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POLLEN MORPHOLOGY OF SOME BALKAN SPECIES FROM GENUS *OXYTROPIS* DC (FABACEAE)

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Summary: The genus *Oxytropis* DC is represented in the Balkan flora with ten species, among them local endemics and relics. Pollen morphology was observed with scanning electron microscopy (SEM), transmission electron microscopy (TEM) and light microscopy (LM). The aim was to examine pollen morphology of *Oxytropis* species growing on the Balkan peninsula as well as to discuss and compare species similarities and their relationships. The results showed that pollen morphology of the selected species was rather homogeneous. The pollen grains were 3-zonocolporate, elliptic or rectangular in equatorial view and triangular-obtuse in polar view. The endopori and ectocolpi varied in shape and dimensions. The taxa could be divided in two groups on the basis of the P/E ratio (sub-prolate and prolate) and ornamentation (perforate and reticulate). In some species the polar area and margins of the colpi were frequently differentiated from the mesocolpium, being with a complete smooth tectum. The similarities and relationships between the species support previous taxonomical decisions.

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Key words: Balkans; Fabaceae; *Oxytropis*; pollen morphology.

INTRODUCTION

The genus *Oxytropis* DC comprises about 300 species distributed in Eurasia and North America, most numerous in Central Asia (Polhill and Raven, 1981) which is considered the center of its origin (Vassilczenko, 1965). *Oxytropis* DC. demonstrates close relations in morphology to *Astragalus* L. and both genera belong to the tribe Galegeae (Broonn) Torrey & Gray, subtribe Astragalinae (Adans.) Benth. of family Fabaceae (Polhill and Raven, 1981; Lock and Schrire, 2005).

In Flora Europaea (Leins and Merxmuller, 1968) the genus *Oxytropis* is presented by 24 species, a lot of them occurring in the Pyrenees, Alps, Carpathians and mountains on the Balkan peninsula. There are several *Oxytropis* species in the Balkans (Leins and Merxmüller, 1968; Greuter et al., 1989), many of them being local endemics and relics. In these areas recently species complexes have been found sometimes

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united in aggregates (Greuter et al., 1989) with complicated taxonomy and not so clear origin. Recently new molecular methods and autecological observations have been applied for examination of the relationships between some *Oxytropis* species (Kozuharova et al., 2007, 2012) and between the morphologically close genera *Astragalus* and *Oxytropis* (Sanderson and Wojciechowski, 1996; Wojciechowski, 2003; Zarre and Azani, 2012).

Data on pollen morphology of *Oxytropis* species are available from some regional pollen morphological studies (Pavlova and Berge, 1994; Perveen and Qaiser, 1998; Ceter et al., 2013). and in several general surveys of the family (Erdtman, 1966; Ferguson and Skvarla, 1981). Pollen is ascribed in general to *Astragalus* - type by Faegri and Iversen (1989) and Moore et al. (1991). The basis for such a conclusion is the 3-zonocolporate pollen and the reticulate ornamentation of the exine. This primary description of the pollen morphology was complemented by Beug (2004) who referred the studied *Oxytropis* species to *Astragalus*-type together with pollen of *Astragalus*, *Colutea*, *Melilotus* p.p. and *Ononis* species.

The main objective of this work was to examine the pollen morphology of *Oxytropis* species growing on the Balkan peninsula as well as to discuss and compare species similarities and their relationships.

MATERIALS AND METHODS

Plant sampling

Pollen from taxa *O. pilosa* (L.) DC, *O. campestris* (L.) DC, *O. urumovii* Jav., *O. purpurea* (Bald.) Markgraf, *O. prenja* (G.Beck) G. Beck in Reichenb., *O. halleri* subsp. *korabensis* (Kummerle & Jav.) Chrtek & Chrtkova, *O. lapponica* (Wahlenb.) J. Gay, *O. neglecta* Ten., *O. dinarica* (Murb.) Westtst. and *O. kozhuharovii* Pavlova, Dimitrov & Nikolova was collected from different mountain areas on the Balkans and the voucher specimens were deposited in the Herbarium of the University of Sofia (SO) and the Institute of Biodiversity and Ecosystem Research (SOM) (Table 1). The plant nomenclature used follows Greuter et al. (1989).

Mixed samples from minimum five flowers of at least five individuals for each taxon were collected for LM, TEM and SEM analyses. The pollen samples were prepared following Erdtman (1966), Skvarla (1966) and Reinold (1963). Measurements and pollen descriptions were made on acetolyzed pollen, prepared in the standard way (Erdtman, 1966). For LM slides were prepared by mounting the pollen in glycerol gelly. The observations were made with OLYMPUS BX-51 microscope under E40, 0.65 and oil immersion (E100, 1.25), using 10x eye piece. For each pollen taxon 25 measurements were carried out on 7 features: polar diameter (P), equatorial diameter (E), colpus length (L), porus length (Plg), porus width (Pls), apocolpium (A) and mesocolpium (M). The P/E ratio was calculated in order to determine pollen shape. The minimal, maximal and average values for each morphological feature are shown in Table 1.

