Bangladesh J. Plant Taxon. **26**(2): 259–268, 2019 (December)

MORPHOLOGICAL, PALYNOLOGICAL AND PHYLOGENETIC RELATIONSHIPS OF *GLAUCIUM* MILL. IN TURKEY

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Key words: Glaucium; *matK*; ITS3-6 DNA; Phylogeny; Subsection; Turkey.

Abstract

Glaucium taxa were investigated in terms of their morphological, palynological and phylogenetical characteristic. The results of this study show differences between the taxa in some of these characteristics, especially in micromorphology and formation of clades in phylogenetic trees based on the *matK* and ITS3-6 DNA sequence data. Based on the findings of the molecular analyses supported by morphological data (stem's trichomes), the genus *Glaucium* of Turkey was divided into subsections *Glabrousae* and *Pubescentae.*

Introduction

Glaucium Mill. (horned poppy), belonging to the family Papaveraceae, is represented by a total of 25 species worldwide, and especially distributed throughout Western, Northern and Eastern Asia, Europe, Northern Africa, and Australia. The distribution of *Glaucium* species relatively widely covers western Asia and the Mediterranean region and is decreased from central Asia to the European countries. As a country, Iran harbors relatively more species of the genus *Glaucium* (17 species) and hence, this country is considered as the hot spot of the genus.

The genus *Glaucium* consists of annual, biennial, and perennial herbaceous plants and grows mostly in saline soils and by the sea. *Glaucium* is represented by a total of 10 taxa in Turkey, namely *G. corniculatum* (L) Rud. subsp. *corniculatum*; *G. corniculatum* (L) Rud. subsp. *refractum* (Nab.) Cullen; *G. grandiflorum* Boiss & Huet var. *grandiflorum*; *G. grandiflorum* Boiss. & Huet var. *torquatum* Cullen; *G. grandiflorum* var. *haussknechtii* (Bornm. & Fedde) Parsa; *G. flavum* Crantz; *G. leiocarpum* Boiss.; *G. acutidentatum* Hausskn. & Bornm.; *G. cappadocicum* Boiss. and *G. secmenii* Yıldırımlı, four (*G. grandiflorum* var. *torquatum, G. acutidentatum, G. cappadocicum, G. secmenii*) of which are endemic (Seçmen *et al.* 1998; Yıldırımlı, 2012). Turkey ranks second with respect to having the maximum number of species following Iran. A lot of chemical studies have been carried out on the genus. Mory (1979) classified 22 species belonging to the genus into two sections, namely *Acropetale* and *Glaucium.* The section *Acropetale* was more primitive than the section *Glaucium.* A micro-macromorphological study on 18 *Glaucium* taxa was carried out by Gran and Sharifnia (2008) based on 28 qualitative and 37 quantitative characters. In the study performed by Vorniceanu *et al.* (2002), the number of chromosomes of *G. flavum* was measured as 2n = 12, and the chromosome lengths of the metaphase stage were measured as 1.30 to 1.78 µm. In this study, the chromosomes were separated into two groups, and five pairs were identified as metacentric and one pair as submetacentric. Ivanovska and Philipov (1996) revealed that the family Papaveraceae had a

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rich content of isoquinoline alkaloids such as aporphine, protopine, protoberberine and proaporphine. In a phytochemical study performed by Vorniceanu *et al.*, 2004, on the species of Papaveraceae, including *G. corniculatum* and *G. flavum*, from which many alkaloids were isolated and identified.

The aim of our study was to identify the widespread distributionof *Glaucium* taxa in Turkey, to generate detailed descriptions identifying its macro-micromorphological and, palynological properties, to reconstruct its phylogeny through molecular studies (Fig. 1).

Fig.1. The areas with dense distribution of *Glaucium* taxa. (= Species distributed in Turkey; = *G. corniculatum*, *G. grandiflorum*, = *G. secmenii*, = *G. flavum*, = *G. leiocarpum*, = *G. acutidentatum* and =*G. cappadocicum*)

Materials and Methods

The specimens of *Glaucium* taxa were collected from natural populations, necessary field data were recorded and photographs were taken during field vists. Aditionally, the specimens of *Glaucium* housed in Turkey's major herbaria were studied and significant characters were recorded. The necessarydrawings of taxa were also constructed (Figs 2-3).

