Taiwania 70(2): 207–218, **2025**
DOI: 10.6165/tai.2025.70.207

Pollen morphology of the *Galium* L. (Rubieae-Rubiaceae) from Pakistan and evaluation of its systematic significance

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(Manuscript received 17 October 2024; Accepted 28 February 2025; Online published 19 March 2025)

ABSTRACT: *Galium* is a typical member of the tribe Rubieae, characterized by its 3 or 4-lobed corolla, rudimentary corolla tubes, corolla tube length and dry mericarps and has a ubiquitous distribution. In this study pollen morphology of 20 species of *Galium* from Pakistani highland was investigated and documented in detail using light and scanning electron microscopy. The genus *Galium* itself is a delimited natural group and its taxa are poorly explored for pollen morphology. Among the investigated species, pollen grains are small to large (P=13.05–21.75 µm, E=17.00–24.20 µm), oblate–spheroidal, suboblate, subprolate and prolate–spheroidal (P/E=0.84–1.16) in shape and 5–10 colpate. Exine sculpturing under SEM showing different ornamentations, including microechinate–scabrate, scabrate–punctate, microreticulate–scabrate, microspine–scabrate, microechinate–punctate, microspine–verrucate, microechinate–verrucate, microspine–perforate, scabrate–verrucate and microechinate–perforate among the investigated taxa. In addition, based on pollen morphometric traits, hierarchical clustering (UPGMA) analysis is performed for correlation between the species. However, pollen traits alone or with the combination of morphological characters are used in the infrageneric relationships and also provide useful information for its evolutionary history.

KEY WORDS: *Galium*, Pakistan, Palynotaxonomy, SEM, UPGMA.

INTRODUCTION

Galium L. is one of the largest genus within the tribe Rubieae (subfamily Rubioideae) and family Rubiaceae and comprises 645 species and approximately 780 taxa included in 16 sections (Natali *et al.*, 1995; Soza and Olmstead, 2010; Tao and Ehrendorfer, 2011; Ehrendorfer and Barfuss, 2014; Ehrendorfer *et al.*, 2018; Ferrer-Gallego, 2020; POWO, 2025). It is the typical group, including perennial subshrubs or annual herbs plants extensively distributed in both the new and old world (Soza and Olmstead, 2010; Elkordy and Schanzer, 2015). Phytoecologically, *Galium* species have a ubiquitous range of distribution and mostly occur in meridional to temperate and subtropical to tropical zones at higher elevations, but some species also in alpine and arctic regions of the world (Ehrendorfer *et al.*, 2005; Chen and Ehrendorfer, 2011; Ferrer-Gallego, 2020). Most of the *Galium* species are diversified in the temperate region of the Western Himalayan, so this region is regarded as center of diversification (Nazimuddin and Ehrendorfer 1987; Mill, 1996; Soza and Olmstead, 2010). In terms of the number of species Flora of the USSR encompassed 93 species followed by China (63 species), Flora Iranica region (60 species) and 32 species were recorded from India (Kirpicznikov,1969;; Ehrendorfer *et al.*, 2005; Chen and Ehrendorfer, 2011; Kumar *et al.*, 2013). Here, we focused on Pakistani highlands hosting 28 species, being 6 are exclusively endemic and mostly distributed to Western Himalayan in northern regions (Nazimuddin and Ehrendorfer, 1987; Nazimuddin and Qaiser, 1989; Ehrendorfer *et al.*, 2005).

Among the Rubiaceae *Galium* is taxonomically very difficult and problematic genus. *Galium* shares many similar morphological features with the other genera in the tribe Rubieae (Yang *et al.*, 2018). The infrasectional taxonomy and classification of *Galium* is very complicated, due to many species of the genus *Asperula* sharing morphological features with *Galium* (Elkordy and Schanzer, 2015). *Galium* is distinguished by its weak quadrangular clambering stem, whorls of leaves, 3 or 4-lobed corolla, rudimentary corolla tubes, corolla tube length and dry mericarps among the tribe Rubieae (Soza and Olmstead, 2010; Son and Chang, 2019). Taxonomically, *Galium* itself very problematic genus and the taxonomic position of its species still uncertain (Elkordy and Schanzer, 2015). The wide range of distribution and polymorphic nature of *Galium* species, often make taxonomic delimitation among the *Galium* taxa (Pobedimova, 1958; Elkordy and Schanzer, 2015).

The first conclusive infrageneric classification of *Galium* was established by Boissier (1881) in the Flora Orientalis with 91 species divided into 3 sections namely, sect. *Eugalium*, sect. *Aparine* and sect. *Cruciata*.

Pobedimova (1958) treated *Galium* in Flora of the USSR and used Boissier infrageneric classification. According to the most recent taxonomic treatments of *Galium* classified into 16 distinct sections (Ehrendorfer *et al*., 2005; Tao and Ehrendorfer, 2011). Several species of *Galium* have been synonymized based on molecular phylogeny (Govaerts *et al*., 2011). In the present study, we highlight five species that have been placed under synonymy i.e., *G. ghilanicum* Stapf (syn: *G. chitralensis* Nazim), *G. tricornutum* Dandy (syn: *G. kurramensis* Nazim), *G. spurium* subsp. *ibicinum* (Boiss. & Hausskn.) Ehrend. (syn: *G. ibicinum* Boiss. & Hausskn), *G. spurium* subsp. *spurium* L. (syn: *G. pauciflorum* Bunge) and *G. cryptanthum* Hemsl. (syn: *G. stewartii* Nazim) from the investigated area (Ehrendorfer *et al*., 2005; Govaerts *et al*., 2011; POWO, 2025). After this treatment, the total of 28 *Galium* species has been reduced to 24 native species occurring in Pakistan (Nazimuddin and Ehrendorfer, 1987; Nazimuddin and Qaiser, 1989; Ehrendorfer *et al.,* 2005; Govaerts *et al*., 2011).

The systematic treatment of *Galium* is very difficult because the plants have a wide range of variations in morphological features that create confusion in identification (Soza and Olmstead, 2010; Ehrendorfer *et al*., 2014; Ferrer-Gallego, 2020). Several taxonomic studies have been conducted on *Galium* for its systematic significance (Puff, 1977; Abdel Khalik *et al*., 2008b; Ehrendorfer, 2010; Elkordy and Schanzer, 2015; Son and Chang, 2019). Among them, micromorphology, anatomy, photochemistry, karyology and molecular study carried out for the taxonomic implications of *Galium* (Kliphuis, 1986; De Block and Robbrecht, 1998; Natali *et al*., 1995; Abdel Khalik *et al*., 2008a; Toni and Mariath, 2011; Govaerts *et al*., 2011; Elkordy and Schanzer, 2015; Ferrer-Gallego, 2020). In addition, pollen morphology in *Galium* also used in taxonomic circumscription (Huysmans *et al*., 2003; Dessein *et al*., 2005; Abdel Khalik *et al*., 2007; Schanzer and Elkordy, 2014; Başer *et al*., 2020; Chouhan *et al*., 2022). Many *Galium* species are still poorly explored for pollen morphology (Perveen and Qaiser, 2007). In the present study, we provide pollen morphology of twenty *Galium* species from Pakistan and we could not access these four species namely, (*Galium subtrinervium* Ehrend. & Schönb. Tem., *G. hoffmeisteri* (Klotzsch) Ehrend. & Schönb. Tem. ex R.R.Mill, *G. vassilczenkoi* Pobed. and *G. humifusum* M.Bieb.). Unfortunately, no collections of these taxa were found in our major herbaria (Ehrendorfer *et al*., 2005).

