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POLLEN MORPHOLOGY AND ITS SYSTEMATIC IMPLICATION ON SOME SPECIES OF *ARTEMISIA* L. FROM GILGIT-BALTISTAN PAKISTAN

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Abstract

This study was accomplished to scrutinize the pollen morphology of 15 species of the genus *Artemisia* of the family Asteraceae from Gilgit-Baltistan region of Pakistan by means of scanning electron microscopy (SEM). Results revealed pollen grains of *Artemisia* species with tricolporate shape, and characterized by globular symmetry (ellipsoid ball shaped from equatorial side and three lobed rounds from polar view) with few exceptions. Additionally, the pollens are marked with reduced spinules on their surfaces which are diagnostic character for the genus *Artemisia*. In this study, seven micromorphological characters of pollen grains of 15 *Artemisia* species *viz.* shape of pollen, arrangement of spinules, exine sculpture, spinules base, equatorial width and polar length, were employed to construct a dendrogram following the consequential cluster analyses. In the dentrogram, four groups within the studied *Artemisia* species have been recognized. The pollen morphology of *Artemisia* could be a good taxonomic marker to cope with its taxonomic delimitations in combination with other floral and molecular attributes.

Introduction

The genus *Artemisia* L. (Asteraceae) possesses five hundred species in the form of herbs and shrubs (Valles and McArthur, 2001). It is a diverse genus of the tribe Anthemideae of family Asteraceae (Martin *et al*., 2003).

Some *Artemisia* species have a remarkable economic status due to their antitumor, antispasmodic, antimicrobial, antiseptic, antimalarial, hepato-protective and antirheumatic activities (Terra *et al*., 2007; Hussain *et al*., 2017).

The basis of taxonomy of *Artemisia* is its capitular morphology (Watson *et al*., 2002). For example, the ray florets in section *Seriphidium* Besser ex Hook are reduced to a membranous vestige. Its capitulum is without ray florets and only possesses the hermaphrodite disc florets. Hence, this capitulum is homogamous. The capitulum in other subgenera (*Abrotanum* Besser, *Dracunculus* Besser, *Absinthium* Mill., *Artemisia* L.) have two types of florets: hermaphrodite or staminate disc florets and ray pistillate florets and this capitulum becomes heterogamous (Bremer and Humphries, 1993).

The taxonomy of this diverse genus is unresolved for many years. It has been years when taxonomists conducted investigations (Bremer and Humphries, 1993; Kornkven *et al*., 1998, 1999; Torell *et al*., 1999; Watson *et al*., 2002; D'Andrea *et al*., 2003; Vallès *et al*., 2008; Sanz *et al*., 2008; Pellicer *et al*., 2010; Garcia *et al*., 2011; Riggins and Seigler, 2012; Haghighi *et al*., 2014;

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Malik *et al*., 2017; Hussain *et al*., 2019b) on this single large genus with 500 species (Torrell *et al*., 1999; Martin *et al*., 2003) and recognized its six subgenera namely *Dracunculus* Besser, *Artemisia* Tourn., *Absinthium* (Mill.) Less., *Tridentatae* (Rydberg) McArthur, *Seriphidium* (Besser) Poljakov and *Pacifica* Hobbs. & Baldwin. In few investigations, the subgenera *Artemisia* and *Absinthium* were merged under one subgenus *Artemisia* (Shultz, 2009) while subgenera *Seriphidium* was separated for the genus *Artemisa* in former studies of Bremer and Humphries (1993), Bremer (1994), Ling (1995) and Ghafoor (2002).

Researchers have approved that the critical anatomical analysis of rarer morphological features with molecular phylogenies are fruitful and this combination could become a power tool to resolve taxonomic issues (Scotland *et al*., 2003; Perveen and Qaiser, 2010) especially the pollen morphology in specific and generic levels of classification of Asteraceae family (Zafar *et al.*, 2007). The significance of pollen morphology in plant systematics has been authenticated by various researchers. Palynological study has been utilized to indicate relationships among the different taxa of Asteraceae family (Mallick, 2015). Stuessy (2009) stated that the data from pollen grains are known to be useful at all levels of the taxonomic hierarchy, and can be helpful in suggesting a relationship.

