Pollen Morphology of Melanoseris Decne. (Cichorieae-asteraceae) From Pakistan and Western Himalayas and Its Taxonomic Significance

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Abstract

Pollen morphological studies of 10 species and 3 varieties of *Melanoseris* Decne. (Cichorieae-Asteraceae) were conducted from Pakistan and Kashmir. Pollen were studied by using Light microscope, Fluorescent microscope and Scanning Electron Microscope. Pollen of 7 taxa were examined for the first time including 4 endemic species to the study area. Pollen grains are usually isopolar, spheroidal to oblate-spheroidal, elliptic to rarely circular in equatorial view, hexagonal in polar view, trizonocolporate and echinolophate with 15 lacunae. Significant variation has been found mainly in the size of pollen, number of echinae and sculpturing of the polar area. All the examined taxa were grouped into two distinct pollen-types viz., *Melanoseris alii* – type and *Melanoseris stewartii* – type. Keys to the pollen types and species of each pollen type are provided. Agglomerative cluster analysis based on the 14 pollen morphological characters, showed that diameter of polar and equatorial axis, pollen size and polar axis were the most valuable variables in explaining total variation and separating the studied taxa. The aim of this study is to analyze the pollen morphological diversity in *Melanoseris* species with the purpose of evaluating additional micromorphological characters for taxonomical delimitation of the studied species and also to find a correlation between the recent phylogenetic studies and the present palynological results.

Introduction

Melanoseris Decne. is a medium sized genus of the subtribe Lactucinae (Cichorieae). The genus is widely distributed in Africa, across SW and Central Asia to E and SE Asia (Kilian et al. 2017). In Pakistan, the genus mostly grows on alpine pastures, moist, grassy and shady places of Kashmir (Western Himalayas), followed by district Gilgit, Astore, Baltistan, Chitral, Hunza, Swat and Rawalpindi (Murree hills) between the elevation of 1200–4500 m (Abid et al. 2017).

It seems that a consensus on the generic circumscription of the genus *Melanoseris* is yet to develop therefore there is a difference of opinion in accepting the number of species of the genus such as Shih and Kilian (2011) recognized ca 60 species whereas The Plant List (2017) accepted ca 30 species and International Plant Name Index IPNI (2022) listed ca 48 species.

This genus was established by Decaisne in 1843 based on *Mulgedium lessertianum* Wall. ex DC., and included two species from the Himalayan region. Later on Edgeworth (1846) accepted 7 species in the genus *Melanoseris* Decne. The genus differed from its closely related genera by having beaked cypselas rather than unbeaked cypselas as in *Cicerbita* Wallr. (formerly under the name *Mulgedium* Cass.) and presence of biseriate pappus with an outer row of minute bristles rather than uniseriate pappus as in *Lactuca* L. (Ghafoor et al. 2017). The genus is considered as one of the oldest genera of the tribe Cichorieae but was not usually recognized as a separate entity during the 19th and 20th century. For this reason, all those species which belonged to *Melanoseris* Decne., were treated under other closely related genera such as *Cicerbita* Wallr., *Prenanthes* L., *Lactuca* L. and *Mulgedium* Cass. However, in the beginning of 21st century, molecular phylogenetic studies clarified the generic circumscription and provided valuable evidences which improved our understanding of some groups within the tribe Cichorieae (Lee et al., 2003, Kilian et al., 2009). Based on these evidences, Shih and Kilian (2011) resurrected this genus as a distinct entity in the Flora of China. Wang et al. (2013) recognized the first lineage of *Melanoseris* using nuclear and plastid DNA datasets from Sino Himalayan region. Kilian et al. (2017) further explored this genus by adding more species from Africa to Asia (at global level) and gave further insights into the circumscription of the subtribe Lactucinae (Cichorieae-Asteraceae) and clearly confirmed that the genus *Melanoseris* Decne., represented a distinct phylogenetic lineage within the subtribe Lactucinae and thus revealed its sister group relationship to *Lactuca* L. On

the basis of the aforementioned molecular phylogentic evidences, Ghafoor et al. (2017) and Abid et al. (2017) also accepted the genus *Melanoseris* for the Flora of Pakistan and reported 16 taxa from Pakistan and Kashmir including 4 endemic species.

The pollen morphological studies have a great value in the taxonomy of the family Asteraceae (Wodehouse, 1926). The pollen of Asteraceae were broadly grouped into two on the basis of gross morphology viz., liguliflorae-type in Lactuceae (Faegri and Iverson, 1975; Moore and Webb, 1978) and tubuliflorae-type other than Lactuceae. Pausinger (1951) divided the tribe Lactuceae (now Cichorieae) on the presence or absence of polar lacunae viz., *Leontodon* type and *Tragopogon* type. Blackmore (1984) dealt with pollen morphology of Lactuceae and recognized 7 pollen types on the basis of distinguishing pollen characters. Chanda and Pal (1990) investigated 44 species of the tribe Lactuceae and also recognized 7 pollen types based on the similarity of pollen characters. Gao (2007) studied the pollen morphology of 8 genera in subtribe Lactucinae and found that it was useful at the species level but not at the generic level. Wang et al. (2009) demonstrated that pollen morphology of subtribes Crepidinae and Lactucinae was difficult to correlate with taxonomic and phylogenetic results. Tremetsberger et al. (2012) provided the first estimate of the age of the tribe Cichorieae based on fossils records of pollen. Peng et al. (2013) studied the pollen morphology of 15 species of *Youngia* and its related genera of the tribe Cichorieae and concluded that pollen characters were neither useful for the delimitation of taxa nor were helpful in resolving the relationship among the groups. On the other hand, Pinar et al. (2016) while studying the pollen morphology of 45 taxa of *Scorzonera* (Cichorieae) concluded that pollen analysis was very useful at the infrageneric and specific level, and could be used for the delimitation of taxa.

Though the literature survey showed that palynological studies had been conducted for the various members of Cichorieae (or Lactuceae) from different parts of the world but the pollen morphology of *Melanoseris* had not been studied exclusively from any part of the world including the area under consideration with the exception of few species which were previously treated under *Prenanthes* L. (*P. brunoniana* by Chanda and Pal 1990) and *Cephalorhynchus* (*C. macrorhiza* by Wang et al. 2009).

In the absence of any comprehensive report on the pollen morphology of the genus *Melanoseris*, a detailed palynological study of 13 taxa belonging to *Melanoseris* was conducted from Pakistan and Kashmir using Light microscope (LM) and Scanning Electron Microscope (SEM) in order to analyze the pollen morphological diversity with the species, obtain additional characters for the delimitation of the taxa and also to correlate the palynological groups with the current phylogenetic studies based on molecular data.

Materials And Methods

Polleniferous material of *Melanoseris* species was obtained from herbarium specimens, housed in BM, E, KUH, M and RAW (codes following Thiers 2016).

Pollen slides were prepared for Light Microscopy (LM) using standard acetolysis techniques Erdtman (1960). Slides were examined by using Nikon Type-102 microscope under (E40, 0.65) and oil immersion (E100, 1.25) using 10x eye piece. Following quantitative and qualitative parameters were taken for each species viz., diameter of polar and equatorial axis (pollen size: L x B), diameter of ora, length of echinae, interspinal distance, exine thickness, size, number, base and apex of echinae, and sculpturing pattern of polar area (Table 1). Mostly five specimens / species from different localities and 10-20 pollen grains/ species were studied. However, in case of less specimens, 5-8 (-10) readings were taken from the available material. Details on the herbarium voucher are included in the list (see Appendix-I).

For SEM, the acetolyzed material was directly transferred with a fine pipette on a metallic stub using double sided cello tape and then coated with gold in a sputtering chamber (Ion- sputter JFC-1100) at 150 A°. The fine details of pollen morphology were examined on a Jeol microscope (JSM-T200 and JSM-6380).

Descriptive terminology used here is based on Erdtman (1952), Blackmore (1984), Blackmore and Pearson (1996) and Punt et al. (2007).