The purpose of our study is to contribute pollen morphology of poorly explored *Galium* species from Pakistan. *Galium* species are very difficult to identify, so these pollen characters provide significant information that can be used with the combination of other morphological characters or alone in the accurate identification of species. Pollen traits are also helpful in the infrageneric classification of *Galium* species at the sectional level. In addition, our findings are providing useful information for the taxonomic treatment of *Galium*. However, the studied features might be used in the phylogenetic and evolutionary relationships of the *Galium* taxa.

MATERIALS AND METHODS

Plant materials

Plant materials used in this study were detached from herbarium specimens of 20 *Galium* species from Pakistan. Some samples were collected from the field along with photographs (Fig. S1-2). Preliminary information including locality, vouchers, elevations, collector and date of collections were provided in Table S1. The coordinates of localities were taken from herbarium specimens and drawing local distribution map from the shape file of Pakistan by using Arc-GIS (version. 10) shown in (Fig. 1). Global distribution status for each species, we assessed Plants of the World Online (POWO). Therefore, valid scientific names and their authority are confirmed from POWO and the International Plant Names Index (IPNI).

Pollen examination (LM) analysis

For the light microscopy floral buds were soaked in 70% ethanol solution and crushed with a glass rod a few drops of glacial 45% lactic acid were added for the expansion of the pollen grains following the protocol of Erdtman *et al*., (1960) with some modification. Pollen grains were stained with one to two drops of glycerin jelly with one percent safranin and mounted on slides and placed coverslip. Pollen micrographs were taken by using a camera (HDCE-X5) connected to a light microscope (CXRII, Labomed Labo America). Scored features, including pollen shape and size, polar and equatorial axes, length and width of colpi, length and width of pore, and size of thickness were measured through the micrometer scale in light microscopy (LM). Measurements of each quantitative character were taken from 20–25 pollen grains. Polar to equatorial ratio (P/E ratio) was determined to follow Khan *et al*., (2024).

Pollen examination (SEM) analysis

Pollen grains were extracted from the dried floral buds without additional treatment and transferred to clean metallic stubs with the help of double adhesive tape and then deposited on the carbon-coated gold probe using ion sputtering equipment of scanning electron microscopy (Model JEOL-5910) installed at the Centralized Resource Laboratory (CRL), University of Peshawar. Pollen grains were observed, and micrographs recordings were saved as TIFF files following the methodology of Johansson (1987). Pollen qualitative characters, including exine sculpturing and decorative coating were observed. For the descriptive terminology, we follow Punt *et al*., (1994) Hesse *et al*., (2009) and Halbritter *et al*., (2018).

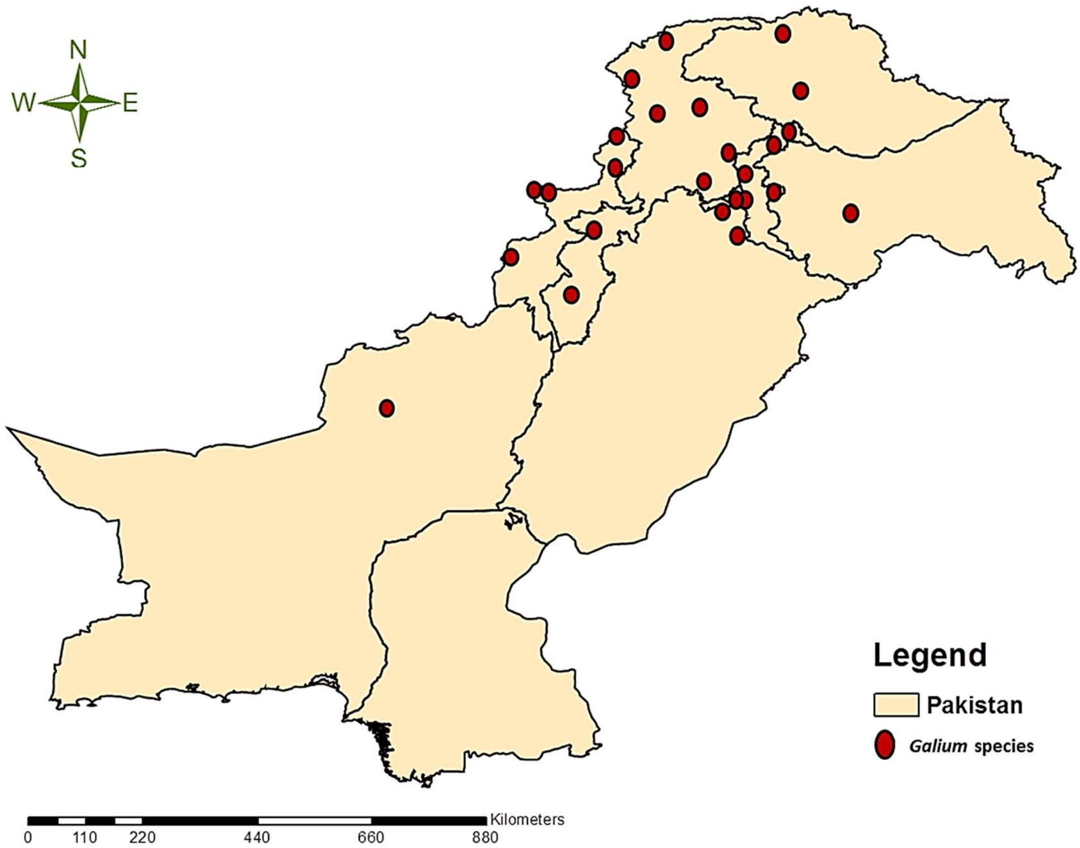

Fig. 1. Map illustrating the occurrence of ***Galium*** species in Pakistan and the colour bubble represents collection sites.

Data analyses

We scored a total of seven characters in quantitative data. Each quantitative character was measured with (maximum, minimum, mean, standard deviation and standard error) through statistical software (SPSS. version 16.0). The mean values of all the scored characters were used in statistical software (Past statistical software version 3.0) for clustering analysis. All the twenty species were clustered in UPGMA (unweighted pair group method using arithmetic average) cladogram to determine the phylogenetic relationship among the taxa.

RESULTS

A total of 20 species of *Galium* were investigated by using light and scanning electron microscopy. Pollen characters of the studied taxa are summarized in Tables 1–2 and pollen micrographs were also illustrated in (Figs. 2–5). Variations among the studied species were determined by using pollen morphometric features (numerical data), including polar and equatorial axes, P/E ratio, length and width of colpi and pore and exine thickness are represented in (Fig. 6). In additithe on, identification key was constructed for the studied taxa using pollen characters.

Polarity and symmetry of Galium pollen grains

Pollen grains of *Galium* are generally monad, isopolar, radially symmetrical, small in size, aperture type is stephanocolpate, aperture orientation is sunken and numbers of colpi also varied among the studied species (Figs. 2–5).

Pollen shape and size

The general pollen grains shape is revealed that they are mostly observed circular, orbicular or lobate in outline at polar view summarized in Table 1. Pollen shape varied among the *Galium* species based on mean values of polar to equatorial ratio. The P/E ratio value ranged from 0.84 in (*G. elegans*) to 1.16 in (*G. tricornutum*). According to Punt *et al*., (1994) mean values of the P/E ratio value can also be assign pollen shape classes such as oblate–spheroidal in (*G. acutum, G. aparine, G. asperifolium, G. asperuloides, G. ceratophylloides, G. cryptanthum, G. ghilanicum, G. serpylloides, G. setaceum, G. subfalcatum* and *G. tenuissimum*), suboblate in (*G. boreale, G. campylotrichum, G. elegans, G. hirtiflorum* and *G. tetraphyllum*), prolate–spheroidal in (*G. saturejifolium* and *G. verum*). The hydrated pollen sizes are varied among the *Galium* species presented in Table 2. Pollen is relatively small in size (10–24 µm) according to Punt *et al*., (1994) pollen size classes. Among the studied taxa,

Table 1. Quantitative pollen characters in investigated species of ***Galium***.