In earlier investigations, Edward (1994) proposed two types of pollen structure in *Asteraceae* namely, the caveate and the anthemoid. The anthemoid type was primarily used for the pollen grains of *Anthemideae*. The anthemoid type of pollen grains contains the basal columellae rising from the foot layer, and one or many shorter layers columellae. There are internal tecta which are present above the basal columellae. While the caveate grains have only internal cavity without basal columellae.

In the subtribe *Anthemideae,* studies based on pollen data confirmed the presence of two major patterns of pollen. These patterns are essential taxonomic characters in *Artemisia* and its closely related genera, i.e. one genus with long spinules (*Anthemis*) and the other with short spinules (*Artemisia*). It could be authenticated that short spinules in the pollen were evolved from long spinules of pollen on the basis of their order of occurrences in the geological past (Martín *et al*., 2003).

In the genus *Artemisia*, investigation of pollen morphology has been started from the time of Wodehouse (1926). The following workers such as Valles *et al.* (1987), Caramiello *et al*. (1989), Lodari *et al*. (1989), Vezey *et al*. (1994), Martin *et al.* (2003), Jiang (2005), and Hayat *et al.* (2010) elaborated the taxonomic importance with different perspectives of the characteristics of *Artemisia* pollens including the structural organization, shape and size, diversity of sculpture in exine, magnitudes of exine aperture etc.

Nevertheless, there is a little information available on the pollen morphology of the genus *Artemisia*, most especially from the north region of Pakistan. This study provides important data on the micromorphological features of pollen grains of some species of the genus *Artemisia* from Gilgit-Baltistan region of Pakistan in order to establish their availability for future taxonomic works. The cluster analysis of these data gives an understanding on the putative relationships among the species of this genus.

Materials and Methods

Study area

Gilgit-Baltistan region of Pakistan bears a diverse climate and this region is well-known for housing an immense biodiversity of plants. This region is located between latitude 35° to 37° East and longitude 72° to 75° North, and has seven districts namely, Gilgit, Baltistan, Ghizar, Ganche, Hunza Nagar, Astore and Diamer. Samples of *Artemisia* species for pollen study were collected

during extensive field surveys over a period of two years (2016-2017) in different areas of Gilgit-Baltistan as already given in our preceding papers (Hussain *et al.*, 2019a,b).

Pollen material

The pollen material employed in this study was obtained from herbarium specimens as well as from fresh samples collected from different regions of Gilgit-Baltistan of Pakistan. The details of origin and collection of studied *Artemisia* species have been provided in Table 1. Primarily, the pollen grains of *Artemisia* species were prepared for scanning electron microscopy (SEM) by the standard methods described by Hayat *et al*. (2010) and Perveen and Qaiser (2010). To separate the pollen grains from anthers, stereo microscope was used.

Table 1. Collection details of *Artemisia* species employed in the present study from Gilgit-Baltistan region of Pakistan.