For TEM analysis acetolysed exines were fixed with 2% osmium tetroxide, pre-stained with uranil acetate and embedded in epon-araldite (Skvarla, 1966). Sections were post-stained by Reinold method (1963).

Table 1. Taxa examined with measurements (μm) of the mean and ranges for the polar (P) and equatorial (E) axes, colpus length (L), porus length (Plg), porus width (Pls), mesocolpium (M), apocolpium (A) and the shape index (P/E).

Taxon, Locality and Voucher specimen	P	E	L	Plg	Pls	M	A	P/E
Ox. campestris	28.6-35.1	22.1-26.0	24.7-28.6	5.2-7.8	3.9-5.2	13.0-18.2	5.2-9.1	1.35
Bulgaria, Pirin Mt., SO 43809	(32.22)	(23.76)	(27.09)	(6.39)	(4.31)	(15.96)	(6.76)	
Ox. campestris	24.7-31.2	15.6-22.1	20.8-27.3	5.2-7.8	2.6-6.5	10.4-15.6	5.2-7.8	1.38
Bulgaria, Pirin Mt. SO 94637	(28.6)	(20.69)	(24.0)	(6.50)	(4.9)	(12.3)	(6.39)	
Ox. pilosa	23.4-29.9	16.9-22.1	19.5-24.7	5.2-7.8	2.6-5.2	9.1-14.3	6.5-7.8	1.43
Bulgaria, P. Skakavitza village SO 94 638	(27.61)	(19.34)	(22.56)	(6.2)	(4.39)	(12.53)	(7.33)	
Ox. pilosa	22.1-30.4	14.4-20.0	17.6-25.5	4.0-6.4	2.4-4.0	8-14.4	4,8-9.6	1.66
Romania, near town Cluj, SO 43872	(29.05)	(17.4)	(21.18)	(5.15)	(3.39)	(10.97)	(5.76)	
Ox. kozuharovii	24.0-28.8	18.4-22.4	20.8-25.6	5.6-8.0	3.2-4.8	12.8-17.6	4.0-7.2	1.31
Bulgaria, Pirin Mt. SO 98085	(27.36)	(20.92)	(22.5)	(7.0)	(4.12)	(15.23)	(5.53)	
Ox. urumovii	24.8-30.4	19.2-22.4	20.8-24.8	4.8-8.0	3.2-5.6	9.8-14.4	5.6-8.8	1.33
Bulgaria, Pirin Mt., SO 94930	(27.0)	(20.25)	(22.56)	(6.81)	(4.76)	(12.57)	(8.64)	
Ox. prenja	27.2-37.6	19.2-27.2	22.4-32.0	6.4-9.6	4.0-8.00	12.8-22.4	4.8-12.8	1.39
Hercegovina, Prenja Mt.SO 43868	(35.84)	(25.66)	(30.59)	(8.03)	(5.92)	(15.8)	(9.02)	
Ox. purpurea	28.8-31.2	16.0-20.8	17.6-24.0	4.8-8.1	3.2-4.8	6.4-14.4	5.6-9.6	1.36
Greece, Pindhos Mt.SO 92790	(28.19)	(19.26)	(21.36)	(5.7)	(3.74)	(11.64)	(6.08)	
Ox. halleri subsp. *korabensis*	25.6-34.4	19.2-24.0	22.4-28.8	5.6-9.6	4.0-8.0	11.2-17.6	5.6-9.6	1.42
Macedonia, Korab Mt.W 09908	(30.0)	(21.12)	(24.96)	(8.38)	(7.12)	(14.88)	(7.13)	
Ox. dinarica	27.2-33.6	18.4-22.4	19.2-26.4	4.8-8.0	3.2-6.4	9.6-14.4	6.4-11.2	1.48
Hercegovina, Velebit Mt., SOM113538	(30.65)	(20.6)	(21.47)	(6.49)	(4.57)	(12.6)	(9.05)	
Ox. neglecta	20.8-24.8	16.0-18.4	15.2-20.0	4.8-8.0	2.4-4.0	10.4-12.8	3.2-8.1	1.37
Croatia, SOM 48 232	(22.72)	(16.57)	(18.68)	(5.66)	(3.04)	(11.64)	(4.76)	
Ox. lapponica	24.0-28.8	17.6-20.8	17.6-24.0	4.8-6.4	2.4-4.8	9.6-12.8	4.8-8.0	1.40
Albania, SO 43883	(26.14)	(18.62)	(20.03)	(5.54)	(3.58)	(10.53)	(6.37)	

Pollen grains for SEM analysis were suspended in a drop of 95% ethanol coated with gold and examined with Jeol microscope JSM-5510. In general, the pollen terminology follows Faegri and Iversen (1989) and Punt et al. (1994, 2007).