Taxa	P (µm) Min-Max=M±SE	E (µm) Min-Max=M±SE	P/E ratio	Clg (µm) Min-Max=M±SE	Clt (µm) Min-Max=M±SE	Plg (µm) Min-Max=M±SE	Plt (µm) Min-Max=M±SE	E (µm) Min-Max=M±SE
G. acutum	16.13–14.05=15.25±1.04	18.03–16.15=17.25±1.07	0.89	3.25–4.25=3.75±0.12	5.00–5.25=4.25±1.03	6.25–4.25=5.25±0.15	3.15–2.20=2.11±0.08	2.25–1.21=1.12±0.13
G. aparine	17.0–16.5=16.00±1.07	15.15–18.03=17.00±1.41	0.95	2.75–5.25=3.90±0.48	3.11–5.50=4.45±0.54	9.00–5.10=7.00±0.09	5.11–4.13=4.14±0.02	1.10–1.50=1.35±0.10
G. asperifolium	20.12–21.03=20.05±1.45	18.05–23.02=21.70±1.45	0.95	3.25–4.25=3.70±0.16	4.00–5.20=4.15±0.17	8.75–7.25=6.19±1.06	3.65–2.34=2.13±0.09	1.50–2.50=2.20±0.17
G. asperuloides	13.12–20.05=20.23±1.12	18.50–26.12=22.23±1.27	0.90	5.25–7.50=4.15±0.37	3.25–4.50=3.80±0.21	7.10–5.25=6.25±0.08	4.11–4.09=4.02±0.03	1.25–2.10=1.06±0.12
G. boreale	17.41–19.05=18.03±1.25	17.15–24.51=21.44±1.25	0.85	4.25–6.00=5.25±0.15	2.25–4.25=3.25±0.20	8.01–6.25=7.05±0.11	5.10–3.89=4.11±0.09	2.20–3.02=2.51±0.11
G. campylotrichum	21.04–23.13=21.15±1.03	25.06–23.09=24.20±1.26	0.87	2.25–5.00=4.25±0.12	5.0–26.25=5.25±0.21	7.25–6.51=5.57±0.45	6.09–4.02=5.14±0.03	1.25–1.20=1.22±0.10
G. ceratophylloides	16.05–21.07=18.10±1.06	20.05–18.50=19.02±1.25	0.94	4.25–5.25=3.12±0.13	4.25–5.25=4.25±0.20	9.09–6.25=7.25±0.12	5.80–3.67=4.89±0.04	2.25–1.24=2.21±0.15
G. cryptanthum	19.51–17.15=18.25±1.07	18.25–20.05=19.02±1.25	0.94	3.25–4.25=2.11±0.12	3.25–2.25=2.98±0.24	7.25–5.25=5.25±0.05	4.10–2.90=3.11±0.05	1.25–1.23=2.25±0.14
G. elegans	11.25–21.20=16.12±1.07	19.20–20.17=19.25±1.05	0.84	3.75–5.00=4.30±0.21	3.75–5.75=4.45±0.34	8.17–4.11=6.75±0.34	5.14–3.48=4.31±0.06	1.25–2.12=1.60±0.12
G. ghilanicum	12.49–16.13=14.25±1.06	17.25–14.14=15.12±1.06	0.93	2.25–5.00=3.50±0.20	2.25–3.25=2.21±0.25	6.25–4.15=7.25±0.50	5.71–4.66=5.10±0.02	3.25–2.51=1.50±0.14
G. hirtiflorum	19.21–14.31=17.09±2.25	19.17–22.34=20.11±1.08	0.85	2.25–3.25=2.10±0.11	2.25–4.25=3.27±0.23	7.13–5.25=6.25±0.21	5.08–4.12=4.92±0.04	1.35–1.25=1.70±0.13
G. saturejifolium	12.17–15.45=13.25±1.04	14.25–10.56=12.89±1.09	1.08	3.25–4.25=2.50±0.12	5.0–25.25=4.25±1.03	8.25–5.25=6.25±0.05	4.22–2.98=3.23±0.14	2.25–1.21=1.20±0.13
G. serpylloides	14.25–15.15=13.05±1.02	16.45–13.25=14.18±1.03	0.92	2.15–5.10=3.10±0.13	3.25–2.25=3.25±1.02	6.25–5.25=4.25±0.45	4.67–3.10=4.13±0.25	2.11–1.20=1.59±0.15
G. setaceum	16.25–20.11=18.15±1.03	17.25–19.67=18.53±1.07	0.97	4.25–3.25=4.00±0.11	2.25–4.25=3.25±1.05	10.25–3.25=6.25±0.04	5.81–4.19=4.99±0.17	2.25–1.41=1.24±0.12
G. spurium	18.25–25.33=21.59±1.03	16.00–28.0=22.00±1.25	0.98	2.75–3.50=3.10±0.14	2.75–3.50=3.10±0.14	11.25–6.51=9.08±0.66	5.22–3.71=4.41±0.31	2.01–3.25=2.65±0.23
G. subfalcatum	21.75–19.15=20.44±0.08	20.05–23.25=21.08±0.04	0.96	2.75–4.50=3.60±0.03	2.25–4.75=3.14±0.04	11.05–6.51=8.45±0.07	5.26-3.88=4.49±0.62	2.75–4.00=3.35±0.23
G. tenuissimum	18.15–19.39=19.03±1.03	18.67–21.34=20.02±0.85	0.95	2.01–5.03=4.25±0.05	2.51–2.11=5.11±0.16	10.25–7.15=8.05±0.06	5.12-4.91=5.09±0.75	2.0–3.15=2.25±0.24
G. tetraphyllum	17.00–20.15=19.05±1.05	20.24–25.00=22.06±0.88	0.88	2.50–3.25=2.90±0.12	2.50–5.07=5.30±1.07	5.0–5.75=5.25±0.13	4.88-3.91=4.23±0.69	2.25–3.25=2.70±0.18
G. tricornutum	13.22–18.05=21.75±0.09	16.25–21.20=18.67±0.08	1.16	5.03–8.01=6.35±0.6	5.01–7.50=5.80±0.45	6.25–10.0=7.18±0.70	5.28-4.89=5.10±0.78	1.25–3.50=1.90±0.04
G. verum	18.25–20.51=19.15±0.07	19.31–17.13=18.03±0.20	1.06	2.11–6.00=5.25±0.4	2.25–6.25=4.25±1.06	7.25–9.25=6.25±0.60	4.63-2.98=3.33±0.86	2.0–2.25=1.25±0.05

Acronyms: P = Polar axis; E = Equatorial axis; Clg = Colpi length; Clt = Colpi width; Plg = Pore length; Plt = Pore width; E= Exine thickness; Min= Minimum; Max= Maximum; M= Mean; SE= Standard error

Table 2. Qualitative and Quantitative pollen characters in investigated species of ***Galium.***