Cluster analysis

The hierarchical clustering was performed choosing the Euclidean distance as the resemblance function and Ward's method for a group linkage method (McCune and Grace 2002) so as to show the group structure in the studied species of *Melanoseris* Decne based on contrasting pollen morphological features. The computation was carried by using the computer program PC-ORD (version 6.0) (McCune and Grace 2002; Peck 2010). A total of 14 pollen features comprising of nine quantitative and five qualitative characters were chosen to make a distinction among the examined species of the genus. The qualitative characters were recorded in binary state (i.e. 1 and 2). However, in some cases multiple character state were also used (i.e. 1, 2 and 3). While in case of absence or presence, characters were coded as 0 and 1 respectively. The characters and character state used for performing cluster analysis are listed in Table 2 and 3a (Supplementary table).

Results

The morphometric data in µm such as diameter of polar axis and equatorial axis, P/E ratio, diameter of ora, length of echinae, interspinal distance, thickness of exine including echinae and number of centrally isolated echinae were noted. The summary of quantitative and qualitative analysis of pollen is presented in Table 1. The selected LM and SEM micrographs are given in Figures 1-5.

Table 1. Summary of Pollen morphology for examined taxa of the genus *Melanoseris* Decne

Name of Taxa	Polar Axis Length	Equatorial Axis Breadth	Pore Diameter	Spine Length	Interspinal Distance	Exine Thickness inclu. Echinae	No. of echinae on the polar area
M. astorensis	37.5 (41.09) 43.75 ± 0.563	40 (43.43) 47.5 ± 0.575	4.375 (5.78) 6.25 ± 0.484	3.75 (5.0) 5.625 ± 0.470	3.25 (5) 5.625 ± 0.357	6.25 (6.875) 7.5 ± 0.424	4- 6
M. alii	42.5 (47.25) 50.0 ± 0.505	46.25 (50) 52.75 ± 0.532	3.75 (4.84) 5 ± 0.284	4.0 (4.5) 5.0 ± 0.208	3.75 (4.5) 5.5 ± 0.265	5.0 (6.16) 7.5 ± 0.408	2 - 4
M. gilgitensis	40.0 (45.35) 48.75 ± 0.685	42.5 (48.21) 52.5 ± 0.731	5 (5.62) 6.25 ± 0.354	4.37 (4.87) 5.0 ± 0.237	3.12 (3.62) 4.37 ± 0.324	5.0 (6.5) 7.5 ± 0.477	2 - 5
M. macrorhiza	27.5 (28.57) 30 ± 0.437	30.0 (31.07) 32.5 ± 0.437	5 (5.62) 6.25 ± 0.354	4.5(5.12) 5.75 ± 0.237	3.12 (3.62)4.37 ± 0.324	5.0 (6.5) 7.5 ± 0.477	2 - 5
M. lessertiana var. *lessertiana*	38.5 (43.5) 48.5 ± 0.597	43.5 (47.37) 51.25 ± 0.630	4.37 (5.83) 7.5 ± 0.428	4.5 (5.25) 6.25 ± 0.350	3.75 (4.5) 5.25 ± 0.414	5.0 (6.5) 8.0 ± 0.465	6 - 8
M. lessertiana var. *lyrata*	37.5 (45.75) 49.0 ± 0.611	41.25 (48.12) 52.5 ± 0.587	3.75 (4.58) 5 ± 0.292	4.37 (5.52) 6.25 ± 0.373	3.5 (4.37) 5.0 ± 0.306	5.0 (6.45) 7.5 ± 0.397	6 - 8
M. lessertiana var. *dentata*	37.0 (42.75) 48.5 ± 0.605	41.25 (48.12) 52.5 ± 0.587	3.75 (4.58) 5 ± 0.292	4.37 (5.52) 6.25 ± 0.373	3.5 (4.37) 5.0 ± 0.306	5.0 (6.45) 7.5 ± 0.397	6 - 8
M. rapunculoides	38.5 (43.0) 47.5 ± 0.735	40.5 (45.25) 50.0 ± 0.739	5 (5.44) 6.52 ± 0.291	6.0 (6.87) 7.5 ± 0.298	2.5 (4.01) 5.0 ± 0.402	5.62 (7.67) 10 ± 0.495	10 – 12
	45.0 (51.47) 56.25	48.75 (53.11)	5 (7.06) 8.75 ± 0.320	3.75 (4.37)	3.125 (3.75) 5.0 ± 0.256	7.5 (8.43) 10 ± 0.327	8 – 10

M. decipiens var. *decipiens*	± 0.619	58.5 ± 0.679		5.0 ± 0.204			
M. decipiens var *multifida*	45.75 (49.62) 53.5 ± 0.707	46.25 (50.87) 55.5 ± 0.792	5 (6.875) 8.75 ± 0.505	3.75 (4.5) 5.25 ± 0.291	3.125 (4.16) 5.0 ± 0.377	7.5 (9.06) 10 ± 0.453	8 – 10
M. brunoniana	43.5 (47.75) 52.0 ± 0.559	46.5 (50.5) 54.5 ± 0.485	3.75 (4.91) 6.25 ± 0.361	4.0 (4.87) 6.25 ± 0.472	2.25 (3.76) 4.5 ± 0.367	6.25 (7.14) 8.75 ± 0.367	12 – 15
M. aitchisoniana	45.0 (47.67) 50.0 ± 0.547	47.5 (51.78) 56.25 ± 0.677	5 (5.625) 6.25 ± 0.353	3.75 (4.5) 5.5 ± 0.579	1.875 (3.12) 4.37 ± 0.506	6.25 (7.625) 8.5 ± 0.372	11-13
M. stewartii	46.5 (48.75) 51.25 ± 0.533	50.0 (51.87) 55.0 ± 0.605	5 (5.72) 6.25 ± 0.320	3.5 (4.75) 6.0 ± 0.516	2.25 (3.31) 4.37 ± 0.440	6.25 (7.65) 8.5 ± 0.430	12 – 14

Table 2: List of characters, scored for cluster analysis

S. No.	Characters : Character state	Symbols
1.	*Pollen grains: small (1), medium (2), large (3)	PG
1.	Minimum diameter of polar axis (µm): less than 30 (1), 31-40 (2), 41-50 (3)	MID
1.	Maximum diameter of polar axis (µm): upto 30 (1), 41-50 (2), 51-60 (3)	MAD
1.	Minimum diameter of equatorial axis (µm): upto 30 (1), 41-44 (2), 46-50 (3)	MIE
1.	Maximum diameter of equatorial axis (µm): upto 35 (1), 41-50 (2), 51-60 (3)	MAE
1.	Number of centrally isolated echinae: 5 or less (1), 6-10 (2), more than 10 (3)	NCE
1.	Average length of echinae (µm): 3.75-5.0 (1), 5.1-5.5 (2), 6.0-6.5 (3)	ALE
1.	Base of the echinae: globose-subglobose (1), conical (2)	BOE
1.	Perforation on equatorial axis: absent (0), present (1)	PEA
1.	Perforation on polar axis: absent (0), present (1)	PPA
1.	Apex of echinae: acute (1), subacute - acuminate (2)	AOE
1.	Minimum interspinal distance (µm): 1.5-2.5 (1), 3-4.5 (2)	MID
1.	Maximum interspinal distance (µm): 4.0-4.5 (1), 5.0-6.0 (2)	MAD
1.	Average thickness of exine (µm): 6.0-6.8 (1), 7.0-8.0 (2), 8.4-9.5 (3)	ATE

***Table 3a: Data matrix of *Melanoseris Decne.* scored for 14 characters presented in Table 2**

Name of Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M. astorensis	2	2	2	2	2	2	1	2	0	0	2	2	2	1
M. alii	2	3	2	3	2	1	1	1	1	1	1	2	2	1
M. gilgitensis	2	2	2	2	2	1	1	1	0	0	1	2	1	1
M. macrorhiza	1	1	1	1	1	1	2	1	1	0	2	2	1	1
M. lessertiana var. lessertiana	2	2	2	2	2	2	2	2	0	0	2	2	2	1
M. lessertiana var. lyrata	2	2	2	2	2	2	2	2	0	0	2	2	2	1
M. lessertiana var. dentata	2	2	2	2	2	2	2	2	0	0	2	2	2	1
M. rapunculoides	2	2	2	2	2	2	3	2	0	0	1	1	2	2
M. decipiens var. decipiens	3	3	3	3	3	3	1	1	1	1	1	2	2	3
M. decipiens var. multifida	3	3	3	3	3	3	1	1	1	1	1	1	2	3
M. brunoniana	3	3	3	3	3	3	1	2	0	0	2	1	1	2
M. aitchisoniana	3	3	3	3	3	3	1	2	0	0	2	1	1	2
M. stewartii	3	3	3	3	3	3	1	2	0	0	2	1	1	2

*Supplementary Table

General pollen characters of the genus *Melanoseris* Decne.

