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Pollen Morphology of *Erythronium* L. (Liliaceae) and its Systematic Relationships

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ABSTRACT

Pollen morphology of three genus of *Erythronium* was studied by the Light Microscopy (LM), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). Sulcus long reaching the ends of the grains, with operculum (E. giganteum, E. sibiricum) or without it (E. caucasicum). With surface latticed ornamentation and large lattice, thickness of muri and size of Lumina in *E. sibiricum* are widely varied. Also, most palynomorphological characteristics of the data transmission electron microscopy (TEM) showed no strong differences between E. caucasicum and E. sibiricum, , but these species are well distinguished from *E. giganteum* according to ectexine thickness (thickness of the tectum and the foot layer), shape and diameter of the caput, height and width of the columella.
KEY WORDS: Caput; Columella; Exine ornamentation; intine; Microrelief; Pollen grain; Tectum.

INTRODUCTION

Takhtajan, 1987 indicated that the genus of *Erythronium* in Tribe *Tulipeae* is of the Liliaceae family. Different sources have considered the species number of this genus varied from 24-30. Baranova (1999) introduced 24 species for this genus, of which 20 species were spread in North America.

Allen et al. (2003) examined the genus of Erythronium, Amana, and Tulipa using the DNA sequences from the chloroplast gene matK and the internal transcribed spacer (ITS) of nuclear ribosomal DNA.

Palynomorphological characters of 20 different pollen species of *Erythronium* were evaluated by different researchers (Ikuse, 1965; Beug (1963); Radulescu, 1973; Nakamura, 1980; Schulze, 1980; Kuprianova, 1983; Takahashi (1987); Kosenko, 1991b, 1992, 1996, 1999; Maassoumi, 2005a, 2005b, 2007). In this study, shape, size, and surface ornamentation of pollen from different species were presented. In the studies which were performed by the Electron Microscopy Scanning (SEM) on the species of this genus, there was an operculum in some species (Takahashi (1987); Kosenko, 1991b, 1996, 1999; Maassoumi, 2005a, 2007).
Takahashi (1987) examined the pollen grains of 19 *Erythronium* species and showed that all these species had monad mode, and the dayad mode is only in the *E. oregonum* Applegate species.

The internal structure of the surface ornamentation can be seen by the Electron Microscopy, especially the Transmission Electron Microscopy (TEM). The shape and thickness of Tectum, shape and diameter of Caput, height and width of Columella, thickness of Foot Layer, the presence or absence of Endexine, the thickness of Intine in the Sulcus zone and below the Exine in a zone without the Sulcus, and the position of various layers of Intine and their thickness, are the evaluable features in the diagnosis of various levels of taxon (subspecies, species, section, subgenus, genus, tribe, subfamily, and family).

The aim of this study is the morphological evaluation of three pollen of *Erythronium* using the Light Microscopy (LM), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) and especially the comparison of their ultrastructure with each another.

MATERIALS AND METHODS

Pollen grains of 3 species were investigated. Pollens for this study were taken from the collections in the Main Botanical Garden Moscow (MHA), Russia; Komarov Botanical Institute of the Russian Academy of Science, St. Petersburg (LE); Biological Faculty of Lomonosov Moscow State University (MW), Russia; In addition fresh material obtained from the Botanical Garden of MGU, Moscow, Russia.

For LM observations, pollens were acetolysed following the technique of the Erdtman (1960) and mounted in glycerin jelly. Slides were prepared for LM by mounting pollen in glycerin jelly. Size measurements were taken based on 25 pollen grains; the values of P (polar axis length) and E (equatorial diameter) were measured and the P/E ratios were calculated. Measurements were recorded using both a 40 × objective, and a crossed micrometer eyepiece graticule.

These evaluations were performed via a Light Microscope of "MIKMED-1 Var. 2-20. LOMO (Russia)" model.

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The pollen data for all species examined are summarized in.

