Pollen morphology of the tribes Gnaphalieae and Calenduleae and their systematic contributions

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Abstract

The tribe Gnaphalieae is a moderately large group within Asteraceae, comprising approximately 178 genera and 2,102 species, predominantly distributed in temperate regions. This study aims to identify and describe pollen types in the tribes Gnaphalieae and Calenduleae, as well as to investigate the relationship among their taxa based on pollen data. The pollen micromorphology of 10 species representing eight genera from these tribes in Iran was examined using light and scanning electron microscopy. The present study showed a noticeable variation in palynological data among Gnaphalieae and Calenduleae studied species. Three pollen types in *Calendula*, *Lasiopogon*, and *Helichrysum*, were identified here among the studied taxa. Clustering and ordination analyses, including WARD tree, PCA and PCoA plots, revealed that, Calenduleae can be distinguished from Gnaphalieae using its tetra-zonocolporate pollen grains and pollen features such as polar axis length, spines height and the distance between spines. Furthermore, palynological data analysis revealed the significance of pollen features in identifying and delimiting species within Gnaphalieae. Similarities in pollen shape and exine ornamentation suggested that, Calenduleae may represent a sister group to Gnaphalieae. The results also indicated that, pollen morphology is a reliable tool for species delimitation within these two tribes.

Keywords: Asteraceae, Calenduleae, Gnaphalieae, pollen micromorphology, SEM

ریخت‌شناسی گرده در قبیله‌های Gnaphalieae و Calenduleae و سهم سیستماتیک آن‌ها

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خلاصه

قبیله Gnaphalieae یک گروه نسبتا بزرگ در کاسنیان شامل تقریبا ۱۷۸ جنس و ۲۱۰۲ گونه است که بیشتر در مناطق معتدل توزیع شده‌اند. هدف از این مطالعه، شناسایی و توصیف انواع گرده در قبیله‌های Gnaphalieae و Calenduleae و همچنین بررسی رابطه بین گونه‌های آن‌ها براساس داده‌های گرده است. ریزریخت‌شناسی گرده ۱۰ گونه که نماینده هشت جنس از این قبیله‌ها در ایران هستند، با استفاده از میکروسکوپ نوری و الکترونی نگاره بررسی شد. مطالعه حاضر تنوع قابل توجهی از داده‌های گرده‌شناسی را در بین گونه‌های مطالعه شده از قبیله‌های Gnaphalieae و Calenduleae نشان داد. سه نوع گرده در *Calendula*، *Lasiopogon* و *Helichrysum*، در بین گونه‌های مورد مطالعه شناسایی شد. تجزیه تحلیل‌های خوشه‌بندی و رسته‌بندی، شامل درخت WARD و پلات‌های PCA و PCoA، نشان داد که Calenduleae را می‌توان با استفاده از دانه‌های گرده تترا-زونوکولپوریت و ویژگی‌های گرده مانند طول محور قطبی، ارتفاع خارها و فاصله بین خارها از Gnaphalieae متمایز کرد. علاوه بر این، آنالیز داده‌های گرده‌شناسی اهمیت ویژگی‌های گرده در شناسایی و جدایی گونه‌ها را درون قبیله Gnaphalieae نشان داد. شباهت‌ها در شکل گرده و تزیینات اگزین نشان داد که Calenduleae ممکن است یک گروه خواهری برای Gnaphalieae باشد. نتایج مطالعه حاضر همچنین نشان داد که ریخت‌شناسی گرده ابزاری قابل اعتماد برای تعیین حدود گونه‌ها در این دو قبیله است.

واژه‌های کلیدی: ریزریخت‌شناسی گرده، Asteraceae، Calenduleae، Gnaphalieae، SEM

Introduction

The paper daisies tribe (Gnaphalieae Cass. ex Lecoq & Juill.: Asteroideae), with about 178 genera and 2102 species, is regarded as a moderately large tribe within Asteraceae (Xu *et al*. 2024, Susanna *et al*. 2020, Smissen *et al*. 2020). Asteraceae is one of the largest families among flowering plants and constitute a significant part of the Iranian flora (Ghahremaninejad *et al*. 2025). Gnaphalieae has evolved from an ancestral area in southern Africa and has established all continents except Antarctica. Additionally, it has smaller distribution centers in the Mediterranean region and South America (Bergh & Linder 2009, Nie *et al*. 2016). The tribe is distinguished from others by its dry, papery involucral bracts, which are often enlarged and showy. In addition, it typically lacks the strap- shaped ligule found in the daisy ray floret (Smissen *et al*. 2020). Although significant advances have been made in understanding of the phylogenetics and evolution of this tribe in recent years, a stable and widely accepted subtribal classification does not yet exist (Anderberg 1991, Bayer *et al*. 2000, 2002, Bergh *et al*. 2018, Mandel *et al*. 2019). According to the latest phylogenetic analyses by Smissen *et al*. (2020), Gnaphalieae is divided into two subtribes: Relhaniinae Less., which includes 124 species across 11 genera that are primarily endemic to African and the much larger Gnaphaliinae Dumort. The latter accounts for over 90% of species diversity, with about 2000 species in 167 genera. They also recovered Relhaniinae as monophyletic and identified it as the sister group to Gnaphaliinae. Phylogenetic studies by Susanna *et al*. (2020) also confirm this classification. Members of Gnaphalieae were traditionally classified within the tribe Inuleae. However, it has only recently been recognized that, this tribe is distinct from the rest of the "old" Inuleae (Anderberg 1989, 1991). According to the latest studies, Gnaphalieae forms a clade with the tribes Calenduleae Cass., Astereae Cass., and Anthemideae Cass. (Funk *et al*. 2009, Jafari *et al*. 2015, Watson *et al*. 2020). Although the exact number of genera and species of Gnaphalieae in Iran is controversial but according to Mozaffarian *et al*. (2018) and POWO (2026), about eight genera and 40 species, distributed in two subtribes (Gnaphaliinae and Relhaniinae), are currently found in Iran.

Calenduleae is one of the smallest tribes within the subfamily Asteroideae, comprising two genera: *Calendula* L. and *Osteospermum* L. This tribe includes approximately 120 species, all of which originated in southern Africa and are distributed across the Mediterranean, Irano-Turanian and Saharo-Arabian regions (Jafari *et al*. 2014, Susanna *et al*. 2020, Watson *et al*. 2020). Calenduleae, despite its small size, is always the subject of debate and disagreement regarding its classification. It is distinguished from other Asteraceae tribes by the presence of appendages on the fruits and lack of pappus. This tribe in Iran is represented by a single genus (*Calendula* L.) and approximately six species (Mozaffarian *et al*. 2018, POWO 2026).

Although various studies on pollen morphology have long been utilized to clarify taxonomic questions within different tribes of the Asteraceae (Hidalgo *et al*. 2008, 2023, Pellicer *et al*. 2009, Atazadeh *et al*. 2020, Lu *et al*. 2022, Ali *et al*. 2023, Pakravan *et al*. 2024, Qu *et al*. 2025), research on the pollen micromorphology of the tribe Gnaphalieae and its relationship to other tribes, such as Calenduleae, remains limited. Consequently, comprehensive palynological studies on these tribes are still lacking. According to Funk *et al*. (2009), the pollen grains of Calenduleae are oblate-spheroidal, tectate and caveate, featuring spinose or spinulose exine sculpture and lacking distinct infratectal bacula. *Calendula* is well characterized palynologically, possessing large pollen grains with prominent long and slender solid spines. Zakymola *et al*. (2017) studied the pollen morphology of six species of the genus *Calendula* in Iran and found that, the pollen grains in this genus were either tri-zonocolporate or tetra-zonocolporate. Skvarla *et al*. (1977) noted that, the pollen grains of many genera within the "Inuleae-Gnaphaliinae" shares a common structure, characterized by a two-layered ectexine consisting of an outer columellate layer and an irregularly interlaced basal layer. They referred to this pollen type as the "inuloid pattern". Subsequently, Anderberg (1991) recognized Gnaphalieae as a distinct tribe and renamed this pollen type the "gnaphalioid type", proposing that, all true Gnaphalieae possess a gnaphalioid sporoderm pattern. Since 1977, only a few researchers have investigated the pollen of Gnaphalieae. Their studies have shown that, pollen morphology and ultrastructure are valuable for classifying genera within the tribe (Anderberg & Källersjö 1988, Anderberg 1991, Moar 1993, El-Ghazaly & Anderberg 1995, Breitwieser & Sampson 1997a, b, Pinheiro *et al*. 2003, Blackmore *et al*. 2007, 2009, Coutinho & Dinis 2009, Osman 2011, Wortley *et al*. 2012, 2015, Elkiran *et al*. 2017, Azizi *et al*. 2023).