Data Analysis

Univariate and multivariate statistical procedures were applied to examine variation among the populations on the basis of pollen-morphological measurements. Cluster analysis using Euclidean distance and Unweighted Pair Group Means Average (UPGMA) was used as computational criteria to determine the similarities between species (Fig. 1). Mean values of the pollen features for the species with more than one population were used.

Ordination by Principal Component Analysis (PCA) was also used to detect the main sources of variation within the data set, to display the results in a non-hierarchical way and to show the relationships between variables (Fig 2). Eigenvalues were extracted from the correlation matrix. PCA assumes linearity which simplifies the mathematical problem by restricting the set of potential bases and formalizing the assumption of continuity in a data set (Shlens, 2003). All statistics were performed using StatSoft – Statistica 7 program.

RESULTS

The pollen grains were 3-zonocolporate, prolate or sub-prolate (P/E varied between 1.31 and 1.66), with well defined endoapertures; elliptic or rectangular-obtuse in equatorial view (Plate 1, A-G); thickenings on the exine were observed in the aperture zone (Plate 1, J-K). Rarely 6-pantocolporate pollen grains were also found (Plate 1, I).

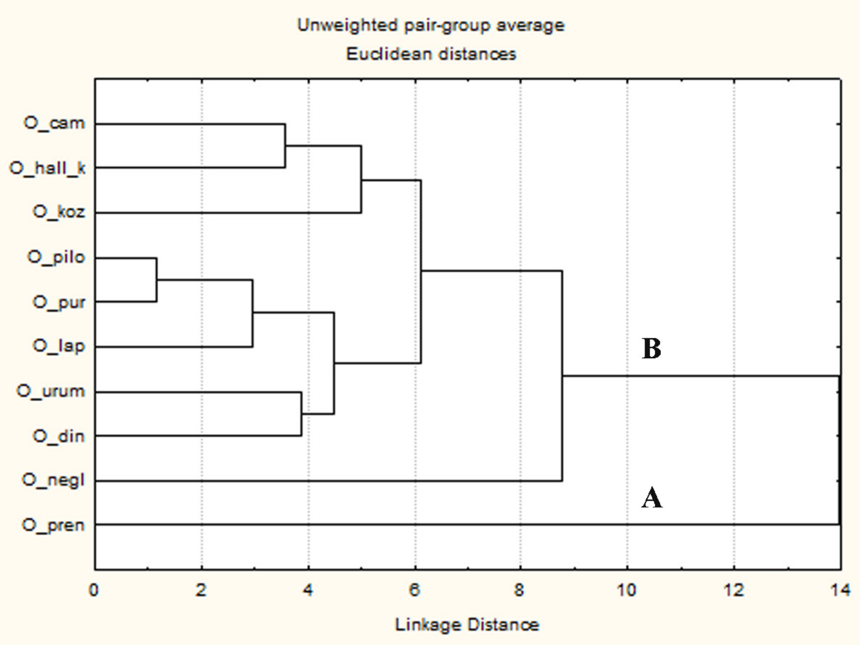

Figure 1. Cluster diagram showing the groups of similarity among the species investigated.

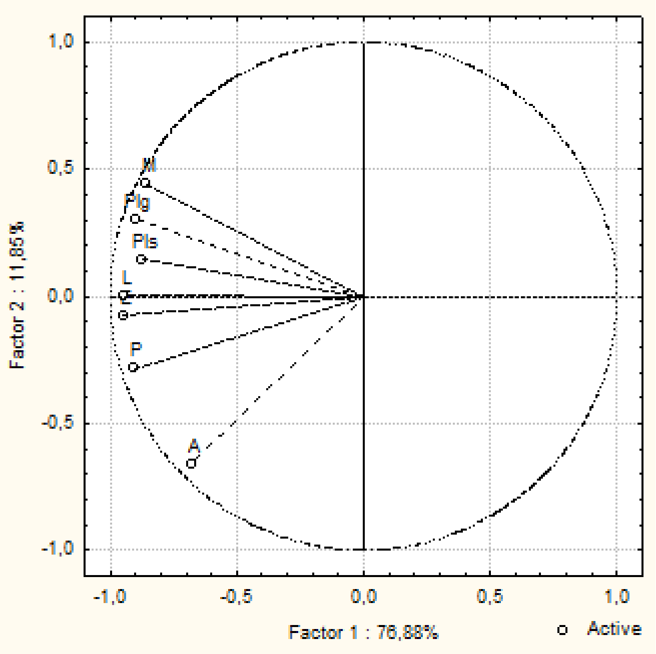

Figure 2. Principal Component Analysis plot variables (vectors). Length of the vectors is proportional to the strength of the correlations between variables and one of the PCs (factors). Variables codes as in the text.

