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Morphological and molecular data reveal a new species of *Allium* (Amaryllidaceae) from SW Anatolia, Turkey

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Abstract

Allium undulatitepalum (Amaryllidaceae) is described as a new species from the Antalya Province of Turkey. It belongs to the section *Melanocrommyum* and is endemic to the south-western region of Turkey. The new species is a close relative of *A. orientale*, but according to results of the ITS sequences, and based on the morphological differences presented in the description, it is clearly different from its relative. A phylogenetic tree, distribution map, illustrations, pollen and seed micro-photographs, karyo-morphology, as well as notes on the biogeography and ecology of the new species are provided.

Key words: Antalya, ITS, *Melonocrommyum*, taxonomy

Introduction

The genus *Allium* Linnaeus (1753: 294) comprises more than 850 species, making it one of the largest petaloid monocotyledonous genera (Keusgen *et al.* 2011). It is a variable group that is widely spread across the Holarctic region from the dry subtropics to the boreal zone (Li *et al.* 2010). The genus *Allium* is represented by 180 species and subg. *Melanocrommyum* (Webb & Berthelot, 1848: 347) Rouy in Rouy & Foucaud (1910: 378) comprises about 33 accepted taxa (Koyuncu 2012, Genç & Özhatay 2014) in the Flora of Turkey.

The classification of the genus *Allium* is taxonomically very complex, often controversial and still in progress. In recent years, improvements in DNA recognition techniques have provided new insights into the intrageneric classification of genus *Allium*. The internal transcribed spacer (ITS) region, including the 5.8S rDNA and the two spacers ITS1 and ITS2, is one of the most commonly used markers for the differentiation of *Allium* species (Dubouzet & Shinoda 1998, Mes *et al.* 1999, Friesen *et al.* 2006, Gurushidze *et al.* 2008, 2010, Ipek *et al.* 2014). Until now the most comprehensive phylogenetic studies of *Allium* subg. *Melanocrommyum* were done by Leibniz-Institute of Plant Genetics and Crop Research team. In the scope of their studies, the ITS region of nuclear ribosomal DNA was sequenced from 195 representative species of *Allium* (Friesen *et al.* 2006), phylogenetic analysis of subg. *Melanocrommyum* was done with multiple individuals of more than 100 species (Gurushidze *et al.* 2008), species level phylogenetic relationships of the subgenus were investigated (Gurushidze *et al.* 2010). Altogether 160 species and subspecies were accepted in the subg. *Melanocrommyum* and as a new classification based on molecular and morphological characters sect. *Melanocrommyum* Webb & Berthelot (1848: 347) was subdivided into nine alliances (Fritsch *et al.* 2010).

During fieldwork (May 2008) in Antalya province, southwestern part of Turkey, the authors collected flowering material of some interesting *Allium* specimens with undulate tepals, on calcareous stony slopes and meadows in Salamut Plateau (Akseki/Antalya). Within these alliances, the new species presented in this study is morphologically related to *A. orientale* Boissier (1854: 25), *A. multibulbosum* Jacquin (1773: 9) and *A. nigrum* Linnaeus (1762: 430). To determine the phylogenetic position of the new species, it was investigated using sequences analysis of ITS regions and compared with the most related taxa contained within different alliances.

As a result of our detailed macro- and micro-morphological studies, we concluded that the morphological characters of these *Allium* specimens from Antalya differ from all other *Allium* species.

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Materials and Methods

The studied specimens of the genus *Allium* were compared with relevant taxonomic literature (Stearn 1980, Kollmann 1984, 1986, Davis *et al.* 1988, Özhatay & Tzanoudakis 2000, Koyuncu 2012, Deniz *et al.* 2013, Özhatay & Genç 2013), and with specimens in the herbaria AEF, AKDU, ANK, BM, E, GAZI, HUB, ISTF, ISTO, K and VANF (acronyms follow Thiers 2015, see also Appendix 1). The overall morphology of the new species was examined by stereo-binocular microscope. The exine sculptures, seed coat details, and pollen surfaces were examined under the scanning electron microscope (SEM). For SEM study, pollen were first treated with 70% alcohol and then dried before mounting on stubs. Seeds and pollen grains were plated with gold and the microphotographs were taken with a Zeiss LEO-1430 Scanning Electron Microscope. Sulcus descriptions were provided and in total 50 pollen grains from each taxon were measured by using a light microscope (LM).

For karyological study, the squash method was used for mitotic metaphase chromosome preparation. Root tips were pretreated in α-monobromonaphthalene at 4°C overnight and then washed with distilled water and finally fixed in Carnoy's solution (3:1 absolute ethanol: glacial acetic acid) for a minimum of 5 hours. The root tips were hydrolysed for 10 minutes in 1N HCl at 60°C, stained using the standard Feulgen technique and squashes were prepared. Slides were made permanent with Entellan, then examined under an Nikon E200 microscope with photographic camera. The nomenclature standard proposed by Levan *et al*. (1964), was applied in the description of the chromosome morphology.