Palynological data supported Anderberg's hypothesis that, *Phagnalon* Cass. and *Aliella* Qaiser & Lack are part of the "basal taxa" within Gnaphalieae (El-Ghazaly & Anderberg 1995). In contrast, *Haastia* Hook.f. was determined not to belong to Gnaphalieae due to its senecioid pollen, single layer of internal tecta and highly bifurcating columellae bases (Breitwieser & Sampson 1997b). Additionally, according to Breitwieser & Sampson (1997a, b), pollen grains provide valuable characteristics for classifying New Zealand Gnaphalieae taxa. They found that, the pollen grains of all studied taxa were spheroidal and tricolporate, which helped clarify some complexities among *Helichrysum* Mill. species. In another study, El-Garf & Osman (2007), evaluated 12 Egyptian species belonging to three tribes (Astereae, Calenduleae, and Eupatorieae) of the Asteroideae using palynological data. Three pollen types, including Calenduleae, Grangeinae and Conyza, were identified and described in this study. Blackmore *et al*. (2009) emphasized the significance of various pollen features, including shape, apertures, exine sculpture and spine size. They investigated the evolution of pollen within the Asteraceae, specifically focusing on the tribe Gnaphalieae. Their results indicated that, Gnaphalieae forms a common clade with Calenduleae based on pollen data, attributed to shared characteristics such as shape, exine ornamentation and spines size in the pollen grains of both tribes. Coutinho & Dinis (2009) investigated the pollen morphology and exine structure of 15 species of Gnaphaliinae using light, scanning electron, and transmission electron microscopy. According to their results, all studied species exhibited a Gnaphalioid exine pattern with a distinctly rough foot layer. They found that, based on pollen data, Inuleae and Gnaphalieae are quite different which does not support the hypothesis that, Inuleae is the sister group of Gnaphalieae. In contrast, Calenduleae, Astereae, and Anthemideae were confirmed as the sister groups of Gnaphalieae. Osman (2011) investigated the pollen morphology of 12 genera, including *Phagnalon*, *Filago* Loefl., *Gnaphalium* L., *Helichrysum*, *Homognaphalium*, *Ifloga* Cass., *Lasiopogon* Cass., *Pseudognaphalium* Kirp., *Flaveria* Juss., *Tagetes* L., *Sphaeranthus* L., and *Senecio* L. from the tribes Gnaphalieae, Helenieae Lindl., Plucheeae Cass., and Senecioneae Cass. (Asteroideae) using light and scanning electron microscopy. He identified two pollen types based on palynological data from the studied taxa: *Senecio* and *Filago* pollen types. The pollen grains exhibited a diversity of shapes, including oblate-spheroidal, spheroidal, suboblate and prolate-spheroidal. The pollen sculpture observed in this study ranged from spiny to spinulate, with perforate bases. Wortley *et al*. (2012), analysed pollen grains of some genera of the two tribes Astereae and Gnaphalieae, including *Allittia* P.S.Short, *Castroviejoa* Galbany, L. Saez & Benedi, *Lorandersonia* Urbatsch, R.P.Roberts & Neubig, *Pembertonia* P.S.Short and *Rhetinocarpha* Paul G.Wilson & M.A.Wilson, and showed that, the studied taxa have the helianthoid pollen type. Their results are consistent with the "Helianthoid" pollen type of Skvarla *et al*. (1977), which is common throughout the subfamily Asteroideae, including the Astereae and Gnaphalieae. In this pollen type, the spines are characterized by a basal part with channels and a solid apical part. Azizi *et al*. (2023) studied the palynological characteristics of 12 Iranian species of *Helichrysum* using SEM. They indicated that, the pollen grains were generally small to occasionally medium-sized, tricolporate, radially symmetrical, isopolar, and exhibited a variety of shapes, including oblate-spheroidal, spheroidal, subprolate, prolate, and prolate-spheroidal, with the latter being the most abundant type. Their results showed that, the exine sculpture was echinate and perforate.

Molecular data provide valuable insights at the genetic level, but pollen morphology remains an important tool for distinguishing infrageneric levels. Its conserved features offer a fundamental approach to understanding phenotypic evolution and resolving taxonomic issues at the tribe, genus and species levels. These stable characteristics underscore the significance of using pollen morphology, especially in the field of plant systematics (Blackmore 2007, Hesse & Blackmore, 2013, Wanninger 2015, Lin *et al*. 2023, Qu *et al*. 2025).

According to Osman (2011), significant pollen features, such as size, shape, exine ornamentation and spine size, provide valuable information for the taxonomic classification of this tribe. These features have long been utilized in systematics and evolution, aided by scanning electron microscopy (Gul *et al*. 2020, Ullah *et al*. 2022, Noroozi *et al*. 2022, Pakravan *et al*. 2024, Atazadeh & Ghahremaninejad 2025). This study represents one of the most comprehensive palynological investigations of Gnaphalieae tribe and its relationship to Calenduleae. It aims to highlight the importance of pollen characteristics for accurate species identification and delimitation. Furthermore, the findings may be integrated with molecular data in the future to address taxonomic questions and classification issues, as well as to elucidate the evolutionary pathways within Gnaphalieae (Noroozi *et al*. 2022). Therefore, the present study intends to: (i) describe and identify pollen types within the tribes Gnaphalieae and Calenduleae based on their features, (ii) explore the relationships among tribes, subtribes, genera and species using palynological data, and (iii) assess the systematic significance of pollen data within both tribes.

Materials and Methods

- Plant materials

Pollen grain samples were collected from 30 specimens representing 10 species, with three specimens of each species across eight genera within the tribes Gnaphalieae (nine species) and Calenduleae (one species). These samples were sourced from fresh material and dried herbarium specimens at T and TUH (herbaria acronyms follow Thiers 2026+) in Iran (Appendix 1). All specimens were thoroughly examined, and only those with healthy flowers and pollen grains were chosen for analysis. All specimens were stored under standardized and controlled conditions. Species names were updated using Plants of the World Online (POWO 2026).

- Pollen analysis

The pollen grain samples were prepared for examination using light microscopy (LM) and scanning electron microscopy (SEM). The previously published protocol (Pakravan *et al*. 2024, Atazadeh & Ghahremaninejad 2025) identified by Erdtman (1952) as the most suitable technique for palynological studies, was followed. Pollen samples were soaked in a mixture of 100% ethanol and glacial acetic acid (3:1) for two weeks to remove dust, then air-dried and mounted directly onto stubs with double-sided tape. For SEM examination, the pollen samples were coated with gold using diode sputtering and observed under scanning electron microscope (Hitachi SU3500, Japan) at 15–20 kV and various magnifications.

For LM studies, pollen grains were separated from the anthers of mature flowers, removed and placed on distinct slides. For each specimen, 20 fully developed pollen grains from 3–5 healthy flowers were selected and measured for the analysis of palynological features, except for *Helichrysum plicatum* DC., for which only 15 grains were measured. The pollen samples were observed using an Olympus BX51 light microscope at various magnifications. The grains were acetolyzed according to Erdtman (1952) and measured for eight palynological features viz., one qualitative feature: pollen shape and seven quantitative features: polar axis, equatorial diameter, the ratio of polar axis to equatorial diameter [P/E], spines height [H], spines diameter [D], the ratio of spines height to spines diameter [H/D], and the distance between spines (Table 1). Notably, two quantitative features (polar axis and equatorial diameter) were measured using a calibrated ocular on the LM, while the remaining five quantitative features (spines height, spines diameter, the ratio of spines height to spines diameter and the distance between spines) were measured from SEM photographs using Digimizer Image Analysis Software Ver. 5.4.9. Qualitative features were scored numerically according to table 1, and both quantitative and qualitative features were included in the statistical analyses. Pollen types were described using the terminology of Erdtman (1943, 1952), Punt *et al*. (2007), Hesse *et al*. (2009), and Halbritter *et al*. (2018). For the quantitative features, the range, mean and standard deviation were calculated for each sample using SPSS Ver. 22 (Allen *et al*. 2014) software.