The exine was thicker in the aperture area. Its structure involved a thin endexine, a foot layer and infratectum with short, straight and unbranched columellae. The ornamentation was tectate, varying in the mesocolpium from perforate (Plate 2, A-C) with puncta or foveolae (*O. purpurea, O. pilosa*) to reticulate (Plate 2, D-I) (*O. urumovii*, *O. campestris*, etc) with lumina partially obscured by processes arising from both the tectum and the foot layer (Plate 3, G). In some species the polar area and margins of the colpi were frequently differentiated from the mesocolpium, being often with a complete smooth tectum. The endopori and the ectocolpi varied in size and shape. The colpi differed in length with lalongate endopori (Plate 3, A-E). The ectocolpi were straight, narrowing at the poles. The sexine was thicker than the nexine. A thinning of the infratectum near the colpus margin was observed while the nexine was considerably thicker in the aperture zone (Plate 3, F).

Differences were observed in the ornamentation in the meso- and apocolpium area (Plate 2, A-B) and ornamentation around the colpus margin. The tectum was interrupted more or less equal to the columellae layer.

The taxa could be divided in two groups on the basis of the P/E ratio: subprolate (P/E<1.33, *O. kozuharovii* and *O. urumovii*) and prolate (P/E >1.33). The second group comprises most of the taxa investigated. The group is rather homogeneous and the differences between species are insignificant. When the ornamentation is taken as a criterion for the species discrimination again two groups are formed: one with perforate and the other with reticulate (diameter of the lumina is about 1 -1.3 µm) pollen grains.

