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Phylogeographic and taxonomic considerations on *Goniolimon tataricum* (Plumbaginaceae) and its relatives from south-eastern Europe and the Apennine Peninsula

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Abstract

Goniolimon species are mainly components of the Eurasian steppe or steppe-like rocky vegetation, with some taxa occurring also in south-eastern Europe and northern Africa. We analysed the variability of: (i) two maternally inherited plastid loci (*rpl*32-*trn*L and 3′*rps*16-5′*trn*K) in 110 individuals of six currently accepted species from the Balkans and one species from the Apennines, to provide new insights into their origin and evolutionary history; and (ii) quantitative morphological characters (14 independent characters and one ratio character) in 641 individuals of three species of which two are morphologically and ecologically similar (*G. italicum* and *G. tataricum*) and the third, *G. dalmaticum*, was frequently misidentified as *G. tataricum* in the past, to provide new taxonomic treatment for proposed *G. tataricum* subspecies. We delineated several quantitative and five qualitative characters studied in a more limited sample as diagnostic for the identification of four subspecies (three newly described and one in a new rank) of *G. tataricum*. The history of westward peripheral populations of this species in the Balkans and the Apennines was rather complex and driven by local geo-historic events. These events facilitated multiple waves of east–west expansion of lineages originating from sources outside of the Balkan Peninsula which periodically diversified and occupied localised areas in the Balkans during the Pleistocene. An initial spread of an ancient *G. tataricum* lineage throughout south-eastern Europe probably occurred during the Messinian Salinity Crisis. Inter- and intraspecific hybridisation/introgression, as well as retention of ancestral polymorphisms, was common in *G. tataricum* and related taxa over time.

Keywords Apennine and Balkan Peninsulas · Divergence time estimates · Evolutionary history · *Goniolimon* · Morphometry · Taxonomy

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Introduction

The genus *Goniolimon* Boiss. (Plumbaginaceae Juss.) comprises c. 25 taxa, in specific or subspecific rank, that are mainly components of the steppe or steppe-like rocky vegetation that occurs predominantly in Asia and Europe (Post

1883; Linczevski 1952; Peng and Kamelin 1996), but also northern Africa (Quézel and Santa 1963; Le Floc'h et al. 2010; Buzurović et al. 2018). Given the current centre of *Goniolimon* diversity (Lledó et al. 2005; Volkova et al. 2017), this genus probably originated in central Asia, and according to the most comprehensive molecular phylogeny of Plumbaginaceae available to date, it is monophyletic (Koutroumpa et al. 2018). Although the age of this genus is still uncertain due to the small number of species used in available molecular dating studies (one *Goniolimon* species, Lledó et al. 2005), Volkova et al. (2017) have recently found that the most recent common ancestor of four *Goniolimon* species dates back to c. 7 million years ago (Myr).

Goniolimon species usually grow on carbonate, siliceous and ultramafic soils and less frequently on sand and loess, at least in south-eastern Europe and the Apennines. They are perennial herbs with woody caudex, characterised by a specific mode of anemochory, since their seeds (enveloped into a calyx) are shed from dry shrubby aboveground parts of the plants that separate from the rosette in the autumn and are carried by the winds (Kubitzki 1993). Diagnostic morphological characters used for their distinction from the genus *Limonium* are styles that are papillose or hairy in the lower part and subspherically capitate stigmas (Boissier 1848; Siebert and Voss 1896). The leaves of *Goniolimon* taxa are organised into basal rosettes, stems are ribbed or winged, their paniculate or sometimes subcorymbose compound inflorescences comprise 2-6-flowered spicules, calyx is infundibuliform with a 5- or 10-lobed limb, and petals are slightly emarginated (Kubitzki 1993). Their heterostylous flowers and dimorphic pollen and stigmas account for the self-incompatibility (Baker 1966; Morretti et al. 2015). Plants are pollinated mainly by bumblebees (Kubitzki 1993) and typically produce a large quantity of seeds that have a high germination rate in in vitro conditions (Petriccione and Marrone 2013). However, in natural populations of *G. italicum* Tammaro, Pignatti & G.Frizzi, poor seed germination has been associated with the prevalence of vegetative over generative reproduction (Petriccione and Marrone 2013). *Goniolimon* taxa have more or less developed salt glands on aboveground organs (leaves and stems) that are common in plants that grow in arid and halophilous environments (Faraday and Thomson 1986; Grigore and Toma 2016). Although reports on chromosome counts in certain *Goniolimon* taxa are sometimes ambiguous, they reveal that the chromosome number in different representatives of this genus is $2n = 32$ (Ančev 1982; Tammaro et al. 1982; Malakhova 1990), $2n = 34$ (Runemark 1974; Strid, 2015) or $2n = 36$ (Pignatti 1972).