Table 1. Qualitative and quantitative characters of pollen grains in the studied species

Qualitative character	**Quantitative character**
Oblate [P/E = 0.5–0.75]	Polar axis [P]
Suboblate [P/E = 0.75–0.88]	Equatorial diameter [E]
Oblate-spheroidal [P/E = 0.88–0.99]	P/E ratio
Spheroidal [P/E = 1]	Spines height [H]
Prolate-spheroidal [P/E = 1.01–1.14]	Spines diameter [D]
Sub-prolate [P/E = 1.14–1.33]	H/D ratio
Prolate [P/E = 1.33–2]	Distance between spines
Per-prolate [P/E = >2]	-

- Statistical analyses

Previous studies (Atazadeh *et al*. 2020, Atazadeh & Ghahremaninejad 2025) demonstrated the effectiveness of clustering and ordination methods for grouping taxa, delimiting species and identifying the most variable pollen features in Asteraceae specimens. Consequently, this study employed the WARD (Minimum Spherical Cluster Method), UPGMA (Unweighted Paired Group Using Average) and PCoA (Principal Coordinate Analysis) for species grouping. Additionally, Principal Component Analysis (PCA) was conducted to identify the most variable palynological features among the studied species (Atazadeh & Ghahremaninejad 2025). The palynological data were standardized (mean = 0, variance = 1) following the method described by Podani (2000), and the paleontological statistics software (PAST) Ver. 6.0 was utilized for the analyses (Hammer *et al*. 2012). To assess the significance of quantitative palynological differences among the studied species, one-way ANOVA and least significant difference tests were performed using SPSS Ver. 22 (Allen *et al*. 2014) for statistical analyses.

Results

- Pollen morphology

The qualitative and quantitative characteristics of the pollen grains were evaluated and described through scanning electron micrographs (Figs 1–3, Appendix 2). The pollen grains of the studied Gnaphalieae and Calenduleae species are monads, tricolporate or tetra-zonocolporate, isopolar and exhibit radial symmetry. According to the terminology of Erdtman (1943), the examined pollen grains of five species are classified as small (15.96–22.93 µm), while five species have medium-sized grains (25.1–42.64 µm). The shape of the pollen grains, determined by the ratio of polar axis to equatorial diameter [P/E] indicates that, four species i.e., *Filago germanica* (L.) Huds. (Figs 1A, B), *Phagnalon persicum* Boiss. (Figs 2K, L), *Pseudognaphalium luteoalbum* (L.) Hilliard & B.L.Burtt (Figs 3C, D), and *Calendula officinalis* L. (Figs 3F, G, H) exhibit a prolate-spheroidal shape. In contrast, three species i.e., *Helichrysum globiferum* Boiss. (Figs 1G, H, I), *H. plicatum* DC. (Fig. 1K), and *Micropus supinus* L. (Figs 2H, I), show a sub-prolate pollen shape. Additionally, two species i.e., *F. griffithii* (A.Gray) Andres-Sanchez & Galbany (Figs 1D, E) and *Leysera leyseroides* (Desf.) Maire (Figs 2E, F), display a prolate pollen shape. Oblate-spheroidal pollen grains were detected only in one species i.e., *Lasiopogon muscoides* (Desf.) DC. (Figs 2B, C). Spines as a diagnostic feature were observed in all studied taxa. The surface of the exine ornamentation was covered with supra-tectal spines, resulting in echinate-perforate pollen grains across all species was observed. Pollen data indicated that, the ornamentation of the aperture membrane was scabrate-perforate in all studied species, with aperture orientations classified as wide, slightly wide and rarely, slender or narrow. The spines were arranged both regularly and occasionally, irregularly; their tips were pointed, sharply pointed, or tapered, while their bases were broad and swollen. In polar view, all studied species exhibited three rows of spines, except for *C. officinalis*, which had 12 rows (Fig. 3H).

- Data analysis

The applied ANOVA test for the palynological features revealed a significant difference ($p<0.001$) in the quantitative features. The data analysis showed that, the average polar axis length ranged from 15.96±0.95 µm in *Lasiopogon muscoides* to 42.64±1.86 µm in *C. officinalis*. Similarly, the average equatorial diameter ranged from 14.59±1.26 µm in *Micropus supinus* to 39.69±1.88 µm in *C. officinalis*. Additionally, the average P/E ratio varied from 0.98 in *L. muscoides* to 1.45 in *Leysera leyseroides*. The average height of spines ranged from 1.21±0.14 µm in *F. griffithii* to 3.77±0.44 µm in *C. officinalis*. Furthermore, the average diameter of the spines varied from 0.94±0.12 µm in *F. griffithii* to 2.2±0.18 µm in the same species. The average H/D ratio varied from 0.84 in *Helichrysum plicatum* to 2.71 in *C. officinalis*. Finally, the distance between spines ranged from 0.67±0.0.08 µm in *F. germanica* to 5.30±1.20 µm in *C. officinalis.*

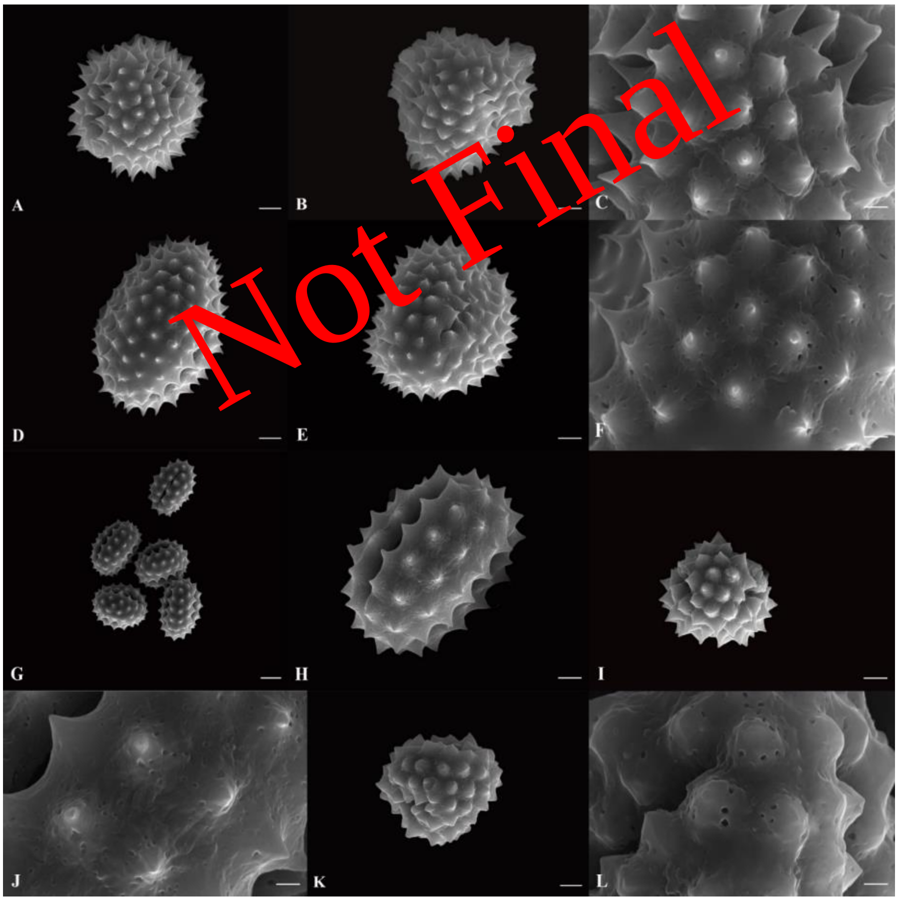

Fig. 1. SEM micrographs of pollen grains in the studied species of Gnaphalieae: A. *Filago germanica*, equatorial view, B. *Filago germanica*, polar view, C. *Filago germanica*, exine sculpture, D. *Filago griffithii*, equatorial view, E. *Filago griffithii*, polar view, F. *Filago griffithii*, exine sculpture, G. *Helichrysum globiferum*, overview of several grains, H. *Helichrysum globiferum*, equatorial view, I. *Helichrysum globiferum*, polar view, J. *Helichrysum globiferum*, exine sculpture, K. *Helichrysum plicatum*, polar view, L. *Helichrysum plicatum*, exine sculpture (Bars: G = 10 µm, E = 3 µm, A, B, D, H, I, K = 2 µm, C, F, J, L = 1 µm).