Pollen grains are usually radially symmetrical, isopolar, oblate to spheroidal (almost spheroidal in *M. macrorhiza*). Polar axis with or without perforations, hexagonal-triangular with rounded, convex or straight sides in outline. Equatorial axis with or without perforations, elliptic –sub-circular in outline. Pollen echinolophate (i.e. echinae situated on ridges) with 15 large lacunae (3 poral, 6 abporal and 6 paraporal) and trizonocolporate. Equatorial ridges narrow with a single central row of echinae, or sometime slightly broad (usually with two rows of echinae) bordering the lacunae. Polar lacunae angular or rounded, abporal lacunae angular and paraporal ones pentagonal. Oral lacunae circular – hexagonal and divided into three lacunae connected by narrow or slightly extensive inter lacunar gaps. Endocolpium circular-elliptic or oval. Polar areas small, medium to large often with 2 -15 scattered or centrally isolated echinae. Exine 5.0-10.0 µm thick including echinae. Tectum echinate, densely or slightly perforated on the ridges, between the ridges and base of the echinae, apex smooth. Echinae 3.25-6.25 µm in length with acute or acuminate- sharply pointed tips and globose (swollen) -conical perforated base. Depressions with or without echinae.

Cluster analysis of the studied species

A dendogram resulting from the hierarchical analysis based on 14 morphological variables of pollen is presented in Figure 6. The horizontal axis indicated the distance between each cluster using the Ward method. The distribution of 13 taxa in disparate cluster showed variation in their pollen morphological characters. The variables related with the size of pollen grain and extents of polar area including number of echinae were found to be significant for the close lineage of studied taxa. The analysis of morphometric data showed that all the assessed taxa were divided into two major clusters viz., cluster I and II. Cluster-I consisted of 5 species (38.46 % of total species), distinguished

by comparatively larger pollen grains. This group was further separated into two subclusters IA and IB with an optimal number of 2-3 taxa in each subcluster. Subcluster IA characterized by having non perforated tectum (equatorial and polar axis not perforated) and 12-15 centrally isolated echinae with conical base, accommodated *M. brunoniana*, *M. aitchisoniana* and *M. stewartii*. Whereas subcluster IB represented by *M. decipiens* var. *decipiens* and var. *multifida*, characterized by perforated tectum (equatorial and polar axis densely perforated) and 6-8 centrally isolated echinae with globose base.

While Cluster II comprised of 8 taxa (61.53 % of total taxa) differed from the former group by the presence of small to medium sized pollen. Due to variation in the pollen morphological characters species of this group was further subdivided into subcluster IIA and IIB with an optimal number of 2-5 taxa in each subcluster. The subcluster IIA distinguished by 6-10 centrally isolated echinae on the polar area, consisted of *M. lessertiana* var. *lessertiana*, var. *lyrata*, var. *dentata*, *M. astorensis* and *M. rapunculoides*. In contrast subcluster IIB was characterized by 2-5 centrally isolated echinae on the polar area. *M. gilgitensis, M. alii* and *M. macrorhiza* fell in this subcluster.

On the basis of morphometric data and cluster analysis all the studied species were grouped into two major pollen types namely *Melanoseris stewartii* - type and *Melanoseris alii* – type.

Key to the Pollen types

1+	Pollen small to medium. Diameter of polar axis upto 50.0 µm and equatorial axis upto 52.0 µm.	**ii-*Melanoseris alii* -type**
-	Pollen large. Diameter of polar axis upto 57.0 µm and equatorial axis upto 60.0 µm.	**i-*Melanoseris stewartii* – type**

1. Pollen type I: *Melanoseris stewartii*- -type

Diagnosis: Pollen grain large, oblate - spheroidal. Echinae not recurved, acute. Polar area with 8-15 centrally isolated upto 6.0 µm long echinae with globose-conical base. Exine 6.25-10.0 µm thick including echinae.

Species included: M. brunoniana (Wall. ex DC.) Kilian and Wang, *M. aitchisoniana* (Beauv.) A. Ghafoor, Qaiser and Roohi Bano, *M. stewartii* (Roohi Bano and Qaiser) A. Ghafoor, Qaiser and Roohi Bano, *M. decipiens* (Hook. f. and Thomson ex C. B. Clarke) N. Kilian and Z.H. Wang

Key to the species of Pollen type I

1+	Polar axis densely perforated, with rounded or convex sides. Echinae upto 5.0 µm long, globose at the base.	1. **M. decipiens**
-	Polar axis not perforated with straight sides. Echina upto 6.0 µm long, conical at the base.	**2**
2+	Equatorial axis slightly perforated on the ridges and ora.	1. **M. brunoniana**
-	Equatorial axis not perforated on the ridges and ora.	**iii- M. aitchisoniana** **iv- M. stewartii**

Pollen description of species of the Pollen type I

Melanoseris decipiens (Hook. f. and Thomson ex C. B. Clarke) N. Kilian and Z.H. Wang including var. *multifida* (Hook.f.) A. Ghafoor, Qaiser and Roohi Bano (Figs. 1a-c, 2c-f)

Equatorial axis 46.5-58.5 µm in diameter, elliptic to sub-circular in outline, densely perforated.

Polar axis 45.0-56.5 µm in diameter, hexagonal with rounded or convex sides in outline, densely perforated.

Paraporal lacunae pentagonal.

Abporal lacunae small, angular, broad towards the pole.

Oral lacunae usually circular

Polar area distinct, medium with 8-10 centrally isolated echinae, single row of echinae or a pair of echinae bordering the lacunae, ridges hardly prominent, perforated at the base of the echinae, on the ridges and between the echinae.

Echinae 3.75-5.25 µm long, globose - subglobose at the base, not sharp at the tip, acute.

Note: The pollen morphological character of both the varieties of *M. decipiens* overlapped and there were no sufficient characters to key them out.

Melanoseris brunoniana (Wall.ex DC.) N. Kilian and Z.H. Wang (Figs. 1d-f, 2g-h)

Equatorial axis 46.5-55.0 µm in diameter, elliptic – sub-circular in outline, slightly perforated on the ridges and ora.

Polar axis 43.0-52.0 µm in diameter, hexagonal with straight sides in outline, not perforated.

Oral lacunae usually circular

Paraporal lacunae pentagonal.

Abporal lacunae small, angular-oval, broad towards the pole.

Polar area distinct, large, with 12-15 centrally isolated echinae, single row of echinae or a pair of echinae bordering the lacunae, ridges prominent, perforated at the base of the echinae, on the ridges and between the echinae.

Echinae 4.0- 6.25 µm long, conical at the base, not sharp at the tip, acute.

Melanoseris aitchinsoniana (Roohi Bano and Qaiser) A. Ghafoor, Qaiser and Roohi Bano (Figs. 1g-i, 2k-l)

Equatorial axis 48.5-56.5 µm in diameter, elliptic – sub-circular in outline, not perforated.

Polar axis 45.5-50.5 µm in diameter, hexagonal with straight sides in outline, not perforated.

Oral lacunae usually circular

Paraporal lacunae pentagonal.

Abporal lacunae small, angular-oval, broad towards the pole.

Polar area distinct, large, with 12-15 centrally isolated echinae, single row or a pair of echinae bordering the lacunae with prominent ridges, slightly perforated at the base of the echinae, on the ridges and between the echinae.

Echinae 3.75- 5.5 µm long, conical at the base, not sharp at the tip, acute.

Melanoseris stewartii (Roohi Bano and Qaiser) A. Ghafoor, Qaiser and Roohi Bano (Figs. 1j-l, 2i-j)

Equatorial axis 50.0-55.5 µm in diameter, elliptic – sub-circular in outline, not perforated

Polar axis 46.5-51.5 µm in diameter, hexagonal with straight sides in outline, not perforated.

Oral lacunae usually circular

Paraporal lacunae pentagonal.

Abporal lacunae small, angular-oval, broad towards the pole.

Polar area distinct, large, with 12-15 centrally isolated echinae, single row or a pair of echinae bordering the lacunae, ridges hardly prominent, perforated at the base of the echinae, on the ridges and between the echinae.

