







Allium goumenissanum (Amaryllidaceae), a new species for Bulgaria and new localities in Greece, with additions to the genetic, cytogenetic and morphological characteristics of the species

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Abstract. *Allium* sect. *Codonoprasum* represents an evolutionarily young and rapidly radiating group of bulbous geophytes, with a significant proportion of polyploids and minor morphological differences between species. In the last 20 years, dozens of new species have been described from the Mediterranean. However, very little new information has been obtained on most of them since their description, especially in terms of cytogenetic, molecular and distribution data. *Allium goumenissanum* is a recently described species of this section, known only from three nearby localities in northern Greece. During the last 10 years, we collected population samples of an unknown species of this section from the southern Balkans, which seemed to be representative of *A. goumenissanum*. A variety of methods (cytogenetic, molecular, morphological and micromorphological) were used to compare the populations of an unknown species with the morphologically similar species *A. goumenissanum* and *A. stamatiadae*. AFLP, ITS sequencing and genome size analyses showed that all populations of the unknown species were in fact *A. goumenissanum* distinct from *A. stamatiadae*. The characterization of the morphology and reproduction of *A. goumenissanum* is completed, and micromorphological and anatomical characteristics of the leaves of both species are newly published. The distribution of *A. goumenissanum*, based on new data, now includes a more extensive area of north central Greece, and the species is found as a new taxon for Bulgaria.

Keywords. *Allium* sect. *Codonoprasum*, *Allium stamatiadae*, AFLP, epidermal micromorphology, distribution, flow cytometry, genome size, ITS, karyology, leaf anatomy, morphology, polyploidy.

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Introduction

Allium L., one of the largest monocotyledonous genera (Friesen *et al.*, 2006; Govaerts *et al.*, 2021), is critical from a taxonomic point of view due to its remarkable polymorphism, accelerated species diversification associated with adaptation to a wide range of habitats and frequent polyploidy (Peruzzi *et al.*, 2017; Han *et al.*, 2020; Duchoslav *et al.*, 2020). Only minor morphological differences are often observed between many closely related species, which are further obscured by the modification or even loss of many diagnostic characters in dry specimens (Don, 1827; Brullo, 2009; Li *et al.*, 2010). This is particularly true for *A.* sect. *Codonoprasum* Rchb. (Reichenbach, 1828), which is one of the largest sections of the genus. This section has the centre of diversity in the Mediterranean region, especially in Greece and Turkey (Wilde-Duyfjes, 1976; Stearn, 1981; Kollmann, 1984, 1986; Pastor & Valdés, 1985; Özhatay, 1990; Brullo *et al.*, 1996, 1998, 2001,

2014; Hanelt, 1996; Trigas & Bareka, 2020). Unclear taxonomic boundaries and misapplication of published names (Wilde-Duyfjes, 1976; Stearn, 1981), which resulted in many described names being overlooked or neglected, together with the gradual discovery of the morphological and phenotypic diversity of section members, have led to a renewed interest in the taxonomy of this section over the last 25 years. These efforts have resulted in the description of dozens of new species, especially in Turkey (e.g. Koçyiğit & Özhatay, 2012; Koçyiğit *et al.*, 2016; Özhatay *et al.*, 2018; Koçyiğit and Kaya, 2020; Armağan, 2021; Brullo & Salmeri, 2021), Greece (e.g. Tzanoudakis & Tan, 2000; Trigas *et al.*, 2010; Kalpoutzakis *et al.*, 2012; Tzanoudakis & Trigas, 2015; Galanos & Tzanoudakis, 2017, 2019; Cattaneo, 2020; Brullo & Salmeri, 2021) and Italy (e.g. Brullo *et al.*, 1997a, b, 2003, 2007, 2014), in addition to the taxonomic re-evaluation of several previously described species (e.g. Brullo *et al.*, 1991, 1994, 1996, 1998, 2008; Salmeri *et al.*, 2016; Vojtěchová *et al.*, 2023).

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Many of the newly described species in this section are considered endemic, as they are reported only from the type locality or several neighbouring sites, and no progress in floristic exploration has been recorded for them since their description. In many of these cases, especially those described from islands (Tzanoudakis & Vosa, 1988), e.g. the southern and south-eastern Aegean islands (e.g. *A. platakisii* Tzanoud. & Kypr.; Tzanoudakis & Kypriotakis, 1993; *A. archeotrichon* Brullo, Pavone & Salmeri; Brullo *et al.*, 1999; *A. symiacum* Galanos & Tzanoud.; Galanos & Tzanoudakis, 2017), there is no doubt that they are truly endemic and taxonomically isolated. In other cases, additional research may reveal that species originally thought to be local endemics actually have a much wider distribution (Galanos & Tzanoudakis, 2017), which in turn has significant implications for their conservation status. This is exemplified by *A. brussalisii* Tzanoud. & Kypr., originally described as a local endemic of Mt. Parnis near Athens (Tzanoudakis & Kypriotakis, 2008), but now considered to be an eastern Mediterranean species reported from all over southern Greece (Kalpoutzakis *et al.*, 2019; Ioannidis & Tzanoudakis, 2022) and Asia Minor (Koçyiğit *et al.*, 2014). The only slight morphological differences between the species of this section, the lack of a more comprehensive taxonomic treatment and key, and the need to work with live plants at the appropriate phenological stage make floristic work considerably more difficult. Moreover, it is not the rule that newly described species of this section are accompanied by at least basic cytogenetic (e.g. genome size) and molecular data (e.g. ITS, ETS, plastid region) in addition to the regularly employed chromosome counting, although they are beneficial for the taxonomy and evolution of the genus *Allium* (e.g. Friesen *et al.*, 2006; Salmeri *et al.*, 2016; Bagheri *et al.*, 2020; Friesen *et al.*, 2022; Khassanov *et al.*, 2023; Vojtěchová *et al.*, 2023), and could be helpful in cases of doubt.

During the last decade, several populations of unknown, late-flowering *Allium* species belonging to the *A. sect. Codonoprasum*, have been collected by us in northern Greece and Bulgaria. Taking into account the recent publications by Trigas & Bareka (2020) and Ioannidis & Tzanoudakis (2022), these populations seem to be morphologically very similar to the recently described *A. stamatiadae* Trigas and *A. goumenissanum* Ioannidis & Tzanoud., known so far from the island of Andros in the Cyclades and from the foothills of Mt. Paiko in northern Greece, respectively. Using additional population samples from the *loci classici* of these species and combining classical (morphology, leaf micromorphology and anatomy; Salmeri, 2019) with cytogenetic (flow cytometry) and molecular approaches (AFLP and ITS), we attempt to determine the taxonomic position of the collected populations, to establish genetic relatedness between the taxa studied and to complete their descriptions. The use of complementary biosystematic tools for species identification (Pyšek *et al.*, 2013) allowed us to assign populations of unknown species to *A. goumenissanum*, and the newly collected

records of this species contributed to a significant expansion of its distribution.

Material and methods

Plant material

During the period 2009–2021, several botanical expeditions were carried out in the central and southern parts of the Balkan Peninsula, in order to collect various representatives of *Allium* sect. *Codonoprasum*. In total, 25 plants from five populations of unknown, late flowering *Allium* (henceforth *Allium* sp.) were collected in the field. To compare the populations of *Allium* sp. with morphologically similar and recently described species, seven and three individuals from the type localities of *A. goumenissanum* (Greece, Mt Paiko, WNW of the village Goumenissa, at the margins of oak forest, elevation 300–400 m asl, 40°57'N, 22°26'E; Ioannidis & Tzanoudakis, 2022) and *A. stamatiadae* Trigas (Greece, Cyclades: Andros island, ca 400 m SW of Vourkoti village, woodland along Vourkoti stream, 37°51'25"N, 24°53'6"E; Trigas & Bareka, 2020), respectively, were collected. All sampled plants were transported, potted and cultivated in the experimental garden (common garden) of Palacký University in Olomouc, Czech Republic (coordinates: 49°34'32"N, 17°17'0"E) and used in selected analyses.