Individuals of the new species and *A. nigrum* collected from Salamut Upland and Demre Province, respectively, were jointly evaluated with *A. orientale, A. atropurpureum* Waldstein & Kitaibel (1800: 16), *A. multibulbosum* and *A. schubertii* Zuccarini (1843: 234) within the molecular studies. Total DNA was extracted from the leaves of the new species and *A. nigrum* living specimens using the CTAB method of Doyle & Doyle (1990). DNA concentration and quality were tested with 1% agarose gel against a DNA standard. ITS region (ITS1, 5.8S rDNA subunit, ITS2) was amplified using a primer combination of ITSA and ITSB (Blattner 1999). Polymerase chain reaction (PCR) amplification was carried out with 1 U of Taq DNA polymerase (Fermentas Life Sciences, Burlington, Canada) in the supplied reaction buffer at 10× concentration, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pM µL-1 of each primer and 40 ng of template DNA in 20 µL reaction volume. The amplifications were performed on a programmable thermocycler (Bionerr, MyGenie™) with the following programme: one cycle of 4 min at 94 °C, 28 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C, and for final extension one cycle of 7 min at 72 °C. PCR products were cleaned up using the GeneJET Gel Extraction Kit (Thermo Scientific Fermentas, Vilnius, Lithuania). Yielded amplicons were sequenced and compared with other closely related species in the subgenus available in the GenBank databases at the National Center for Biotechnology Information (NCBI) using BLAST similarity search tool. Sequencing process was carried out at Akdeniz University Faculty of Agriculture (Antalya) and Iontek (İstanbul) Laboratories Turkey, as direct sequencing from PCR products. The sequences of ITS regions were aligned by using CodonCode Aligner 3.7.1 software (CodonCode Corporation, Centerville, MA, USA). Phylogenetic dendrogram was based on sequence alignments by maximum likelihood method with 1000 bootstrap (BS) replicates using the program MEGA5 (Tamura *et al.* 2011). The entire ITS sequences of *A. undulatitepalum* and *A. nigrum* were deposited in the NCBI genbank database under Accessions Numbers KP881223–KP881228.

Description of the new species

Allium undulatipetalum İ.Genç & N.Özhatay *sp. nov.* (Figs. 1, 2, 3, 4A–F)

Allium undulatitepalum is related to *A. orientale* Boiss. and it differs from the latter species by bulb globose (not ovoid); leaves 2–5 cm wide (not 0.8–2 cm wide); inflorescence semispherical (not fastigiate-semispherical); perigone segments after anthesis only reflexed (not reflexed and twisted outwards), 6–7 × 3.5–6 mm (not 5–6 × 2–3 mm), edges often undulate (not straight), white with green midvein (not pinkish white-pink with green or pinkish midvein); ovary purplish black in every stage (not green to turn purple); capsule globose (not elliptic to pear shaped); seed testa cells angularly round (not elliptic-oblong), well convex and with many prominent verrucae (not one or two-rowed with less prominent verrrucae).

Type:—TURKEY. C3 Antalya: Akseki, Çaltılıçukur Village, Salamut Plateau, calcareous stony and grassy slopes close to *Cedrus libani* forests, 36°53N, 31°55E, 1600 m, 9 May 1982, *T. Ekim*, *M. Koyuncu s.n.* (holotype ISTE 54419!; isotypes AEF!).