Taxa	Latitude	Longitude	Location	Voucher specimen no	Collectors	Date
Artemisia annua L.	N-35'54.949	E-74'18.508	Barmas Paen Gilgit	PMNH-41582	Adil Hussain and Tanseer	24-06-2016
A. austriaca (Vaill.) L. *	N-36'01.609	E-74'33.255	Bagrote Valley Gilgit	PMNH-41643	Adil Hussain and Tabeer	15-08-2016
A. chamaemelifolia Vill.*	N-36'09.622	E-74'11.622	Naltar Valley Gilgit	PMNH-41630	Adil Hussain and Tanseer	04-08-2016
A. chinensis L.*	N-35'26.585	E-75'27.011	Shangrilla skardu	PMNH-41722	Adil Hussain and Tanseer	02-10-2017
A. campestris L.	N-36'08.708	E-74'12.397	Naltar Valley Gilgit	PMNH-41619	Adil Hussain and Tabeer	02-08-2016
A. gmelinii Weber ex Stech.	N-36'08.967	E-74'12.112	Naltar Valley Gilgit	PMNH-41621	Adil Hussain and Tanseer	02-08-2016
A. herba-alba Asso.	N-35'54.061	E-74'12.762	Kargah Nala Gilgit	PMNH-41599	Adil Hussain and Tanseer	25-07-2016
A. indica Willd.	N-36'15.250	E-73'24.240	Yasin Ghizer	PMNH-41694	Adil Hussain and Amar	11-08-2017
A. maritima L. Ex Hook f	N-35'52.660	E-74'25.594	Minawar Gilgit	PMNH-41616	Adil Hussain and Tanseer	31-07-2016
A. montana Pamp.*	N-35'30.883	E-75'40.115	Hashupi Shigar Skardu	PMNH-41708	Adil Hussain and Tanseer	28-08-2016
A. pontica L.*	N-36'02.121	E-74'35.227	Bagrote Valley Gilgit	PMNH-41642	Adil Hussain and Tanseer	14-08-2016
A. rutifolia Var.	N-36'08.708	E-74'12.397	Naltar Valley Gilgit	PMNH-41618	Adil Hussain and Tanseer	02-08-2016
A. scoparia Waldst. & Kit.*	N-35'26.665	E-75'26.960	Kachura Lake Skardu	PMNH-41714	Adil Hussain and Tanseer	30-08-2016
A. tournefortiana Rachb.	N-35'25.493	E-75'44.507	Shigar Valley Skardu	PMNH-41704	Adil Hussain and Tanseer	27-08-2016
A. vulgaris L.	N-36'20.508	E-74'52.277	Shishkat Hunza Nagar	PMNH-41646	Adil Hussain and Tanseer	10-07-2016

The voucher specimen numbers for each species have been obtained from Pakistan Museum of Natural History (PMNH) Islamabad Pakistan. * Rare *Artemisia* species from Gilgit-Baltistan region of Pakistan

Scanning electron microscopy (SEM)

For SEM analysis, the pollen grains were acetolysed and directly transferred to the sticky carbon disc on metal stub and coated with platinum in a sputtering chamber (Pelco Auto sputter Coater SC-7, Ted Pella Inc). Philips XL30 TMP (FEI Company) electron microscope was used to

analyze the samples at 5, 10 and 20 kV (Hussain *et al.*, 20019a), at the core electron microscopy core laboratory, Tupper Hall, University of California Davis California USA.

Light microscopy (LM)

Micromorphological observations of pollen grains were done with OLYMPUS/BX-51 light microscope at Department of Plant Sciences, University of California Davis California USA. Observations for equatorial diameter (E), polar diameter (P) and P/E ratio were taken according to Reitssma (1970) and Hayat *et al.* (2010).

Cluster analysis

A data matrix was generated from the recorded micromorphological characteristics of the pollens of *Artemisia*. This data matrix was then employed for cluster analysis by means of UPGMA method with EUCLIDEAN in the MVSP software version 3.21 (Kovach, 2007).

Results and Discussion

In this study, the characteristics of pollen of 15 *Artemisia* species have been examined in detail using scanning electron microscopy. Results of this study found some variation in the pollen structure of the investigated *Artemisia* species. The characteristics of pollen includes equatorial (E) and polar (P) measurements, polar and equatorial ratio (P/E), pollen shape, spinules presence/absence and the ornamentation of exine. The quantitative characteristics of the examined species are given in Table 2 and SEM micrographs on pollen structures are presented in Figs 1-3. The equatorial and polar views of the *Artemisia* pollen are given in Figs 1 and 2, respectively. The sculpture of exine surface of *Artemisia* pollens are evident from Fig. 3, where the presence of tiny spinules can also be seen. Similarly in the spinule densities was varied among different species of *Artemisia*. These spinules are very unique in all investigated *Artemisia* species. Few investigated species showed some degenerative tendency in their structure. For example, the spinules in *A. chinensis* were found to be loosely arranged (Fig 3i) as compared to the spinules of other *Artemisia* species pollen (Fig. 3).

Table 2. Quantitative characteristics of pollen of different *Artemisia* species from Gilgit-Baltistan region of Pakistan.