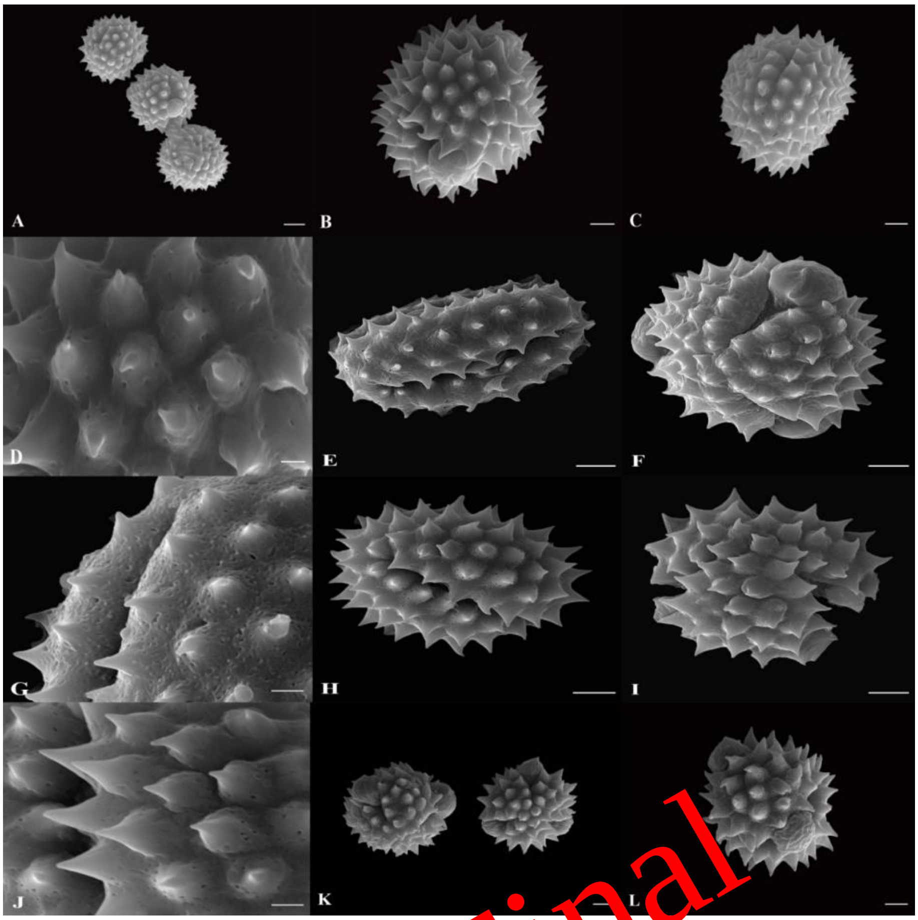

Fig. 2. SEM micrographs of pollen grains in the studied species of Gnaphalieae: A. *Lasiopogon muscoides*, overview of three grains, B. *Lasiopogon muscoides*, equatorial view, C. *Lasiopogon muscoides*, polar view, D. *Lasiopogon muscoides*, exine sculpture. E. *Leysera leyseroides*, equatorial view, F. *Leysera leyseroides*, polar view, G. *Leysera leyseroides*, exine sculpture, H. *Micropus supinus*, equatorial view, I. *Micropus supinus*, polar view, J. *Micropus supinus*, exine sculpture, K. *Phagnalon persicum*, overview of two grains, L. *Phagnalon persicum*, equatorial view (Bars: A, E, K = 10 µm, F, H, I = 5 µm, B, C, G, J, L = 2 µm, D = 1 µm).

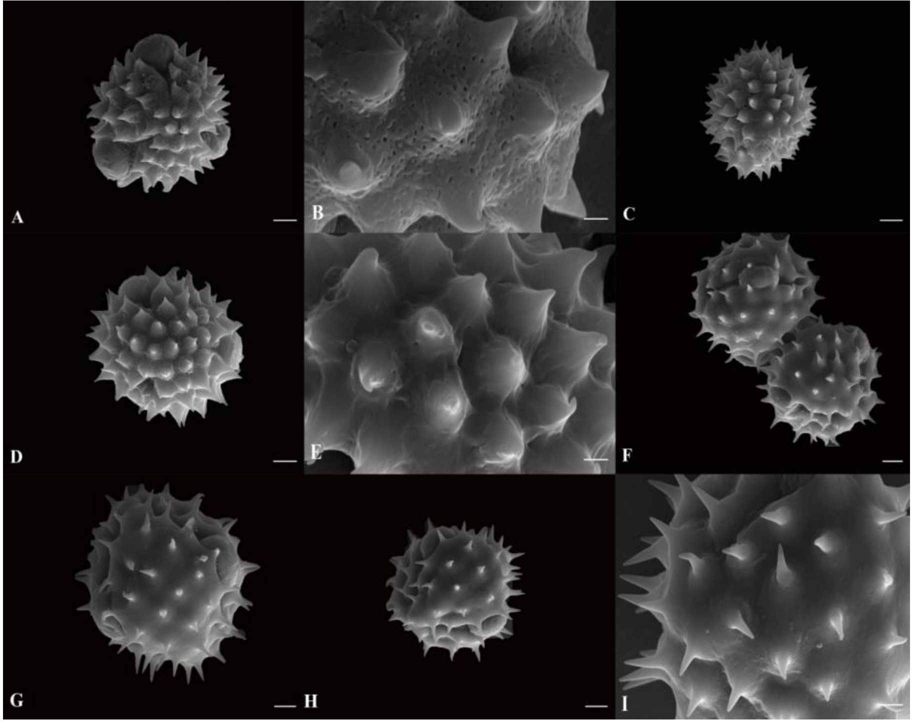

Fig. 3. SEM micrographs of pollen grains in the studied species of Gnaphalieae and Calenduleae: A. *Phagnalon persicum*, polar view, B. *Phagnalon persicum*, exine sculpture, C. *Pseudognaphalium luteoalbum*, equatorial view, D. *Pseudognaphalium luteoalbum*, polar view, E. *Pseudognaphalium luteoalbum*, exine sculpture, F. *Calendula officinalis*, overview of two grains, G. *Calendula officinalis*, equatorial view, H. *Calendula officinalis*, polar view, I. *Calendula officinalis*, exine sculpture (Bars: F, G, H = 10 µm, A, C, D, I = 2 µm, B, E = 1 µm).

- Significance of clustering and ordination methods

The results of various clustering and ordination methods, including the WARD tree, PCA and PCoA plots, are presented in Figures 4–6. Although a UPGMA analysis was conducted in this study, it did not yield significant differences in species delimitation; therefore, only the WARD tree is included here.

The PCA of palynological features revealed that, the polar axis, equatorial diameter, spines height, the ratio of spines height to spines diameter [H/D] and the distance between spines were the most variable features among the studied taxa. These features are taxonomically significant for delimiting both the tribes Gnaphalieae and Calenduleae, as well as for delineating subtribes, genera and species within Gnaphalieae. This indicates that, pollen size and various aspects of spine morphology can serve as important taxonomic tools for distinguishing and delimiting tribes and identifying pollen types in both Gnaphalieae and Calenduleae. Overall, the palynological features demonstrated that, the first two PCA components accounted for approximately 74% of the total variation.