For SEM, seed and pollen samples were mounted on stubs using double-sided adhesive tape, coated with gold using a POLARON SC7620 sputter, and then examined and photographed with LEO 440 SEM. Seed analysis was performed according to Stearn (1996) and pollen analysis was performed according to Punt and Hoen (2007).

DNA Isolation: The leaf pieces (30 mg) of ten *Glaucium* and two *Papaver* taxa were grinded with the help of microtube pestle in combination with liquid nitrogen in the different 1.5 ml mücrotubes.. Total genomic DNA isolation of the grinded leaf samples were performed with the “Gene MATRIX Plant and Fungi” kit according to the manufacturer’s protocol. Isolated DNA concentration of each sample was quantified by Nano Drop ND-1000 spectrophotometer. Stock DNAs were kept at –20 °C.

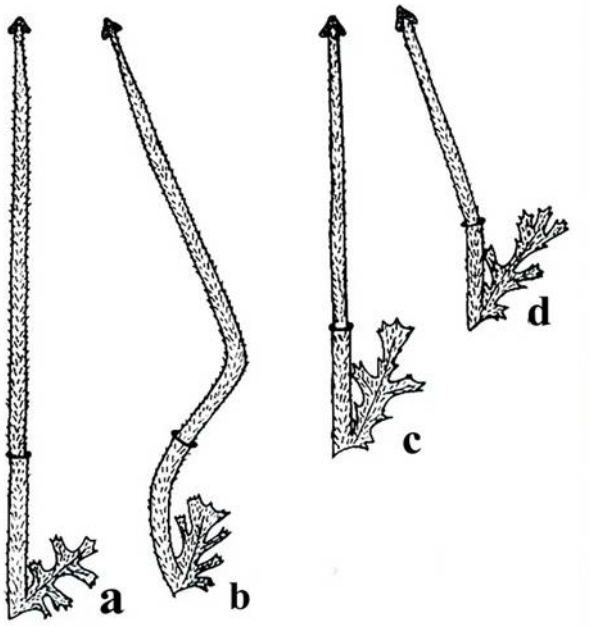

Fig. 2. Fruits of *Glaucium* taxa of Turkey. a. *G. grandiflorum* var. *grandiflorum*; b. *G. grandiflorum* var. *torquatum*; c. *G. corniculatum* subsp. *corniculatum*; d. *G. corniculatum* subsp. *refractum.*

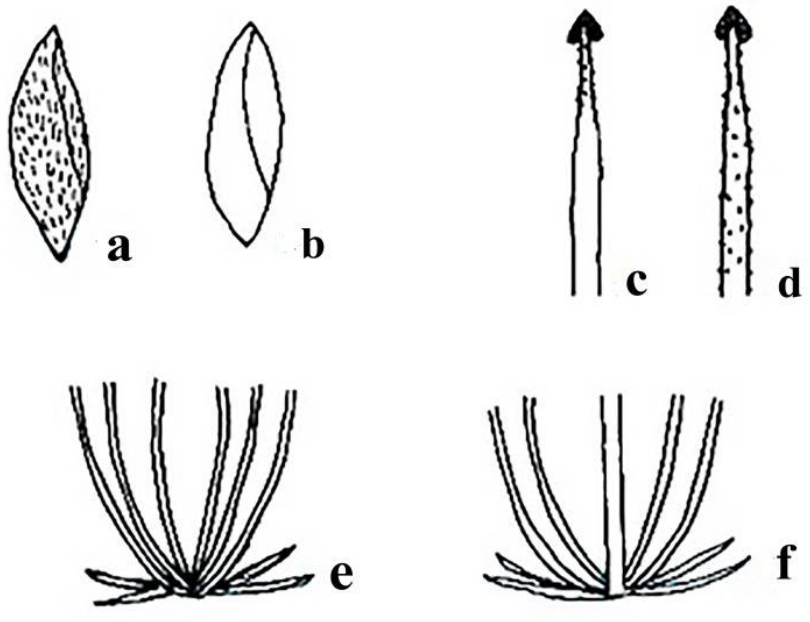

Fig. 3. Plant parts of some *Glaucium* taxa used for diagnosis: a. Sepal of *G. grandiflorum* var. *grandiflorum*;b. Sepal of *G.leiocarpum*; c. Fruit of *G. leiocarum*; d. Fruit of *G. flavum*; e. Stem of *G. grandiflorum* var. *haussknechtii*; f. Stem of *G. grandiflorum* var. *grandiflorum.*