In addition, individuals of several related species of *Allium* sect. *Codonoprasum* (*A. paniculatum* L., *A. marginatum* Janka, *A. fuscum* Waldst. & Kit.) with no published DNA sequences were used for DNA extraction and together with selected published DNA sequences of 20 representatives of *A. sect. Codonoprasum* and outgroup *Allium* sect. *Allium* obtained from GenBank were included in the selected molecular analyses. A survey of analysed taxa, populations and analyses is available in Supplementary Material S1. Voucher specimens were deposited in the Herbarium of Palacký University in Olomouc (OL).

Molecular methods

Total genomic DNA was extracted from ca. 70 mg of fresh leaves following the CTAB protocol (Doyle & Doyle, 1987) with minor modifications. The quality of isolated DNA was checked using agarose electrophoresis and the DNA concentration was measured using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, USA).

AFLP fingerprinting was performed for 98 individuals from 28 populations with 1 to 5 individuals per population, including all 5 populations of *Allium* sp., the type population of *A. stamatiadae*, and populations of closely related species *A. paniculatum*, *A. fuscum* and *A. marginatum*. Unfortunately, plants from the type population of *A. goumenissanum* were not available when doing AFLP. Nineteen individuals were replicated to allow the determination of the error rate, and two blanks (DNA replaced with water) were included to test for contamination. The AFLP procedure followed the

protocol of Vos *et al.* (1995) with modifications described in Schönswetter *et al.* (2009). Additionally, 0.25 U of polymerase was used in the pre-selective and selective amplifications, and 0.4 U for the NED-labelled primer combination. Twelve selective primer combinations were initially screened. Finally, the three primer combinations for selective PCR (fluorescent dye in brackets) were used: EcoRI (FAM)-ACA/MseI-CATG, EcoRI (VIC)-ACG/MseI-CAAC, and EcoRI (NED)-AAC/MseI-CAGG. Electropherograms were visualised in Genographer 1.6.0 (Montana State University, USA) for fragment scoring and problematic profiles were removed. The remaining profiles were scored using Peak Scanner 2.0 (Applied Biosystems, Foster, USA), using default peak detection parameters. Automatic binning and scoring were performed using RawGeno 2.0.1 (Arrigo *et al.*, 2009) in R 2.15.2 (R Core Team, 2012), with the following settings: scoring range = 100–500 bp, minimum intensity = 80 relative fluorescence units, minimum bin width = 1 bp, and maximum bin width = 1.5 bp. Fragments with a reproducibility of less than 85 % based on a single sample comparison were discarded. The error rate (Bonin *et al.*, 2004) was calculated by RawGeno 2.0.1. Fragments present or absent in only one individual were removed from the data set. Principal coordinate analysis (PCoA) based on the Jaccard distance matrix between individuals was calculated in Canoco 5 (ter Braak & Šmilauer, 2012). A Neighbor-Net network based on uncorrected P distances was calculated using SplitsTree CE (Huson & Bryant, 2006). To evaluate the support for the major branches of the Neighbor-Net network, a bootstrap analysis with 1000 pseudoreplicates was performed.

On the basis of published sequence data for *Allium* sect. *Codonoprasum*, a nuclear ribosomal internal transcribed spacer region (nrITS) was selected for sequencing from 11 individuals from five populations of *Allium* sp., 7 individuals from the type population of *A. goumenissanum*, 3 individuals from the type population of *A. stamatiadae*, and 2 individuals each from *A. paniculatum*, *A. fuscum* and *A. marginatum* (Supplementary Material S1). The PCR reaction was performed using GoTaq® G2 DNA Polymerase (Promega Corp., Madison). Each reaction mixture of a total volume of 15 µl contained 1 µl of genomic DNA, 5X Green GoTaq® Reaction Buffer, 3mM MgCl₂, 0.1 mM forward and reverse primers (ITS1, ITS4, White *et al.*, 1990) and dNTP, and 0.08 µl of GoTaq® G2 DNA Polymerase (5 U/µL). Reaction conditions for ITS loci included an initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles comprising 95 °C for 30 s, 52 °C for 45 s, and 72 °C for 45 s, with a final extension at 72 °C for 5 min. After the PCR, products were visualised on agarose gel, purified using enzymatic purification protocol (Kim & Blackshaw, 2001) and bidirectionally sequenced by Macrogen Europe (Amsterdam). The sequences were aligned and edited in Geneious 7.1.7 (Kearse *et al.*, 2012). Nucleotide sequences were deposited in GenBank using BankIt (<https://www.ncbi.nlm.nih.gov/WebSub/>).

Phylogenetic and molecular evolutionary analyses of both newly sequenced and published DNA sequences of several *Allium* species, including various representatives of *A.* sect. *Codonoprasum* obtained from GenBank,

were performed using MEGA version 11 (Tamura *et al.*, 2021) for Maximum Parsimony (MP), and MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2001) as implemented in the graphical user interface siMba (Mishra & Thines, 2014) for Bayesian Analysis (BA). Model selection was conducted using ModelFinder (Kalyaanamoorthy *et al.*, 2017) within IQ-TREE (Nguyen *et al.*, 2015). As a result, we used Tamura-Nei model with gamma distribution for MP tree (pairwise deletion, 1000 bootstrap replicates; Felsenstein, 1985). BA was performed under the General Time Reversible model, run for 5 M generations, sampled every 1000th generation, and 3000 trees have been discarded as burn-in. All other settings were set as default.

Chromosome counts and flow cytometry

Young roots were harvested from the pot-grown plants of populations 5 and 6 (Supplementary Material S1), pretreated with 8-hydroxyquinoline (0.002 M) for 4 h in the dark at room temperature, fixed in a cold mixture of ethanol and acetic acid (3:1) overnight and then stored at 4 °C until use. Selected root tips were hydrolysed in 5 N HCl for 25 minutes, stained with Schiff reagent for 40 minutes and squashed in 45% acetic acid (Lillie, 1951). The preparations were photographed and counted using an Olympus CX-31 light microscope.

DNA ploidy (relative genome size, RGS; Suda *et al.*, 2006) and genome size (absolute genome size, AGS; Greilhuber *et al.*, 2005) were estimated by flow cytometry. Samples were prepared according to the protocol described by Duchoslav *et al.* (2010) and following the methods of Vojtěchová *et al.* (2023). *Triticum aestivum* L. ‘Saxana’ (2C = 34.24 pg) served as the primary internal standard; other internal standards were calibrated against *Triticum aestivum*, i.e. *Secale cereale* L. ‘Daňkovské’ (2C = 16.19 pg) and *Vicia faba* L. ‘Inovec’ (2C = 26.92 pg; Doležel *et al.*, 2007). In general, only histograms with a coefficient of variation (CV) of less than 5 % were accepted. For each sample, the fluorescence intensity of 3000 and 5000 particles was recorded for RGS and AGS estimation, respectively. For RGS estimates, individual plants or mixed samples of up to four plants per population were measured. Sample measurements for AGS were averaged per plant over three replicates on different days, and if the variation in AGS between days exceeded 2 %, the measurement was repeated (Doležel *et al.*, 2007). Forty-two and two plants were analysed for RGS and AGS, respectively, including plants from type localities of *Allium goumenissanum* and *A. stamatiadae*.