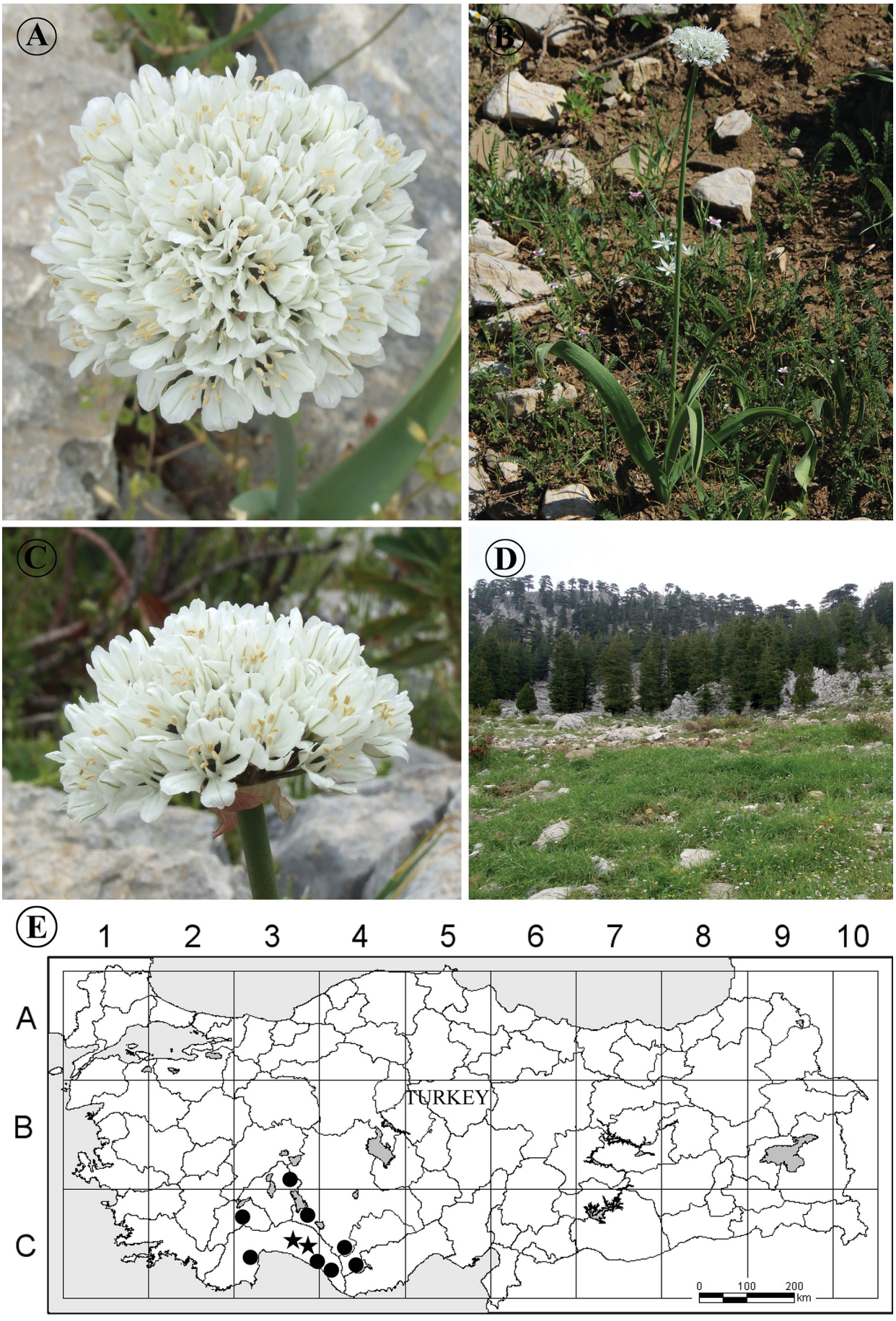

FIGURE 1. *Allium undulatitepalum*: A. Top view of inflorescence, B. Habit, C. Side view of inflorescence, D. Habitat, E. Distribution map of *A. undulatitepalum* (★) and *A. orientale* (●) (Photos: İ.G.Deniz & İ.Genç).

Bulb globose, 2–3 cm in diameter, outer tunics blackish, disintegrating, inner tunics white. Scape 20–50 cm long above ground, slightly flexuous, cylindrical, 2–3 mm in diameter, basal part green or flushed carmine. Leaves 3–5, linear-lanceolate, 2–5 cm wide and 7–25 cm long, green, with narrow white margin, smooth. Spathe most often completely split in 2–3 triangular parts, initially adpressed to the pedicels and later deflexed, faintly purplish with somewhat darker veins. Inflorescence semispherical at flowering time, subspherical in fruit, dense, 4–8.5 cm in diameter. Pedicels cylindrical, up to 2 cm long, almost equal, greenish or slightly carmine-flushed. Perigone nearly campanulate. Tepals obovate-orbiculate, obtuse at apex, often undulate, 3.5–6 mm wide and 6–7 mm long, white with green midvein. Filaments 4/5 as long as tepals, fleshy, basally united, triangular, white. Anthers yellow. Ovary depressed-globose with three furrows, dark purplish-black. Style cylindrical. Capsule globose, with three longitudinal furrows, 5–6 mm wide. Seeds ovate to broadly ovate, rugose, blackish, 3–4.2 mm long. The seed testa cells angularly round, convex, with many prominent verrucae and omega-like (Ω) undulations with moderate wavelength. Flowering in May–June, fruiting in June–August. 2n = 16.

Distribution, habitat and ecology:—*Allium undulatitepalum* is a local endemic restricted to the Salamut, Güzle and Çimi Plateaus in Antalya, southwestern Anatolia (Fig. 1E). It is a territory belonging to the Mediterranean floristic region. The new species colonizes only calcareous stony and grassy slopes close to *Cedrus libani* forests, between 1400–1700 m of elevation (Fig. 1D).

Etymology:—The species epithet is derived from its undulate tepals representing the one of the main characters which distinguish it from other similar species (Fig. 2).