Echinae 3.5-6.0 µm long, conical at the base, not sharp at the tip, acute.

2. Pollen type II: *Melanoseris alii*- type

Diagnosis: Pollen grain usually small- medium, oblate – spheroidal or spheroidal. Polar area usually with 2-8 centrally isolated echinae. Echinae upto 7.0 µm in length, globose-conical at the base. Exine 5.0-10.0 µm thick including echinae.

Species included: *M. astorensis* (Roohi Bano and Qaiser) A. Ghafoor, Qaiser and Roohi Bano, *M. alii* (Roohi Bano and Qaiser) N. Kilian and Z.H. Wang, *M. gilgitensis* (Roohi Bano and Qaiser) A. Ghafoor, Qaiser and Roohi Bano, *M. lessertiana* (Wall.ex DC.) Mamgain and Rao, *M. rapunculoides* (DC.) Edgew and *M. macrorhiza* (Royle) N. Kilian

Key to the Pollen type II

1+	Polar area with 2-5 centrally isolated echinae. Echinae upto 5.0 µm long.	**2**
-	Polar area with 6-8 centrally isolated echinae. Echinae more than 5.0 µm long.	**4**
2+	Pollen small. Diameter of polar axis 27.0 - 30.0 µm and equatorial axis 31.0-32.5 µm.	**i- M. macrorhiza**
-	Pollen medium. Diameter of polar axis 37.5-50.0 µm and equatorial axis 40.0-52.5µm.	**3**
3+	Tectum densely perforated in equatorial and polar axis. Echinae swollen-globose at the base. Polar region with a single row of echinae bodering the lacunae.	**iv- M. alii**
-	Tectum not perforated in equatorial and polar axis. Echinae conical at the base. Polar region with a double rows of echinae bodering the lacunae.	**ii- M. gilgitensis**
4+	Echinae upto 6.5 µm long. Polar area hexagonal with straight sides. Exine upto 7.5 µm thick. Ridges prominent in polar axis, either with a single row of echinae or pair of echinae bordering the lacunae	**5**
-	Echinae upto 7.5 µm long. Polar area hexagonal with rounded or convex sides. Exine more than 7.0 µm thick. Ridges not prominent in polar axis.	**vi- M. rapunculoides**
5+	Echinae upto 5.0 µm long, not sharp, with 4-6 centrally I isolated echinae	**v- M. astorensis**
-	Echinae upto 6.25 µm long, more or less sharp, with 6-8 centrally isolated echinae	**iii- M. lessertiana**

Pollen description of species of the Pollen type II

i- ***Melanoseris macrorhiza*** (Royle) N. Kilian (Figs. 3j-l, 4e-f)

Equatorial axis 30.0-32.5 µm in diameter, subcircular in outline, perforated.

Polar axis 28.0-30.0 µm in diameter, hexagonal with convex sides in outline, not perforated.

Paraporal lacunae pentagonal

Abporal lacunae small, oval-subangular

Oral lacunae perforated, usually circular

Polar area with 2-5 central isolated echinae, rows of echinae bordering the adjacent lacunae and asymmetric concavities are also present, ridges not prominent, highly perforated at the base of the echinae and between the echinae.

Echinae 4.0-5.5 µm long, globose at the base, sharply pointed at the tip, recurved, acuminate.

Melanoseris gilgitensis (Roohi Bano and Qaiser) A. Ghafoor, Qaiser and Roohi Bano (Figs 3g-i, 4c-d)

Equatorial axis 42.5-52.5 µm in diameter, elliptic – subcircular in outline, not perforated.

Polar axis 40.0-48.75 µm in diameter, hexagonal with convex sides in outline, not perforated.

Paraporal lacunae pentagonal.

Abporal lacunae small, oval-subangular.

Polar area with 2-5 centrally isolated echinae, ridges not prominent, perforated at the base of the echinae, on the ridges and between the echinae.

Echinae 4.37-5.0 µm long, subglobose at the base, not sharp at the tip, acute.

Melanoseris alii (Roohi Bano andQaiser) N. Kilian and Z. H. Wang (Figs. 3d-f, 4a-b)

Equatorial axis 46.25-52.5 µm in diameter, elliptic – subcircular in outline, perforated.

Polar axis 42.5-50.0 µm in diameter, roughly hexagonal – subcircular in outline, perforated.

Paraporal lacunae pentagonal.

Abporal lacunae small, oval.

Polar area hardly distinct with 2-4 central isolated echinae, bordering with a single row of echinae, ridges slightly prominent, densely perforated at the base of the echinae, on the ridges, and between the echinae.

Echinae 5.0-5.5 µm long, swollen – globose at the base, not sharp at the tip, acute.

iv-***Melanoseris astorensis*** (Roohi Bano and Qaiser) A. Ghafoor, Qaiser and Roohi Bano (Figs. 3a-c)

Equatorial axis 40.0-47.5µm in diameter, elliptic – sub-circular in outline, not perforated.

Polar axis 37.5-43.5 µm in diameter, hexagonal with rounded or convex sides in outline, not perforated.

Paraporal lacunae pentagonal.

Abporal lacunae large, oval-subangular, broad towards the pole.

Polar area distinct with 4-6 central isolated echinae, a single row of echinae bodering the lacunae, ridges prominent, perforated at the base of the echinae, on the ridges and between the echinae.

Echinae 4.0-5.5 µm long, conical at the base, not sharp at the tip, acute.

v-***Melanoseris lessertiana*** (Wall.ex DC.) Mamgain and Rao (Figs. 4g-l, 5a-f)

Equatorial axis 41.0-52.5 µm in diameter, elliptic – sub-circular in outline, not perforated.

Polar axis 37.5-50.0 µm in diameter, hexagonal with rounded or convex sides in outline, not perforated.

Paraporal lacunae pentagonal.

Abporal lacunae large, oblong-angular, broad towards the pole.

Polar area each with 6-8 centrally isolated echinae, single row or a pair of echinae bordering the lacunae, ridges prominent, perforated at the base of the echinae, on the ridges and between the echinae.

Echinae 4.3-6.25 µm long, conical at the base, more or less sharp at the tip, subacute - acuminate.

Species included: Melanoseris lessertiana (Wall.ex DC.) Mamgain and Rao var*. lessertiana*, *Melanoseris lessertiana* (Wall. ex DC.) Mamgain and Rao var*. lyrata* (Decne.) A. Ghafoor, Qaiser and Roohi Bano, *Melanoseris*

lessertiana (Wall.ex DC.) Mamgain and Rao var. *dentata* DC.

Note: Due to overlapping in qualitative and quantitative pollen characters in all the three varieties of *M. lessertiana* no workable key was possible.

Melanoseris rapunculoides (DC.) Edgew. (Figs. 4m-n, 5g-i)

Equatorial axis 40.5-50.0 µm in diameter, oblate-spheroidal, elliptic – subcircular in outline, not perforated.

Polar axis 38.5-47.5µm in diameter, hexagonal with convex sides in outline, not perforated.

Paraporal lacunae pentagonal.

Abporal lacunae large, ovate-oblong, broader towards the pole.

Polar area distinct, large with 10-12 centrally isolated echinae, ridges not prominent, perforated at the base of the echinae, on the ridges and between the echinae.

Echinae 6.0-7.5 µm long, conical at the base, more or less sharp at the tip, acute.

Discussion

Melanoseris Decne., is a stenopalynous genus, as all the examined species had almost similar oblate-spheroidal, isopolar, echinolophate and trizonocolporate pollen. However, imperative and variable pollen characters were the size of pollen (diameter of polar and equatorial axis), followed by pollen perforation, size and number of centrally isolated echinae on the polar area. Primarily, on the basis of the size of pollen all the studied species could be segregated into *M. alii*-type and *M. stewartii*-type.