Phenology, morphology, epidermal micromorphology and leaf anatomy

Phenological observations and morphometric analyses were carried out on 12 individuals from four *Allium* sp. populations cultivated in the common garden according to procedures described by Vojtěchová *et al.* (2023). Thirty-eight morphological characters considered taxonomically important were measured, mostly using an adjustable

ruler or calliper, or scored on fresh material (Table 1). The data obtained were compared with the original description of the morphologically similar species *A. goumenissanum* (Ioannidis & Tzanoudakis, 2022), *A. stamatiadae* (Trigas & Bareka, 2020) and additionally with *A. rausii* Brullo, Guglielmo, Pavone, Salmeri & Terrasi (Brullo *et al.*, 2003).

Leaves were examined at the beginning of senescence, tepals and ovaries at the time of stigma receptivity, and stamens just before anther maturity and pollen release. During flowering, the position of the spathe valves was recorded. Reproductive parts, capsules and seeds were examined on at least three randomly selected flowers, fruits or seeds per plant. Size measurements of these characters were made from their digitised scans in ImageJ 1.50i (Rasband, 2021).

Finally, leaf blades were preserved in 70% ethanol for subsequent anatomical study.

Transverse sections of leaf blades from several individuals of *Allium* sp. and plants from type populations of *A. goumenissanum* and *A. stamatiadae* cultivated in the common garden were made manually with a razor blade and then stored in glycerol. Epidermal characteristics were studied by direct observation of epidermal slides of the central part of the adaxial side of leaf blade. The mesophyll was removed by boiling for 20 minutes at 100 °C in 80% lactic acid. The epidermal part of the leaf was then peeled off and a microscopic preparation was made. The character and distribution of micropapillae on the leaf margins were observed and the density of clavate papillae per 2 mm of the leaf was calculated.

Table 1. Comparison of morphological characters of cultivated accessions of newly sampled populations of *Allium goumenissanum* (formerly named as *Allium* sp.) with three related Greek autumn-flowering species of *Allium* sect. *Codonoprasum* (*A. goumenissanum*, *A. stamatiadae*, *A. rausii*). Typical values, ranges and descriptions are taken from the original descriptions (Brullo *et al.*, 2003; Trigas & Bareka, 2020; Ioannidis & Tzanoudakis, 2022). Descriptive statistics of all quantitative morphological characters and their ratios ((minimum–) 10% quantile – mean – 90% quantile (–maximum)) for cultivated accessions (*) are reported. Characters that have not been evaluated in the respective source are marked with a dash (–).

Character	<i>Allium goumenissanum</i> (original description)	<i>Allium goumenissanum</i> (formerly named as <i>Allium</i> sp.) *	<i>Allium stamatiadae</i> (original description)	<i>Allium rausii</i> (original description)
Bulb shape	ovoid	ovoid	ovoid to subglobose	ovoid
Bulb size (mm)	15 × 18	–	12–23 × 9–22	15–20 × 12–19
Bulb (outer tunics)	coriaceous, brown to reddish-brown	coriaceous, mostly brown, weakly reddish-brown	coriaceous, pale greyish-brown	coriaceous, dark brown
Bulb (inner tunics)	membraneous, whitish	membraneous, whitish	membraneous, whitish	membraneous, whitish
Bulblets	present, growing laterally or up to the upper part of the bulb, short-stalked, brownish to reddish	present, growing laterally or up to the upper part of the bulb, short-stalked (1–3 cm), brownish to reddish	present, mainly at the upper part of the bulb and the base of the stem, long-stalked, light brown to yellowish	–
Scape height (cm) and insertion	50–60, inserted centrally to the bulb	(34–) 35–43–61 (–62), inserted centrally to the bulb	25–55, inserted externally to the bulb	30–50, –
Leaf shape	fistulous, canaliculated, ribbed abaxially, glabrous	fistulous, pentagonal to canaliculated, ribbed abaxially, glabrous	fistulous, convex and ribbed abaxially, ± flat adaxially, glabrous	semicylindric, glabrous
Leaf sheathing of stem	1/2–2/3	1/2–2/3	1/2–3/4	1/3–1/2
Number of leaves	3–4	(5–) 5–5–7 (–7)	4–5	5–6
Leaf length (cm)	–	(10–) 11–15–18 (–20)	35–50	10–20
Leaf width (mm)	3–4	(2.0–) 2.1–2.9–3.5 (–3.7)	4–10	2–4
Shape of spathe	opposite	opposite	slightly reflexed	erect
Longer spathe valve length (cm)	15 (–20)	(7–) 8–12–17 (–18)	14–35	8–16
Shorter spathe valve length (cm)	8 (–12)	(4–) 4–7–11 (–12)	9–24	5–12
Density of papillae per 2 mm of leaf margin at the base of the 2 nd uppermost leaf	–	(0–) 0–1.3–11 (–11)	–	–
Density of papillae per 2 mm of leaf margin in the middle of the 2 nd uppermost leaf	–	(0–) 0–1.5–12 (–12)	–	–
Inflorescence	lax, fastigiate, subspherical	weakly lax, subspherical	lax, subhemispheric	lax, subhemispheric
Number of flowers	50–60	(30–) 31–56–97 (–98)	25–70	up 70
Pedicel length (mm)	8–20 (–30)	(17–) 18–21–25 (–26)	10–24	15–35
Tepal shape	outer elliptic and slightly mucronate, the inner obovate rounded	outer elliptic and slightly mucronate, the inner obovate and usually rounded	oblong to narrowly elliptic, rounded and often shortly apiculate at apex	elliptical, rounded and apiculate at the apex

Character	<i>Allium goumenissanum</i> (original description)	<i>Allium goumenissanum</i> (formerly named as <i>Allium</i> sp.) *	<i>Allium stamatiadae</i> (original description)	<i>Allium rausii</i> (original description)
Tepal colour	reddish–brown with greenish–white margin	reddish–brown with greenish–white margin	whitish tinged with brown	greenish–yellow tinged with brown
Tepal length (mm)	(5–) 6	(5.0–) 5.7–6.0–6.5 (–6.6)	5.5–6.5	6–7
Tepal width (mm)	(2–) 3	(2.0–) 2.3–2.5–2.6 (–2.9)	2.2–2.5	2.5–2.7
Ovary shape and colour	oblong–obovate, greenish, gradually narrowed and whitish towards the base and slightly rounded and yellowish in the upper part	oblong–obovate to cylindrical, gradually narrowed towards the base, greenish to yellowish, yellowish to greenish and papillose in the upper part	obovoid–cylindrical, narrowed at base, truncate and papillose above, yellowish and greenish in the upper part	obovoid–cylindrical, smooth, yellowish and dark green in the upper part
Ovary length (mm)	4	(3.8–) 3.9–4.3–4.7 (–4.8)	3.0–4.0	3.5–4.5
Ovary width (mm)	2	(1.6–) 1.8–2.0–2.2 (–2.3)	1.5–2.0	2.0–2.2
Style length (mm)	3	(2.0–) 2.2–2.5–3.0 (–3.1)	1.0–2.2	0.5–1.0
Stamen	included	included or partly exerted	included	included
Stamen filament colour	white	white	white	white
Stamen filament length (mm)	–	(2.7–) 2.9–3.7–4.1 (–4.4)	–	–
Anther shape and colour	purple, oblong–ovate	whitish changing to purple, rounded at the apex	oblong–elliptical, rounded to apiculate at the apex, whitish	ovate–elliptical, yellow
Anther length (mm)	1.2	(1.1–) 1.1–1.2–1.3 (–1.3)	0.7	1.3
Anther width (mm)	0.6	(0.6–) 0.6–0.7–0.8 (–0.8)	0.3–0.4	0.8
Capsule shape	pear-shaped	pear-shaped	obovoid to subglobose	subglobose, narrowing at the base
Capsule length (mm)	5.5	(4.9–) 5.2–5.8–5.9 (–6.1)	5.4–6.5	4.5–6.0
Capsule width (mm)	4.5	(4.2–) 4.3–4.6–4.7 (–4.8)	4.3–5.4	4.5–5.0
Seed length (mm)	3.5	(3.2–) 3.2–3.8–4.2 (–4.2)	3–4	–
Seed width (mm)	2	(1.4–) 1.5–1.8–2.1 (–2.2)	2	–