Although a comprehensive phylogenetic inference for the *Goniolimon* is still lacking, representatives of this genus have been used in several molecular phylogenies at various taxonomic levels, along with other species of interest (e.g.

Meimberg et al. 2000; Lledó et al. 2005; Koutroumpa et al. 2018). However, only one *Goniolimon* species, *G. speciosum* (L.) Boiss., has been studied to date from a biogeographic/phylogeographic aspect (Volkova et al. 2017). The authors analysed the variability at nuclear and plastid loci in *G. speciosum* individuals sampled from populations across the entire distribution range of the species (China, Kazakhstan, Mongolia and Russia) and demonstrated its exceptionally complex evolutionary history, which is concordant with that of several other steppe species and genera with wide Eurasian distribution that have been studied to date (*Clausia aprica* (Stephan) Korn.-Tr., Franzke et al. 2004; *Capsella* sp., Hurka et al. 2012; genera *Dontostemon* Andrz. ex C.A. Mey. and *Clausia* Korn.-Tr., Friesen et al. 2016).

Six *Goniolimon* species are currently present in the Balkans, and they represent an important component of the exceptionally rich Balkan's flora (Stevanović et al. 1995; Jakovljević et al. 2011; Tomović et al. 2014). They are characterised by different ranges of distribution that sometimes extend over Asian steppe as well. This is the case with *G. tataricum* (L.) Boiss. which is widely distributed throughout the Balkans, but the main part of its distribution range is in the large steppe vegetation belt in Russia and Ukraine (Linczevski 1952). Two species characterised by a less widespread distribution, *G. incanum* (L.) Hepper (= *G. collinum* (Griseb.) Boiss.) and *G. besserianum* (Schult.) Kusn., are less abundant in the Balkans and are more common in nearby eastward areas (Pignatti 1972; Bokhari and Edmondson 1982). The remaining *Goniolimon* species are characterised by a limited range of distribution and are endemic to the Croatian Dalmatia (*G. dalmaticum* Rchb.f., Buzurović et al. 2016) and some parts of Greece (*G. heldreichii* Halácsy and *G. sartorii* Boiss., Pignatti 1972). As already mentioned, *G. italicum* is present in the localised area in the central Apennines (Tammaro et al. 1982). Therefore, populations of *Goniolimon* species from the Balkans and the Apennines represent either extrazonal marginal (peripheral, range edge) populations of the species having wider range of distribution (*G. tataricum*, *G. besserianum*, *G. incanum*) or localised and isolated ones.

Available data on genetic features of marginal populations of numerous plant species demonstrate that these usually small-sized and fragmented populations are generally subjected to different regimes of natural selection when compared to central populations (Lesica and Allendorf 1995; Lenormand 2002; Eckert et al. 2008). Furthermore, they are usually characterised by low levels of genetic diversity, due to smaller effective population sizes, more severe effects of random genetic drift and reduced interpopulation gene flow due to isolation (Crow 1993; Kramer et al. 2008). In addition, in species having both vegetative and generative reproduction, the prevalence of the former reproduction mode in less favourable environments (typical for peripheral