For SEM studies unacetolysed pollen grains were attached to aluminum stubs and sputter coated (Eiko IB-3 ION COATER) with Au/Pb and examined under Hitachi S-405 A scanning electronic microscope (Fig. 1)

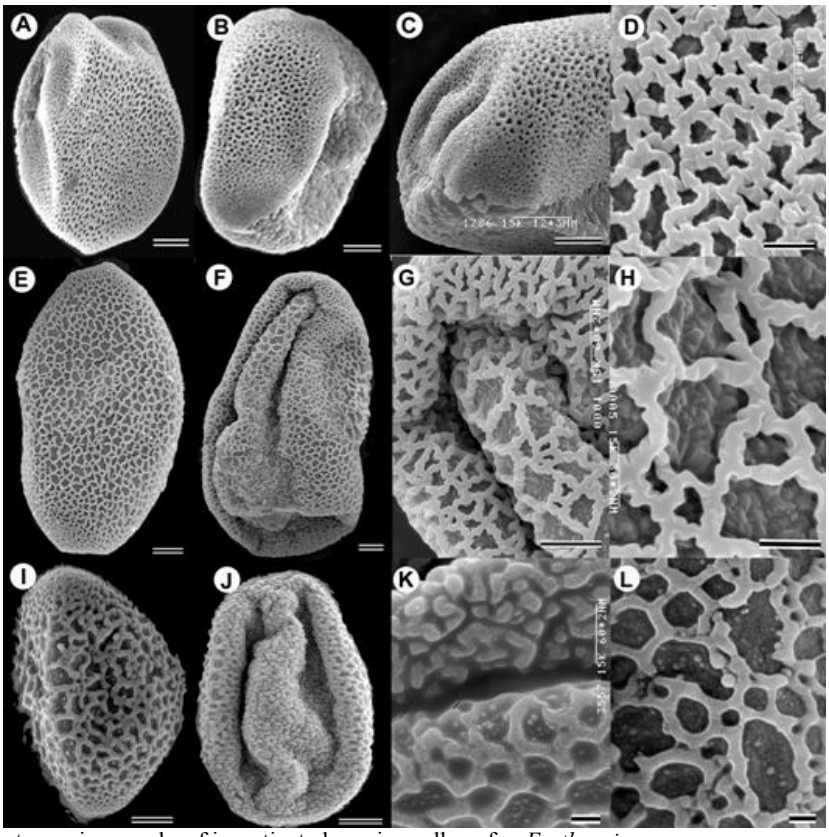

Fig. 1. Scanning electron micrographs of investigated species pollen of *Erythronium*:
A-D *E. caucasicum* Voronov:
(A) In distal view;
(B) In view where the sulcus is seen;
(C) Tuberculate ornamentation of sulcus membrane at the distal surface and latticed ornamentation at the proximal surface;
(D) Latticed ornamentation with high magnification;
(E) The general view of pollen grain from the proximal surface;
(F) The general view of pollen grain from the distal surface with operculum;
(G) The view of some part of pollen grain from the distal surface with higher magnification; and the latticed ornamentation of operculum surface and the sulcus edge are determined;
(H) The macroreticulate ornamentation with high magnification;
(I) The general view of pollen grain from the proximal surface;
(J) The general view of pollen grain from the distal surface of the operculum;
(K) The view of some parts of pollen grain from the distal surface with higher magnification, and the latticed ornamentation of operculum surface and sulcus edge are determined;
(L) The large latticed ornamentation with more or less discontinuous Muri and varied Lumina in terms of size.
Scale bar: (A, B, C, E, F, I, J) = 10 µm, (G) = 4 µm, (D, K, L) = 2 µm, (H) = 2 µm.