The clustering and ordination analyses demonstrated that, pollen data are a valuable tool for delimiting tribes and subtribes. These methods effectively separated the tribes Gnaphalieae and Calenduleae. Furthermore, *Leysera leyseroides*, the only representative of the subtribe Relhaniinae, could be clearly distinguished from all species in the subtribe Gnaphaliinae based on pollen features such as the polar axis and especially the distance between spines.

The SEM images (Figs 1–3), clearly illustrate the significant diversity in both quantitative and qualitative characteristics of pollen grains among the studied taxa. Notably, differentiation exists even among species within the same genus, such as *Filago*. The WARD tree analysis indicates that, *F. griffithii* (formerly *Cymbolaena griffithii* and now considered synonymous with *F. griffithii*) and *F. germanica*, while belonging to the same genus, exhibit differences in several features, including pollen shape, size, P/E ratio and distance between spines. As a result, they are classified into two distinct pollen types in the description of new types within Gnaphalieae. In contrast, the two species of the genus *Helichrysum*, *H. globiferum*, and *H. plicatum*, displayed similarities in most pollen features and were grouped together in the WARD tree, PCA and PCOA plots. This shows that, the palynological data are informative and useful for delineating genera and species.

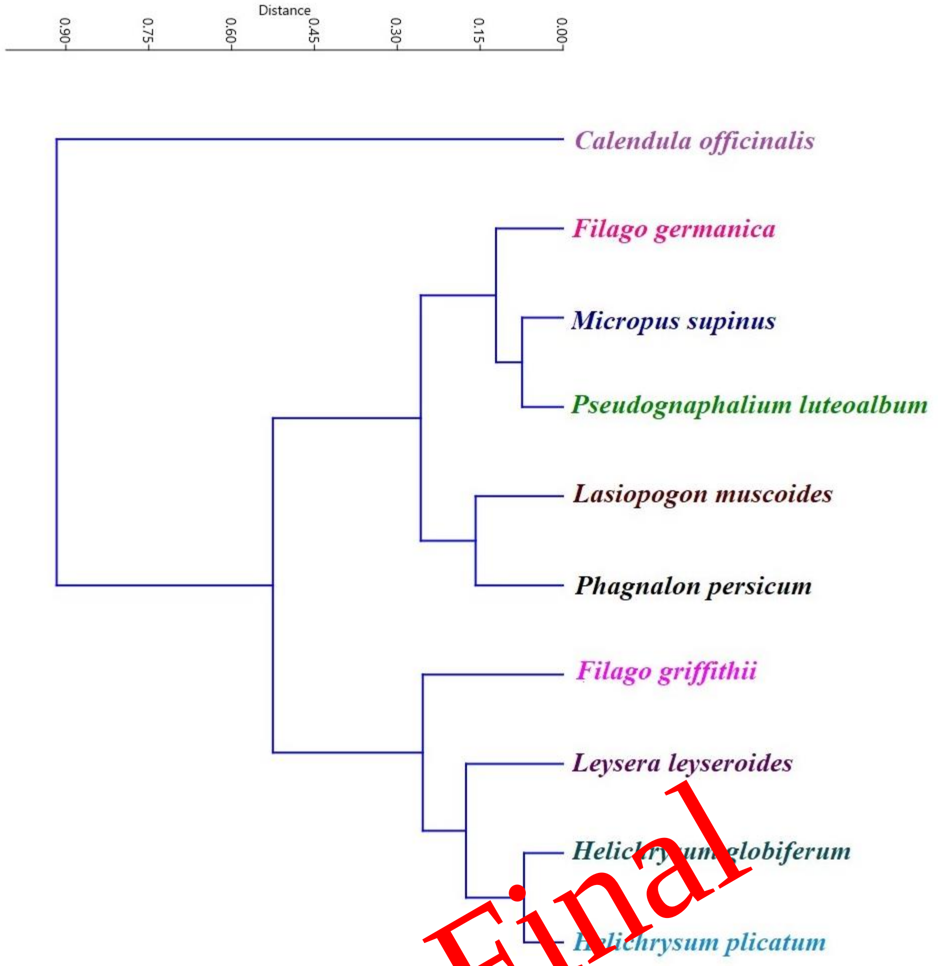

Fig. 4. WARD tree of the studied species of Gnaphalieae and Calenduleae based on pollen data (species according to Appendix 1).

Fig. 5. PCA plot of the studied species of Gnaphalieae and Calenduleae based on pollen data (species according to Appendix 1): A. Pollen shape, B. Size of polar axis, C. Size of equatorial diameter, D. P/E ratio, E. Spines height, F. Spines diameter, G. H/D ratio, H. Distance between spines.

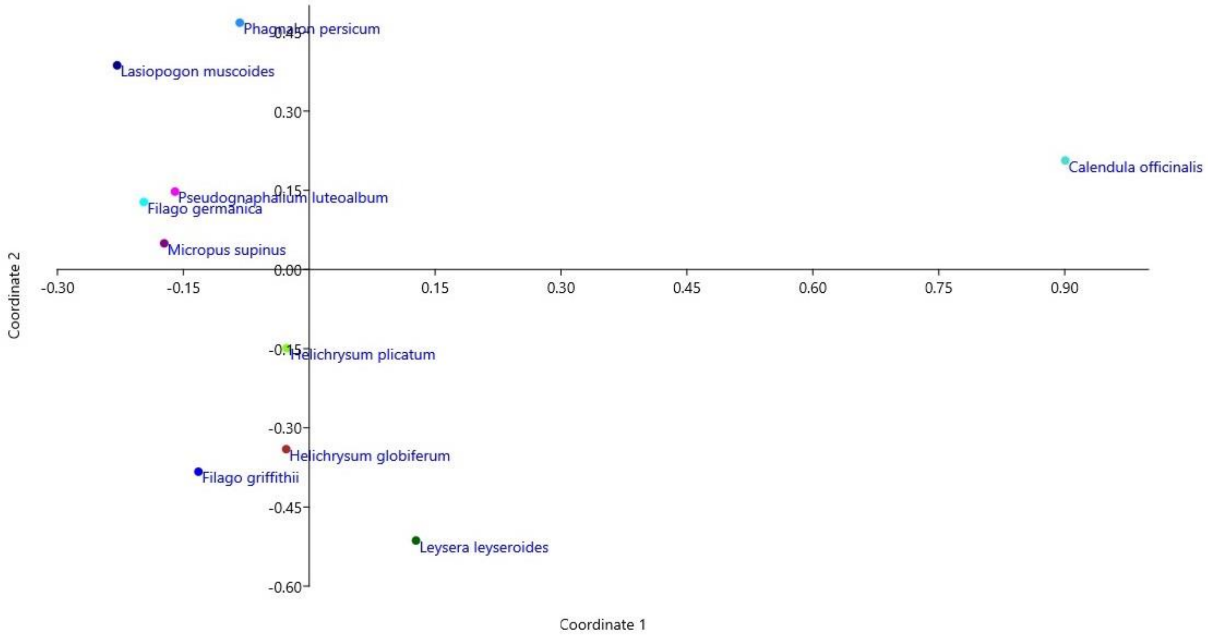

Fig. 6. PCoA plot of the studied species of Gnaphalieae and Calenduleae based on pollen data (species according to Appendix 1).

- Relationships among species based on pollen features

In the WARD tree, the studied species were divided into two main clusters based on pollen data. The first cluster included *C. officinalis*, the only representative of the tribe Calenduleae. This species was clearly distinguished from all species of the tribe Gnaphalieae by significant pollen features, such as polar axis, equatorial diameter, spines height and the distance between spines, allowing it to be identified as a separate pollen type. The second cluster includes all species of Gnaphalieae, which are classified into two main subsets based on meaningful features such as pollen size [P], spines height and the distance between spines. The first subset comprises *Filago germanica*, *Micropus supinus*, *Pseudognaphalium luteoalbum*, *Lasiopogon muscoides*, and *Phagnalon persicum* from subtribe Gnaphaliinae. This group consists of species with small-sized grains and features prolate-spheroidal, sub-prolate (only in *Micropus supinus*) and oblate- spheroidal (only in *Lasiopogon muscoides*) pollen shapes. The species in this subset are grouped together due to overlapping ranges in features such as polar axis (15.96–22.93), spines height (1.61–2.49) and the distance between spines (0.67–1.08). Therefore, they can be considered a separate pollen type within Gnaphalieae. The second group includes species such as *F. griffithii*, *Helichrysum globiferum*, and *H. plicatum* from subtribe Gnaphaliinae and *Leysera leyseroides* from subtribe Relhaniinae. The species in this subset have medium-sized grains. These species exhibit prolate and sub-prolate shapes. They are related to one another due to overlapping ranges in features, including polar axis (25.1–32.99), P/E ratio (1.22–1.45), spines height (1.21–1.52), and the distance between spines (1.13–2.61). Consequently, they can be classified as a distinct pollen type within Gnaphalieae.