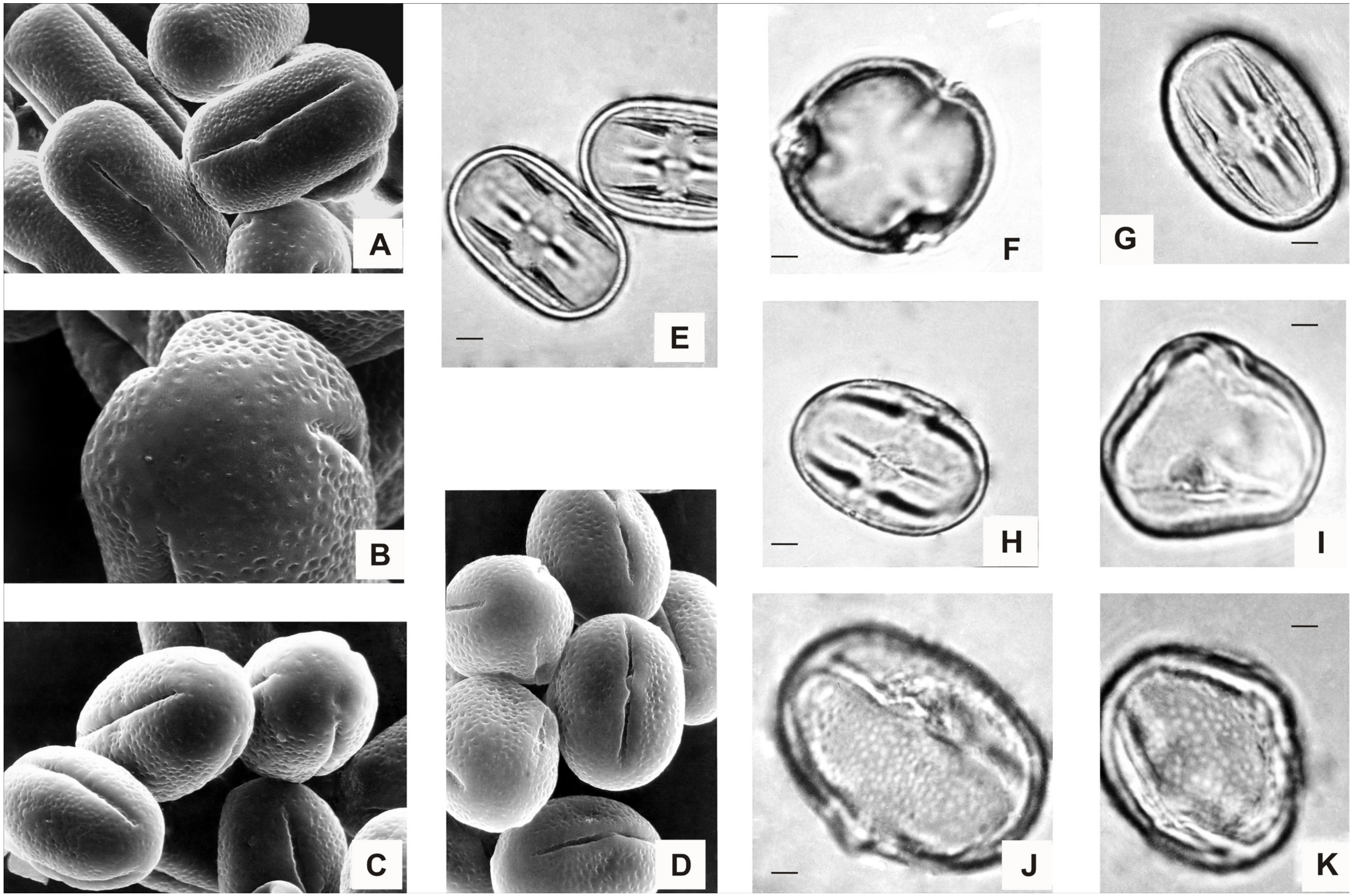

Plate 1. (A-B) *O. halleri* subsp. *korabensis,* SEM 2000x, 5000x; (C) *O. prenja,* SEM 2000 x; (D) *O. kozhuharovii,* SEM 2000 x; (E) *O. dinarica*, LM 1280x; (F) *O. kozhuharovii,* LM 1280x; (G) *O. lapponica,* LM 1280x; (H-I) *O. pilosa,* LM 1280x; (J) *O. prenja,* LM 1280x; *O. kozhuharovii,* LM 1280x. Scale bar for LM photos is 5µm.