Taxa	Size of hydrated pollen (µm)	Size	Dispersal unit	Outline at polar view /Amb	Aperture type	Pollen type	Pollen class - Colpate	Pollen shape	Aperture orientation	Exine sculpturing
G. acutum	15–17	Small	Monad	Orbicular	Colpate–6	Stephanocolpate	6–8	Oblate–spheroidal	Sunken	Scabrate–punctate
G. aparine	16–17	Small	Monad	Circular	Colpate–5	Stephanocolpate	4–6	Oblate–spheroidal	Sunken	Microspine–perforate
G. asperifolium	20–21	Small	Monad	Circular	Colpate–7	Stephanocolpate	5–7	Oblate–spheroidal	Sunken	Scabrate–verrucate
G. asperuloides	20– 22	Small	Monad	Lobate	Colpate–8	Stephanocolpate	6–9	Oblate–spheroidal	Sunken	Microreticulate–scabrate
G. boreale	18–21	Small	Monad	Orbicular	Colpate–6	Stephanocolpate	5–7	Suboblate	Sunken	Microechinate–scabrate
G. campylotrichum	21–24	Small	Monad	Orbicular	Colpate–7	Stephanocolpate	4–8	Suboblate	Sunken	Microechinate–perforate
G. ceratophylloides	18–19	Small	Monad	Circular	Colpate–6	Stephanocolpate	4–7	Oblate–spheroidal	Sunken	Microspine–scabrate
G. cryptanthum	18–19	Small	Monad	Lobate	Colpate–5	Stephanocolpate	5–8	Oblate–spheroidal	Sunken	Microechinate–scabrate
G. elegans	16–19	Small	Monad	Orbicular	Colpate–7	Stephanocolpate	6–9	Suboblate	Sunken	Scabrate–punctate
G. ghilanicum	14–15	Small	Monad	Orbicular	Colpate–9	Stephanocolpate	7–10	Oblate–spheroidal	Sunken	Microspine–scabrate
G. hirtiflorum	17–20	Small	Monad	Circular	Colpate–9	Stephanocolpate	5–9	Suboblate	Sunken	Microechinate–punctate
G. saturejifolium	13–12	Small	Monad	Orbicular	Colpate–7	Stephanocolpate	4–7	Prolate–spheroidal	Sunken	Microechinate–scabrate
G. serpylloides	13–14	Small	Monad	Circular	Colpate–8	Stephanocolpate	5–8	Oblate–spheroidal	Sunken	Microspine–verrucate
G. setaceum	18–18	Small	Monad	Lobate	Colpate–5	Stephanocolpate	4–7	Oblate–spheroidal	Sunken	Microechinate–punctate
G. spurium	21–22	Small	Monad	Orbicular	Colpate–7	Stephanocolpate	6–8	Oblate–spheroidal	Sunken	Microechinate–verrucate
G. subfalcatum	20–21	Small	Monad	Orbicular	Colpate–6	Stephanocolpate	4–7	Oblate–spheroidal	Sunken	Microechinate–scabrate
G. tenuissimum	19–20	Small	Monad	Lobate	Colpate–9	Stephanocolpate	6–10	Oblate–spheroidal	Sunken	Microreticulate–scabrate
G. tetraphyllum	19–22	Small	Monad	Orbicular	Colpate–7	Stephanocolpate	5–8	Suboblate	Sunken	Microechinate–scabrate
G. tricornutum	21–18	Small	Monad	Lobate	Colpate –6	Stephanocolpate	5–8	Subprolate	Sunken	Microechinate–verrucate
G. verum	19–18	Small	Monad	Orbicular	Colpate–9	Stephanocolpate	5–10	Prolate–spheroidal	Sunken	Microspine–verrucate

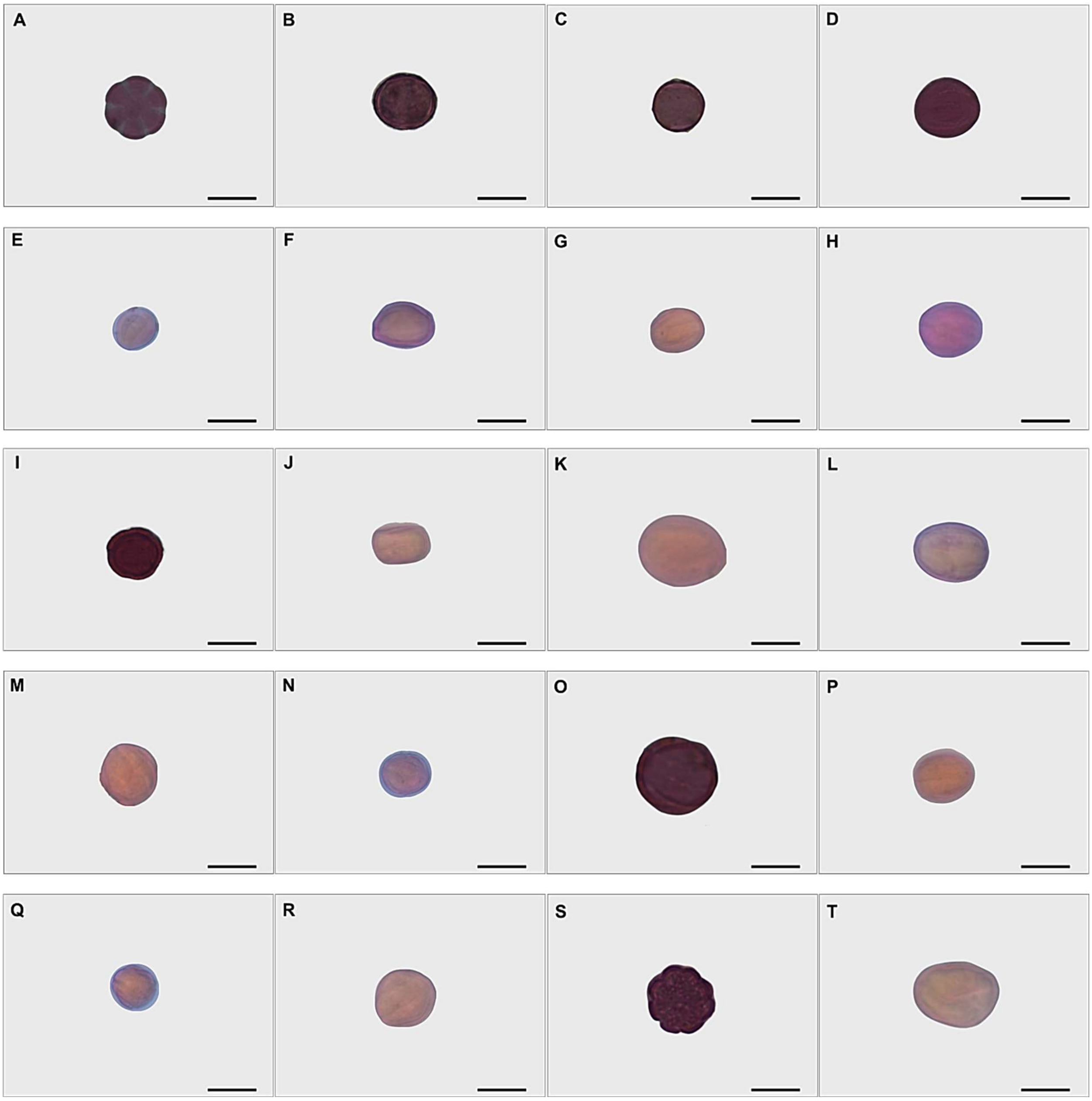

Fig. 2. Pollen micrographs of ***Galium*** species (LM); Pollen general view: Magnification: 100×, **A. *G. acutum*, B. *G. aparine*, C. *G. asperifolium*, D. *G. asperuloides*, E. *G. boreale*, F. *G. campylotrichum*, G. *G. ceratophylloides*, H. *G. cryptanthum*, I. *G. elegans*, J. *G. ghilanicum*, K. *G. hirtiflorum*, L. *G. saturejifolium*, M. *G. serpylloides*, N. *G. setaceum*, O. *G. spurium*, P. *G. subfalcatum*, Q. *G. tenuissimum*, R. *G. tetraphyllum*, S. *G. tricornutum*, T. *G. verum*.**

small size pollen 13–12 µm (*Galium saturejifolium*), while comparatively large size 21–24 µm was observed in (*G. campylotrichum*). Therefore, the average value of the polar axis ranges from 13.05 µm (*Galium serpylloides*) to 21.75 µm in (*G. tricornutum*) and equatorial axis is 17.00 µm (*G. aparine*) to 24.20 µm (*G. campylotrichum*).