Al-Ghazaly (1980), Feuer and Tomb (1977), Chanda and Pal (1990), Pinar et al. (2016), Abid and Qaiser (in Press) also used the size of pollen for the delimitation of various species of the tribe Cichorieae. Al- Ghazaly (1980) observed a direct correlation between the size of pollen and plant habit and stated that annual species had smaller pollen compared to perennials. However, in the present studies all the perennial species had medium to large pollen with the exception of *M. macrorhiza* which had smaller pollen grains. Wang et al. (2013) also reported almost similar sized pollen in *Cephalorrhynchus macrorhiza* (now *Melanoseris macrorhiza*). In addition, special importance was also paid to perforation of tectum for the differentiation of species within each pollen type. For instance, dense perforation on the equatorial and polar regions of *M. macrorhiza*, *M. alii* and *M. decipiens* were easily segregated from the other species of *Melanoseris*. However, due to significant differences in their pollen size these species fell into pollen type I (*M. decipiens*) and pollen type II (*M. macrorhiza*, *M. alii*). While, in rest of the species perforation was only at the base of the echinae and between the echinae of the pollen. Skvarla et al. (1977) found that tectal elements in some Asteraceae were perforated (known as internal formina) and well-developed in some species, particularly at the base or in the lower portion of each echinae. Perforation at the base of echinae and between the echinae were considered as a good and important pollen characters for discriminating taxa in Asteraceae (Stix, 1960; Salgado-Labourian, 1982; Mesfin et al. 1995; Piner and Dinmez, 2000, Coutinho and Davis, 2007; Kodek et al. 2012 and Ceter et al. 2013). Waller (1976) concluded that complex exine sculpturing seemed to be associated with entomophily whereas pollen with smooth surface were characteristic of anemophilous plants, as pollen sculpturing give support to attach the pollen to the body of insects. Length of echinae was also a significant character for the segregation of studied species, such as echinae were upto 7.5 µm long in *M. rapunculoides* compared to upto 6.5

µm long echinae in *M. decipiens, M. brunoniana, M. stewartii* and *M. aitchinsoniana*. While in the rest of the species, the echinae were smaller and upto 5.0 µm long. Tomb et al. (1974) also utilized spine length as an important diagnostic character in Cichorieae. Wageniz (1976) stated that reduction of the spine length was an important evolutionary trend in the pollen morphology of Asteraceae. Wang et al. (2009) reported that the plesiomorphic state seemed to produce shorter spines of less than 3.0 µm high. Moreover, the size of polar area and number of centrally isolated echinae on the polar area also varied among our species. Polar area was smaller with 2–5 echinae (in *M. macrorhiza, M. alii* and *M. gilgitensis*), medium with 6–8 echinae (in *M. lessertiana* and *M. astorensis*) and moderately large with 12–15 echinae (in *M. brunoniana, M. stewartii and M. aitchinsoniana*). Wang et al. (2009) and Peng et al. (2013) also used the size of polar area and number of echinae to differentiate species and genera of the tribe Cichorieae. In *M. macrorhiza* the exine thickness was the lowest and the highest was in *M. rapunculoides* (Table 1). As far as evolutionary trend of the genus is concerned, the mixture of ancestral and derived character states are met in the pollen of *Melanoseris* such as small pollen (ancestral)-medium-large (derived), echinolophate (derived), trizonocolporate (ancestral) and oblate-spheroidal (derived), echinae 3.25–7.5 µm long (derived), polar area small-medium-large (derived).

A dendrogram resulting from the cluster analysis significantly supported the distinction of each pollen type. All the examined species were divided into Cluster-I and II (Fig. 6).

The Cluster-I (*Melanoseris stewartii*- type) represented the assemblage of 5 morphologically closely allied taxa (i.e., 3–8 or few flowered species with biseriate involucral phyllaries). Inferred from pollen morphology this pollen type was characterized by comparatively larger pollen grains (Table-1). However, occurrence of *M. decipiens* on the separate lineage with short branch showed variation in the pollen morphological characters among the species. The main difference was that the pollen were distinctly and densely perforated on the equatorial and polar axis. In contrast, perforations were confined to the lower portion of the echinae and between the echinae of the polar region in *M. brunoniana*, *M. aitchinsoniana* and *M. stewartii*. The present pollen morphometric data and cluster analysis congruent the recent phylogenetic analysis of Kilian et al. (2017) based on nrITS and plastid DNA tree. For instance, the Western himalayan species *M. decipiens* and *M. brunoniana* had same chromosomes number (2n = 16) and formed a well-supported sister clade to the polytomous main clade of J3 (Kilian et al., 2017). Moreover, *M. decipiens* was morphologically distinct from the other species by 6–8 florets per capitulum (v/s 5 florets per capitulum), linear and not trunacate cypselas (v/s oblong and truncate), shortly discolourous beak (v/s not beaked). Interestingly, the pollen and general (vegetative and floral) morphological characters in *M. brunoniana*, *M. aitchinsoniana* and *M. stewartii* were almost similar and showed considerable overlapping in the pollen characters (Table 1, 3a (Supplementary table). Slight differences were observed in the number of centrally isolated echinae and perforation of polar area. Therefore, the aforementioned species deeply nested with a small distance on the same cluster (Fig. 6). It was an evident from cluster analysis *M. aitchinsoniana* showed close affinities with *M. stewartii* by sharing almost same qualitative and quantitative pollen characters (Table 1) However, *M. aitchinsoniana* was also morphologically nearer to *M. brunoniana* as both had similar cypselas characters rather with *M. stewartii* (Abid et al. 2017). In this case, it was obvious that morphologically related species might not have similar pollen characters.

The Cluster-II (represented *Melanoseris alii* type) accommodated species having medium pollen grains excluding *M. macrorhiza*. Species belonging to this pollen type had capitula with many florets except in *M. rapunculoides* (few florets). However, difference was observed in the number of central echinae on the polar area therefore these species appeared into subclusters i.e., II-A (central echinae 6–8) and II-B (central echinae 2–5). *M. astorensis*, *M. lessertiana* and *M. rapunculoides* belonged to subcluster II-A, could be differentiated on the basis of the length of echinae, and exine thickness (see key to pollen types). The recent molecular studies of Wang et al. (2013) and Kilian

et al. (2017) showed that *M. lessertiana* formed a well-supported clade with *M. qinghaica* (not present in our area) instead of *M. astorensis* and *M. rapunculoides*. *M. astorensis* was not included in their phylogenetic studies so at present it was difficult to recognize the molecular relationship of this species. Moreover, *M. lessertiana* and *M. astorensis* had apparently close affinities in pollen and general morphological characters compared to *M. rapunculoides*. Moreover, *M. rapunculoides* clearly differed in number of pollen characters (such as length of echinae, thickness of exine and perforation of polar axis) from *M. astorensis* and *M. lessertiana*. *M. lessertiana* could be easily identified from the last two species by the following floral characters such as floral axis 10–15 cm long (rather than less than 10 cm long), phyllaries biseriate (rather than 3–4 seriate), 5 florets per capitulum (rather than 25–30 florets), cypselas upto 12 mm long with concolorous beak (rather than upto 8 mm long with discolorous beak). In this case, pollen morphological characters showed good correlation with general morphological characters.

Among the three species of subcluster II-B, *M. macrorhiza* occurred on a distinct branch but linked partially with *M. alii* and *M. gilgitensis* as most of the pollen variable were overlapping. However, the former species could be easily distinguished by smaller pollen grains (27.5–30.0 µm long and 31.0-32.5 µm broad) while in the remaining species pollen grains were medium (37.0–56.0 µm long and 46.0–62.0 µm in broad). Moreover, the presence of sharply pointed and acuminate echinae also distinguished *M. macrorrhiza* from rest of the species of the genus. Wang et al. (2013) included this species in their molecular studies and analyzed that *M. macrorhiza* formed a well-supported cluster with *M. violifolia* and *M. lessertiana* in the ITS phylogeny. Whereas in the plastid phylogeny all three species appeared in different clades (M-1, M-3 and M-4). Morever, Kilian et al. (2017) in their molecular analysis revealed that among the four terminal clades (or species group) of the main polytomy *M. macrorhiza* belonged to one of them (*Melanoseris macrorhiza* group of clade-J3).

M. alii and *M. gilgitensis* appeared on the same cluster with a short branch but clearly keyed out on the basis of pollen characters. In the former species (*M. alii*) equatorial and polar axis of the pollen were densely perforated and echinae with globose base. While in the later species (*M. gilgitensis*) equatorial and polar axis of the pollen lacked perforation and echinae with conical base. Moreover, these species clearly differed on the basis of vegetative and cypselas characters (Bano and Qaiser, 2015; Abid et al. 2017). In this case, pollen morphology showed well-supported relation with the gross morphology between the species.