Taxa	Polar (μm)	Equatorial (μm)	P/E Sphericity
Artemisia annua	15.60	18.79	0.83
A. austriaca	17.20	16.49	1.04
A. campestris	15.55	15.75	0.98
A. chamaemelifolia	20.30	19.04	1.06
A. chinensis	24.24	12.01	2.01
A. gmelinii	16.86	14.90	1.13
A. herba-alba	22.58	18.64	1.21
A. indica	17.65	14.37	1.22
A. maritima	15.59	14.49	1.07
A. montana.	15.45	16.54	0.93
A. pontica	17.86	14.65	1.21
A. rutifolia	16.70	17.43	0.95
A. scoparia	15.12	17.36	0.87
A. tournefortiana	19.08	19.59	0.97
A. vulgaris	15.33	16.93	0.90

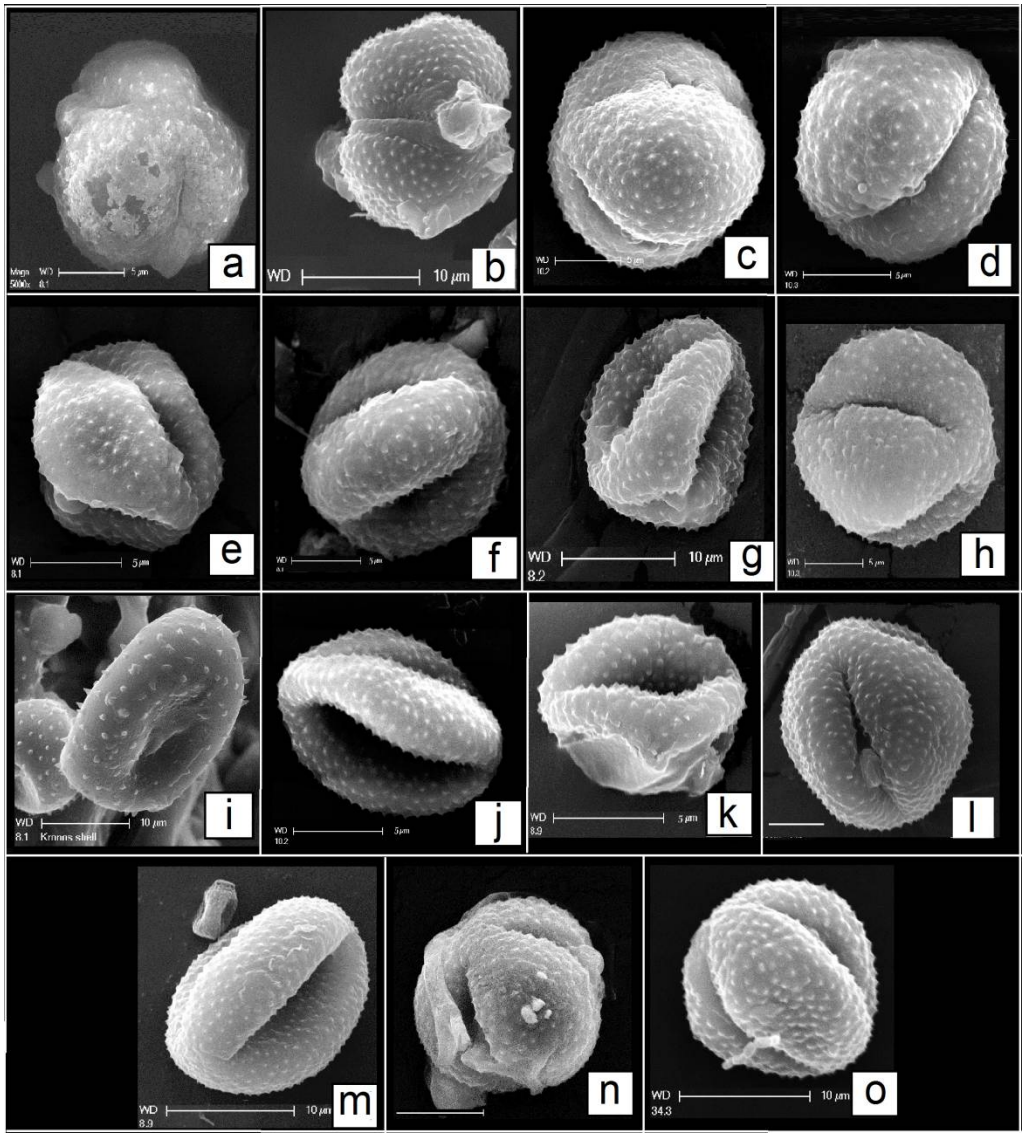

Fig. 1. Scanning electron micrographs showing the equatorial view of pollens of *Artemisia* species: a, *A. annua*; b, *A. maritima*; c, *A. rutifolia*; d, *A. campestris*; e, *A. chamaemelifolia*; f, *A. tournefortiana*; g, *A. indica*; h, *A. scoparia*; i, *A. chinensis*; j, *A. austriaca*; k, *A. gmelinii*; l, *A. herba-alba*; m, *A. pontica*; n, *A. vulgaris;* o, *A. montana*. Scale bar = 2-10µm.