Results and discussion

Populations of Allium sp. belong to A. goumenissanum based on molecular and cytogenetic analyses

A total of 716 AFLP fragments were scored for 98 individuals from which high-quality, reproducible AFLP fingerprints were obtained. The average replicate error rate was 6.5% and the average number of loci per individual was 134. The Neighbor-Net network (Figure 1A) showed that accessions from all populations of *Allium* sp. form a homogeneous genetic group that is clearly separated from other species by a clear split with 100% bootstrap support. Within this group, each population is clustered separately. Accessions of *A. stamatiadae* from *locus classicus* formed another distinct genetic group with 100% support being most closely related to the former group. The first three PCoA axes confirmed the results of the Neighbor-Net analysis. Five well-separated genetic clusters were distinguished in the PCoA ordination space, corresponding to five taxonomic groups (Figure 1B).

Unfortunately, samples of *A. goumenissanum* from *locus classicus* were not available when the AFLP analysis was performed, so to test the phylogenetic position of the *Allium* sp. populations, the nrITS region was selected for sequencing, combining 27 newly sequenced accessions of five taxa and additional sequences of 15 species obtained from GenBank. The nucleotide alignment used for phylogenetic reconstruction is provided as Supplementary Material

S2. The maximum parsimony and Bayesian-inference-based phylogenetic trees were congruent, with well resolved topology. Figure 2 depicts the Bayesian tree. The accessions of *Allium* sp., *A. goumenissanum* and *A. stamatiadae* belong to a strongly supported clade that is weakly supported as a sister to a strongly supported clade formed by summer-flowering species of the *A. paniculatum* complex (*A. paniculatum*, *A. fuscum*, *A. marginatum*, *A. oleraceum* L.; Vojtěchová *et al.*, 2023), but also late summer-autumn flowering species typical for moist to wet, and usually saline sites near the sea coast (*A. savii* Parl., *A. telmatum* Bogdanović, Brullo, Giusso & Salmeri and *A. pseudotelmatum* Duchoslav & Jandová; Brullo *et al.*, 1994; Bogdanović *et al.*, 2008; Jandová *et al.*, 2017). Within the former lineage, two subclades are weakly supported, one representing accessions of *A. stamatiadae* and the second representing *A. goumenissanum* and all accessions of *Allium* sp. Two accessions of population n° 5 (*Allium* sp.) are nested separately within this subclade, but with weak support.

There are four SNP positions distinguishing between sequences of *A. stamatiadae* and *A. goumenissanum* in the nrITS region. Heterozygous state was observed at five SNPs in accessions from some populations of *Allium* sp. but also in a plant of *A. goumenissanum* from the type locality (Supplementary Material S3). This nucleotide additivity pattern is indicative of the uncompleted process of concerted evolution to homogenise all nrDNA repeats in hybrids and allopolyploids (Álvarez & Wendel, 2003), though the same pattern is also known to occur in some diploid species and their autopolyploids (Wang *et al.*, 2023b).

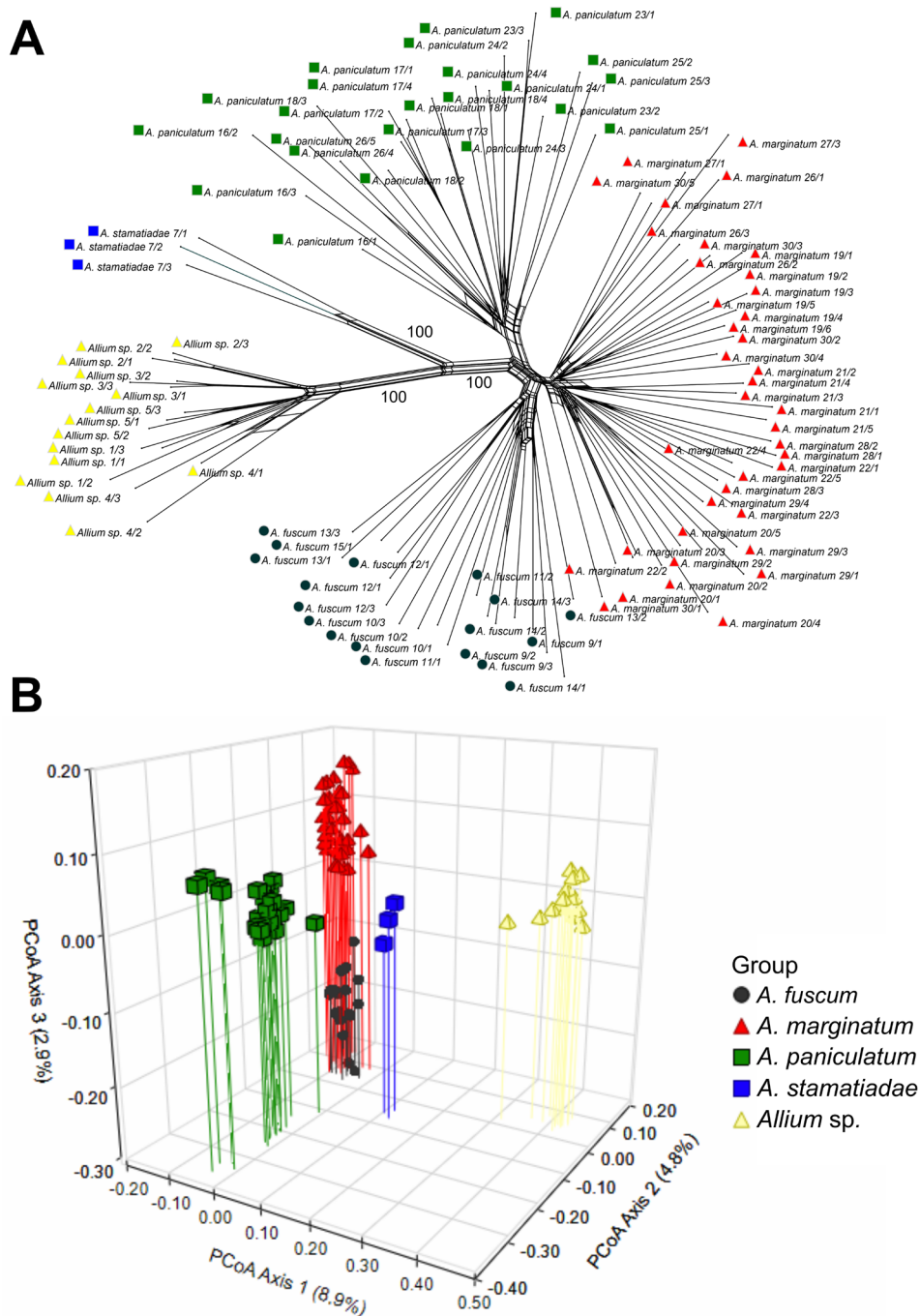


Figure 1. Results of molecular analyses of 98 individuals of the selected taxonomic groups based on 714 AFLP loci. A, Neighbor-net diagram. Bootstrap supports (in %) for the three splits concerning *Allium stamatiadae* and *Allium sp.* are reported. Population codes are explained in Supplementary Material S1; B, The first three axes (explained variation in parentheses) of the PCoA. The colours of points indicate taxonomic groups (black, *A. fuscum*; red, *A. marginatum*; green, *A. paniculatum*; blue, *A. stamatiadae*; yellow, *Allium sp.*).

