3:148–346.
- Montazerolghaem S, A Susanna, JA Calleja, V Mozaffarian, MR Rahiminejad 2017 Molecular systematics and phylogeography of the genus *Echinops* (Compositae, Cardueae-Echinopsinae): focus on the Iranian centre of diversification. *Phytotaxa* 297:101–138.

- Osman AK 2009 Contributions to the pollen morphology of tribe Cardueae (Cichorioideae-Compositae). *Feddes Rep* 120:145–157.
- Palazzesi L, O Hidalgo, VD Barreda, F Forest, S Höhna 2022a The rise of grasslands is linked to atmospheric CO<sub>2</sub> decline in the late Palaeogene. *Nat Commun* 13:293.
- Palazzesi L, J Pellicer, VD Barreda, B Loeuille, JR Mandel, L Pokorny, CM Siniscalchi, MC Tellería, IJ Leitch, O Hidalgo 2022b Asteraceae as a model system for evolutionary studies: from fossils to genomes. *Bot J Linn Soc* 200:143–164.
- Partridge AD 1978 Palynology of the Late Tertiary sequence at site 365, leg 40, deep sea drilling project. Pages 953–961 in HM Bolli, WBF Ryan, eds. *Initial Reports of the Deep Sea Drilling Project 40*. Texas A & M University, Ocean Drilling Program, College Station, TX.
- Penn O, E Privman, H Ashkenazy, G Landan, D Graur, T Pupko 2010 GUIDANCE: a web server for assessing alignment confidence scores. *Nucleic Acids Res* 38:W23–W28.
- Perveen A 1999 Contributions to the pollen morphology of the family Compositae. *Turk J Biol* 23:523–535.
- Pla Dalmau JM 1961 Polen. Estructura y Características de los Granos de Polen. *Precisiones Morfológicas Sobre el Polen de Especies Recolectadas en el N.E. de España. Polinización y Aeropalinología*. Talleres Gráficos DCP, Gerona.
- Porras R, JM Muñoz Álvarez 2000 Breeding system in the cleistogamous species *Centaurea melitensis* (Asteraceae). *Can J Bot* 77:1632–1640.
- POWO 2022 Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. <http://www.plantsoftheworldonline.org/>.
- Punt W, PP Hoen 2009 The northwest European pollen flora, 70: Asteraceae—Asteroideae. *Rev Palaeobot Palynol* 157:22–183.
- Punt W, PP Hoen, S Blackmore, S Nilsson, A Le Thomas 2007 Glossary of pollen and spore terminology. *Rev Palaeobot Palynol* 143:1–81.
- R Core Team 2019 R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.
- Revell LJ 2012 phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol Evol* 3:217–223.
- Robinson H 1992 The Asteraceae of the Guianas. III. Vernoniae and restoration of the genus *Xiphochaeta*. *Rhodora* 94:348–361.
- Sánchez-Jiménez I, GA Lazkov, O Hidalgo, T Garnatje 2010 Molecular systematics of *Echinops* L. (Asteraceae, Cynareae): a phylogeny based on ITS and *trnL-trnF* sequences with emphasis on sectional delimitation. *Taxon* 59:698–708.
- Sánchez-Jiménez I, J Pellicer, O Hidalgo, S Garcia, T Garnatje, J Vallès 2009 Chromosome numbers in three Asteraceae tribes from inner Mongolia (China), with genome size data for Cardueae. *Folia Geobot* 44:307–322.
- Sanders H 2021 Polyploidy and pollen grain size: is there a correlation? *Grad Rev* 1:153–161.
- Sarkissian TS, LD Harder 2001 Direct and indirect responses to selection on pollen size in *Brassica rapa* L. *J Evol Biol* 14:456–468.
- Shao J, Q Quan, W Cai, L Guan, W Wu 2012 The effect of floral morphology on seed set in *Carthamus tinctorius* Linnaeus (Asteraceae) clones of Sichuan province in China. *Plant Syst Evol* 298:59–68.