Pollen apertures

In contrast, pollen grains of *Galium* have a simple aperture and zonocolpate. Pollen aperture orientation is sunken between the colpi in the examined taxa (Figs. 2–5). Among the all studied taxa, pollen having more than three colpi is described as a stephanocolpate type, but number of apertures varied within the *Galium* species and here we categorized as colpate–5 (*G. aparine* and *G. setaceum*), colpate–6 (*G. acutum, G. boreale*, *G. ceratophylloides, G. subfalcatum* and *G. tricornutum*), colpate–7 (*G. asperifolium*, *G. campylotrichum*, *G. elegans, G. saturejifolium*, *G. spurium* and *G. tetraphyllum*), colpate–8 (*G. asperuloides* and *G. serpylloides*), and colpate–9 was observed among *G. ghilanicum, G. tenuissimum* and *G. verum*. Colpi are

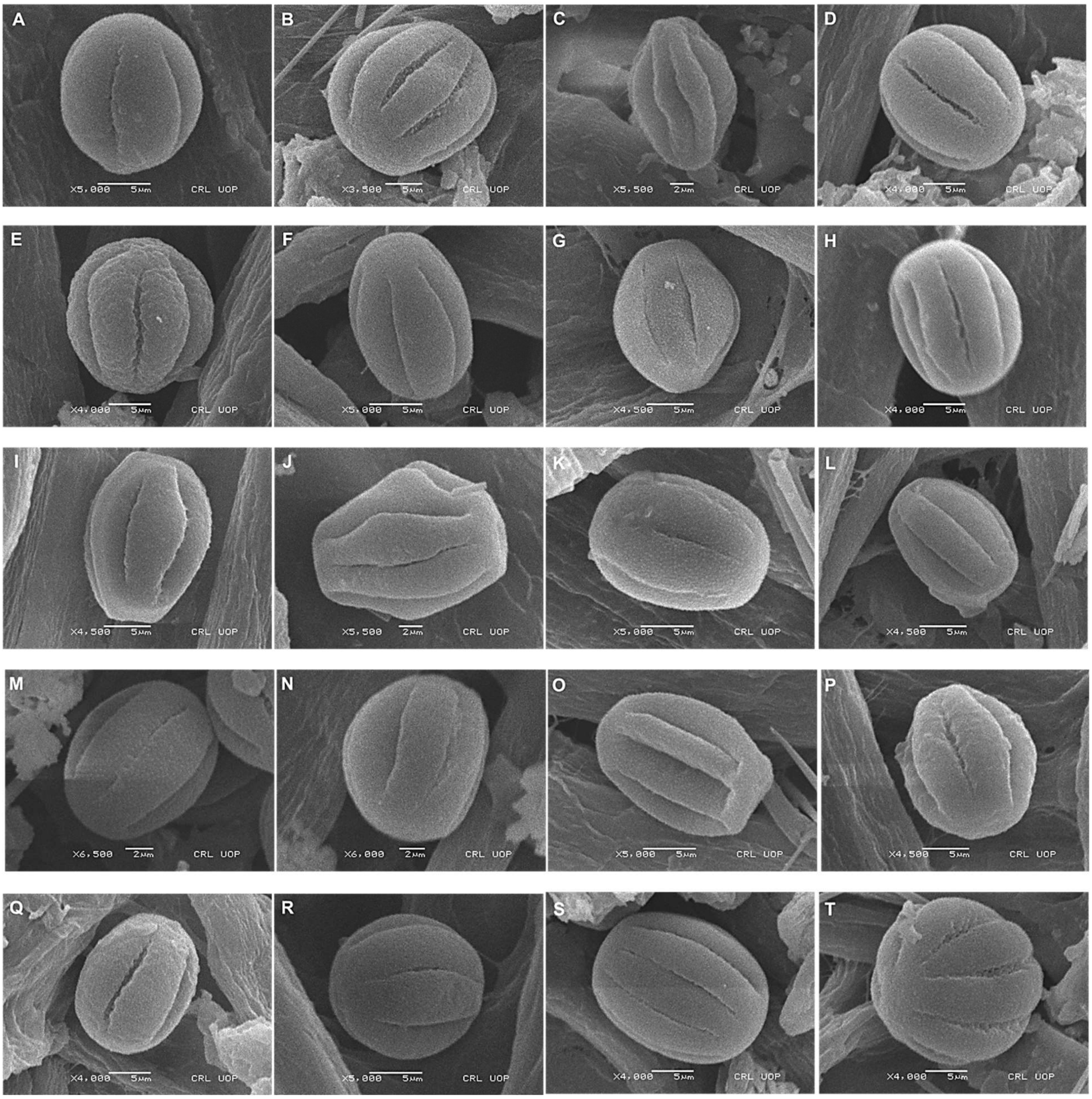

Fig. 3. Pollen micrographs of ***Galium*** species (SEM); Equatorial view and only in 20T with slightly oblique polar view: **A. *G. acutum*, B. *G. aparine*, C. *G. asperifolium*, D. *G. asperuloides*, E. *G. boreale*, F. *G. campylotrichum*, G. *G. ceratophylloides*, H. *G. cryptanthum*, I. *G. elegans*, J. *G. ghilanicum*, K. *G. hirtiflorum*, L. *G. saturejifolium*, M. *G. serpylloides*, N. *G. setaceum*, O. *G. spurium*, P. *G. subfalcatum*, Q. *G. tenuissimum*, R. *G. tetraphyllum*, S. *G. tricornutum*, T. *G. verum*.**

slightly narrow at the polar view and widest at the equatorial view. The number of colpi also varies within the species and ranges between 4–6 in *G. aparine* and 7–10 was scored in *G. ghilanicum*. The average length of colpi ranged from 2.10 µm in *G. hirtiflorum* to 6.35 µm in *G. tricornutum* and the width of colpi ranged from 3.10 µm in *G. spurium* to 5.80 µm in *G. tricornutum*. Similarly, the average length of pore ranged from 4.25 µm in *G. serpylloides* to 9.08 µm in *G. spurium* and the width of colpi ranged from 2.11 µm in *G. acutum* to 5. 14 µm was measured in *G. campylotrichum*.

Pollen exine sculpturing/exine ornamentation

The investigated species showed different patterns of exine ornamentation and thickness (Figs. 2–5). The size and thickness of the exine may vary between the species. Based on the exine thickness variation, we determined that the average value of exine thickness ranged from 1.12 µm in *Galium acutum* to 3.25 µm, which was also calculated in *G. subfalcatum* Table 2. Generally, *Galium* pollen at scanning electron microscopy shows variation in size and density of tectum perforations, which have been used in distinction investigated species. Exine sculpturing