From LM and SEM observations, the shape of pollen grain was found to be homogeneous with few exceptions throughout the genus and confirms the monophyly of genus *Artemisia* (Hayat *et al*., 2010) as presented the monophyly of *Artemisia* in molecular studies of Torrell *et al*. (1999), Watson *et al*. (2002), Hussain *et al*. (2019a,b). The general features of *Artemisia* pollen recorded in the present study are in high concordance with Jiang (2005) and Hayat *et al*. (2010) who found approximate symmetry or globular, 3 lobed spheres in the equatorial view while ellipsoid in the polar side with tricolporate structure in different species of *Artemisia.*

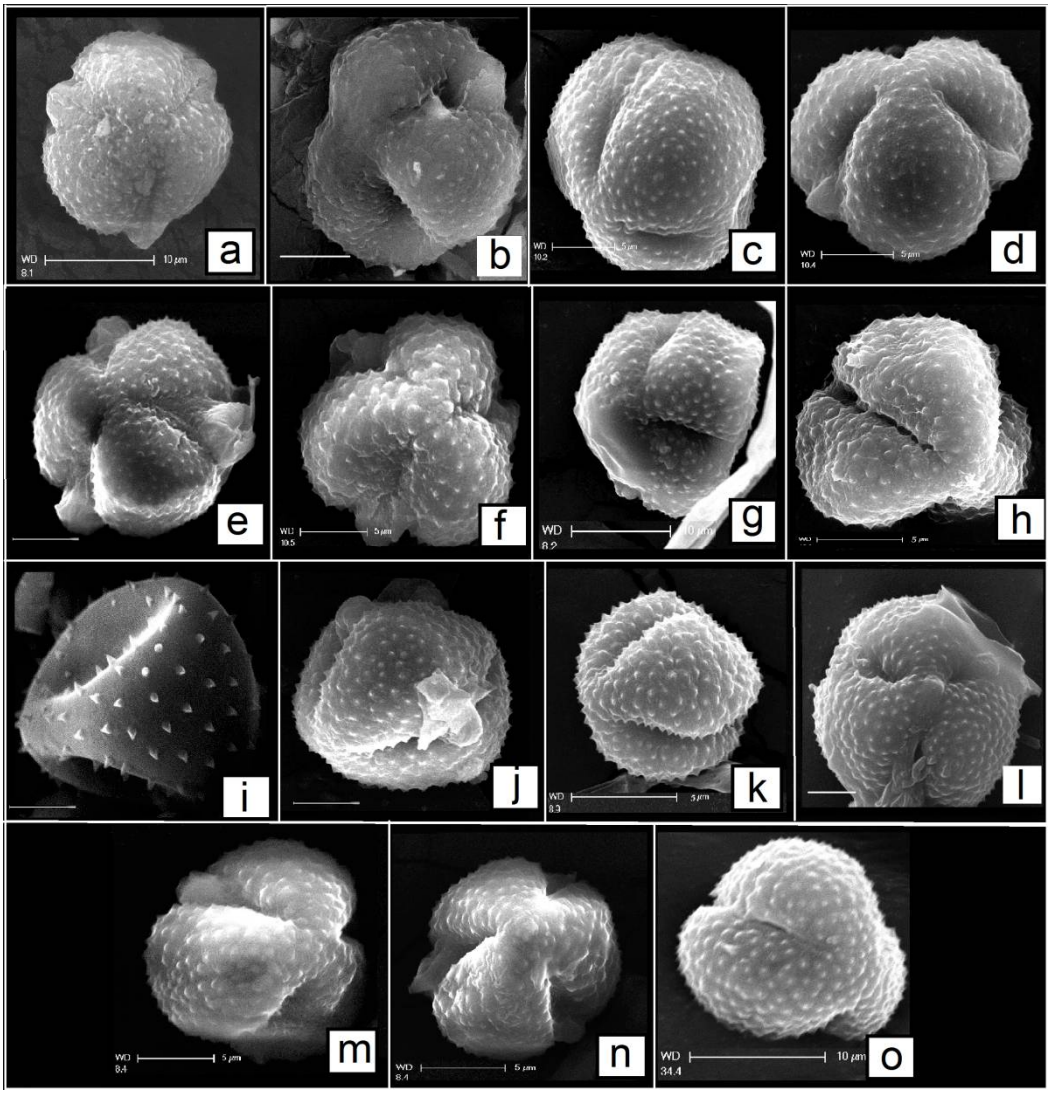

Fig. 2. Scanning electron micrographs showing the polar view of pollens of *Artemisia* species: a, *A. annua*; b, *A. maritima*; c, *A. rutifolia*; d, *A. campestris*; e, *A. chamaemelifolia*; f, *A. tournefortiana*; g, *A. indica*; h, *A. scoparia*; i, *A. chinensis*; j, *A. austriaca*; k, *A. gmelinii*; l, *A. herba-alba*; m, *A. pontica*; n, *A. vulgaris;* o, *A. montana*. Scale bar = 2-10 μm.

Pollen characteristics among the investigated species look similar except in *Artemisia chinensis.* The division of the genus *Artemisia* into subgenera by means of floral morphology and molecular studies could not be recognized by pollen data, because the pollen morphology of the species investigated is very similar in shape, size as well as in exine sculpture with few exceptions. The family Asteraceae is eurypalynous, and the genera of this family possess zonocolporate type of pollen (Sachdeva and Malik, 1986). An important character of pollen is spine present in the exine that can be utilized as diagnostic character in the genera of Asteraceae (Pinar and Donmez, 2000). On the other hand, the morphology of pollen of different Asteraceae

genera previously investigated showed that the exine feature of pollen is very significant in taxonomy and classification based on phylogeny (Mbagwu and Edeoga, 2006). This study also validates that the spinule present in *Artemisia* pollen is very crucial marker for species delimitation and classification.