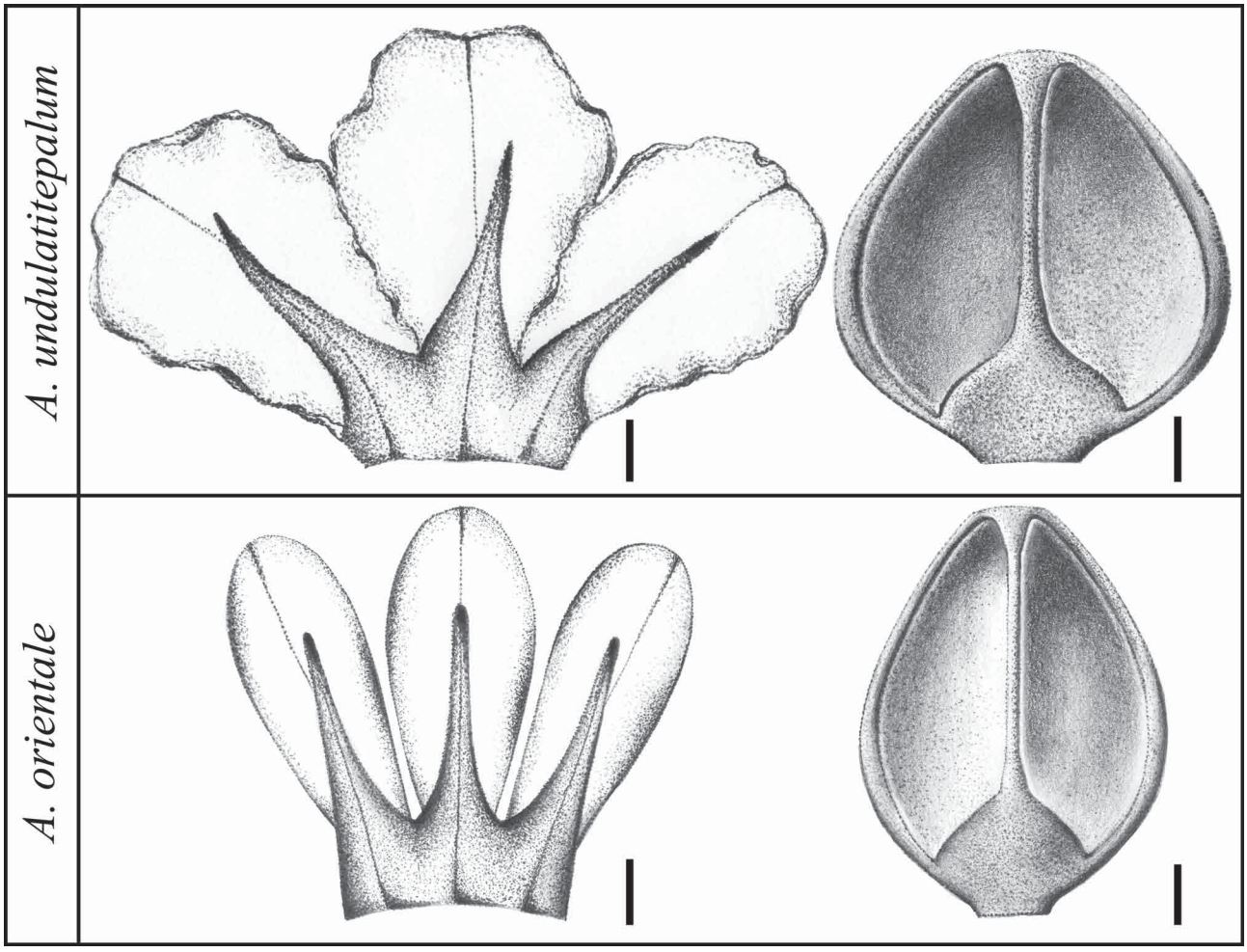

FIGURE 2. Shape of tepals, filaments, and capsule valves of *Allium undulatitepalum* and *A. orientale* (all scale bars 1 mm).

Molecular analysis:—The ITS sequences of *A. undulatitepalum* had ≥ 99% nucleotide identity with *A. orientale* and also ≥ 96% with *A. nigrum* according to the BLAST analysis. The *A. orientale*, *A. multibulbosum* and *A. nigrum* alliances are related to each other morphologically, but occur in different clades (Fig. 3). *Allium undulatitepalum* is closely related to, but separate from *A. orientale*, occurring in the same clade. These molecular results supported the data obtained from the morphological studies with reliable bootstrap values. For ITS studies, alongside of *A.*

undulatitepalum accessions some sequences of related taxa in the subgenus were retrieved from NCBI nucleotide database and compared. According to records of the NCBI and Fritsch *et al.* (2010), all *A. orientale* specimens were collected from Antalya (FM177375–177377) and some *A. multibulbosum* (FM177370) specimens from İzmir Province in Turkey, respectively. The accessions FM177368–177370 were cited as *A. nigrum* in Gurushidze *et al.* (2008), then these samples were considered as *A. multibulbosum* (Fritsch *et al.* 2010, Fragman-Sapir & Fritsch 2011) (Fig. 3). *A. nigrum* was included in molecular studies with specimens collected in Demre County, Antalya. Phylogenetic relationships among the studied *Allium* species, based on ITS sequences were in agreement with previous studies (Gurushidze *et al.* 2008, Fritsch *et al.* 2010).