It is concluded from the foregoing discussion that the present palynological results almost corroborated with the aforementioned molecular studies. The morphometric pollen data together with statistical analysis provided useful information for the delimitation of studied species of the genus. The present results also showed that morphologically closely related species may or may not have similar pollen.

Declarations

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COMPETING INTERESTS STATEMENT:

The authors have declared that no competing interest's statement exists.

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Figures

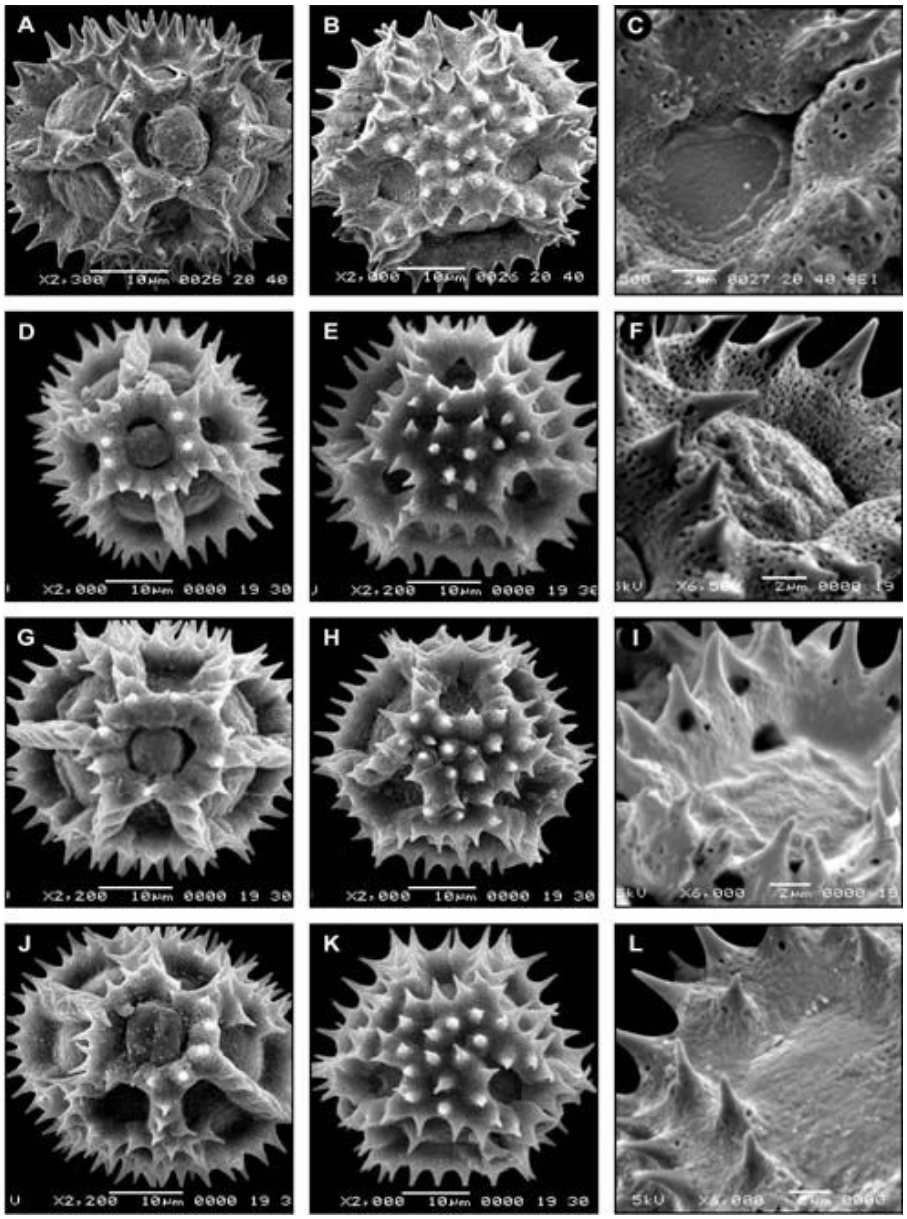

Figure 1

Scanning Electron Micrographs (SEM) of the pollen grains: *Melanoseris decipiens* var. *multifida*: **a** equatorial view, **b** polar view, **c** exine pattern. *Melanoseris brunoniana*: **d** equatorial view, **e** polar view, **f** exine pattern. *Melanoseris aitchisoniana*: **g** equatorial view, **h** polar view, **i** exine pattern. *Melanoseris stewartii*: **j** equatorial view, **k** polar view, **l** exine pattern (scale bar: c, f, i, l = 2 µm; a, b, d, e, g, h, i, k = 10 µm).

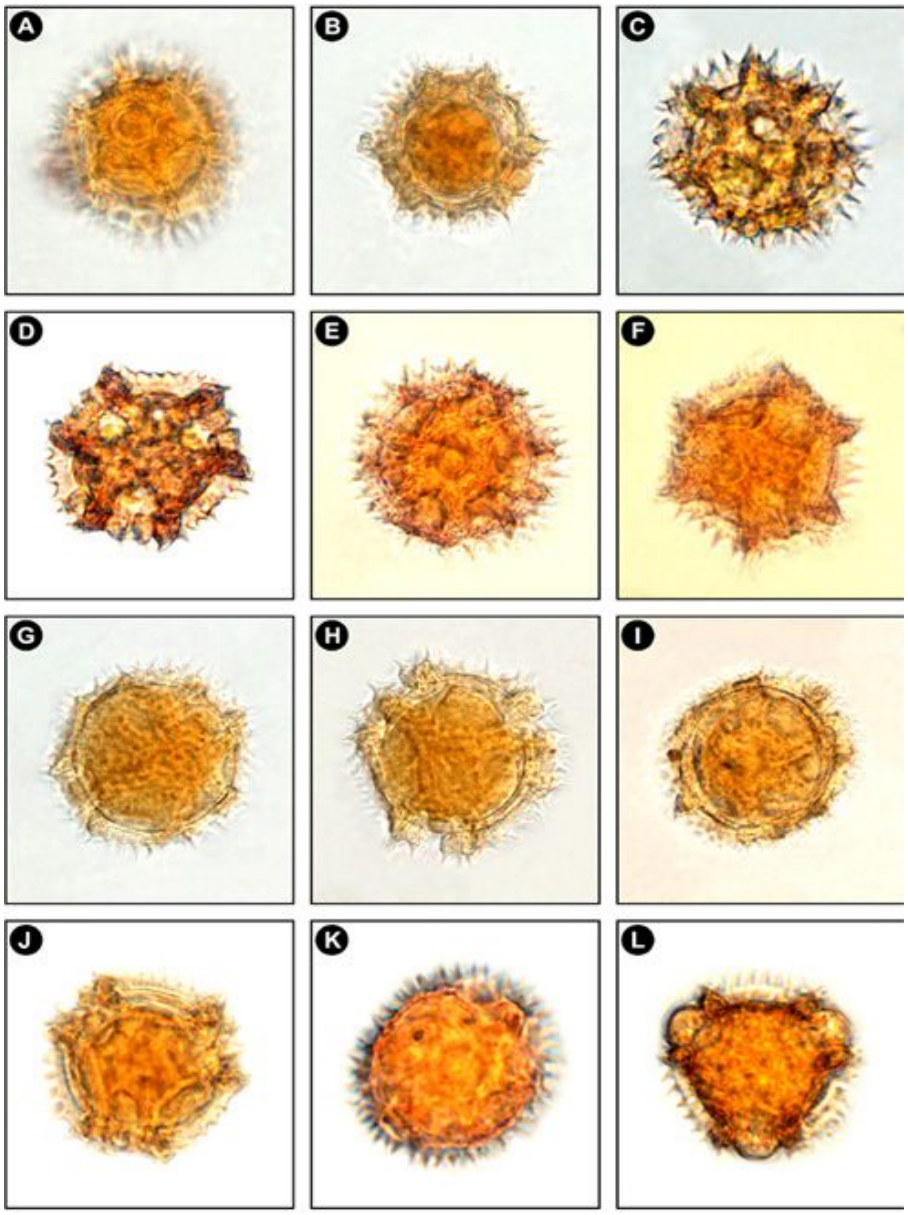

Figure 2

Light Micrographs (LM) of the pollen grains at 100 x: *Melanoseris rapunculoides*: **a** equatorial view, **b** polar view. *Melanoseris decipiens* var. *decipiens*: **c** equatorial view, **d** polar view. *Melanoseris decipriens* var. *multifida*: **e** equatorial view, **f** polar view. *Melanoseris brunoniana*: **g** equatorial view, **h** polar view. *Melanoseris stewartii*: **i**, equatorial view, **j** polar view. *Melanoseris aitchisoniana*: **k** equatorial view, **l** polar view.