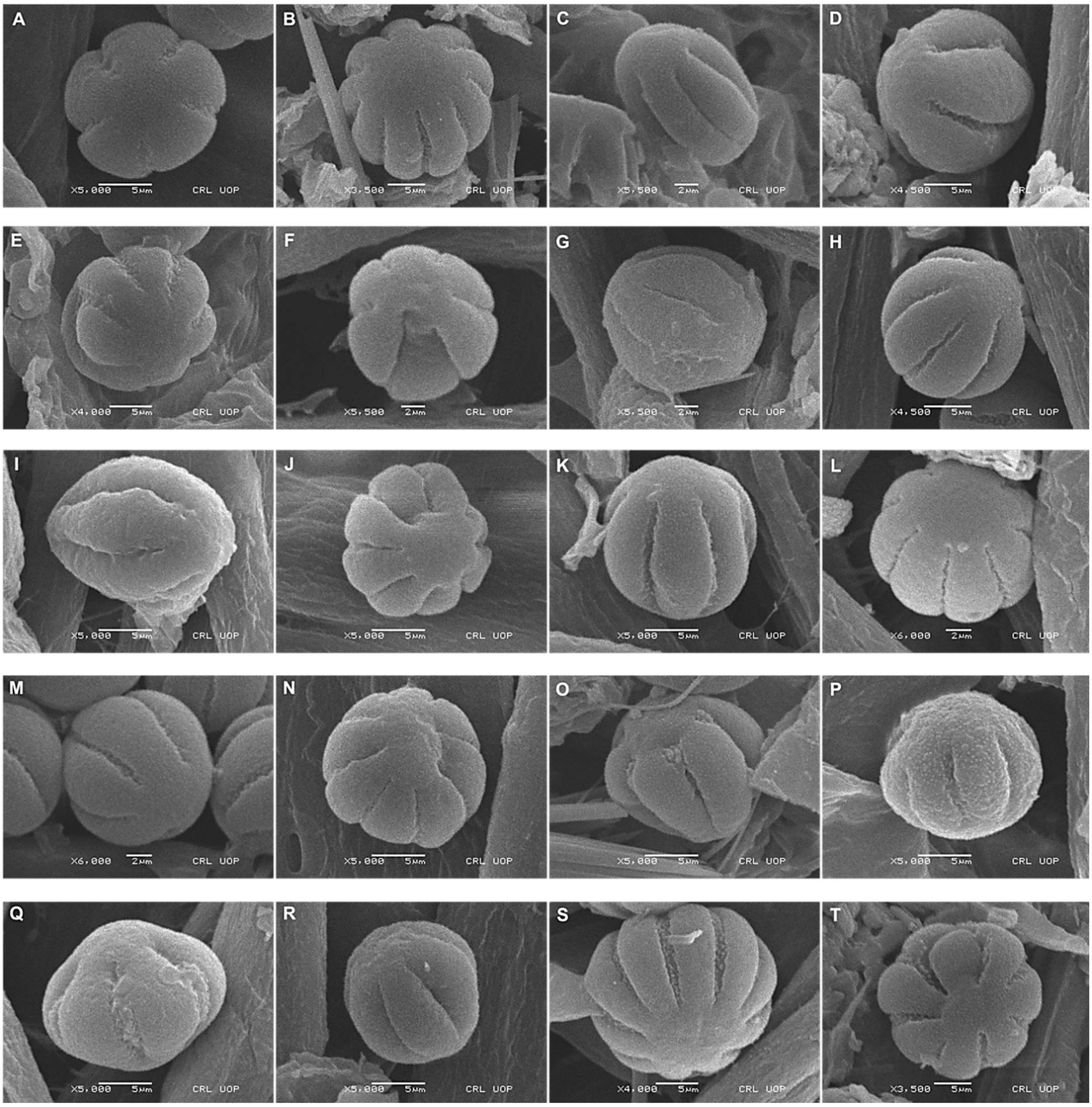

Fig. 4. Pollen micrographs of ***Galium*** species (SEM); Obliquely polar view: **A. *G. acutum*, B. *G. aparine*, C. *G. asperifolium*, D. *G. asperuloides*, E. *G. boreale*, F. *G. campylotrichum*, G. *G. ceratophylloides*, H. *G. cryptanthum*, I. *G. elegans*, J. *G. ghilanicum*, K. *G. hirtiflorum*, L. *G. saturejifolium*, M. *G. serpylloides*, N. *G. setaceum*, O. *G. spurium*, P. *G. subfalcatum*, Q. *G. tenuissimum*, R. *G. tetraphyllum*, S. *G. tricornutum*, T. *G. verum*.**

microechinate–scabrate in (*G. boreale, G. saturejifolium, G. subfalcatum* and *G. tetraphyllum*), scabrate–punctate in (*G. acutum* and *G. elegans*), microreticulate–scabrate in (*G. asperuloides* and *G. tenuissimum*), microspine–scabrate in (*G. ceratophylloides* and *G. ghilanicum*), microechinate–punctate in (*G. hirtiflorum* and *G. setaceum*), microspine–verrucate in (*G. serpylloides* and *G. verum*), microechinate–verrucate in (*G. tricornutum* and *G. spurium*), microspine–perforate in (*G. aparine*), scabrate–verrucate in (*G. asperifolium*) and microechinate–perforate was observed in *G. campylotrichum*.

Hierarchical clustering (UPGMA) analysis

Clustering analysis showed a relationship between the species based on scored morphometric characters (Fig. 7). The UPGMA dendrogram nested all the *Galium* species with little variation. All the species are separated at the terminal of UPGMA dendrogram branches. The main cladistic tree of (UPGMA) dendrogram comprising two main clades, including seventeen species are correlated in (Clade-I) and three species are represented in (Clade-II), respectively. The first clade is divided into two subclades i.e. subclade–I only consists of *Galium tricornutum*,