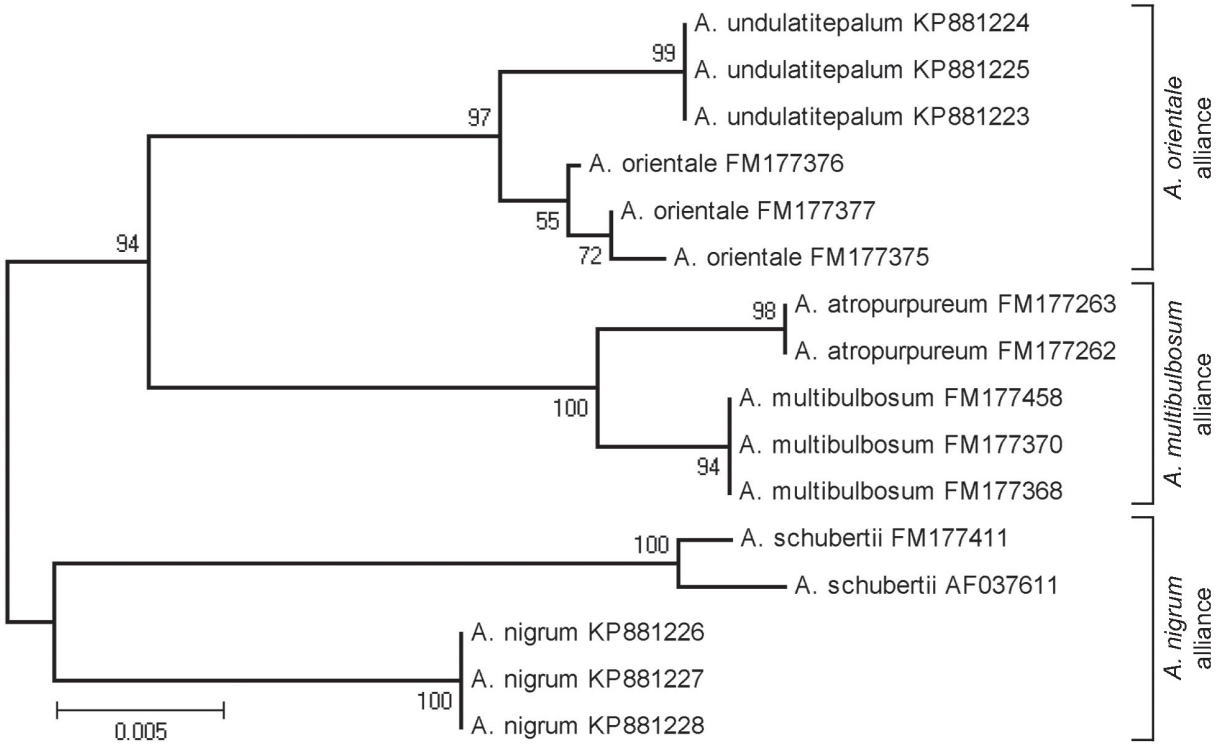

FIGURE 3. Maximum likelihood tree of ITS sequences with bootstrap values from 6 *Allium* species (GeneBank accession numbers are given behind the *Allium* names, abbreviations starting with "KP" are listed in the 'specimens examined' section).

Karyotype:—In the present study, the somatic chromosome number of *A. undulatitepalum* was determined as 2n = 16 (Fig. 4F). The karyotype formula of the new species consists of twelve chromosome pairs with centromeres in median position and four pairs with centromeres in submedian position (12m + 4 sm^{sat}). The shortest chromosome pairs is 11.42 µm long, the longest is 15.13 µm and the haploid chromosome length is 107.1 µm. The basic chromosome number of the species in sect. *Melanocrommyum* is x = 8. The majority of Turkish species have 2n = 16 chromosomes (Genç 2010). In the present study, the somatic chromosome number of *A. undulatitepalum* was determined to be 2*n* = 16 (Fig. 4F). *Allium orientale* is similar to the new species in its diploid character 2n = 16 (Bartolo *et al.* 1984, Pogosian & Seisums 1992, Genç *et al.* 2013) but different cytotypes of *A. orientale* were reported as being triploid or tetraploid 2n = 24, 32 from Cyprus (Tzanoudakis 1999). In the present study, the karyotype formula of *A. undulatitepalum* was determined as 12m + 4sm^{sat}. Conversely, according to Genç *et al.* (2013), *A. orientale* has 10m + 6 sm^{sat} chromosomes. *Allium undulatitepalum* shows also chromosomes larger than *A. orientale.*