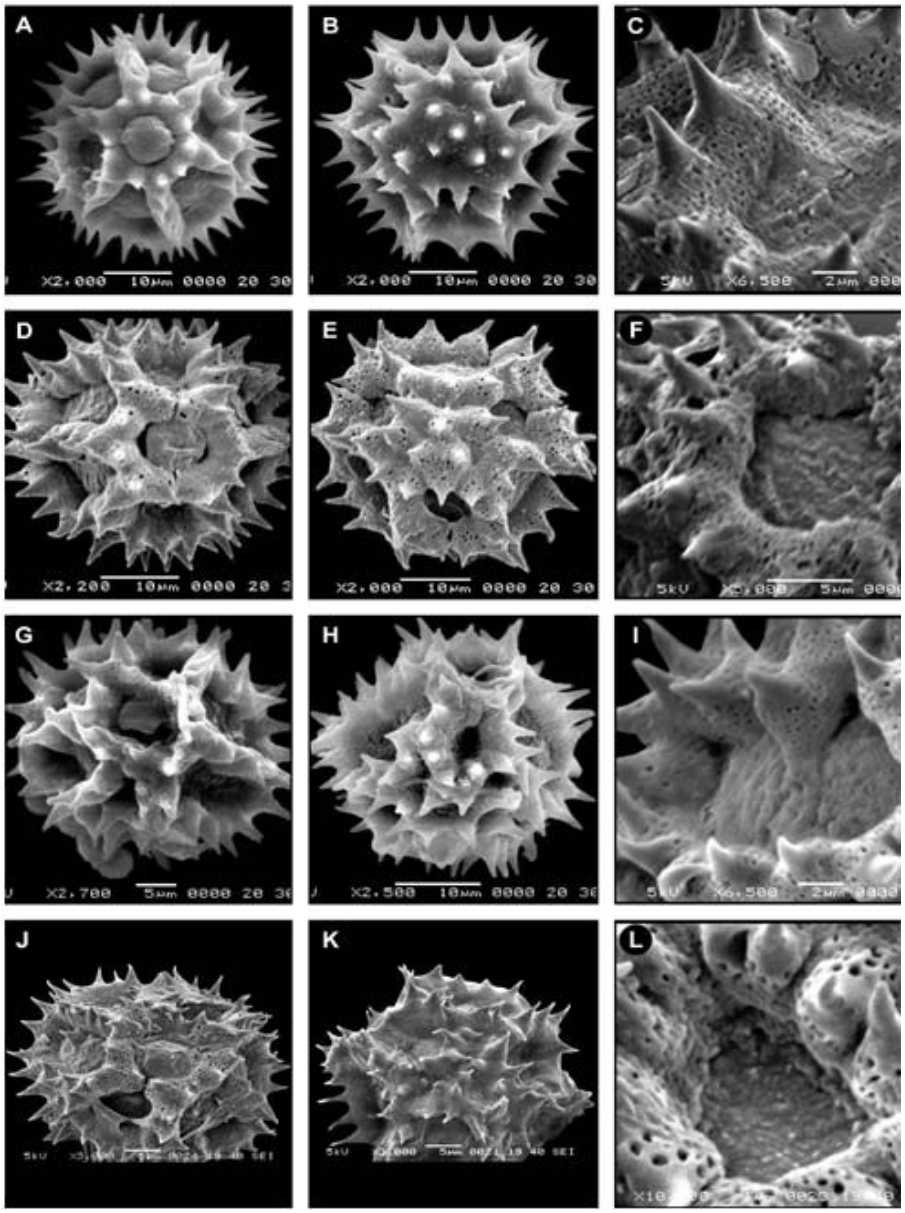

Figure 3

Scanning electron micrographs (SEM) of the pollen grains. *Melanoseris astorensis*: **a** equatorial view, **b** polar view, **c** exine pattern. *Melanoseris alii*: **d** equatorial view, **e** polar view, **f** exine pattern. *Melanoseris gilgitensis*: **g**, equatorial view, **h** polar view, **i** exine pattern. *Melanoseris macrorhiza*: **j** equatorial view, **k** polar view, **l** exine pattern (scale bar: c, l = 2 μm; a, f, l = 5 μm; b, d, e, g, h, j, k= 10 μm).

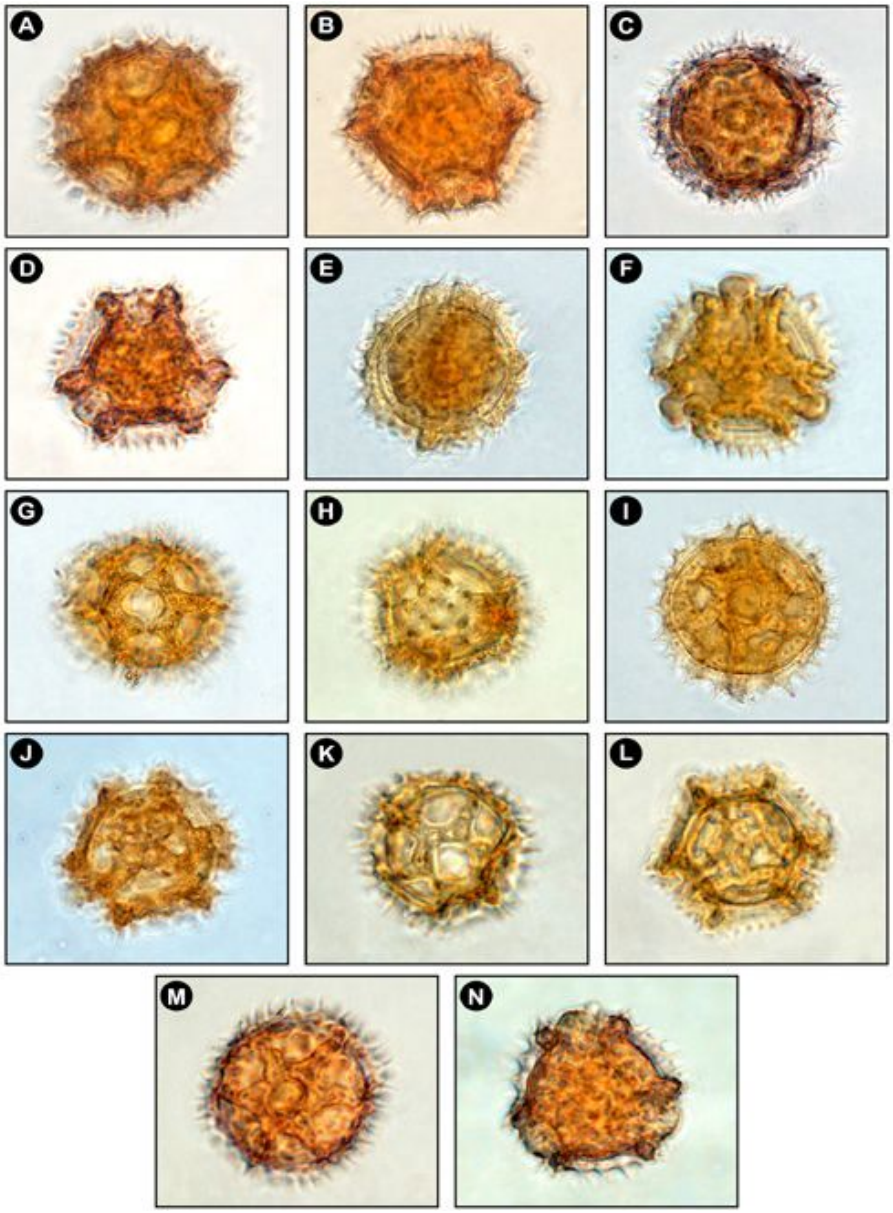

Figure 4

Light Micrographs (LM) of the pollen grains at 100 x: *Melanoseris alii*: **a** equatorial view, **b** polar view. *Melanoseris gilgitensis*: **c** equatorial view, **d** polar view. *Melanoseris macrorhiza*: **e** equatorial view, **f** polar view. *Melanoseris lessertiana* var. *lessertiana*: **g** equatorial view, **h** polar view. *Melanoseris lessertiana* var. *lyrata*: **i** equatorial view, **j** polar view. *Melanoseris lessertiana* var. *dentata*: **k** equatorial view, **l** polar view. *Melanoseris astorensis*: **m** equatorial view, **n** polar view.