PCR amplification and sequencing: matK and ITS3-6 sequences of ten Turkish *Glaucium* taxa were analyzed. The *matK* region of the chloroplast DNA was amplified with the *matK*_390f and *matK*_1326r primers and ITS3-6 region of the nuclear DNA was amplified with the ITS-3F and ITS-6R primers (Cuénoud *et al*., 2002). PCR study was performed with a total 50 µl standard reaction volume for each sample. Optimum amplification conditions were obtained with 100 ng genomic DNA, 1 × reaction buffer, 2.5 mM $MgCl_2$, 20 µM dNTPs, 0.4 µM for *matK*_390f and *matK*_1326r primers, 2 U Hot Start Taq DNA polymerase (SolisBioDyne) and a PCR mix was prepared in accordance with the seamounts. Amplification was performed in a Techne Progene Thermal Cycler (Barloworld Scientific, Staffordshire, U. K.). The reaction mixtures were heated in an initial step of 94 °C for 15 min and then subjected to 35 cycles of the following program: 95 °C for 45 s, 57 °C for 45 s, and 72 °C for 1 min. After the last cycle, the temperature was maintained at 72°C for 10 min. The amplification products were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide and the product sizes were determined on gels by nucleotide size marker (100 bp ladder; Solis BioDyne). The PCR products were sequenced with a Big Dyecycle sequencing kit (Applied Biosystems, Foster City, California) using an ABI 3130 XL genetic analyzer (Applied Biosystems).

Sequence analysis: The amplified fragments were in duplicate conditions. Alignment of the *matK* sequences was generated using the MUSCLE algorithm of MEGA 6 software with default settings (Edgar, 2004; Tamura *et al.*, 2011). Ends of the alignment were trimmed to make all the sequences in the final data set equal in length. The evolutionary history and molecular phylogenetic analysis were inferred using the maximum likelihood (ML) method based on the Tamura–Nei model via MEGA6 software (Tamura *et al.*, 2011). The percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances (pair-wise distances) were computed, using the maximum composite likelihood model, in units of the number of base substitutions per site (Tamura *et al.*, 2004). The analysis involved 20 nucleotide sequences.

Results and Discussion

A total of 10 *Glaucium* taxa were analyzed in terms of their morphological, palynological, and phylogenetic characters. Although some of the morphological characters of the taxa examined were following the information contained in Flora of Turkey (Cullen, 1965), it was noticed that some of their properties were different. In addition, the data yielded from Mory's (1979) study and those yielded as a result of our measurements were compared. In this comparison, the major similarity was observed in terms of the morphological and palynological characters. In a micro-macromorphological study performed by Gran and Sharifnia (2008) of 18 *Glaucium* taxa, the species *G. haussknechtii* has been recognized as synonymous with *G. grandiflorum* based on the analyses of 28 qualitative and 37 quantitative characters. As a result of our detailed analyses of morphological, seed, pollen and phylogenetic data, it was revealed that these two species were different.

In this study the *Glaucium* taxa were divided into two groups with respect to stem hairs. Taxa with pubescence stems were *G. corniculatum* subsp. *corniculatum* and *G. corniculatum* subsp. *refractum, G. grandiflorum* var. *grandiflorum, G. grandiflorum* var. *torquatum, G. grandiflorum* var. *haussknechtii* and *G. secmenii*, while the taxa with hairless stems were *G. flavum, G. leiocarpum, G. acutidentatum* and *G. cappadocicum.*

The petals of the taxa included in the hairy group were red, or reddish-orange, whilethose with hairless group were yellow or yellowish-orangethe seeds were separated by thin prominent sections. The testa outline of the seeds of taxa with hairy stemswere clearly arch shaped, and

curved (undulate) (Figs 4-5); while that of the taxa with hairless stemswere smooth or less curved (Figs 4-5).

SEM analysis showedthat the taxa included in the hairy group were variable in pollen shape. Pollens of *G. corniculatum* subsp. *corniculatum* and *G. grandiflorum* var. *grandiflorum* were suboblate, and that of *G. grandiflorum* var. *torquatum* was prolate. The taxa of *G. corniculatum* subsp. *refractum, G. grandiflorum* var. *haussknechtii* and *G. secmenii* included in the hairy group had spheroidal pollens (Fig. 5, Table 1).