Pollen characters:—*A. undulatitepalum* and *A. orientale* have monosulcate pollen grains and the exine ornamentation is perforate, striate and rugulate in both species (Fig. 4E, 4G). Also, the pollen shape (based on LA/SA ratio) is prolate in distal view, and circular in polar view (Fig. 4D). According to LM measurements, *A. undulatitepalum*: long axis (28.3–)32.66(–36.75) ± 1.96 µm, short axis (14.7–)18.54(–24.15) ± 2.32 µm; *A. orientale*: long axis (28.35–)32.57(–36.75) ± 1.74 µm, short axis (16.8–)20.05(–23.1) ± 1.64 µm. Results of the SEM and LM studies indicate that sulcus extends from distal face to polar points but does not reach the proximal face and that sulcus ends are rounded in both species. Palynological characters and measurement data from *A. undulatitepalum* and *A. orientale* are very similar and are consistent with the results of previous studies conducted on different species of the sect. *Melanocrommyum* (Özhatay & Koçyiğit 2009, Deniz *et al.* 2013).

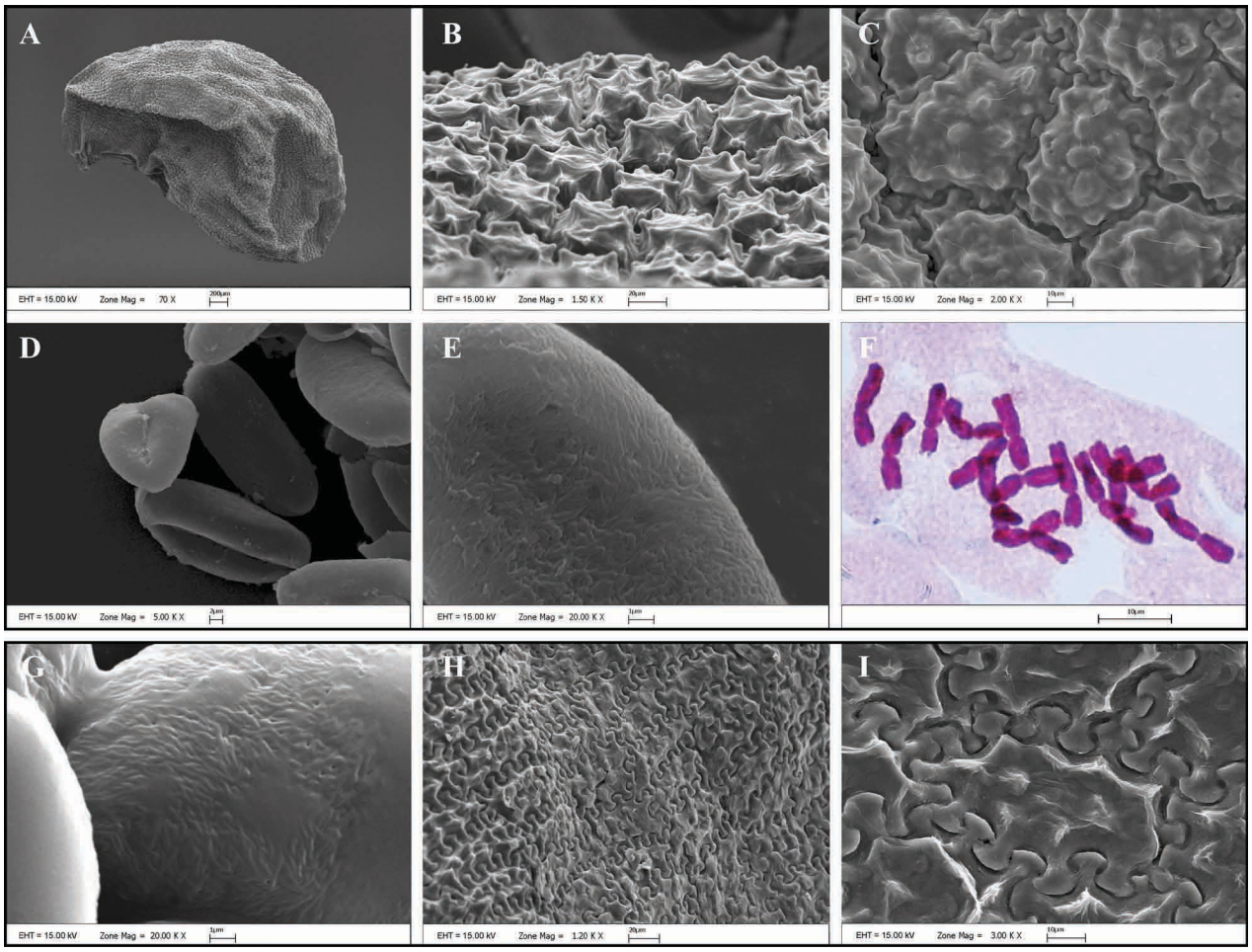

FIGURE 4. *Allium undulatitepalum* (A–F) and *A. orientale* (G–I): A. Seed, B. Periclinal walls, C & I. Testa cells and undulation of anticlinal walls, D. Pollen grains, E & G. Exine ornamentation, F. Mitotic metaphase chromosomes 2n = 16, H. Periclinal and anticlinal walls (Specimens examined: A–C *Deniz* 5827, D–E *Deniz 5547,* F *Genç 1182*, G *Genç 1360*, F *Genç 1363*).