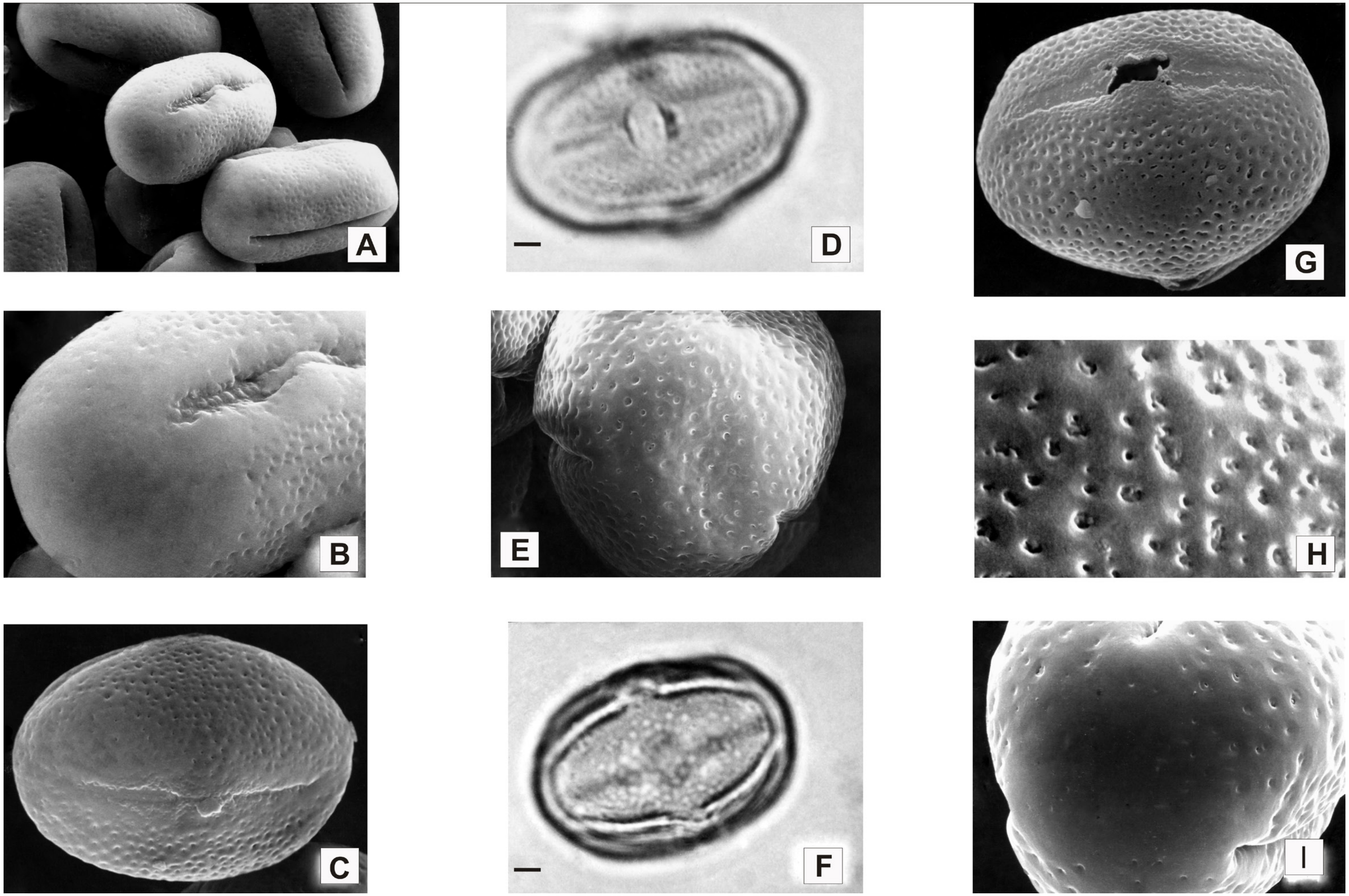

Plate 2. (A-B) *O. purpurea,* SEM 2000 x, 5000x; (C) *O. pilosa,* SEM 2000 x; *O. neglecta,* LM 1280x; (E) *O. halleri* subsp. *korabensis,* SEM 5000x; (F) *O. kozhuharovii,* LM 1280x; (G) *O. urumovii,* SEM 2000x; (H) *O. campestris,* SEM 7500x; (I) *O. prenja,* SEM 5000x. Scale bar for LM photos is 5µm.