The DNA content can be a useful additional marker for species identification and discrimination between closely related taxa (Suda *et al.*, 2007; Prančl *et al.*, 2014; Koblrová *et al.*, 2022; Vojtěchová *et al.*, 2023) and for identification of ploidy levels within polyploid complexes (Duchoslav *et al.*, 2010, 2013; Popelka *et al.*, 2019). The relative genome size (RGS) values of all newly recorded Greek populations of *Allium sp.* were similar to each other and similar to the RGS

values of *A. goumenissanum* individuals sampled from the *locus classicus* (Figure 3), which were found to be triploid ($2n = 3x = 24$, Supplementary Material S4; see also Ioannidis & Tzanoudakis, 2022). Only population n° 5 had slightly lower RGS than other populations, but triploid chromosome number was also confirmed in this population (Supplementary Material S4). The low intra-population variation of RGS also suggests that the populations studied are cytotype-uniform.

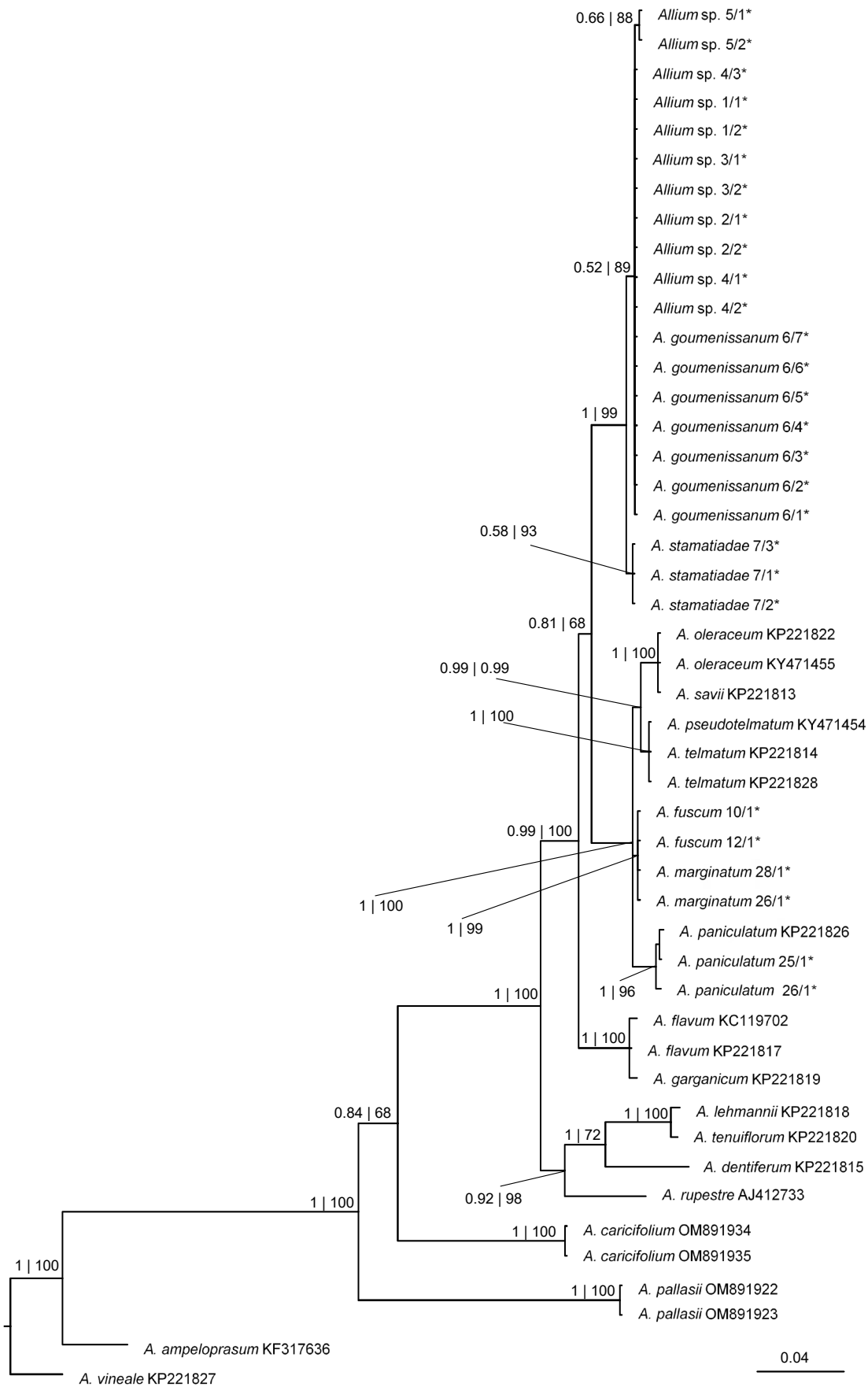


Figure 2. Evolutionary relationships among 47 *Allium* spp. accessions based on Bayesian analysis of nrITS region. On the phylogenetic tree, the numbers on branches denote statistical support in Bayesian Analysis (Bayesian posterior probability values ≥ 0.5) and Maximum Parsimony (Bootstrap support values $\geq 50\%$), respectively. Individuals newly sequenced in this study are denoted by *. For population codes (population/accession) and GenBank identities see Supplementary Material S1.

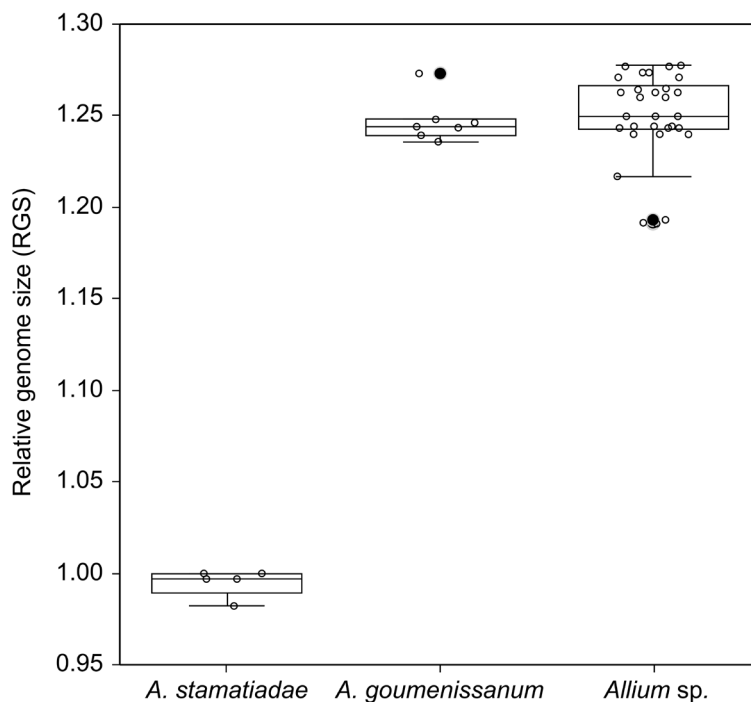


Figure 3. Box- and dot-plots of relative genome size (RGS) of plants from type localities of *Allium stamatiadae* and *A. goumenissanum*, and from localities of *Allium sp.* *Triticum aestivum* served as an internal standard in FCM for all populations except for *A. stamatiadae* where *Secale cereale* was used and later recalculated to *T. aestivum*.