populations) may further promote the loss of genetic diversity (e.g. Beatty et al. 2008) which may be the case in *G. italicum* (Petriccione and Marrone 2013). On the other hand, it is well known that the Balkan Peninsula served as a glacial refugium for numerous plant taxa (Hewitt 1996; Médail and Diadema 2009), and available studies demonstrate that even the small-sized populations of a species present in this region over a long time may be characterised by high levels of genetic diversity (e.g. Lewis and Crawford 1995; Medrano and Herrera 2008; Aleksić and Geburek 2014) and even marked genetic structure (Aleksić et al. 2017). Therefore, predictions on genetic layout of peripheral/isolated populations of *Goniolimon* species from the Balkans are not straightforward, and it is very likely that their evolutionary history differs from that observed in populations of *G. speciosum* which has a wide distribution range extending over the large Asian steppe belt (Volkova et al. 2017).

In addition, available chorological data based on floristic records from the Balkans, Apennines and Africa are questionable, as discussed by Buzurović et al. (2016, 2018). For instance, *G. dalmaticum*, described by Reichenbach (1854–1855), was erroneously recorded in the floras and checklists of Albania (Vangjeli 2015; Barina et al. 2018), Bosnia and Herzegovina (Bjelčić 1967), Bulgaria (Ančev 1982), Croatia (Nikolić 2015; Bogdanović 2015), North Macedonia (Micevski and Matevski 1995) and Greece (Dimopoulos et al. 2013). These records, which actually refer to *G. tataricum*, led to the false impression that *G. dalmaticum* is rather widely distributed in the Balkans. Furthermore, Buzurović et al. (2018) described a new species in northern Africa, *G. africanum* Buzurović, Bogdanović & Brullo, rejecting the presence of *G. tataricum* in this continent. These findings highlight the need for the taxonomic revision of the Balkans *Goniolimon* taxa.

In order to shed light on the origin and evolutionary history of *Goniolimon* species in south-eastern Europe, we have analysed the variability of two maternally inherited plastid intergenic spacers (*rpl*32-*trn*L and 3′*rps*16-5′*trn*K) in 110 individuals belonging to six *Goniolimon* species from the Balkan Peninsula and one from the Apennine Peninsula. Furthermore, we have carried out morphometric analyses of two morphologically and ecologically similar species, *G. tataricum* from the Balkans and *G. italicum* from the Apennines, to resolve ambiguities regarding their relationship and current taxonomic treatments.

Materials and methods

Study species and sampling

We studied seven currently accepted *Goniolimon* species from the Balkan and the Apennine Peninsula, namely *G.*

besserianum, *G. dalmaticum*, *G. heldreichii*, *G. incanum*, *G. italicum*, *G. sartorii* and *G. tataricum* s.l. (Pignatti 1972; Bokhari and Edmondson 1982; Tammaro et al. 1982). According to the available literature, the distribution range of *G. tataricum* extends over western Kazakhstan, European Russia, Transcaucasia, Ukraine, Romania, Bulgaria, Serbia, North Macedonia, Greece, Croatia and Bosnia and Herzegovina (Răvărut 1960; Bjelčić 1967; Ančev 1982; Bokhari and Edmondson 1982; Ranđelović and Stamenković 1986; Micevski and Matevski 1995; Buzurović et al. 2013, 2016). Populations of this species are rather common in the Balkans, where they typically occur at steppe-like habitats, i.e. at xerophilous pastures and rocky grounds in hilly regions at 60–1400 m a. s. l. (Micevski and Matevski 1995). Populations of *G. besserianum* and *G. incanum* are abundant in Bulgaria, Romania, Moldova, Ukraine and western Russia (*G. besserianum*, Linczevski 1952; Pignatti 1972) and in Bulgaria, Greece, Romania, Turkey and possibly in Lebanon and Syria (*G. incanum*, Pignatti 1972, Greuter et al. 1989). In the Balkans, they occur on steppe-like sites (xerophilous pastures and rocky grounds), sands and sometimes on saline soils. Although today both *G. heldreichii* and *G. sartorii* grow in limited areas in Greece, they have different habitat preferences; namely, the former is found on steppe-like habitats and the latter on halophilous rocky sites near the Aegean Sea in the eumediterranean zone, similarly as *G. dalmaticum*, which is endemic to the Croatian Dalmatia (Buzurović et al. 2016). *Goniolimon italicum* is found in a localised area in the central Apennines, in habitats similar to those that are typical for *G. heldreichii* and *G. tataricum* (Tammaro et al. 1982).