For TEM pollen grains were fixed in 1% osmium tetroxide and stained with a solution of Uranyl-acetate in 70 % alcohol and lead citrate (Reynolds, 1963), then dehydrated in an ethanol series and embedded in Epon mixture(Epon 812, Epon Härter DDSA, Epon Härter MNA) according to the standard method of Weakley (1977). Ultrathin sections of the pollen grains were obtained by a glass knife (LKB 8800 Ultratome III), and lead citrate ((Reynolds, 1963). Observations were made using a JEOL JEM_100B transmission electronic microscope. All measurements on TEM micrographs have been made in standard vision in several pollen grains. Height and width of caput thickness and its form, length of columella (from under foot layer until down of caput) and width of columella, foot layer thickness in ultrastructural exine of all pollen grains in *Erythronium* were measured (Fig. 1-3)
Descriptive terminology follows Kremp (1967) and Punt et al., (2007)

RESULTS

All considered species are monad, heteropolar, bilaterally symmetrical, monosulcate at the level of distal, boat-shaped, and flattened-spheroidal, the outline is as the oblate-spheroidal form from the perspective of polar view and as an elliptical form from the perspective of equator view, and Sulcus reaches the ends of the grains.

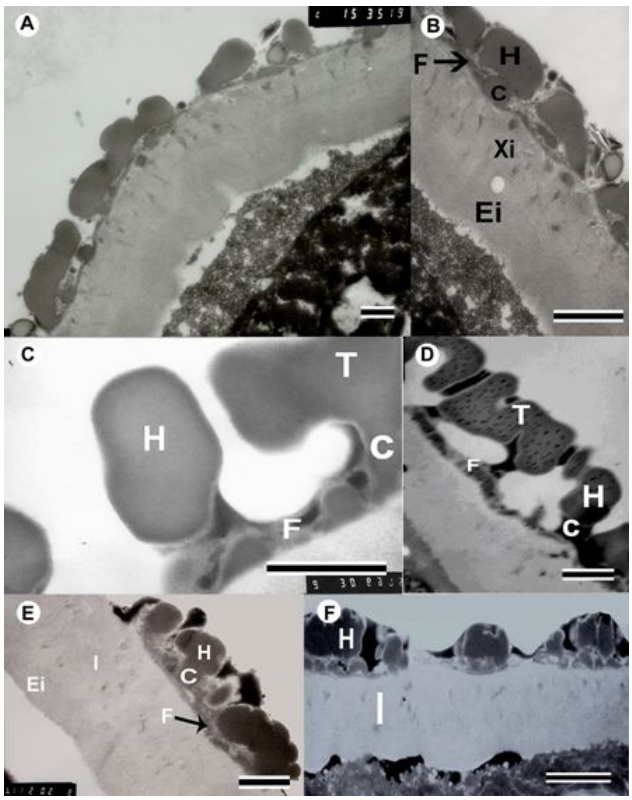

Fig. 2 Transmission electron micrographs of Sporoderm in pollen of investigated species of *Erythronium*: (A, B) *E. caucasicum* Voronov; (C,D) *E. giganteum* Lindl. (*E. grandiflorum* Pursh); (E, F) *E. sibiricum* (Fisch. & Mey.) Krylov
(C) = columellae, (H) = caput, (Ei) = endintine ,(F) = foot layer; (I) = intine, (T) = tectum , (Xi) = exintine, Scale bar: (A- F) = 1 µm

Sporoderm is made of Tryphine, Ectexine, and Intine. Tryphine evenly fills the muri of network.

Erythronium caucasicum Voronov

Pollen grains large, monosulcate without operculum (Fig. 1A, 1B, 1C). The length of the polar axis of 50.0 - 65.4 - 75.0 mm, equatorial diameter 75.0-89.3 - 110.0 mm; the ratio P / E = 0.77.