Absolute genome sizes (AGS) were estimated for one plant from each of the type localities of *A. goumenissanum* (40.8 pg) and *A. stamatiadae* (33.6 pg). They represent the prime estimates for the species studied and clearly differ from each other. The AGS of *A. goumenissanum* is 1.21 times that of *A. stamatiadae*. This suggests that *A. goumenissanum* is not a recent neotriploid derived from the closely related diploid *A. stamatiadae* supposing genome size additivity in neoautopolyploids (Rebernig *et al.*, 2012; Soltis *et al.*, 2012). Compared with rare AGS estimates for other closely related species, the AGS of triploid *A. goumenissanum* is similar to that found in triploids of *A. oleraceum* (Duchoslav *et al.*, 2013), *A. paniculatum* (Vojtěchová *et al.*, 2023) and *A. carinatum* L. (Šmarda *et al.*, 2019). On the contrary, the recorded AGS value of *A. stamatiadae* is similar to the AGS of diploids of *A. carinatum* (Baranyi & Greilhuber, 1999) and *A. fuscum* (Vojtěchová *et al.*, 2023). It appears that the AGS is similar between taxa of the same ploidy within this group of closely related species. This could suggest strong phylogenetic signal in AGS and minor effect of selective pressures on changes in AGS. However, this hypothesis needs to be proven by analysing the evolution of genome size and phylogenetic relationships of a larger number of taxa in this section, as previous studies on AGS evolution in *Allium* showed contradictory results (Gurushidze *et al.*, 2012; Wang *et al.*, 2023a).

Considering the different ploidy levels and the genome size of *A. stamatiadae* and *A. goumenissanum/Allium sp.*, the positions of the studied populations on the AFLP network and the nrITS tree, and the observed karyotype of *A. goumenissanum* (Ioannidis & Tzanoudakis, 2022), we can conclude that all accessions of *Allium sp.* belong to *A. goumenissanum*

and that *A. goumenissanum* is either an allotriploid derived from two unknown parental diploid or diploid and tetraploid species, one of which was probably also the ancestor of *A. stamatiadae*, or an autotriploid derived from unknown diploid species.

Morphology, epidermal micromorphology and leaf anatomy: common garden observations

The morphology of accessions of *Allium sp.* cultivated in a common garden was compared with the published descriptions of *A. goumenissanum*, *A. stamatiadae* and *A. rausii*, representing the morphologically closest species. The morphology of the accessions studied was mostly consistent with the published description of *A. goumenissanum* (Table 1), confirming the results of the cytogenetic and molecular analyses and illustrating the stability of most morphological characters under different environmental conditions. Only the ranges of some quantitative characters (e.g. number of leaves and number of flowers) were found to be wider in cultivated accessions compared to the original description. With regard to qualitative characters, we observed slight differences in some characters between cultivated accessions and original description of *A. goumenissanum*, mainly concerning the shape and colour of the ovary and the position of the stamens (Table 1). In addition, purple anthers have been reported as a diagnostic character distinguishing *A. goumenissanum* from closely related species (Ioannidis & Tzanoudakis, 2022), but we found that in cultivated accessions this is typical of well-developed anthers, whereas young anthers are whitish. *Allium stamatiadae* and *A. rausii* were indeed similar to *A. goumenissanum* mainly in quantitative characters, but differed in many qualitative characters, e.g. shape of leaf and spathe, colour of tepals and anthers (Table 1).

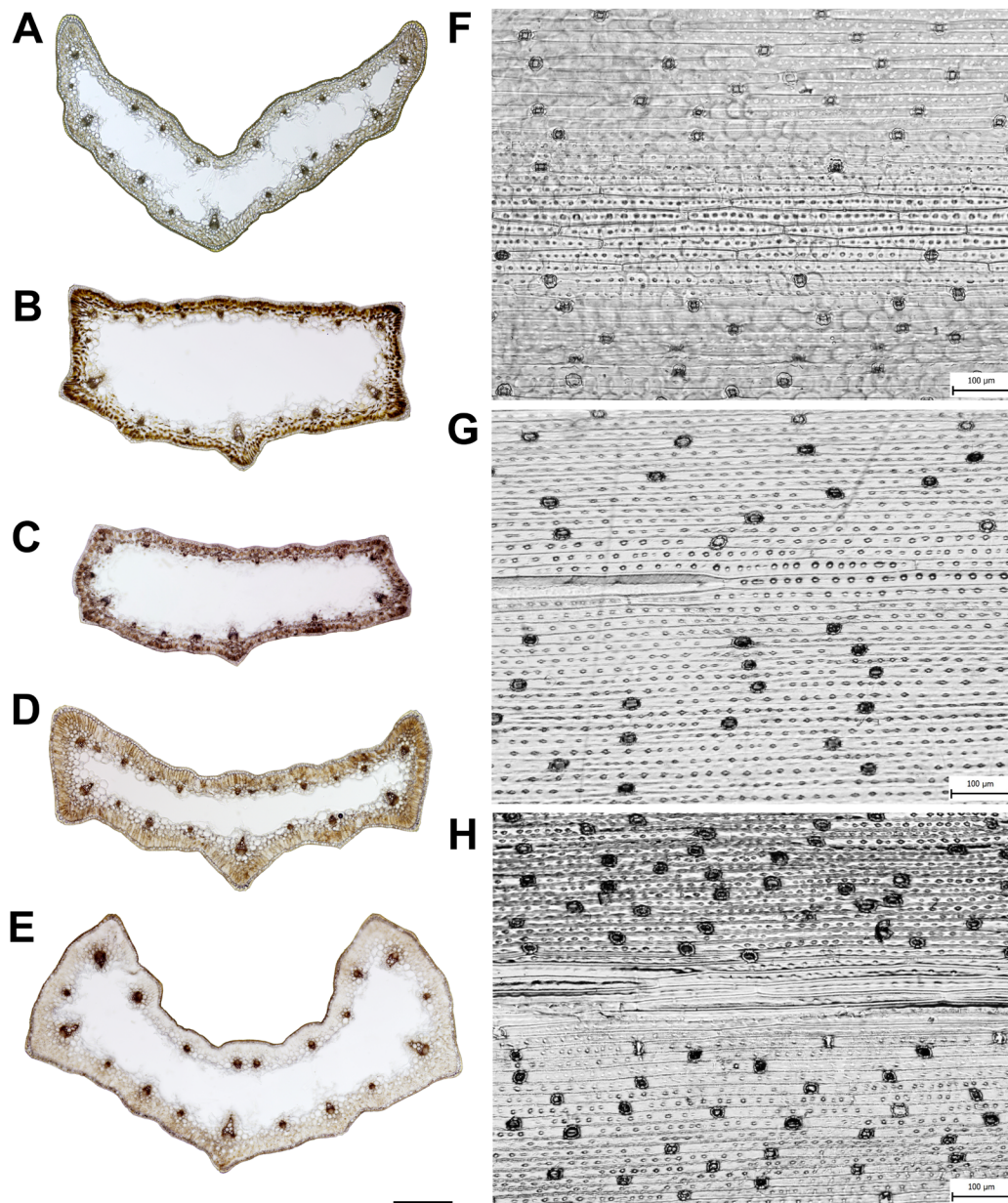


Figure 4. Morphology and anatomy of leaves. A–E, Light micrographs of transverse sections; F–H, Dermograms of the adaxial leaf surface of the leaves of cultivated accessions originating from type populations of *A. stamatiadae* (A, F), *A. goumenissanum* (B, G), and newly discovered populations of *A. goumenissanum* (formerly named as *Allium* sp.) (C–E, H). Scale bar = 500 µm (A–E), 100 µm (F–H). Photographs by K. Vojtěchová.