- Description of pollen types

Based on the WARD tree, PCA and PCOA plots, as well as the current pollen characteristics, one can identify three distinct pollen types in the examined Gnaphalieae and Calenduleae species, described below:

1. *Calendula*

Pollen grains tetra-zonocolporate, medium-sized, 42.41 >P<42.64 µm, 38.84 >E<39.69 µm, 1.06 >P/E<1.09, prolate-spheroidal in equatorial view, exine ornamentation echinate-perforate, subtriangular in polar view, spines with a height of 3.60–3.77 µm and a diameter of 1.39–1.49 µm, 2.45 >H/D<2.71, distance between the spines 4.80–5.30 µm.

Spines slender, conical with sharply pointed tips, occasionally recurved and shiny; slightly swollen at base, regularly distributed. *Calendula officinalis* belongs to this type.

2. *Lasiopogon*

Pollen grains tricolporate, small-sized, $15.96 > P < 22.93$ µm, $14.59 > E < 22.62$ µm, $0.98 > P/E < 1.27$, prolate-spheroidal, sub-prolate (only in *Micropus supinus*) and oblate-spheroidal (only in *Lasiopogon muscoides*) in equatorial view, exine ornamentation echinate-perforate, circular or subtriangular in polar view, spines with a height of 1.61–2.49 µm and a diameter of 1.2–2.2 µm, $1.05 > H/D < 1.38$, distance between the spines 0.67–1.08 µm. Spines conical with pointed or sharply pointed tips, rarely recurved; broad, swollen and perforated at base, regularly or irregularly distributed. *Filago germanica*, *Micropus supinus*, *Pseudognaphalium luteoalbum*, *Lasiopogon muscoides*, and *Phagnalon persicum* from subtribe Gnaphaliinae belong to this type.

3. *Helichrysum*

Pollen grains tricolporate, medium-sized, $25.1 > P < 32.99$ µm, $18.11 > E < 22.71$ µm, $1.22 > P/E < 1.45$, prolate and sub-prolate in equatorial view, exine ornamentation echinate-perforate, circular in polar view, spines with a height of 1.21–1.52 µm and a diameter of 0.94–0.75 µm, $0.84 > H/D < 1.33$, distance between the spines 1.13–2.61 µm. Spines conical with pointed, sharply pointed or tapered tips, rarely recurved; broad, swollen and perforated at base, regularly distributed. *Filago griffithii*, *Helichrysum globiferum*, and *H. plicatum* from subtribe Gnaphaliinae and *Leysera leyseroides* from subtribe Relhaniinae belong to this type.

Discussion

This study is one of the most comprehensive palynological investigations of Gnaphalieae and its relationship with Calenduleae. The findings aim to enhance the identification of pollen types based on the pollen features of their respective subtribes in Iran.

According to the results, *L. muscoides* and *C. officinalis* exhibit the smallest and largest pollen sizes [P], respectively, among the analyzed species. This confirms the findings of Osman (2011), who also recognized *L. muscoides* as having the smallest pollen among the representatives of the tribe Gnaphalieae. Notably, in the present study, pollen size was regarded as a distinguishing feature among the studied species, leading to the classification of members from both tribes into three main groups. It was interesting to observe significant variations in pollen shapes among the studied species of Gnaphalieae, including oblate- spheroidal, prolate-spheroidal, sub-prolate, and prolate. These findings confirm the results of Osman (2011) and Azizi *et al*. (2023). Furthermore, the examined pollen grains of all Gnaphalieae taxa were tricolporate, isopolar and echinate-perforat. Features such as pollen size [P/E], spines height and spacing were particularly distinctive. This shows that, the pollen grains possess taxonomically informative characteristics for the classification of Gnaphalieae. These findings confirm the results of Breitwieser & Sampson (1997a) and Osman (2011).

Calendula officinalis, belonging to Calenduleae, is distinguished from members of the tribe Gnaphalieae by its tetra-zonocolporate pollen grains and pollen features such as, polar axis, spines height and the distance between spines. However, it still exhibits similarities with Gnaphalieae taxa in features like pollen shape, P/E ratio and exine ornamentation. Therefore, it can be considered a sister group to Gnaphalieae, confirming the results of Coutinho & Dinis (2009) and Blackmore *et al*. (2009). Additionally, although *L. leyseroides*, the only representative of the small subtribe Relhaniinae, was distinguished from all species in the subtribe Gnaphaliinae based on pollen features such as the polar axis and the distance between spines, and was located further away from them in PCA and PCoA plots, it also displayed similarities to Gnaphaliinae in spines size (H, D, and H/D). This supports the findings of Coutinho & Dinis (2009) and

Smissen *et al*. (2020), who considered Relhaniinae to be the sister group of Gnaphaliinae.

Although pollen features were effective in delimiting genera and species, the results showed some inconsistencies. For example, the two species of the genus *Helichrysum* were clustered together in the WARD tree. In contrast, the two species of the genus *Filago*, namely, *F. griffithii* (formerly *Cymbolaena griffithii* and now considered synonymous with *F. griffithii*) and *F. germanica*, were positioned very far apart in the analyses and classified into two distinct pollen types. Therefore, the pollen results do not support this synonymy. This incongruence may stem from the adaptation of certain pollen characteristics, such as shape and size, to varying environmental conditions and biogeographical patterns. Increasing evidence suggests that, environmental factors significantly affect pollen morphology (Yang *et al*. 2020, Noroozi *et al*. 2022, Hidalgo *et al*. 2023, Atazadeh & Ghahremaninejad 2025). Following Hidalgo *et al*. (2023), which demonstrates a link between pollen morphology and climate, one can infer that, current global changes might also affect the phenotypic spectrum of pollen and, consequently, pollination success. Additionally, Lopez-Vinyallonga *et al*. (2009) indicate that, many morphological and micromorphological features may have evolved in response to environmental adaptations and different biogeographical patterns, rendering them homoplasious features. Thus, a combination of these factors could explain the heterogeneity of relationships among species in Gnaphalieae. However, further studies are needed to establish demonstrating the influence of environmental conditions on pollen morphology within this tribe. Future research that connects pollen datasets with habitat information of the studied species may deepen our understanding of how ecological environments influence pollen features. Specifically, one can find out whether shifts in longitude and latitude, changes in temperature and humidity, affect the evolution of pollen characteristics or not. Yang *et al*. (2020) and Noroozi *et al*. (2022) suggest that, habitat humidity may serve as a selective force influencing pollen morphology in angiosperms, noting significant correlation between pollen shape and environmental adaptations in members of the Lamiid clade and the Boraginaceae, respectively. Considering the above points, the classification of pollen grains from the studied taxa of Gnaphalieae into two distinct groups; each with different pollen sizes and diverse shapes; could be related to climatic conditions and ecological adaptations. However, further studies are needed to explore this relationship.