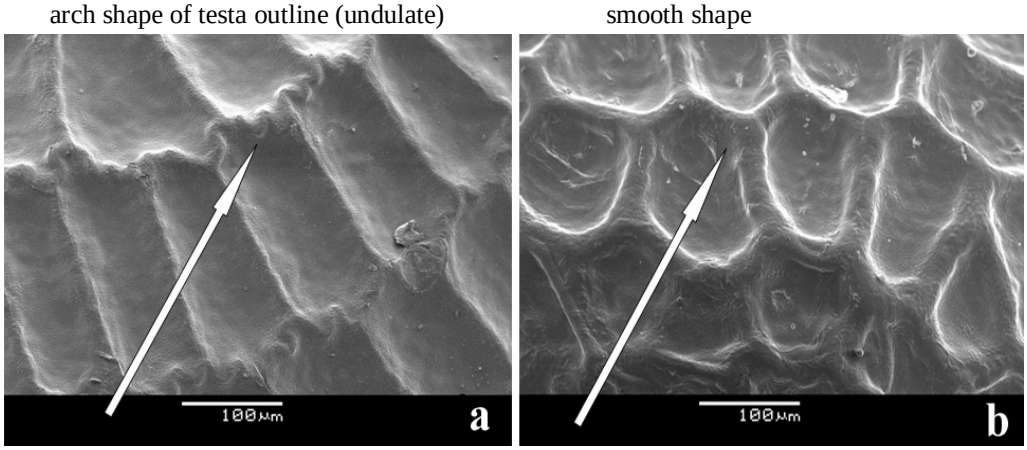

Fig. 4. Testa outline of the seeds of taxa with hairy, i.e. *Pubescentae* (a) and hairless i.e. *Glabrousae* (b) stem.

It was observed that in the ML tree based on *matK* DNA sequences (Fig. 6), the *Glaucium* taxa were resolved into the moderately to strongly supported *G. flavum-G. leiocarpum-G. acutidentatum-G. cappadocicum* clade with glabrous stem and the weakly supported or unsupported clade of rest of the taxa studied (*G. corniculatum* subsp. *corniculatum*, *G. corniculatum* subsp. *refractum*, *G. grandiflorum* var. *grandiflorum*, *G. grandiflorum* var. *torquatum*, *G. grandiflorum* var. *haussknechtii*, *G.* and *G. secmenii*) with pubescent stem. In the ML tree based on ITS3-6 DNA sequences (Fig. 7), the *G. flavum-G. leiocarpum-G. acutidentatum-G.cappadocicum* clade was strongly resolved, but clade of rest of the taxa studied was unsupported though *G. secmenii* and *G. corniculatum* subsp. *refractum* were resolved in to a moderately supported clade.

The results of phylogenetic analyses showed that the *Glaucium* taxa were grouped into two main clades in the ML trees based on the *matK* and ITS3-6 DNA sequences (Figs 6-7), which is in compatible with the hairness of their stems, petal color and testa outline of the seeds. The taxa included in these two sub-clades were also compatible with ovary tubercle. However, we weren't able to observe the formation of these two sub-clades when we analyzed the ITS3-6 DNA sequences snd the sub-clades of *Glaucium* taxa based on these morphological characters were not clearly supported by pollen characters, like pollen lenght (P),equatorial width (E), P/E ratio and pollen shape etc., that were mostly overlapping. For example, in *G. grandiflorum* var. *grandiflorum* pollens were suboblate, and P/E was 1.51, *G. grandiflorum* var. *torquatum*pollens were prolate and P/E was 0.78, and in *G. corniculatum* subsp. *Corniculatum* pollens were suboblate, and P/E was 0.83. In all other *Glaucium* taxa, pollens were spheroidal, and P/E values falled within the P/E range of above-mentioned taxa.