Exine ornamentation are at a reticulate type; the ornamentation around the Sulcus have the pitted type; muri of networks have continuous mode, thickness of Network muri is equal to 0.5µm-0.8µm; the Lumina size is equal to 0.3-3.0 µm; and the shape of Lumina varied from round to elliptical, rhombic, rounded-angular, much elongated, and round-twisting (Tab. 1, Fig.1D)

Ectexine (with 0.8-1.3 µm thickness) include: Tectum (with 0.7-0.9 µm thickness), columellae (length of columellae is 0.2-0.5 µm and the width of columellae is 0.4-1.1 µm), and foot layer (0.1-0.2 µm thickness). The shape of caput is flattened-spheroidal to rectangular with 0.5-0.7 µm thickness and 0.8-1.4µm width. Tiny microreliefs on the surface of the muri are smooth (Tab. 2, Fig. 2A, 2B, 3C).

Erythronium giganteum Lindl. (*Erythronium grandiflorum* Pursh)

Pollen grains very large, monosulcate with operculum (Fig. 1E, 1F). The length of the polar axis of 62.5 - 80.3 - 92.5 mm, equatorial diameter 97.5 - 135.3 - 163.8 microns, the ratio P / E = 0.59.

The thickness of Intine below the Exine in a zone with no sulcus is equal to 1.0 µm (Fig. 2A, 2B, 3C) and the thickness in the sulcus zone is equal to 4.4 µm (Fig. 3A, 3B). Intine is composed of three layers, which are: Exintine which is a layer with about 0.8-2.5 µm thickness with high electron density and a lot of channels inside; Mesintine, a layer with about 0.4 -2.4 µm thickness with low electron density, and Endintine, which is a layer with 0.8 -1.3µm thickness with a more electron density (Tab. 2, Fig. 2B, 3B, 3C).

The Exine ornamentation is in macroreticulate form, the ornamentations around the sulcus are latticed, the muri of networks has the continuous mode, the muri thickness of networks is equal to 0.6-1.6 µm, and the Lumina size is equal to 2.2-4.6 µm (Tab. 1, Fig.1G, 1H).

Ectexine (with 1.5-2.3 µm thickness) includes: Tectum (with 0.7-1.5 µm thickness), Columellae (the length of columellae is 0.3-0.5 µm and the width is 0.3-0.7 µm), and Foot Layer (with 0.2-0.5µm thickness).
The caput has the flattened-spheroidal to peroblate shape with 1.2 - 1.6 µm thickness and 0.8 - 1.6 µm width. The microreliefs on the surface of the muri are almost smooth (Tab. 2, Fig 2C, 2D).

The thickness of Entine below Exine, in an area with no sulcus is equal to 1.4-2.0 µm (Fig. 3D, 3E). Entine is composed of two layers, which are: Exintine, which is a thin layer with 0.6 - 0.8µm thickness and low electron density; and Endintine which is a thick layer with 1.2 - 1.4 µm thickness and more electron density.