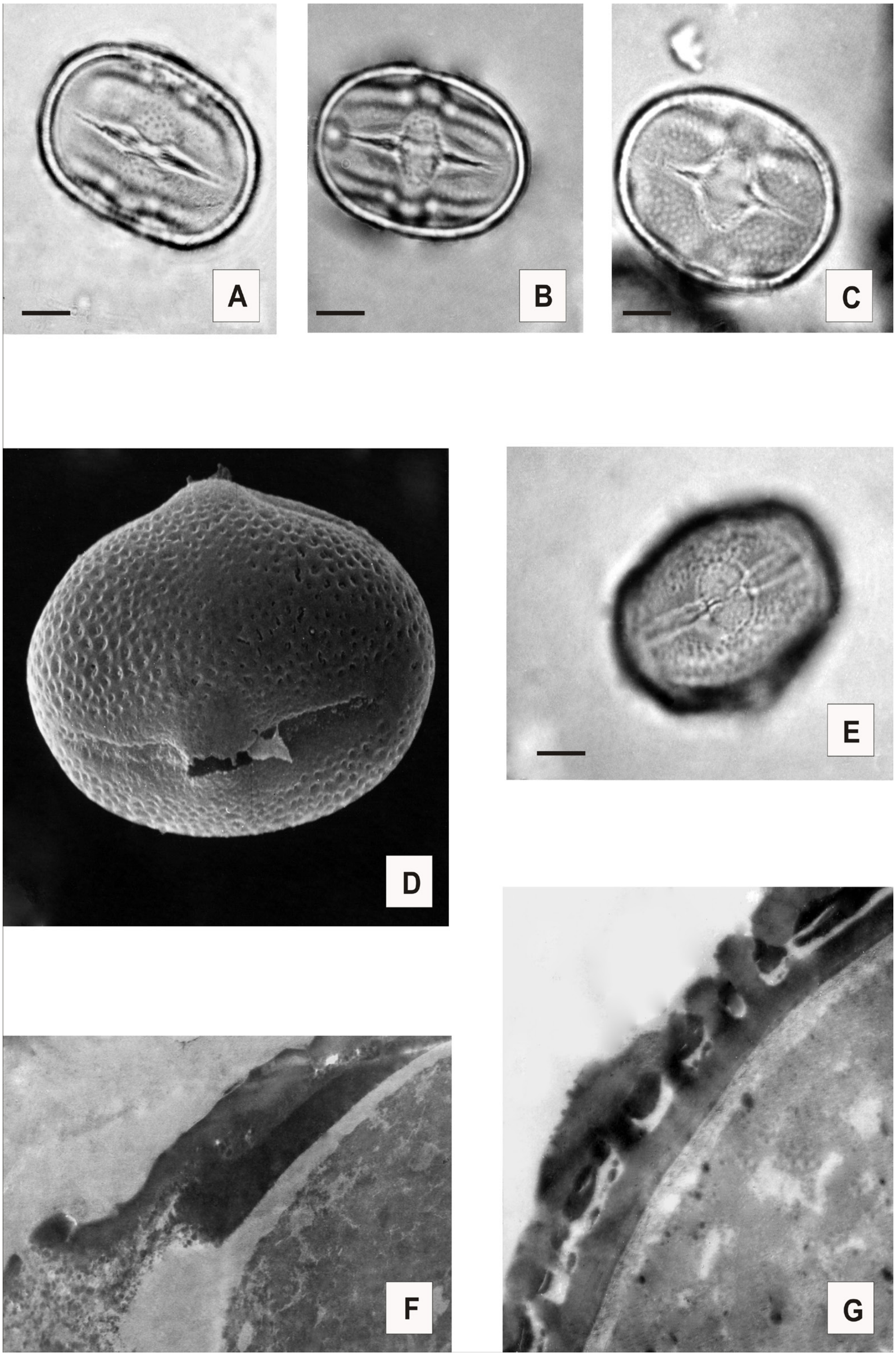

Plate 3. (A) *O. purpurea,* LM 1280x; (B); *O. dinarica*, LM 1280x; (C, E) *O. halleri* subsp. *korabensis,* LM 1280x; (D) *O. urumovii,* SEM 2000x; (F) *O. kozhuharovii,* TEM 15000x; (G) *O. pilosa,* TEM 15800 x. Scale bar for LM photos is 5µm.

The dendrogram obtained by the hierarchical cluster analysis (Fig. 1) reveals *O. prenja* as an outlier (cluster A) with the longest pollen grains (P = 35.84 µm). The second cluster (B) group includes all other species, separated in subgroups. *O. neglecta* is an outlier in this group because of its shortest polar diameter. A very high degree of similarity (lowest Euclidian distances) was established for the pollen grains of *O. pilosa* and *O. purpurea.* They formed a separate group on the basis of the perforate tectum. The pollen grains of *O. campestris* and *O. halleri* subsp. *korabensis* showed high similarity and could be easily distinguished from the other pollen grains by their shorter colpi. These two species stand closer to *O. kozhuharovii* compared to *O. urumovii* and *O. dinarica.* The same results were confirmed from the PCA when species populations (cases) were plotted.

Principal component analysis (PCA) was performed also to see the loadings for each variable and their contribution to each of the PCs (Fig. 2). Percentage eigenvalues and factors loadings on the axes performing the PCA on all investigated populations are reported in Table 2. The horizontal axis PC1 explains 76.88% of the variance but the vertical PC2 explains 11.85% of the variance. Two-dimensional scattergram performed plotting the first against the second axis showed that the polar diameter, equatorial diameter and apocolpium with negative coordinates and correlations with factor 1 contributed to species divergence. The positive coordinates and correlations with factor 2 are presented from all other characters. The studied traits were quite variable which was obvious from their situation on the factor plane. The porus width is the most conservative feature in all species populations. Most of the pollen characters were very closely correlated. They demonstrated significant positive correlations at p< 0.001 such as P and E (0.92), P and L (0.9), E and L (0.95), Plg and Pls (0.93), and M and Plg (0.88).

Table 2. Percentage eigenvalues, variance explained by first three components and factor loadings of the variables.

	Variables code	1	2	3
Eigenvalue (%)		76.88	11.85	7.53
Cumulative (%)		76.88	88.73	96.26
Variables				
Polar diameter	P	-0.90	-0.28	-0.23
Equatorial diameter	E	-0.95	-0.08	-0.26
Colpus - length	L	-0.94	0.00	-0.25
Porus - length	Plg	-0.90	0.30	0.29
Porus - width	Pls	-0.87	0.14	0.41
Mesocolpium	M	-0.86	0.44	-0.13
Apocolpium	A	-0.68	-0.66	0.26

DISCUSSION

The pollen grains of the studied *Oxytropis* species were generally small, tricolporate, with little variation or specialization and closely resembled *Astragalus* species confirming previous pollen characteristics (Ferguson and Skvarla, 1981). The tectum was very restricted, being predominantly perforate (*O. pilosa, O. purpurea*) or finely reticulate (*O. campestris*, *O. urumovii*, *O. kozhuharovii*). This result synchronizes with the evolutionary trends, suggesting specialization in the genera of the tribe and delimiting species as well. The smooth tectum around the colpus area and apocolpium in *Galegeae* pollen is a feature considered indicative of a tendency towards specialization (Ferguson and Skvarla, 1981). However, we do not completely confirm this result although for some primitive species like *O. pilosa* it is true. There are pollen grains (*O. purpurea, O. prenja*) characterized by such smooth area around the colpus margin and apocolpium but considered primitive on the basis of their perforate tectum. Quite often on the background of the primitive morphological syndrome in some closely related *Astragalus* species (Pavlova et al., 1995) are observed at the same time primitive and advanced features in the structure of the exine as elements of heterobathmy. Obviously, this phenomenon is also true for the *Oxytropis* species. Even regarding separate pollen morphological features the evolutionary rate has been rather quick and has led into a strong divergence of closely related species.