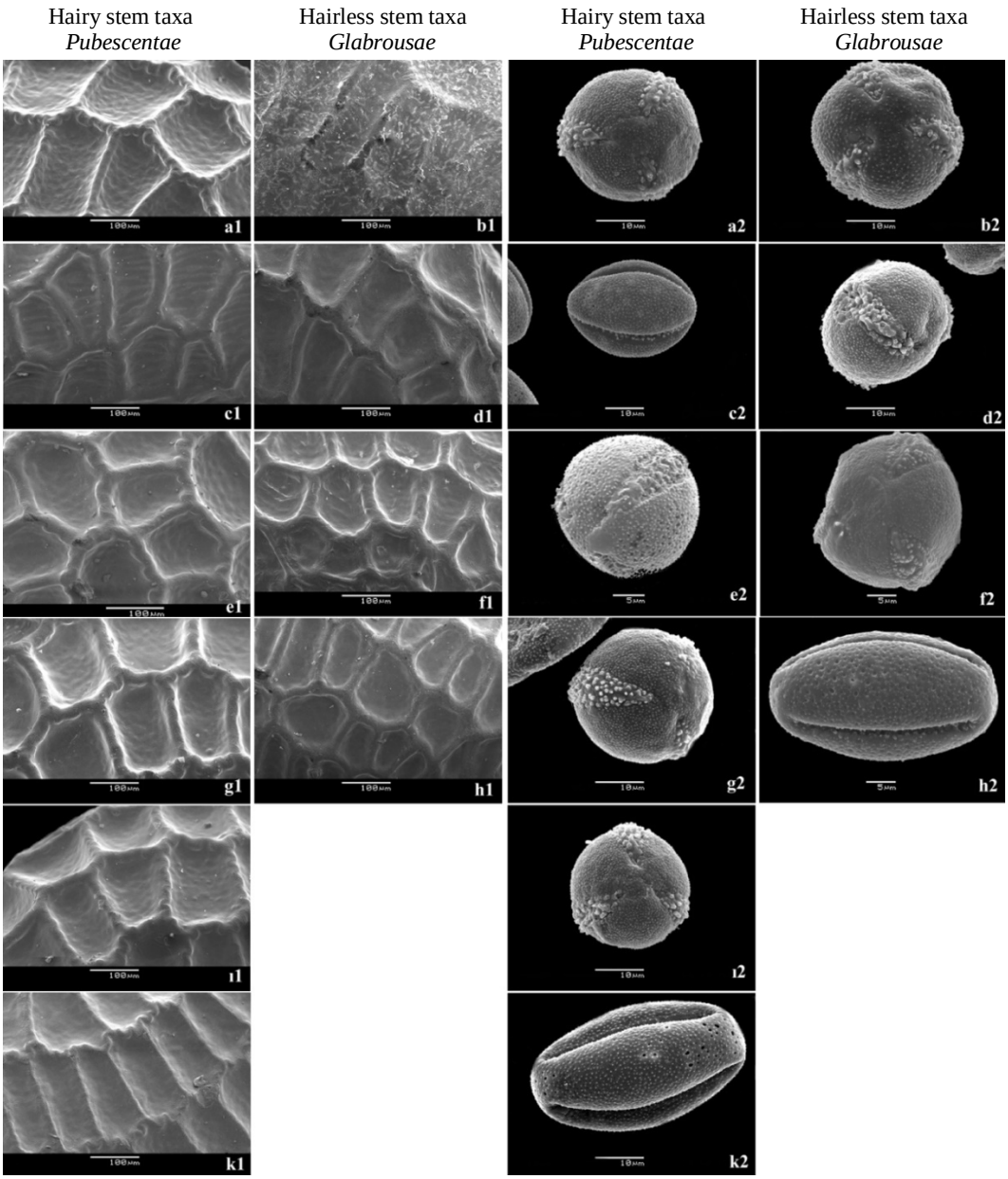

Fig. 5. *Glaucium* seeds (a1, b1, c1, d1, e1, f1, g1,h1, ı1, k1) and pollen (a2, b2, c2, d2, e2, f2, g2,h2, ı2, k2) SEM views, a1, a2: *G. corniculatum* subsp. *corniculatum* b1, b2: *G. flavum* c1, c2: *G. corniculatum* subsp. *refractum* d1, d2: *G. leiocarpum* e1, e2: *G. secmenii* f1, f2: *G. acutidentatum* g1, g2: *G. grandiflorum* var. *grandiflorum* h1, h2: *G. cappadocicum* ı1, ı2: *G. grandiflorum* var. *torquatum* k1, k2: *G. grandiflorum* var. *haussknechtii*.