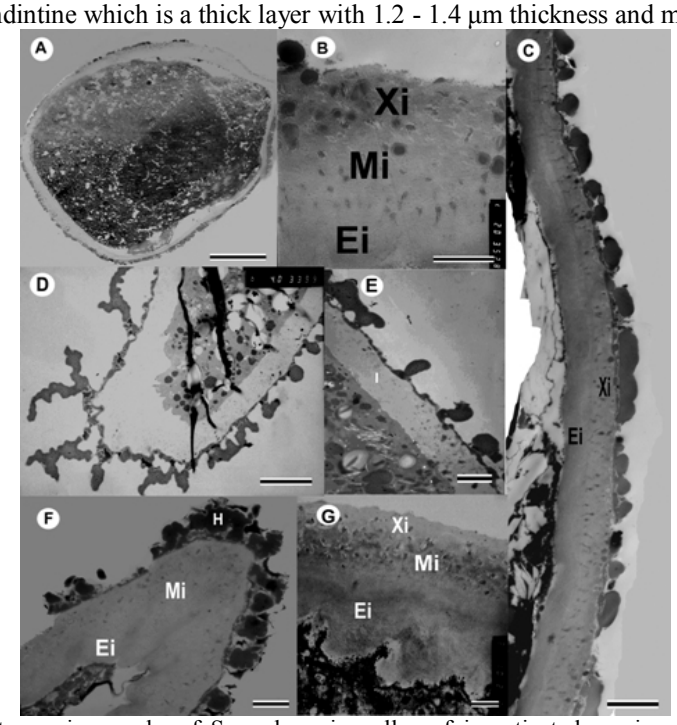

Fig. 3 Transmission electron micrographs of Sporoderm in pollen of investigated species of *Erythronium*: (A- C) *E. caucasicum* Voronov; (D, E) *E. giganteum* Lindl. (*E. grandiflorum* Pursh); (F, G) *E. sibiricum* (Fisch. & Mey.) Krylov
(H) = caput, (Ei) = endintine, (I) = intine, (Mi) = mesintine , (Xi) = exintine,
Scale bar: (A) = 10 µm, (B, F, G) = 1 µm, (C, E) = 2 µm, (D) = 20 µm

Erythronium sibiricum (Fisch. & Mey.) Krylov

Pollen grains large, monosulcate with operculum (Fig. 1I, 1J). The length of the polar axis of 41.3 - 47.8 - 60.0 mm, equatorial diameter 66.3 - 77.8 - 97.5 microns, the ratio P / E = 0.61.

The Exine ornamentation is in reticulate form, the ornamentations around the sulcus is latticed, the muri of networks has the discontinuous mode, the muri thickness of networks is equal to 0.1-2.1 µm, and the Lumina size is equal to 0.5–11.3µm (Fig.1K, 1L).

Ectexine (with 0.8 - 1.3 µm thickness) includes: Tectum (with 0.4-0.8 µm thickness), Columellae (the length of columellae is 0.1-0.4 µm and the width is 0.2-0.5 µm), and Foot Layer (with 0.1-0.3µm thickness).
The caput is flattened-spheroidal to mushroom-shaped, with 0.6-0.8 µm thickness and 0.7 - 0.9 µm width. The microreliefs on the surface of muri are rough and tubercular (Fig 2E, 2F).

The thickness of Entine below Exine, in an area with no sulcus is equal to 1.5-2.0 µm (Fig. 2E, 2F), the thickness in an area with sulcus is equal to 0.4 µm (Fig. 3F, 3G). Entine is composed of three layers, which are: Exintine, which is a thin layer with 0.2 - 1.0µm thickness and low electron density; Mesintine which is a layer with 0.8 - 1.5µm thickness and more electron density, it is fibrillar with lots of small channels inside; Endintine which is a thick layer with 2.5 - 3.0 µm thickness and more electron density (Fig. 3F, 3G).

DISCUSSION

Allen et al. (2003) indicated in their phylogenetic investigations that three genus including *Erythronium*, *Tulipa,* and *Amana* were in a branch, and the pollen similarities showed their resemblance (Maassumi 2005b; Kosenko, 1992, 1999). Maassoumi (2005a) showed that the European species of *E. dens-canis* L. is similar to *Amana latifolia* (Makino) Honda in terms of shape of pollen and surface pattern of Exine.

In addition, by the Transmission Electron Microscopy (TEM) on studied samples *(E. caucasicum* Voronov, *E. giganteum* Lindl., *E. sibiricum* (Fisch. & Mey.) Krylov.), and evaluating the micrographs provided by Takahashi (1987) about the species of *E. californicum* Purdy, *E. japonicum* Decne, *E. klamathense* Applegate, *E. montanum* S. Watson, *E. rostratum* W. Wolf, it can be found that these species are different in

terms of thickness Exine (tectum thickness, foot-layer thickness, the height and width of the columella), the shape and diameter of Caput, and the microrelief of sculptural elements. The shape of Caput in American species *E. klamathense* Applegate is similar to *E. caucasicum* Voronov, and also the shape of Caput in *E. montanum* S. Watson is similar to *E. giganteum* (*E. Grandiflorum Pursh*).