Seed characters:—The seeds are ovate to broadly ovate, blackish and rugose in *A. undulatitepalum* (Fig. 4A). When compared from an identical midpoint position on the dorsal surface of the seeds, the dominant shape of *A. undulatitepalum* testa cells is angularly rounded while those of the related *A. orientale* are elliptic-oblong (Fig. 4C, 4I). The new species presented in the study has depressed, omega-like (Ω) undulated anticlinal walls and strongly convex, prominently well developed verrucate periclinal walls (Fig. 4B, 4C). On the other hand, *A. orientale* has one or two rowed and less prominent verrucae on the periclinal walls (Fig. 4H, 4I). It is reported that the seeds of subg. *Melanocrommyum* are black, generally ovate and somewhat larger than those of most *Allium* species belonging to other groups (Neshati & Fritsch, 2009). Many species of subg. *Melanocrommyum* share convex periclinal walls with verrucate sculptures and S-, U- and omega-like (Ω) undulated anticlinal walls. Transitions also occurred between these types (Fritsch *et al.* 2006, Neshati & Fritsch 2009, Celep *et al.* 2012).

Taxonomic relationships:—*Allium nigrum* and *A. orientale* are widely distributed and variable members of the sect. *Melanocrommyum*. They were long considered as a taxonomically problematic species. The neotypification of *A. nigrum* was defined by Seisums (1998), some variants occurring in Israel were described as new species separate from *A. nigrum* and *A. orientale* by Fragman-Sapir & Fritsch (2011), and useful morphological qualitative-quantitative characters separating *A. nigrum* and *A. cyrilli* were presented by Peruzzi *et al.* (2012). As a result of these studies, *A. multibulbosum* was accepted as a valid species different from *A. nigrum* again. Also, it was understood that *A. orientale* has a more narrow distribution in Turkey. Due to this confusion, some herbarium specimens of *A. undulatitepalum* were identified as *A. nigrum* or *A. orientale* in the past. As a result of our studies and investigations, *A. undulatitepalum* is morphologically related to *A. orientale*. The detailed morphologic differences between *A. undulatitapalum* and *A. orientale* are presented in Table 1. At the first glance, *A. undulatitepalum* is clearly distinguishable from *A. orientale* by broad and undulate tepals, the dark purplish-blackish ovarium in every stage, the semispherical and dense inflorescence, and by broad leaves.

TABLE 1. Diagnostic morphological characters of *A. undulatitepalum* and *A. orientale*.

Characters	*A. undulatitepalum*	*A. orientale*
Bulb	Globose	Ovoid
Scape	20–50 cm	20–40 cm
Leaves	3–5, 2–5 cm wide	3–7, 0.8–2 cm wide
Inflorescence	Semispherical	Fastigiate-semispherical
Perigone segments	After anthesis reflexed, 6–7 × 3.5–6 mm, white with green midvein, edges often undulate	After anthesis reflexed and twisted outwards, 5–6 × 2–3 mm, pinkish white-pink with green or pinkish midvein, edges straight
Ovary	Purplish black in every stage	Green to turn purple
Capsule	Globose	Elliptic to pear shaped
Seed testa cells	Angularly round, well convex and many prominent verrucae	Elliptic-oblong, convex, one or two rowed, less prominent verrucae

Additional specimens examined of *Allium undulatitepalum* (paratyes):—TURKEY. C3 Antalya: Manavgat, Güzle Upland, meadows, 1400 m, 7 May 1982, *T. Ekim*, *M. Koyuncu 5267b* (ISTE 54418!); Akseki, Çaltılıçukur Village, Salamut Upland, 1600 m, 9 May 1982, *T. Ekim*, *M. Koyuncu 5285a* (ISTE 54420!); Akseki, Çimi Village, Çimi Upland, Aldürbe area, 1500–1700 m, 22 May 1983, *M. Koyuncu 6035*, *S. Erik* (AEF 19392!); Akseki, Çaltılıçukur Village, Salamut Plateau, calcareous slopes 1640 m, 7 May 2008, *İ. Genç et al. 1182* (ISTE 91484!); *ibidem*, 13 June 2012, *H. Sümbül*, *C. Aykurt*, *İ.G. Deniz 4578* (AKDU!; NCBI Accession No: KP881225); *ibidem*, 14 May 2014, *İ.G. Deniz 5545* (AKDU!; NCBI Accession No: KP881223); *ibidem, 5546* (AKDU!; NCBI Accession No: KP881224), *ibidem, 5547* (AKDU!); *ibidem,* 5 August 2014, *İ.G. Deniz 5827* (AKDU!).