It was foundthat *G. grandiflorum* var. *grandiflorum* and *G. grandiflorum* var. *torquatum* constituted a small sub-clade and the difference between them was minimal. They were also morphologically separated from each other in terms of the position of the fruit's pedicel, and the pollen's shape. The fruit's pedicel of *G. grandiflorum* var. g*randiflorum* was vertical, and the

pollen's shape was suboblate while the fruit' pedicel of *G. grandiflorum* var. *torquatum* was curved, and the pollen' shape is prolate.

G. corniculatum subsp. *refractum* and *G. secmenii* constituted a sub-clade in the ML tree based on ITS3-6 DNA sequences, but not in that based on *matK* DNA sequences.Both taxa's fruit pedicels were curved and their pollens were spheroidal. To know the relationship between these two taxa needs further study.

Table 1. The comparative pollen properties of *Glaucium* taxa.

Pollen properties Taxa	Pollen lenght (P) Min-max Mean (SD) µm	Equatorial width (E) Min-max Mean (SD) µm	P/E ratio	Pollen shape	
G. corniculatum subsp. *corniculatum* (465-2)	24-35 32,96 (2.12)	37-41 39,53 (1.19)	0.83	Suboblate	
G. corniculatum subsp. *refractum* (467)	28-33 30,73(1.28)	32-35 33,73(1.01)	0.91	Spheroidal	
G. secmenii (587)	19-30 24,5(2,59)	20-34 25,5(2,75)	0.91	Spheroidal	*Pubescentae*
G. grandiflorum var. *grandiflorum* (416)	27-37 29,1 (2.00)	34-46 37,06(2.21)	0.78	Suboblate	
G. grandiflorum var. *torquatum* (354-1)	29-34 29,5 (1.49)	18-23 18,5(1.13)	1,51	Prolate	
G. grandiflorum var. *haussknechtii* (456)	28-35 30,26(1.55)	32-40 34,26 (1.76)	0.88	Spheroidal	
G. flavum (460)	31-37 33,4 (1.67)	33-40 37,46 (1.67)	0.89	Spheroidal	*Glabrousae*
G. leiocarpum (415)	30-37 33 (1.41)	28-38 34.5 (3,53)	0.94	Spheroidal	
G. acutidentatum (440)	25-31 29.5 (1.87)	28-40 31.9 (2.10)	0.92	Spheroidal	
G. cappadocicum (449)	25-36 30,3 (1.88)	26-37 33,06(2.06)	0.91	Spheroidal	

Mory (1979) divided *Glaucium* taxa into two sections (*Acropetale* and *Glaucium*). *Glaucium* taxa into two sections are supported by our study, and our all *Glaucium* taxa belong to Mory's section *Glaucium*. In our research, it was determined Mory's section *Glaucium* we can be divided into two sub-sections.

Since these subsections clearly differ from each other, it was concluded that the nomenclature should be cited as Subsection *Glabrousae* K. Yıldız & Mungan and Subsection *Pubescentae* K.Yıldız & Mungan.

According to these results, the identification key of *Glaucium* species that have grown in flora of Turkey was performed as follows:

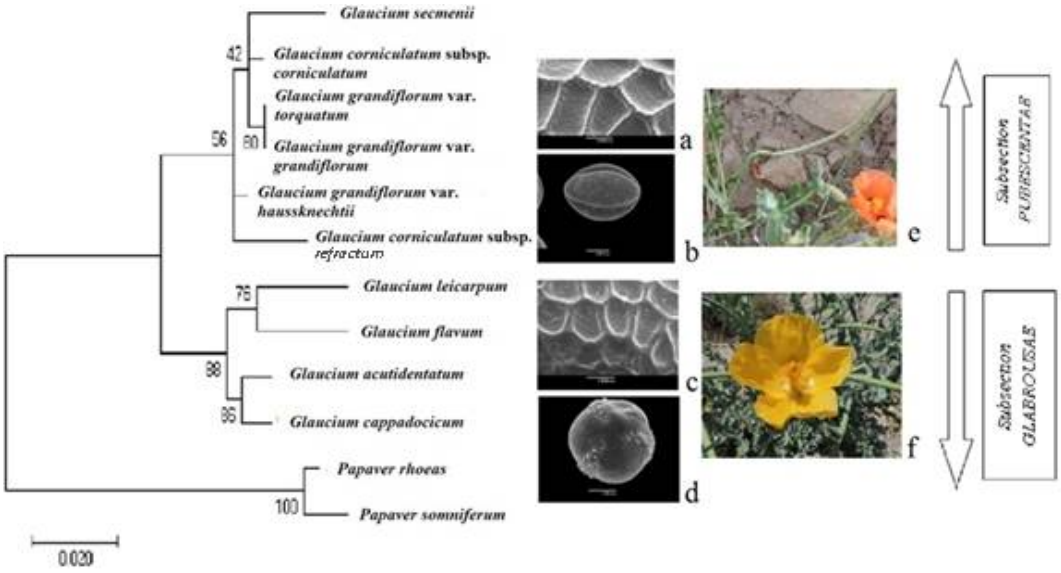

Fig. 6. ML tree based on *matK* DNA sequence of the Subsections *Pubescentae* and *Glabrousae*. *Pubescentae*; a. Seed surface b. Pollen e. morphological appearance, and *Glabrousae*; c. Seed surface d. Pollen f. morphological appearance.

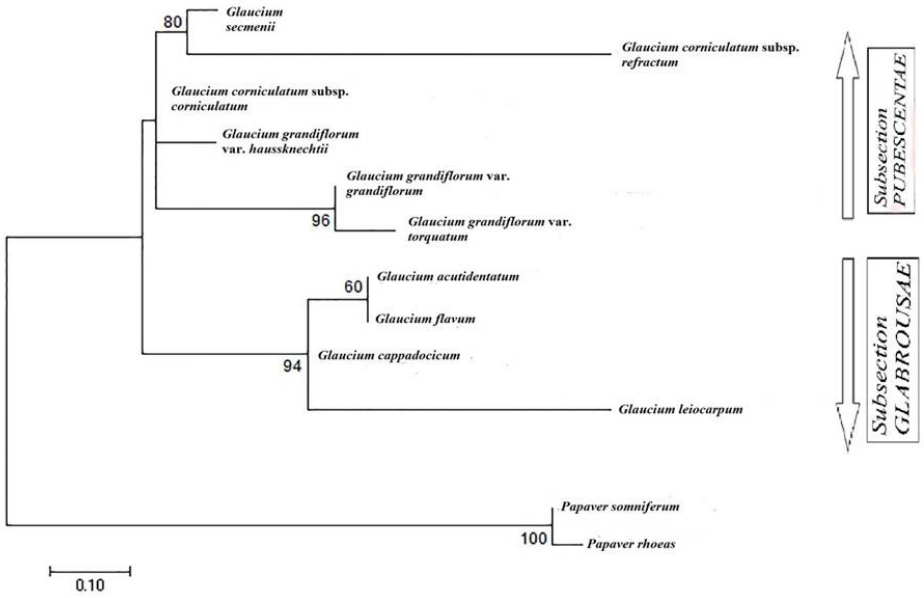

Fig. 7. ML tree based on ITS3-6 DNA sequences of the subsections *Pubescentae* and *Glabrousae*.

1. Stem and ovary pilose with adpressed or subspreading hairs
   2. Fruiting pedicels shorter than the leaves subtending them
      3. Sepals 1-2.7 cm; petals 1.5-3.5x1.4-2.8…..……..…….1. *corniculatum*
3. Sepals 0.7-2.5 cm; petals 1.2-2.2x 1-2.1……2. *secmenii*
2. Fruiting pedicels exceeding the leaves subtending them……...3. *grandiflorum*
1. Stem glabrous, Ovary tuberculate or glabrous never pilose
4. Ovary papillose- tuberculate, at least near the apex
   5. Upper leaves sinuate-dentate with obtuse or rounded lobes; fruit neither torulose nor attenuate at the apex, petals yellow…… 4. *flavum*
   5. Upper leaves pinnatifid, with acute segments; fruit somewhat torulose, attenuate at the apex, ……..……..……..……5. *leiocarpum*
4. Ovary smooth, etuberculate
6. Radical leaves deeply pinnatifid; sepals greyish-black…..... 6. *acutidentatum*
6. Radical leaves obovate-runcinate, dentate; sepals green……7. c*appadocicum*

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(Manuscript received on 7 July, 2019; revised on 8 December, 2019)