In the present study, pollen morphology played as a reliable discriminating marker for delineating the tribes Gnaphalieae and Calenduleae. The data analysis showed the significance of pollen characteristics in identifying and delimiting species within Gnaphalieae. This finding confirms the results of Blackmore *et al*. (2009), who emphasized the importance of pollen features, particularly spines size, in defining unresolved taxonomic groups within various Asteraceae tribes. Additionally, other studies (El-Garf & Osman 2007, Osman 2011, Wortley *et al*. 2012, Qu *et al*. 2025) highlighted various aspects of spine features, such as height, diameter and spacing, as effective taxonomic markers for distinguishing different pollen types and improving infrageneric classification within the Asteraceae tribes. Furthermore, these microstructural features provide reliable evidence for resolving systematic issues even within Gnaphalieae (El-Ghazaly & Anderberg 1995, Breitwieser & Sampson 1997a, b). Accordingly, results derived from the present study, identified three distinct pollen types in *Calendula*, *Lasiopogon*, and *Helichrysum* within the studied taxa of Gnaphalieae and Calenduleae. The pollen features, such as polar axis, spines height and the distance between spines, play a key role in identifying these three types; representatives of each groups did not overlap in these features and were completely distinct from one another. These pollen types are presented in two tribes for the first time. Skvarla *et al*. (1977) and Wortley *et al*. (2012) identified a general Helianthoid pollen type for the subfamily Asteroideae, which encompasses the tribes Astereae and Gnaphalieae. In contrast, the present study provides a detailed analysis of the pollen characteristics specific to the tribe Gnaphalieae, resulting in the recognition of two distinct pollen types within this tribe. Furthermore, the description of Calenduleae pollen type in El-Garf & Osman (2007), who examined two genera within Calenduleae including *Calendula* and *Osteospermum*, aligns with the *Calendula* pollen type identified in the present study. In addition, classification of pollen grains from the genera *Filago*, *Pseudognaphalium* and *Lasiopogon* as a separate pollen type, as described in Osman's (2011) palynological data, aligns with the findings of the present study. However, the grouping of pollen grains from certain *Helichrysum* species with *Phagnalon* into a distinct pollen type in Osman's (2011) study contradicts results derived from the present study. Therefore, the identification of these three new pollen types through palynological analyses highlights the impressive diversity of pollen grains within the two tribe. This discovery underscores the importance of pollen types as key taxonomic markers for delimiting Gnaphalieae and Calenduleae species and emphasizes their value in discussions about the evolution and systematics of these tribes.

The present study revealed significant variation in pollen data among the examined Gnaphalieae and Calenduleae species. According to previously published literature (Noroozi *et al*. 2022, Atazadeh & Ghahremaninejad 2025, Qu *et al*. 2025), the pollen data and practical palynological information from this study can be integrated with molecular data using reliable markers in the future. This integration aims to address taxonomy and classification issues and clarify the true evolutionary pathways of Gnaphalieae and Calenduleae. Furthermore, future research on pollen morphology in these two tribes should focus on expanding taxon sampling across various ecological ranges, thereby enhancing our understanding of the role of pollen in ecological adaptations and evolutionary processes (Zhang *et al*. 2024). In addition, transmission electron microscopy (TEM) could be employed to perform detailed analyses of pollen microstructures, revealing subtle differences between species and improving taxonomic accuracy (Gabarayeva *et al*. 2024).

Conclusion

In the present study, several genera from the tribes Gnaphalieae and Calenduleae that had not been previously comprehensively investigated using pollen data were carefully evaluated. Through clustering and ordination methods, three distinct pollen types in *Calendula*, *Lasiopogon*, and *Helichrysum* were identified here among the examined taxa. This study also demonstrated that, pollen morphology is a reliable tool for delineating the studied species and identifying pollen types. Significant variation in pollen data was revealed among examined Gnaphalieae and Calenduleae species. The classification of pollen grains from these taxa in two distinct groups (each characterized by different pollen sizes and diverse shapes), may be related to climatic conditions and ecological adaptations. Therefore, combining these palynological results with molecular and phytogeographic data, alongside broader taxon sampling, will undoubtedly aid researchers in the future, in resolving taxonomic complexities and better understanding the evolutionary pathways of the studied specimens within the tribe.

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Appendix 1. Investigated species of Gnaphalieae & Calenduleae in Iran along with their related data. Three samples were selected from each species

No.	Taxon	Tribe	Subtribe	Locality	Altitude (m)	Longitude (E)	Latitude (N)	Voucher No.
1	*Filago germanica* (L.) Huds.	Gnaphalieae	Gnaphaliinae	Mazandaran Prov.: Behshahr	3	53° 32' 53"	36° 42' 08"	15664 (T)
2	*F. griffithii* (A.Gray) Andres-Sanchez & Galbany	"	"	Lorestan Prov.: Cham-e Divan village	979	48° 00' 20"	33° 29' 25"	23678 (TUH)
3	*Helichrysum globiferum* Boiss.	"	"	Fars Prov.: Marvdasht	1610	52° 80' 25"	29° 87' 42"	12376 (T)
4	*H. plicatum* DC.	"	"	E. Azarbaijan Prov.: Tabriz, Golzar village	1360	46° 14' 33"	38° 04' 39"	8057 (T)
5	*Lasiopogon muscoides* (Desf.) DC.	"	"	Yazd Prov.: Bafq	992	55° 24' 21"	31° 36' 27"	13215 (T)
6	*Leysera leyseroides* (Desf.) Maire	"	Relhaniinae	Kerman Prov.	1759	57° 05' 00"	30° 17' 42"	34553 (TUH)
7	*Micropus supinus* L.	"	Gnaphaliinae	Isfahan Prov.: Kashan	940	51° 25' 28"	33° 59' 40"	20691 (TUH)
8	*Phagnalon persicum* Boiss.	"	"	Fars Prov.: Shiraz, Pir-bano	1553	52° 28' 32"	29° 29' 37"	16547 (T)
9	*Pseudognaphalium luteoalbum* (L.) Hilliard & B.L.Burtt.	"	"	Mazandaran Prov.: Ramsar, Javaher-deh	1765	50° 28' 31"	36° 51' 17"	16516 (T)
10	*Calendula officinalis* L.	Calenduleae	-	N. Khorasan Prov.: Esfarayen, Sarigol Protected Area	1528	57° 40' 28"	36° 58' 38"	91 (T)

Appendix 2. Evaluated features of pollen grains in the studied species of Gnaphalieae in Iran (Codes are mentioned in table 1). P: Polar axis, E: Equatorial diameter, H: Spines height, D: Spines diameter [range: X, mean values (standard deviation)]. Three samples were selected from each species