Samples were collected over several years (from 2010 to 2017), during the summer season when plants were in full anthesis (Table 1). Sampling was carried out so as to cover the full distribution ranges of the studied species within the south-eastern Europe and the Apennines, and thus, species characterised by wider distribution ranges were represented by a larger number of populations (Fig. 1, Table 1). In each population, individuals were sampled along transects. Plant material for molecular analyses (young leaves) was dried with silica gel and kept in freezer prior to the analyses, while the material used for morphometric analysis (five spikelets per plant) was fixed in a 70% ethanol − 85% glycerol solution (ratio 1:1) and kept at room temperature prior to the analyses. Voucher specimens from all populations were deposited in BEOU, BEO and ZAGR. Herbarium acronyms are according to Thiers (2019) (see Table 1).

The sample that was used for molecular analyses comprised 110 individuals from 37 populations representing all seven currently accepted species from south-eastern Europe and the Apennines (Pignatti 1972; Bokhari and Edmondson 1982; Tammaro et al. 1982). The overall sample for morphological analysis comprised 641 individuals

**Table 1** *Goniolimon* taxa used in the study, sampling localities, sample sizes and voucher numbers

Species	Country: locality	Pop. acr.	Long. (N)	Lat. (E)	Alt. (m a. s. l.)	Sample size: morph./mol.	Voucher
G. besserianum	Bu: Kavarna, Topola	BT	43.40988	28.26549	17	0/1	BEOU-40952
	Rm: Târguşor-Gura Dobrogei	GD	44.46727	28.46960	59	0/1	BEO-82743
	Rm: Jurilovca, Capul Doloşman	JU	44.76107	28.93661	31	0/2	BEO-82742
G. dalmaticum	Ct: Pag island	PA	44.51571	14.95523	1	0/1	BEO-82733
	Ct: Vir island, Rastovac	VI	44.32667	15.02562	4	0/3	BEO-82732
G. heldreichii	Gr: Karditsa, Loutrá Kaḯtsis	KL	39.13111	22.16028	400	0/2	BEOU-45790
G. incanum	Bu: Aytos	AJ	42.71056	27.27305	171	0/1	BEOU-40972