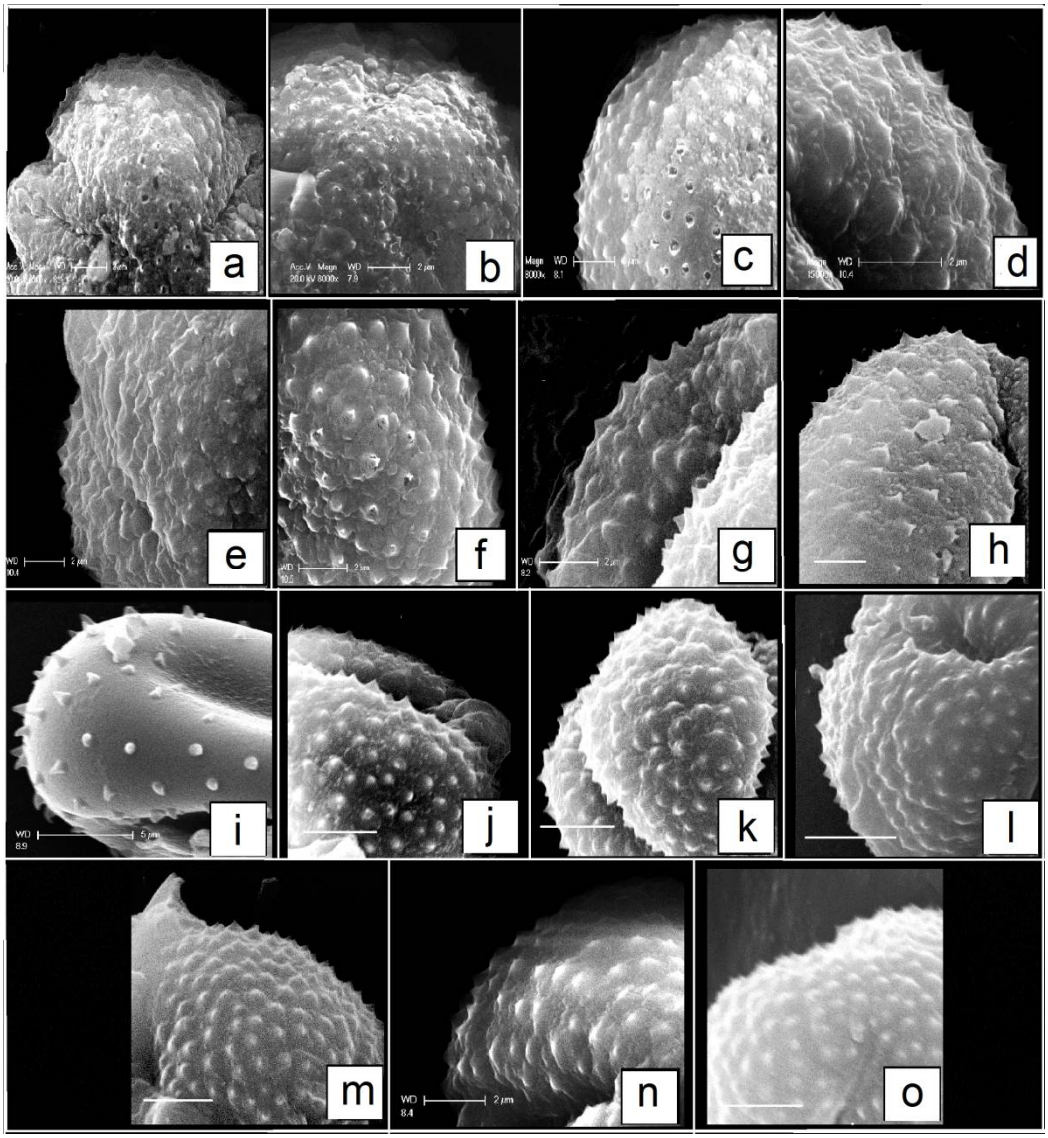

Fig. 3. Scanning electron micrographs showing the exine sculpture view of pollens of *Artemisia* species: a, *A. annua*; b, *A. maritima*; c, *A. rutifolia*; d, *A. campestris*; e, *A. chamaemelifolia*; f, *A. tournefortiana*; g, *A. indica*; h, *A. scoparia*; i, *A. chinensis*; j, *A. austriaca*; k, *A. gmelinii*; l, *A. herba-alba*; m, *A. pontica*; n, *A. vulgaris;* o, *A. montana*. Scale bar = 2-10 µm.

A total of seven micromorphological characters of the pollens of different *Artemisia* species, studied by LM and SEM, were selected for cluster analysis using UPGMA (Table 3). The data matrix based on pollen features of different *Artemisia species* used for cluster analysis is provided in Table 4.

Table 3. Pollen characters and character states for the cluster analysis of *Artemisia.* The numbers in brackets are the codes for character states. The code of plesiomorphic character state is always 0.

Sl. No.	Characters	Character states
1	Pollen type	*Anthemis* (0), *Artemisia* (1)
2	Pollen shape	Globular (0), Oblate (1)
3	Spinules arrangement	Dense (0), Loose (1)
4	Exine sculpture	Granular (0), Sinuolate (1)
5	Spinules base	Stretching and outward extending (0) Normal* (1)
6	Polar length	>26µm (0), >23-26µm (1), >22-23µm (2), >21-22µm (3), >20-21µm (4), >19-20µm (5), >18-19µm (6), >17-18µm (7), 16-17µm (8), 15-16µm (9)
7	Equatorial width	>21µm (0), >20-21µm (1), >19-20µm (2), >18-19µm (3), >17-18µm (4), >16-17µm (5), >15-16µm (6) 14-15µm (7)

Table 4. Data matrix used in cluster analysis of *Artemisia* species based on pollen features.