No.	Taxon	Shape	P (µm)	E (µm)	P/E ratio	H (µm)	D (µm)	H/D ratio	Distance between spines
1	*Filago germanica* (L.) Huds.	5	17.62–18.33 x = 18.14 (0.25)	17.21–17.73 x = 17.53 (0.22)	1.03	1.53–1.75 x = 1.68 (0.07)	1.15–1.28 x = 1.21 (0.04)	1.38	0.54–0.76 x = 0.68 (0.08)
2	*F. germanica* (L.) Huds.	5	17.45–18.74 x = 18.15 (0.42)	17.44–17.87 x = 17.62 (0.18)	1.03	1.38–1.81 x = 1.61 (0.16)	1.10–1.42 x = 1.27 (0.14)	1.26	0.52–0.85 x = 0.71 (0.13)
3	*F. germanica* (L.) Huds.	5	17.28–18.24 x = 17.79 (0.41)	15.98–18.04 x = 17.33 (0.83)	1.02	1.45–1.82 x = 1.66 (0.15)	1.02–1.34 x = 1.20 (0.09)	1.38	0.55–0.75 x = 0.67 (0.08)
4	*F. griffithii* (A.Gray) Andres-Sanchez & Galbany	7	24.98–26.30 x = 25.46 (0.57)	18.09–8.35 x = 18.21 (0.12)	1.39	1.04–1.42 x = 1.21 (0.14)	0.78–1.06 x = 0.94 (0.12)	1.28	0.84–1.34 x = 1.13 (0.19)
5	*F. griffithii* (A.Gray) Andres-Sanchez & Galbany	7	24.95–25.83 x = 25.20 (0.42)	18.30–18.43 x = 18.35 (0.05)	1.37	1.09–1.45 x = 1.23 (0.12)	0.8–0.99 x = 0.98 (0.11)	1.25	1.05–1.71 x = 1.27 (0.25)
6	*F. griffithii* (A.Gray) Andres-Sanchez & Galbany	7	25–25.99 x = 25.61 (0.34)	17.87–18.50 x = 18.11 (0.24)	1.41	1.13–1.46 x = 1.26 (0.11)	0.76–1.14 x = 0.98 (0.11)	1.28	1.02–1.36 x = 1.18 (0.13)
7	*Helichrysum globiferum* Boiss.	6	26.63–27.67 x = 27.15 (0.4)	20.41–21.41 x = 20.88 (0.39)	1.30	0.99–1.36 x = 1.20 (0.13)	1.25–1.55 x = 1.37 (0.11)	0.87	1.74–2.38 x = 2.06 (0.26)
8	*H. globiferum* Boiss.	6	27.24–27.73 x = 27.49 (0.25)	20.38–21.89 x = 21 (0.6)	1.30	1.18–1.73 x = 1.44 (0.19)	1.34–1.66 x = 1.50 (0.15)	0.96	1.76–2.31 x = 2.04 (0.19)
9	*H. globiferum* Boiss.	6	26.67–28.21 x = 27.38 (0.68)	20.40–21.48 x = 20.96 (0.39)	1.30	1.06–1.46 x = 1.24 (0.17)	1.17–1.67 x = 1.44 (0.15)	0.86	1.7–2.31 x = 2.07 (0.23)
10	*H. plicatum* DC.	6	23.49–25.68 x = 25.1 (0.74)	19.24–21.74 x = 20.52 (1.06)	1.22	1.23–1.77 x = 1.43 (0.2)	1.39–2.06 x = 1.62 (0.25)	0.88	1.84–2.19 x = 2.01 (0.15)
11	*H. plicatum* DC.	6	24.57–25.64 x = 25.31 (0.69)	19.50–21.15 x = 20.46 (0.86)	1.23	1.2–1.67 x = 1.48 (0.2)	1.36–2.07 x = 1.75 (0.3)	0.84	1.25–2.46 x = 2.10 (0.43)
12	*H. plicatum* DC.	6	23.82–25.61 x = 25.19 (0.89)	19.34–20.62 x = 20.14 (0.69)	1.25	1.37–1.84 x = 1.52 (0.18)	1.47–1.87 x = 1.67 (0.16)	0.91	1.94–2.30 x = 2.11 (0.14)
13	*Lasiopogon muscoides* (Desf.) DC.	3	14.50–16.84 x = 15.96 (0.95)	15.18–17.29 x = 16.02(1.11)	0.99	1.71–1.99 x = 1.83 (0.1)	1.57–1.66 x = 1.63 (0.04)	1.12	0.71–0.83 x = 0.77 (0.05)
14	*L. muscoides* (Desf.) DC.	3	14.70–16.89 x = 16 (0.93)	15.53–16.62 x = 16.17 (0.57)	0.98	1.45–2.12 x = 1.85 (0.25)	1.42–1.79 x = 1.59 (0.12)	1.16	0.61–1.14 x = 0.84 (0.22)

15	*L. muscoides* (Desf.) DC.	3	14.63–16.95 x = 16.06 (1.04)	15.50–16.72 x = 16.09 (0.61)	0.99	1.73–1.97 x = 1.88 (0.08)	1.59–1.76 x = 1.67 (0.06)	1.12	0.7–1.02 x = 0.81 (0.14)
16	*Leysera leyseroides* (Desf.) Maire	7	32.24–33.90 x = 32.99 (0.85)	21.10–23.94 x = 22.71 (1.14)	1.45	0.95–1.77 x = 1.36 (0.29)	0.78–1.51 x = 1.04 (0.25)	1.30	1.6–3.46 x = 2.46 (0.61)
17	*L. leyseroides* (Desf.) Maire	7	31.32–33.84 x = 32.30 (0.91)	20.58–24.27 x = 22.60 (1.42)	1.42	0.66–1.82 x = 1.30 (0.44)	0.84–1.5 x = 1.08 (0.26)	1.20	2.07–3.19 x = 2.61 (0.5)
18	*L. leyseroides* (Desf.) Maire	7	31.18–33.72 x = 32.24 (1.04)	20.32–24.67 x = 22.34 (1.80)	1.44	1.02–1.89 x = 1.38 (0.37)	0.72–1.32 x = 1.03 (0.23)	1.33	2.10–2.78 x = 2.44 (0.28)
19	*Micropus supinus* L.	6	17.31–20.36 x = 18.87 (1.52)	13.58–16.28 x = 14.93 (1.13)	1.26	1.78–1.88 x = 1.83 (0.04)	1.23–1.64 x = 1.46 (0.14)	1.25	0.81–1.33 x = 1 (0.19)
20	*M. supinus* L.	6	16.89–20.25 x = 18.60 (1.45)	13.75–15.76 x = 14.59 (1.26)	1.27	1.45–1.78 x = 1.66 (0.11)	1.43–1.56 x = 1.53 (0.06)	1.08	0.68–1.10 x = 0.92 (0.17)
21	*M. supinus* L.	6	17.32–19.95 x = 18.65 (1.28)	13.51–16.40 x = 15.03 (1.30)	1.24	1.59–1.95 x = 1.74 (0.13)	1.26–1.76 x = 1.55 (0.15)	1.12	0.79–1.19 x = 1 (0.16)
22	*Phagnalon persicum* Boiss.	5	20.89–23.93 x = 22.41 (1.52)	20.17–22.38 x = 21.20 (0.90)	1.05	2.36–2.60 x = 2.49 (0.09)	1.82–2.46 x = 2.10 (0.23)	1.18	0.55–1.49 x = 1.06 (0.29)
23	*Ph. persicum* Boiss.	5	20.71–24.14 x = 22.93 (1.92)	20.82–23.99 x = 22.62 (1.35)	1.01	2.15–2.82 x = 2.44 (0.3)	2.10–2.54 x = 2.20 (0.18)	1.10	0.77–1.18 x = 0.91 (0.14)
24	*Ph. persicum* Boiss.	5	20.35–24.11 x = 22.81 (2.12)	21.24–23.28 x = 22.47 (0.86)	1.01	1.91–3 x = 2.37 (0.4)	1.35–2.79 x = 2.14 (0.51)	1.10	0.85–1.35 x = 1.06 (0.17)
25	*Pseudognaphalium luteoalbum* (L.) Hilliard & B.L.Burtt.	5	18.22–21.97 x = 19.68 (2)	16.34–19.06 x = 17.59 (1.37)	1.11	1.60–1.97 x = 1.76 (0.15)	1.24–1.78 x = 1.64 (0.22)	1.07	0.66–1.53 x = 1.06 (0.33)
26	*P. luteoalbum* (L.) Hilliard & B.L.Burtt.	5	18.86–21.87 x = 20.23 (1.52)	17.46–19.33 x = 18.39 (0.93)	1.10	1.38–2.09 x = 1.83 (0.25)	1.45–1.73 x = 1.58 (0.13)	1.15	0.57–2.20 x = 1.08 (0.59)
27	*P. luteoalbum* (L.) Hilliard & B.L.Burtt.	5	17.76–22.58 x = 20.09 (2.41)	16.72–18.62 x = 18 (0.86)	1.11	1.44–2.24 x = 1.86 (0.29)	1.54–2.14 x = 1.77 (0.25)	1.05	0.60–1.43 x = 0.95 (0.24)
28	*Calendula officinalis* L.	5	40.15–43.93 x = 42.41 (1.58)	38.58–41.87 x = 39.69 (1.88)	1.06	2.94–4.22 x = 3.60 (0.53)	1.23–1.63 x = 1.42 (0.13)	2.53	4.20–5.56 x = 4.80 (0.66)
29	*C. officinalis* L.	5	40.68–43.31 x = 42.49 (0.94)	36.51–42.34 x = 39.13 (1.88)	1.08	2.98–4.02 x = 3.77 (0.44)	1.23–1.58 x = 1.39 (0.12)	2.71	3.77–6.50 x = 5.30 (1.20)
30	*C. officinalis* L.	5	40.55–44.12 x = 42.64 (1.86)	37.41–40.93 x = 38.84 (1.88)	1.09	3–4.21 x = 3.66 (0.54)	1.39–1.63 x = 1.49 (0.1)	2.45	4.64–5.73 x = 5.24 (0.44)