	Bu: Kardzhali	KR	41.62170	25.46418	483	0/1	BEOU-41017
	Bu: Nessebar	NE	42.70486	27.89777	63	0/4	BEOU-40960
	Gr: Alexandroupoli	AL	40.93646	26.05817	80	0/2	BEOU-45785
	Gr: Porto Lagos	PL	41.00885	25.10161	2	0/1	BEOU-45783
	Tu: Mudanya	MU	40.37735	28.71546	6	0/1	BEOU-34930
	Tu: Vezirhan	VE	40.23421	30.01923	279	0/1	BEOU-34929
G. italicum	It: Capestrano, Collelungo	KC	42.28144	13.77230	381	21/1	BEOU-45792
	It: Ocre, Fossa Raganesca*	FR	42.30134	13.47421	808	29/4	BEOU-45791
G. sartorii	Gr: Porto Rafti, Koroni	PR	37.86984	24.03413	1	0/2	BEOU-45782
G. tataricum	Bu: Blagoevgrad-Kočerinovo	BL	42.07186	23.03872	405	0/5	BEOU-38502
	Bu: Bobošєvo	BO	42.12659	23.02028	402	30/5	BEOU-38501
	Bu: Dragoman	DR	42.90534	22.95265	810	30/4	BEOU-38500
	Bu: Kresna, Tisata	TI	41.73156	23.15570	196	30/2	BEOU-38497
	Bu: Kožuh, Rupite	RP	41.46075	23.25825	227	28/4	BEOU-38498
	Gr: Kato Nevrokopi-Granitis	KN	41.31585	23.87238	596	30/4	BEOU-38499
	Mk: Gevgelija, Moin	GE	41.13601	22.45659	116	26/2	BEOU-38504
	Mk: Katlanovo, Kisela voda	KA	41.89229	21.68761	247	30/5	BEOU-38503
	Mk: Mariovo, Vitolište	MA	41.16959	21.78956	707	30/4	BEOU-38505
	Mk: Ovče Pole, Bekirlija	BR	41.69339	21.95679	204	30/4	BEOU-40788
	Mk: Ovče Pole, Bogoslovec	OP	41.78377	22.02112	310	30/5	BEOU-38506
	Mk: Sivec, Prisad	SI	41.41837	21.60009	896	30/4	BEOU-34932
	Mk: Žeden, Rašče	RS	42.05144	21.25481	315	30/5	BEOU-38495
	Rm: Cluj, Apahida	AP	46.80720	23.73037	319	17/3	BEO-82741
	Sr: Bela Palanka, Čiflik	CI	43.22217	22.42146	380	30/4	BEOU-34927
	Sr: Prokuplje, Hisar	HI	43.22496	21.57676	340	28/4	BEOU-34926
	Sr: Raška, Gubavac	GU	43.28166	20.61782	483	25/6	BEOU-34924
	Sr: Mt Rujan, Mali Orljak	RU	42.25921	21.76590	712	0/3	BEOU-31360
	Sr: Mt Vujan	VJ	43.98512	20.44412	500	30/4	BEOU-34923
"*G. croaticum*"	Ct: Grabovac	GR	43.43043	16.98615	325	12/0	BEO-82735
"*G. croaticum*"	Ct: Zadvarje*	ZA	43.43103	16.88793	207	18/4	BEO-82734
"*G. graecum*"	Gr: Serres*	SE	41.10008	23.53718	169	24/1	BEOU-45784