The shape of the leaf cross section and the structure of the leaf surface are considered taxonomically important characters in *A. sect. Codonoprasum* (Zahariadi, 1975; Brullo *et al.*, 1996; Koçyiğit & Özhatay, 2011). Differently shaped leaf cross sections were found between *A. stamatiadae* and *A. goumenissanum* cultivated in the common garden. *Allium stamatiadae* has a convex leaf shape, (slightly) ribbed abaxially and more or less flat adaxially (Figure 4A), in accordance with the original description (Trigas & Bareka, 2020). On the other hand, the leaves of *A. goumenissanum* (Figure 4B–E, 5E, H, I) had an overall pentagonal shape with two distinct ribs on the margins of the adaxial side and one in the middle vein of the abaxial side. This pentagonal cross-section was found to be variable both within and between individuals, ranging from a pentagonal shape with flat adaxial side to the canaliculate cross-section, which is

also mentioned in original description of *A. goumenissanum* (Ioannidis & Tzanoudakis, 2022; see Table 1). On the other hand, no obvious differences were found in the leaf surface as well as the anatomical structure of leaf blades between studied species (Figure 4), detailed description available in Supplementary Material S5.

The phenology of cultivated accessions of *A. goumenissanum* observed over five years in the Central European climate was similar to that reported for *A. goumenissanum* at the *locus classicus* in Greece (Ioannidis & Tzanoudakis, 2022). In particular, intensive development of daughter bulbs (bulblets) was observed in spring (March–April). Usually one or two newly formed, whitish bulblets on 1–5 cm long stalks grew either laterally or to the top of the bulb in adult plants (Figure 5C). Mature bulblets have a brownish to reddish

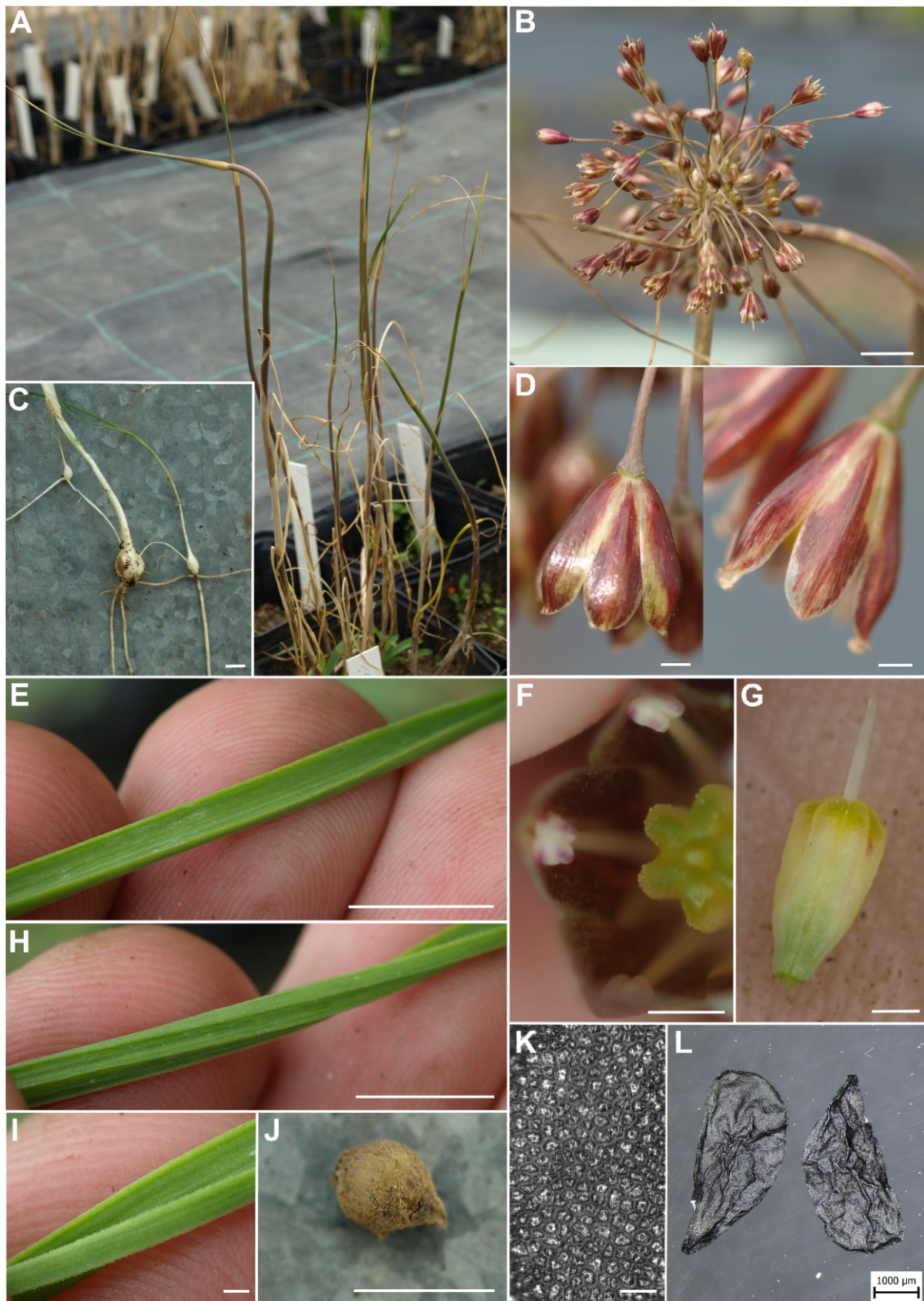


Figure 5. Habitus and morphology of cultivated accessions of newly discovered populations of *Allium goumenissanum* (formerly named as *Allium* sp.). A, Habitus (late July); B, Inflorescence; C, Mother bulb with two daughter bulbs (bulblets) on 2–5 cm long stolons and contractile roots (middle March); D, Flowers; E, Adaxial side of leaf; F, Anthers; G, Pistil; H, Abaxial side of leaf; I, Papillae on the margin of leaf; J, Mature daughter bulb (bulblet) in late September; K – L, Sculpture of testa and overall shape of seeds. Scale bar = 1 cm (B, C, E, H, J), 1 mm (D, F, G, I, L), 100 μ m (K). Photographs by M. Duchoslav.