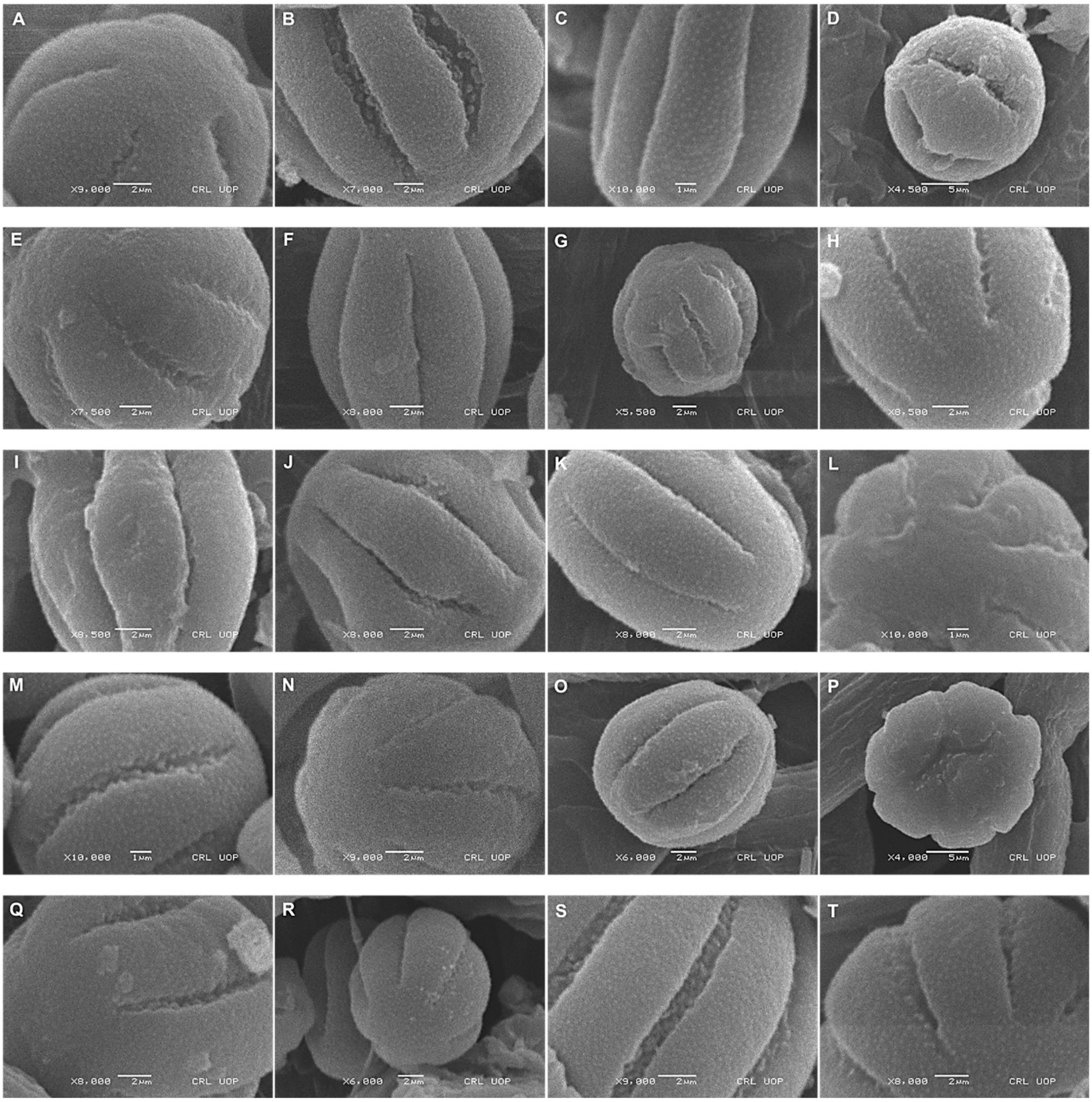

Fig. 5. Pollen micrographs of ***Galium*** species (SEM); Exine sculpturing view: **A.** ***G. acutum***, **B.** ***G. aparine***, **C.** ***G. asperifolium***, **D.** ***G. asperuloides***, **E.** ***G. boreale***, **F.** ***G. campylotrichum***, **G.** ***G. ceratophylloides***, **H.** ***G. cryptanthum***, **I.** ***G. elegans***, **J.** ***G. ghilanicum***, **K.** ***G. hirtiflorum***, **L.** ***G. saturejifolium***, **M.** ***G. serpylloides***, **N.** ***G. setaceum***, **O.** ***G. spurium***, **P.** ***G. subfalcatum***, **Q.** ***G. tenuissimum***, **R.** ***G. tetraphyllum***, **S.** ***G. tricornutum***, **T.** ***G. verum***.

while the subclade-II consists of sixteen species nested at the primary and secondary subclade-II. Primary (subclade-II) includes ten species namely; *G. boreale*, *G. tenuissimum*, *G. ceratophylloides*, *G. setaceum*, *G. hirtiflorum*, *G. cryptanthum* and *G. verum*, while the secondary branch consists of three species namely; *G. aparine*, *G. elegans* and *G. acutum*. The secondary (subclade-II) consists of the primary branch, including four species namely; *Galium asperifolium*, *G. asperuloides*, *G. campylotrichum* and *G. tetraphyllum*, while the secondary branch is composed of *G. spurium* and *G. subfalcatum*. Therefore, clade-II consists of three species namely; subclade-I composed of only *G. ghilanicum* and subclade-II comprising *G. saturejifolium* and *G. serpylloides*. The UPGMA dendrogram revealed a relationship among the investigated *Galium* species and provided significant information on the artificial correlation. The scored features used in the hierarchical tree (UPGMA) did not provide a strong correlation among the species, because overall Galium species showed high homogeneity in pollen.

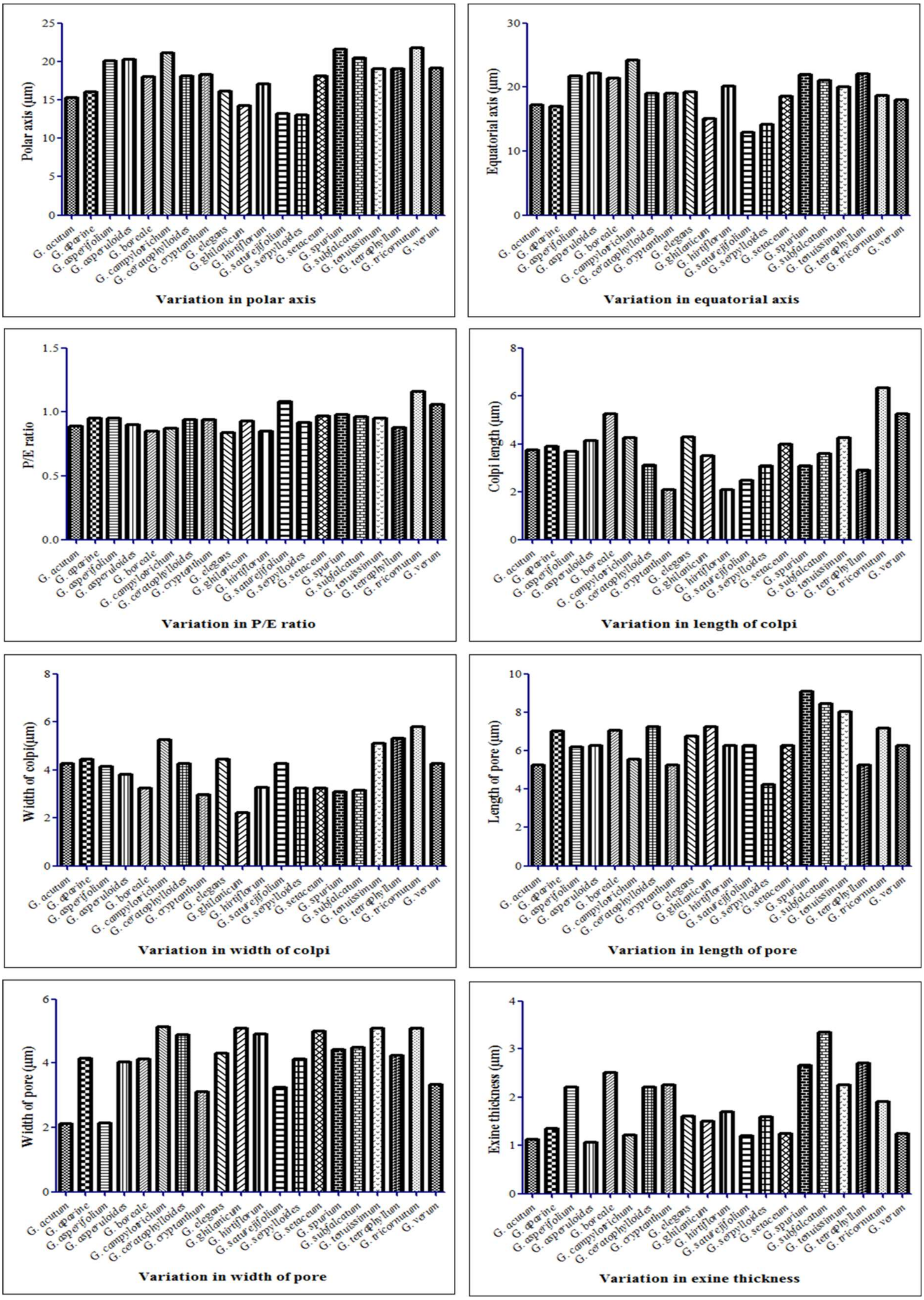

Fig. 6. Morphometric variations among the investigated ***Galium*** species.