Pop. acr. population acronym; *Long.* longitude; *Lat.* latitude; *Alt.* altitude; *Sample size: morph./mol.* number of individuals used for morphometric analyses and for molecular analyses; *BH* Bosnia and Herzegovina; *Bu* Bulgaria; *Ct* Croatia; *Gr* Greece; *It* Italy; *Mk* North Macedonia; *Rm* Romania; *Sr* Serbia; *Tu* Turkey

*Type localities

from 24 populations belonging to three species, *G. dalmaticum*, *G. italicum* and *G. tataricum* s.l., of which two are morphologically and ecologically similar (*G. italicum* and *G. tataricum* s.l.), and the third, *G. dalmaticum*, has frequently been misidentified as *G. tataricum* in the past. The analysis of *G. italicum* and *G. tataricum* s.l.

included 586 individuals from 22 populations belonging to these two species. Altogether, we analysed 14 independent quantitative and one derived ratio characters (Table 2). Qualitative characters (calyx tube indumentum, calyx lobe shape and outer, middle and inner bract indumentum), estimated in a more limited sample of *G.*
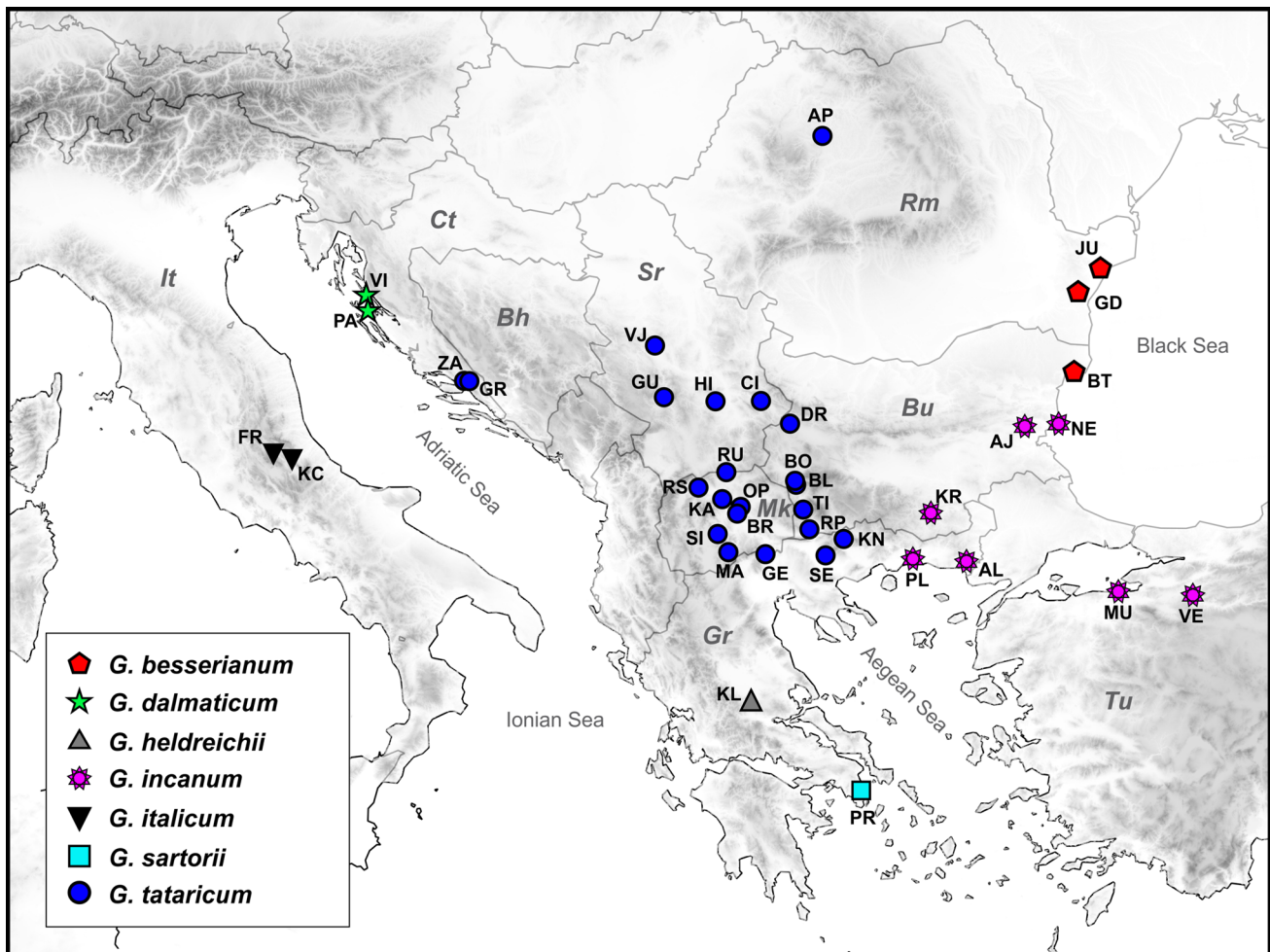
Fig. 1 Distribution of the studied *Goniolimon* taxa from SE Europe and the Apennine Peninsula and locations of sampled populations. Acronyms of populations are given in Table 1

italicum and *G. tataricum*, were used for assembling the identification key for proposed *G. tataricum* subspecies. To facilitate the assessment of taxonomic patterns in *G. tataricum* s.l., we tagged individuals from population SE from Greece as "*G. graecum*" and those from two populations from Croatia (ZA and GR) as "*G. croaticum*".