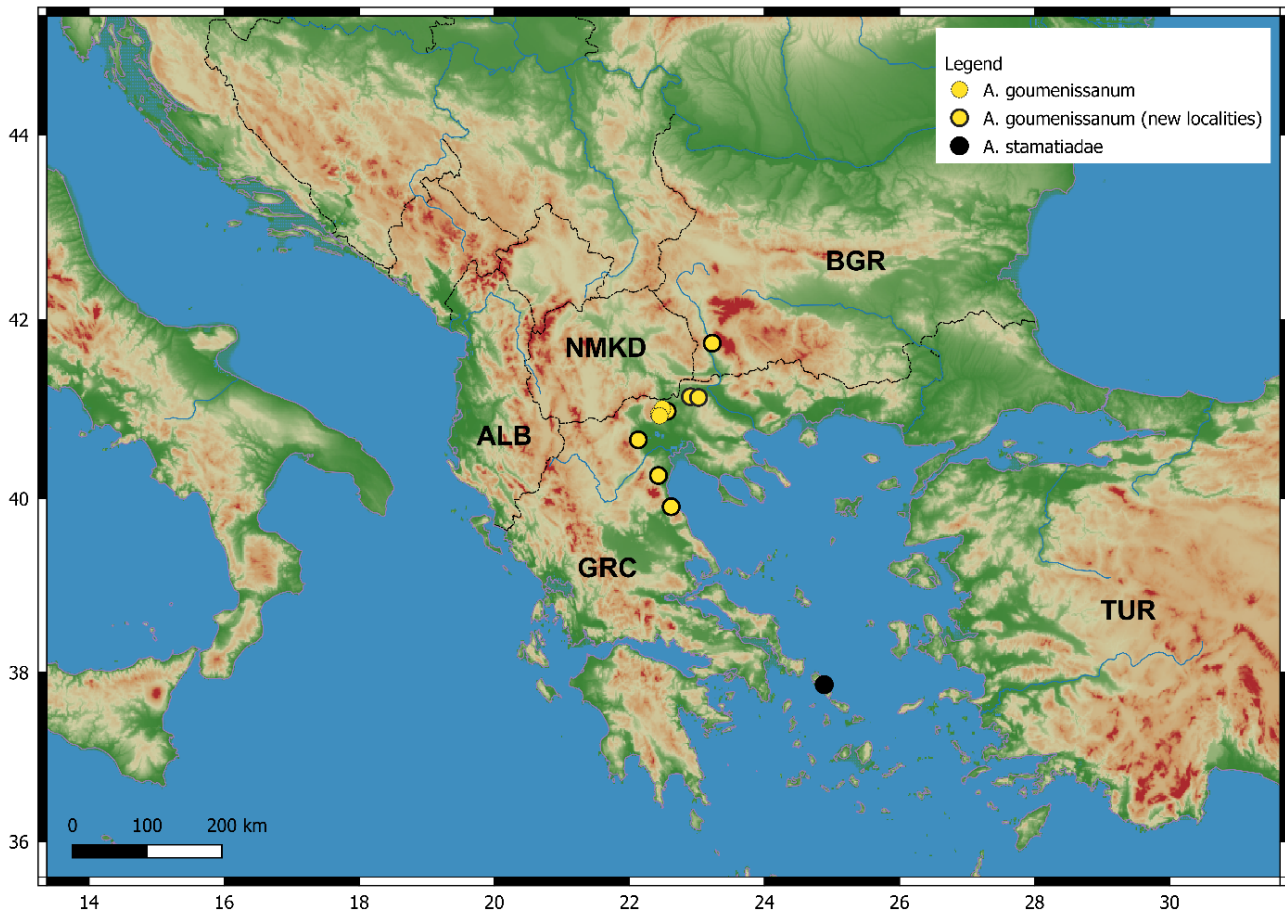


Figure 6. Currently known distribution of *Allium stamatiadae* and *A. goumenissanum* (including localities from *loci classici* and newly observed localities, formerly named as *Allium* sp.). For a detailed description of localities, see Supplementary Material S1.

outer tunic (Figure 5J). Bulblets can either grow above ground through leaf sheaths, potentially spreading far from the mother plant, or settle in the soil close to the mother plant. Consequently, clusters of plants usually develop under both natural (Ioannidis & Tzanoudakis, 2022) and cultivated conditions. A similar pattern of bulblet development, resulting in the clustered spatial pattern of individuals, has been observed in some other polyploid *Allium* species of *A. sect. Codonoprasum* (e.g. *A. oleraceum* L.; Duchoslav, 2001, 2009, Duchoslav *et al.*, 2010; Fialová & Duchoslav, 2014) and *sect. Allium* (e.g. *A. vineale* L.; Håkansson, 1963; Duchoslav, 2001). The flowering period lasted from mid-August to the end of September.

Cultivated plants also produced aborted, poorly developed and well-developed seeds (Figure 5L). The semi-ovoid black seeds have a distinctly sculptured surface (Figure 5K). Testa cells are usually irregularly many-edged (usually 4–6 edged), loose, the periclinal walls bear several verrucae with frequently one larger central verruca, with depressed and striate anticlinal walls. This pattern has been found in several species of *A. sect. Codonoprasum*, e.g. *A. pallens* L. (Celep *et al.*, 2012; Yusupov *et al.*, 2022). Seed set was usually low, about 0–10 seeds per inflorescence and about 0–3 seeds per flower. Seed production despite the triploid nature of the species is somewhat surprising, as triploid *Allium* species/cytotypes are usually seed sterile (e.g., Levan, 1933; Karpavičienė, 2017) due to

meiotic aberrations caused by odd chromosome number (Levan, 1933). Only one of fifty 1.5-year-old seeds, kept at room temperature and then treated under optimal conditions for germination of representatives of *A. sect. Codonoprasum*, i.e. dark moist cold treatment at constant 7°C (Åström & Hægström, 2004; Fialová *et al.*, 2014), germinated. This low germination percentage is not evidence of seed non-viability, but rather evidence of loss of seed viability. In related species of *A. sect. Codonoprasum*, only a small proportion of seeds older than one year were found to be viable (Specht & Keller, 1997; Fialová *et al.*, 2014). Our data therefore suggest the potential for sexual reproduction even in this triploid species. Unfortunately, we were not able to repeat the experiment with fresh seeds, as most of the plants were destroyed by late frost in 2022.

Distribution and habitats

In conclusion, all available evidence suggests that the populations of *Allium* sp. belong to the recently described species *A. goumenissanum*. The currently known distribution of *A. goumenissanum* thus increased from three neighbouring localities near the village of Goumenissa in the province of Kilikis (northern continental Greece; Ioannidis & Tzanoudakis, 2022), covering an area of about 20 km², to the 10 populations covering an area of about 2800 km² (a minimum convex polygon) in northern Greece, but also in southwestern

Bulgaria (Figure 6), for which this is a new species record (Stoyanov *et al.*, 2022). In terms of habitat conditions, the newly discovered populations occupied a variety of forest and non-forest habitat types, including *Quercus pubescens* Will., *Ostrya carpinifolia* Scop. and *Platanus orientalis* L. forests and their margins, which were also reported as habitats of the type population (Ioannidis & Tzanoudakis, 2022). Species was also found in peach orchards, shrubs on upper terraces of river floodplains and in the grassy vegetation of road ditches. Many sites were located near small streams or in river floodplains. The anthropogenic nature of some of the habitats occupied by *A. goumenissanum* confirms previous observations that polyploids of *A. sect. Codonoprasum* often show a tendency towards anthropochory and colonisation of anthropogenic habitats (Hanelt, 1996; Brullo *et al.*, 2008; Duchoslav *et al.*, 2010, 2020), suggesting that the species is actually more common but probably overlooked.

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Authorship contribution

KV: Formal analysis, Research, Methodology, Writing; LK: Research, Methodology, Writing; RK: Research; MK: Formal analysis, Research, Methodology, Writing; VI: Research; DT: Research; MD: Conceptualization, Formal analysis, Research, Methodology, Writing.

Conflict of interest

None

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Supplementary Material

- S1.** A survey of analysed taxa, populations, analyses, and GenBank accession number of submitted sequences.
- S2.** The nucleotide alignment on nrITS used for phylogenetic reconstruction. For a detailed description of localities, see S1.
- S3.** Details of SNPs at several positions of nrITS in the studied accessions. For a detailed description of localities, see S1.
- S4.** Mitotic metaphase chromosomes of *Allium goumenissanum* from type population (n° 6) and population n° 5 (formerly named as *Allium* sp.). For information on the location of the populations, see S1.
- S5.** Description of leaf surface and the anatomical structure of leaf blades of *Allium goumenissanum* and *A. stamatiadae*.