Sl. No.	Taxa	1	2	3	4	5	6	7
1	*A. annua*	1	1	0	1	1	9	3
2	*A. austriaca*	1	1	1	0	0	7	5
3	*A. campestris*	1	0	1	1	0	9	6
4	*A. chamaemelifolia*	1	1	0	1	1	4	2
5	*A. chinensis*	1	1	1	0	1	1	1
6	*A. gmelinii*	1	1	0	1	1	8	7
7	*A. herba-alba*	1	1	1	0	1	9	7
8	*A. indica*	1	1	1	1	1	7	7
9	*A. maritima*	1	1	1	0	1	9	3
10	*A. montana*	1	0	0	0	1	9	5
11	*A. pontica*	1	1	0	0	1	7	7
12	*A. rutifolia*	1	0	1	0	0	8	4
13	*A. scoparia*	1	0	1	1	0	9	4
14	*A. tournefortiana*	1	1	1	1	1	5	2
15	*A. vulgaris*	1	0	0	1	0	9	5

In the dendrogram, four groups have been recognized within the genus *Artemisia*. Species like *A. chinensis, A. chamaemelifolia* and *A. tournefortiana* were clustered in group 1. *A. herba-alba, A. pontica, A. gmelinii* and *A. indica* were placed in group 2. *A. austriaca, A. scoparia, A. rutifolia, A. montana, A. campestris* and *A. vulgaris* were designated in group 3. The rest two species *A. annua* and *A. maritima* were placed in group 4 (Fig. 4).

The pollen morphological evolutions have the ability to develop more and more degenerative structures (Hayat *et al.*, 2010). Few characters of pollen like, lobular pollen shape, arrangement dense spinules, broad spinule base, granular exine sculpture, large pollen size, thick exine and broad colpus width, are the plesiomorphic characteristics of *Artemisia* pollen. On the other hand,

in apomorphic condition these pollen characteristics have ability to be transformed to oblate pollen, lose arrangement of spinules, without prominent spinule base, sinuolate exine sculpture, small pollen size and volume, reduced exine thickness and thin colpus.

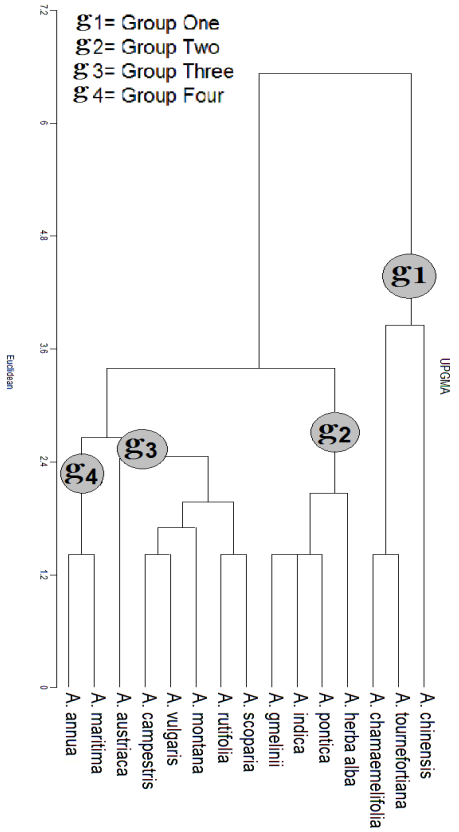

Fig. 4. Dendrogram based on cluster analysis of pollen micromorphological characters of different species of the genus *Artemisia*.

Studies authenticated one reason behind this evolution is the patterns of pollination from entomophily to anemophily. While, climate changing patterns with high latitude to low latitude during the relocation from North Temperate Zone and low evaluation moist regions during the glacial epoch are other major cause of this evolution in pollen of *Artemisia* (Jiang *et al*., 2005).

Our results are in accordance with the findings of Hayat *et al*. (2010) and Martin *et al*. (2003) who proposed pollen morphology as a diagnostic feature for *Artemisia* and recognized as an excellent taxonomic marker. However, our results are not in agreement with Jiang *et al*. (2005) suggesting that the grouping of *Artemisia* species based on their pollen morphology is very difficult task. We also propose that the arrangement patterns of spinules (dense/loose) are also a good taxonomic character for species delimitation.

This study concludes that the pollen characters of genus *Artemisia* could be taxonomically crucial traits for few species with in the genus. Together with the data from molecular studies, phytochemistry, karyology and phytogeography, the micromorpholigical traits of pollen can be useful for subgeneric classification of the genus *Artemisia*.

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