The delimitation of the *Oxytropis* species on the basis of their pollen characteristics is difficult, although some clear groups of species could be recognized. The results confirm the data for the similarities of the pollen grains between *Oxytropis* and *Astragalus* species which are united in one pollen type *Astragalus* (Beug, 2004).

The shape, to some extent the size and the sculpturing of the tectum considered of significance as secondary characters in pollen morphology of Fabaceae (Ferguson and Skvarla, 1981) varied within species of the studied group. As it was previously reported for the studied *Oxytropis* species in Bulgaria (Pavlova and Berge, 1997) pollen size did not appear to correlate with the ploidity of the species. The mean values for polar and equatorial diameters in the diploid species (*O. urumovii*, *O. pilosa*, *O. dinarica*) are very close to the tetraploid (*O. kozhuharovii, O. halleri*) and hexaploid (*O. campestris*). The same fact was reported for *Astragalus* species from Turkey (Pinar et al., 2009) and could be concluded for all members of the tribe *Galegeae*.

Being more conservative the features of the aperture system are of great value for the characterization of the pollen type at a level of tribes and genera suggesting relationships (Ferguson and Skvarla, 1981). The trend of increasing the number of apertures in some of the genera in Fabaceae was stated by Ferguson and Skvarla (1981) and Guinet and Ferguson (1989). There are a few data showing variation of the aperture in *Astragalus* (Pavlova et al., 1994, Pinar et al., 2009) and *Oxytropis* (Pavlova and Berge, 1997). The fertile 6-pantocolporate pollen grains found for some species (*O. pilosa, O. purpurea, O.*

prenja) in this study confirm previous data (Pavlova and Berge, 1997). The percentage of such pollen grains in the samples varied, but it was always higher in *O. pilosa*. Such "mixed pollen", i.e. pollen in which some of the grains are isodiametric, was also reported for some species from *Trigonelleae* (Small et al., 1981). Obviously, this phenomenon is frequent in some species of Fabaceae, but without taxonomical value. The reason for heteromorphy of the pollen grains could be a result of polyploidy, hybridization or abnormalities during the meiosis process (Beug, personal communication). As this phenomenon is found in diploid species (often in *O. pilosa* and rarely in *O. purpurea)* it is most appropriate to be a result of anomalies. However, the appearance of 6-pantocolporate pollen grains in polyploid species of the genus *Oxytropis* is also possible bearing in mind that polyploidy and hybridization are the main evolutionary mechanisms in the genus (Vassilczenko, 1965).

The pollen wall stratification is also of the greatest value in the tribal classification, being not so different at species level (Ferguson and Skvarla, 1981). As a result of this study we can confirm an evolutionary trend for increasing the height of the columellae which is associated with a reduction in the endexine and retention of the foot layer. Higher columellae are related to the reticulate ornamentation and correlate closely with specialization in macromorphology. The same evolutionary trend is characteristic for the representatives of *Astragalus* in Bulgaria (Pavlova et al., 1994, 1995).

The analysis of the pollen morphology is of significance for some taxonomical decisions especially above species rank. The results of the statistical methods evaluating the similarity between studied species are in accordance with some taxonomical decisions adopted in Flora Europaea (Leins and Merxmüller, 1968) and in regional studies (Chrtek and Chrtkova, 1983; Pavlova et al., 1999; Kozuharova, 2007) for closely related and critical species. The decision for the taxonomical position and relations between *O. campestris*, *O. urumovii,* and *O. dinarica* (Chrtek and Chrtkova, 1983; Kozuharova, 2007) were confirmed by the pollen morphological data of the studied populations. *Oxytropis urumovii* and *O. dinarica* are clustered together showing a high degree of similarity and separated from *O. campestris*. The pollen morphology of the last species is more similar to the one of the Balkan endemic *O. halleri* subsp. *korabensis* and both taxa form a separate cluster. Near to this group is the Bulgarian endemic *O. kozhuharovii*. Opposite to the statement of Kozuharova et al. (2007) considering *O. kozhuharovii* more closely related to *O. prenja* than to *O. halleri,* our pollen morphological data separate *O. prenja* very clearly on the basis of the largest pollen grains (P x E = 35.84 x 25.66 μm). In general, pollen morphological data provide additional reliable information in solving taxonomical problems discussing similarities and relationships between species.

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