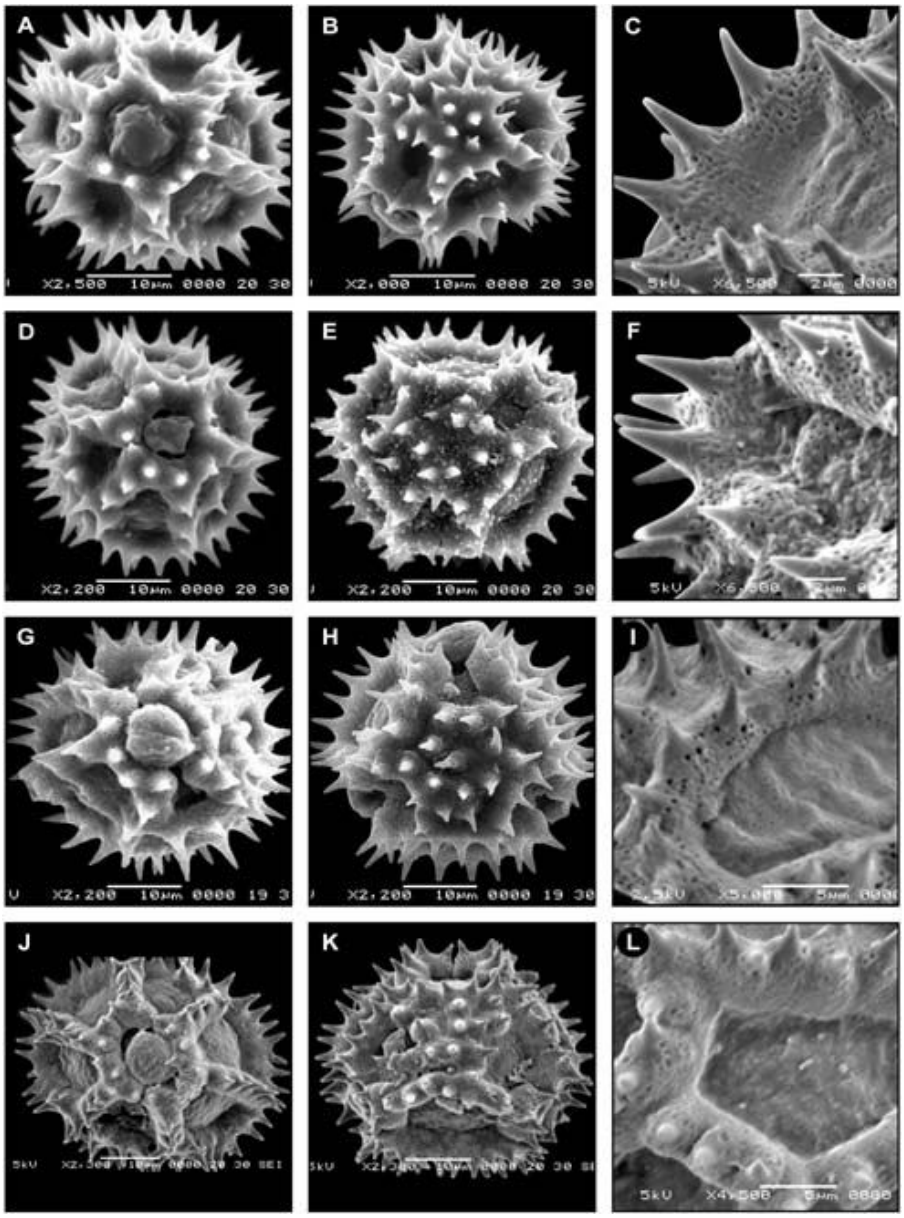

Figure 5

Scanning electron micrographs (SEM) of the pollen grains: *Melanoseris lessertiana*: **a** equatorial view, **b** polar view, **c** exine pattern. *Melanoseris lessertiana* var. *lyrata*: **d** equatorial view, **e** polar view, **f** exine pattern. *Melanoseris rapunculoides*: **g** equatorial view, **h** polar view, **i** exine pattern. *Melanoseris decipiens* var. *decipiens*: **j** equatorial view, **k** polar view, **l** exine pattern (scale bar: c, f = 2 μm; i, l = 5 μm; a, b, d, e, g, h, j, k = 10 μm).

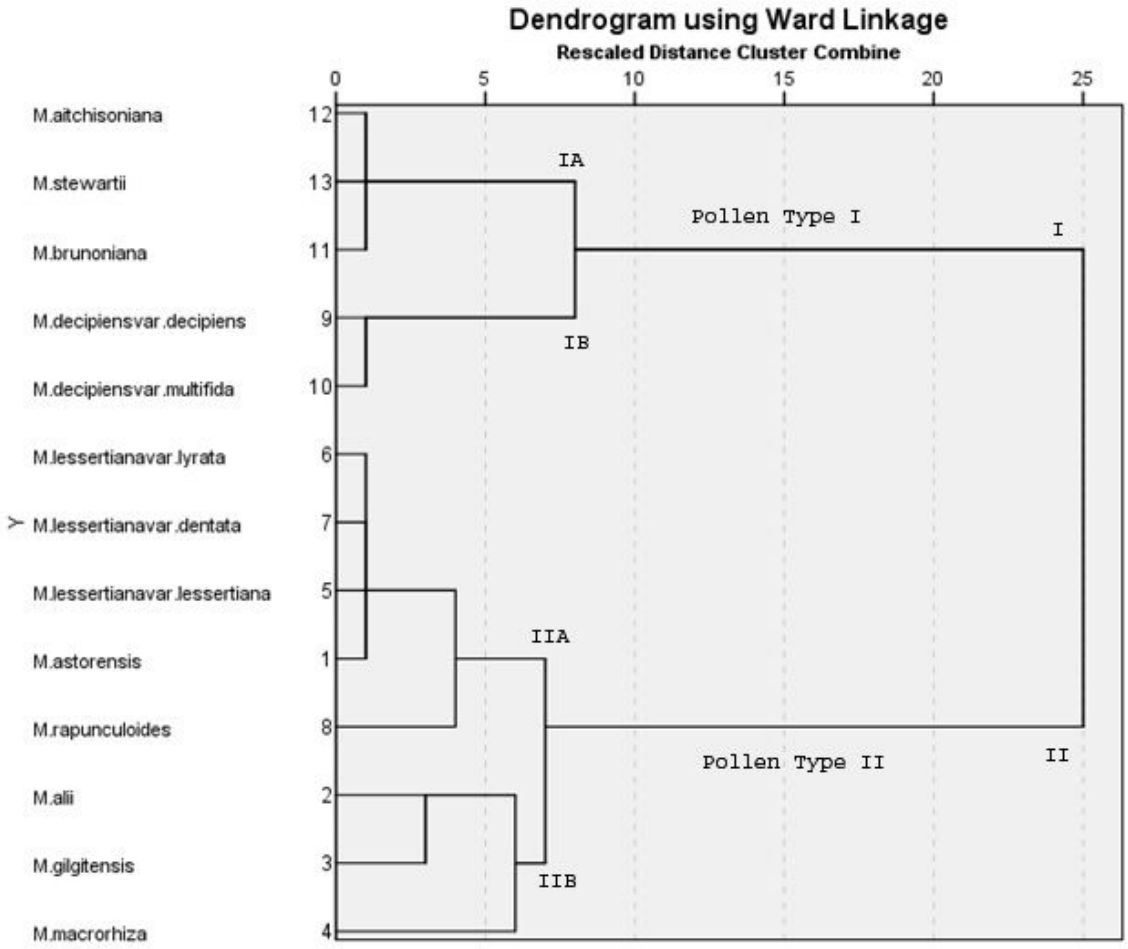

Figure 6

Dendogram showing the relationship of the studied species of *Melanoseris* Decne.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- APPENDIXI.docx