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APPENDIX 1. Additional specimens examined of taxa related to *A. undulatitepalum*

Allium orientale:—CYPRUS. Cultivated fields, sea level, 26 February 1934, *s.c. s.n.* (K!).TURKEY. C3 Antalya: Cilicicus, s.d., *Aucher-Eloy 2188* (epitype G photo!); Termessos, s.d., *V. Horton* 1162 (K!); Termessos National Park, 900 m, 28 October 1976, *T. Baytop*, *H.J. Leep 36223* (ISTE!); *ibidem*, 26 April 1976, *A. Baytop 34712* (ISTE!); *ibidem*, 30 April 1979, *A. Baytop 41805* (ISTE!); *ibidem*, 22 April 1974, *G. Dökmeci*, *E. Tuzlacı*, *Y. Doğantan 27654* (ISTE!); *ibidem*, 8 May 1983, *K. Alpınar 50309* (ISTE!); *ibidem*, 23 May 2009, *İ.G. Deniz*, *İ. Genç 1363* (ISTE 91576!); Hisarçandır, openings in *Pinus brutia* forest, 960 m, 15 May 2014, *İ.G. Deniz 5555* (AKDU!); Akseki-Konya road, 4.5 km from Akseki junction, 1138 m, 7 May 2008, *İ. Genç 1193* (ISTE 91485!); Akseki, E of Süleymaniye Village, 1394 m, 22 May 2009, *İ.G. Deniz*, *İ. Genç 1358* (ISTE 91572!); Between Hisarçandır-Sarıçınar, 3 km to Hisarçandır, 1097 m, 23 May 2009, *İ.G. Deniz*, *İ. Genç 1360* (ISTE 91573!); Isparta: Davras Mountain, Sav Village, 23 May 1966, *A. Baytop 9652* (ISTE!); C4 Konya: Between Beyşehir-Akseki, Küpe Mountain, Teke Upland, 1350–1450 m, 19 May 1984, *M. Koyuncu 6877* (ISTE 54424!).

Allium nigrum:—CYPRUS. Plantae per insulam Cypro lectae, 6 April 1862, *T. Kotschy s.n.* (BM!). GREECE. Iter Aegaeum, Rhodos, Montes Akramiti, in agris incultis vallis longitudinalis, ca. 600 m, 19 May 1935, *K.H. & F. Rechinger 7452* (BM!); Flora Graeca Exsiccata, in Chio, prope Sephi, *T.G. Orphanides 841* (K!). ITALY. 2 km NE of Fiesole, 360 m, 20 May 1961, *s.c. s.d.* (K!). TURKEY. C1 Aydın: Kuşadası District, 20 May 1974, *G. Ertem 27426* (ISTE!); C2 Antalya: Demre, Davazlar, Hoyran Village, fields, 460 m, 8 April 2012, *İ.G. Deniz 4411* (AKDU!; NCBI Accession No: KP881226); *ibidem, 4412* (AKDU!; NCBI Accession No: KP881227); *ibidem, 4413* (AKDU!); ibidem, 11 April 2014, *İ.G. Deniz 5497* (AKDU!; NCBI Accession No: KP881228); Muğla: d. Bodrum, Musgebi to Karatoprak, fields, 50–100 m, 12 April 1965, *Davis 40963* (K!); Between Kaş-Kale, 38 km to Kaş, Hoyran District, 500 m, 29 June I980, *M. Saraçoğlu 44041* (ISTE!); C4 İçel: Silifke, Sarıaydın Village, 1800 m, 6 May 1981, *T. Baytop 46198* (ISTE!); C5 İçel: Tarsus-Namrun road, Belçınar Village, fields, 950 m, 25 May 1977, *M. Koyuncu* (HUB 6137!, AEF!); C6 Hatay: Açana District near Antakya, 27 April 1967, *G. Clark s.n.* (K!).

A. multibulbosum:—GREECE. Iter Graecum, Insula Mytilina (Midilli), in Lapidosis trachyticis ad Philia, ca. 300 m, 18–24 May 1935, *K.H. & F. Rechinger 5901* (BM!). TURKEY. B1 İzmir: Yamanlar Mountain, Karagöl, s.d., *Alava & Bocquet 5088* (K!); Manisa: Manisa Mountain, 650 m, 25 May 1973, *Seçmen & Leblebici 16436* (EGE!); Balıkesir: Between Doyuran-Þahinderesi, 1000 m, 21 May 1992, *N. & E. Özhatay 64273* (ISTE!); C2 Burdur: Ören, Karanlık Canyon, 1350 m, 8 June 1996, *N. & E. Özhatay*, *H. Duman 72315* (ISTE!).