- Siljak-Yakovlev S, B Godelle, V Zoldos, J Vallès, T Garnatje, O Hidalgo 2017 Evolutionary implications of heterochromatin and rDNA in chromosome number and genome size changes during dysploidy: a case study in *Reichardia* genus. *PLoS ONE* 12:e0182318.
- Siniscalchi CM, RMB Souza-Souza, B Loeuille, JR Pirani, V Gonçalves-Esteves 2017 The systematic value of pollen morphology in *Chresta* Vell. ex DC. (Vernoniae, Asteraceae). *Rev Palaeobot Palynol* 244:182–191.
- Skvarla JJ, ML DeVore, WF Chisoe 2005 Lophate sculpturing of Vernoniae (Compositae) pollen. *Rev Palaeobot Palynol* 133:51–68.
- Souza-Souza RMB, B Loeuille, CBF Mendonça, RL Esteves, V Gonçalves-Esteves 2016 Pollen morphology of the genus *Paralychnophora* (Vernoniae–Asteraceae). *Palynology* 40:280–288.
- Stix E 1960 Pollenmorphologische untersuchungen an Compositen. *Grana Palynol* 2:41–104.
- 1964 Polarisationsmikroskopische untersuchungen am sporoderm von *Echinops banaticus*. *Grana Palynol* 5:289–297.
- 1970 Beitrag zur morphogenese der pollenkörner von *Echinops banaticus*. *Grana* 10:240–242.
- Susanna A, BG Baldwin, RJ Bayer, JM Bonifacino, N Garcia-Jacas, SC Keeley, JR Mandel, S Ortiz, H Robinson, TF Stuessy 2020 The classification of the Compositae: a tribute to Vicki Ann Funk (1947–2019). *Taxon* 69:807–814.
- Tadesse M 2004 Asteraceae (Compositae). Pages 1–408 in I Hedberg, I Friis, S Edwards, eds. *Flora of Ethiopia and Eritrea*. Vol 4, pt 2. The National Herbarium, Addis Ababa and Department of Systematic Botany. EMPDA, Uppsala.
- Tahmasebi G, AA Azimi, BD Hashemloian, M Yosfrad, N Jalilian 2022 Manna formation, cytogenetic and pollen morphology in *Echinops polygamus* Bunge. *J Posit Sch Psychol* 6:2459–2466.
- Tormo MR, JL Ubera 1988 Contribución a la palinología de *Echinops* L. (Compositae) en la Península Ibérica. *An Asoc Palinol Leng Esp* 4:25–32.
- 1995 Tipos polínicos de la tribu Cardueae en la Península Ibérica. *Monogr Jard Bot Cordoba* 2:5–52.
- Torres C 2000 Pollen size evolution: correlation between pollen volume and pistil length in Asteraceae. *Sex Plant Reprod* 12:365–370.
- Van Breedam J, P Huybrechts, M Crucifix 2022 Modelling evidence for late Eocene Antarctic glaciations. *Earth Planet Sci Lett* 586:117532.
- Vogel S 2015 Vertebrate pollination in Compositae: floral syndromes and field observations. *Stapfia* 103:5–26.
- Vonhof MJ, LD Harder 1995 Size-number trade-offs and pollen production by Papilionaceous legumes. *Am J Bot* 82:230–238.
- Vural C, MK Biter, YD Mehmet 2010 A new species of *Echinops* (Asteraceae) from Turkey. *Turk J Bot* 34:513–519.
- Wagenitz G 1955 Pollenmorphologie und Systematik in der Gattung *Centaurea* L. s. 1. *Flora Allg Bot Ztg* 142:213–279.
- Wickham H 2016 ggplot2: elegant graphics for data analysis. 2nd ed. Springer, New York.
- Wodehouse RP 1935 Pollen grains. Hafner, New York.
- Zhang C, C-H Huang, M Liu, Y Hu, JL Panero, F Luebert, T Gao, H Ma 2021 Phylotranscriptomic insights into Asteraceae diversity, polyploidy, and morphological innovation. *J Integr Plant Biol* 63:1273–1293.