The obtained data indicate the similarity of two species *E. caucasicum* and *E. sibiricum* in terms of Ectexine thickness and the Tectum to foot layer ratio (T/F) (Tab. 2), and different layers of Entine (Fig. 3B, 3G). In addition, these two species are different in terms of Exine ornamentation and operculum, muri thickness, Lumina size (Tab. 1, Fig 1), microreliefs on the surface of the muri, shape and thickness of caput, and the height and width of columellae (Tab. 2, Fig. 2A, 2B, 2E, 2F).

According to the obtained data, the height of columellae in the studied species *(E. caucasicum* Voronov, *E. giganteum* Lindl., *E. sibiricum* (Fisch. & Mey.) Krylov) was low (0.1-0.5 µm) (Tab. 2, Fig. 2). Moreover, despite the fact that Takahashi (1987) considered the *E. japonicum* Decne species with no columella, the TEM micrograph of this species showed short columellae. Therefore, the short columella is a characteristic of *Erythronium* genus.

In addition, lack of Endexine in *Erythronium* indicates its similarity to the genus *Tulipa, Amana,* and *Gagea* (Maassoumi, 2005a).

Moreover, Allen et al. (2003) placed two species including *E. japonicum* Decne and *E. caucasicum* Voronov in closed branches by drawing the evolution of *Erythronium* species in terms of morphological features, and the pollen morphological data also showed the similarity of these two species. The similarities included shape of Caput, the low height of Columella, and high T/F ratio. The T/F ratio for the *E. japonicum* Decne species which is calculated by the micrograph provided by Takahashi (1987) and for *E. caucasicum* Voronov is almost equal to each other (T/F=6). In addition, by evaluating the T/F ratio using the micrographs provided by Harly (2003) about the *Tulipa,* and also this ratio in the sections and subgenus of studied species in this genus (Maassoumi, 2008) it can be observed that the obtained ratio is close to this ratio in studied *Erythronium* species.

Kosenko (1990, 1996) indicated that the operculum in some *Tulipa* species is as a valuable trait in terms of evolution, but Kosenko (1991b, 1996, 1999) has not considered this trait for *Erythronium.* However, Kosenko (1991b, 1999) showed that *E. caucasicum* Voronov can be with operculum or without it. While, in the pollens of this species, studied by Maassoumi (2007), the operculum was not seen. Also, in this study the operculum is observed for *E. sibiricum* (Fisch. & Mey.) Krylov, but Kosenko (1991b, 1999) has not reported the operculum for this species.

According to the Pollen characteristics among different genus of Liliaceae Juss Family, the *Erythronium* genus shows advanced features (Maassumi 2005b). Moreover, the studied species of *E. giganteum* Lindl is similar to type I- Martagon with *Lilium* genus in terms of some pollen traits including the size, and Exine ornamentation (Baranova, 1985). In addition, our data on the TEM data and Kosenko (1992) showed that the Caucasian *E. caucasicum* observed similarity with *Fritillaria camschatcensis* (L). Ker. Gawl.

Conclusions

The analysis of the results obtained show, that endexine in the investigated species of *Fritillaria* is very thin or absolutely non-existent.

The form and diameter of columella caput can be used as a good pollen-morphological characteristics for semitectate type of tectum.

Attributes of ultrastructure have not shown any correlation with subdivision into subgenera *Fritillaria*. However still there is not enough data from TEM for final conclusions.

The available Palynological data provide evidence for the heterogeneous character of the genus *Korolkowia* with respect to the exine ornamentation.

Appendix

Specimen examined

The following specimens were included in the study:

Erythronium caucasicum Voronov Russia: Krasnodar region, Krasnaya Polyana, 05/15/1966, I.L Krylov. *Erythronium giganteum* Lindl. USA: Oregon, in April. 1880, Thomas P. Howell. *Erythronium sibiricum* (Fisch. & Mey.) Krylov. Russia: Moscow, Moscow State University Botanical Garden, 06.06.2002, EV Klyuyko

FOOTNOTES

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