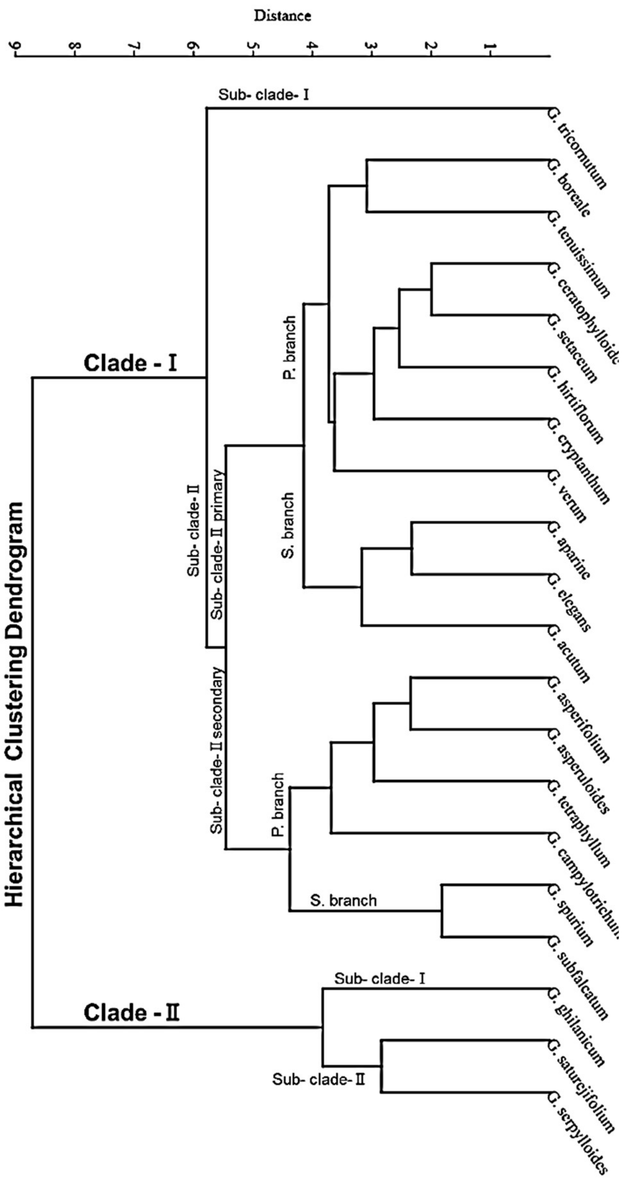

Fig. 7. Hierarchical clustering (UPGMA) analysis for the investigated ***Galium*** species.

DISCUSSION

The present study deals with palynotaxonomic implications for the systematics of poorly explored *Galium* taxa from Pakistan. Pollen among the *Galium* exhibited by orbicular, circular and lobate outline and varied in aperture types. Among the tribe Rubithe eae, genus *Galium* is poorly studied, despite its pollen morphology. Pollen morphology has been demonstrated within *Galium* taxa for its taxonomic circumscription (Huysmans *et al*., 2003). Pollen morphology alone or with the combination of other morphological features was used for taxonomic delimitation in several *Galium* taxa (Robbrecht, 1982; Abdel Khalik *et al*., 2007; Perveen and Qaiser, 2007; Başer *et al*., 2020). Generally, *Galium* has more than three colpi described as stephanocolpate. Pollen grains of *Galium* was exhibited by monads, 6–9 zonocolpate, oblate–spheroidal, prolate–spheroidal and exine sculpturing is scabrate–perforate with scabrate aperture membrane (Başer *et al*., 2020). Some features within pollen qualitative and quantitative data are used as descriptive characters in the morphological description of *Galium* species (Parsapanah and Faghir, 2021). Among the *Galium* species, pollen features were used as a reliable characters for the accurate identification (Huysmans *et al*., 2003; Vinckier *et al*., 2000; Minareci *et al*., 2010). Sometimes, pollen morphology is used to distinguish *Galium* species from the other genera within the Rubieae (Toni and Mariath, 2011; Başer *et al*., 2020). Pollen features are used as supportive characters in the infrageneric classification of *Galium*.

The general pollen morphology of *Galium* taxa comprising circular to orbicular or lobate outline. In *Galium* taxa, the pollen shapes are classified into three classes, including prolate–spheroidal, oblate–spheroidal or subprolate were assessed based on the value of P/E ratio. Başer *et al*., (2020) studied three different types of pollen shapes, i.e., prolate, spheroidal and oblate–spheroidal among the six species of *Galium* species. Pollen shape is spheroidal to subulate was studied in eighteen taxa of *Galium* for its systematic implication (Huysmans *et al*., 2003). There are four types of pollen shapes viz. suboblate, prolate–spheroidal, subprolate–spheroidal, oblate–spheroidal, were observed in *Galium* species (Perveen and Qaiser, 2007). Pollen shapes classified as, prolate–spheroidal, oblate–spheroidal and spheroidal to suboblate and used as a reliable character in *Galium* taxa (Abdel Khalik *et al*., 2007). Huysmans *et al*., (2003), described simple type (zonocolpate) pollen apertures and their number varies from 5–9 in *Galium* species. The pollen aperture number ranged from 6–7 was recognized as most frequent numbers among the *Galium* species (Başer *et al*., 2020). In some *Galium* species, aperture numbers varied from 6–7 and the colpi were narrow long with acute ends (Perveen and Qaiser, 2007). In our finding, pollen aperture numbers vary among the taxa and are used as a key feature for the segregation of species.

The sizes of pollen grains are obtained from the mean values of polar to equatorial diameters and compared with previously published data. Abdel Khalik *et al*., (2007) pollen sizes were used to distinguish species; *G. aparine* (21–23 μm), *G. spurium* (18–17 μm), *G. tricornutum* (22–23 μm) and *G. ceratopodium* (18–18 μm). Huysmans et al. (2003) also measured pollen sizes in *Galium* species viz. *G. aparine* (18–17 μm), *G. boreale* (20.5–19.5 μm), *G. palustre* (18–15.5 μm), *G. spurium* (21–20 μm), *G. tricornutum* (24–22 μm) and *G. verum* (21–19 μm). The value of pollen size was used as ranged polar to equatorial diameters (P 16.89–21.66 μm, E 14.17–21.42 μm) among the *Galium* species (Perveen and Qaiser, 2007). The average polar diameter value ranged between from 14.21 µm (*G. galiopsis*) to 18.68 µm (*G. incanum* subsp. *elatius*), while the equatorial ranged from 12.07 µm (*G. humufisum*) to 18.94 µm (*G. mite*) (Başer *et al*., 2020). Pollen sizes were used as diagnostic features in the morphology of *Galium* and other taxa (Khan et al., 2017; Khan et al., 2018; Al-Taie and Sadek 2023). Pollen sizes were used as a significant feature in the identification of species among the *Galium* section *Platygalium,* despite their taxonomic value (Schanzer and Elkordy, 2014).

The exine sculpturing has not been described previously for the *Galium* pollen grains. Exine ornamentation was most useful trait among the *Galium* for evaluation of its systematic value (Faegri and Iversen, 1964; Huysmans *et al*., 2003, 200; De Toni and Mariath, 2011; Schanzer and Elkordy, 2014). The scabrate–perforate with scabrate type aperture membrane was reported in *G. aparine* and *G. spurium* (Abdel Khalik *et al.,* 2007). The scabrate–punctate and spinulose–punctate ornamentations were described in the *G. acutum, G. chitralense*, *G. serpyllioides, G. setaceum, G. asperifolium* and *G. tetraphyllum* (Perveen and Qaiser, 2007). In *Galium* species, scabrate–echinate type ornamentation was used as a descriptive feature for their taxonomic circumscription (Wikström *et al*., 2015). Başer *et al*., (2020), was observed scabrate–perforate type pattern among the six *Galium* species. In our results, we described microechinate–perforate, microechinate–punctate, microechinate–scabrate, microechinate–verrucate, microreticulate–scabrate, microspine–perforate, microspine–scabrate, microspine–verrucate, scabrate–punctate and scabrate–verrucate among the *Galium* and also represents an informative trait for species delimitation.

CONCLUSION

Our study contributes data to the palynotaxonomy of *Galium*. Pollen traits were alone or with a combination of other morphological features used for the easy and accurate identification of *Galium* species. Pollen morphology of *Galium* was poorly explored, despite for systematic significance. The variations among the scored traits have been providing significant information for distinguishing of analyzed species. In addition, the pollen morphometric data was used in hierarchical clustering (UPGMA) analysis for the phylogenetic relationships among the species. Our, hierarchical clustering (UPGMA) analysis not provide natural relationships among the investigated taxa, like molecular phylogeny, but it can be used for artificial relationships.

ACKNOWLEDGMENTS

The authors would like to extend their sincere appreciation to ALP project 'Ex-situ conservation of wild edible and medicinal plants from poorly explored bio-diversity hotspot of Pakistan' (CS-245). The authors would like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP2025R301), King Saud University, Riyadh, Saudi Arabia. The authors also thank the Centralized Resource Laboratories (CRL), Department of Physics, University of Peshawar, for providing SEM facilities for this work.

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