It should be noted that samples of *G. tataricum* sensu stricto from the area cited in protologue, the so-called Tataria (currently part of the Russian Federation) (Linné 1753) (lectotype designated by Edmondson in Jarvis 2007), are not included in the current analysis. Nevertheless, our comprehensive survey of the herbarium material, available on the websites presenting numerous European herbaria (which included also samples from "Tataria"), as well as the thorough examination of herbarium sheets borrowed from several herbaria (C, H, LE, MA, P), suggests that nominal subspecies of *G. tataricum* is certainly present in the Balkan Peninsula.

Molecular analyses

DNA extraction, PCR amplification and sequencing

Plant tissue of each individual (20–30 mg of silica gel dried leaves) was homogenised with TissueLyser II (Qiagen, Valencia, CA, USA) and used for extraction of the total genomic DNA following Aleksić et al. (2012). Two plastid (cpDNA) intergenic spacers, 3′*rps*16-5′*trn*K and *rpl*32-*trn*L, were amplified with forward (F) and reverse (R) primers published by Shaw et al. (2007). PCRs were carried out in a total volume of 25 µl, containing 50 ng template DNA; 2.5 µl 10×KAPA Taq Buffer B with 1.5 mM Mg (Kapa Biosystems, Boston, USA); 0.2 mM dNTPs; 0.1 µM of each F and R primer; 0.80% BSA (bovine serum albumin, Promega, St. Louis, USA) and 1U/µl of KAPA Taq DNA polymerase (Kapa Biosystems, Boston, USA). PCR amplification of both loci was performed in a Eppendorf Mastercycler ep

Table 2 Summary of morphometric analysis of quantitative characters and outcomes of ANOVA

No.	Morphological characters (mm)	Goniolimon tataricum subsp. tataricum				Goniolimon tataricum subsp. italicum				Goniolimon tataricum subsp. graecum				Goniolimon tataricum subsp. croaticum				ANOVA	
		Min	Max	LQ	UQ	Min	Max	LQ	UQ	Min	Max	LQ	UQ	Min	Max	LQ	UQ	F	p
1	Calyx length	5.9	9.2	7.3	8.1	7.9	10.2	8.4	9.0	6.2	7.7	6.8	7.2	7.9	10.0	8.7	9.4	**105.65**	**0.0000**
2	Calyx rib length	4.2	6.8	5.2	5.7	5.9	7.2	6.2	6.6	5.1	6.3	5.4	5.9	5.3	7.2	5.8	6.7	**158.22**	**0.0000**
3	Calyx lobe length	1.1	2.6	1.4	1.7	1.2	1.9	1.4	1.6	0.8	1.2	0.9	1.0	1.9	2.9	2.4	2.5	**90.08**	**0.0000**
4	Inner bract length	4.0	7.4	5.4	6.3	5.1	7.2	5.5	6.2	4.9	6.6	5.5	6.0	5.8	7.4	6.5	7.1	**20.94**	**0.0000**
5	Inner bract width	1.7	4.3	2.4	2.8	2.1	3.1	2.4	2.6	2.4	3.4	2.6	2.8	2.1	3.0	2.5	2.8	**3.74**	**0.0110**
6	Middle cusp of inner bract length	1.3	3.4	2.1	2.5	1.9	3.1	2.1	2.6	2.0	2.8	2.1	2.2	2.3	3.0	2.6	2.8	**13.88**	**0.0000**
7	Lateral cusp of inner bract length	0.8	2.3	1.4	1.7	1.1	2.2	1.5	1.8	1.2	1.6	1.3	1.5	1.6	2.1	1.7	1.9	**14.90**	**0.0000**
8	Middle bract length	3.1	8.0	5.2	6.1	4.8	7.5	5.3	6.3	4.3	6.8	5.1	5.7	6.0	7.4	6.4	6.8	**19.97**	**0.0000**
9	Middle bract width	1.0	2.3	1.5	1.8	1.3	2.3	1.6	1.9	1.4	2.2	1.6	1.8	1.3	2.1	1.6	1.8	**5.22**	**0.0014**
10	Cusp of middle bract length	0.9	2.8	1.6	2.0	1.7	3.1	2.0	2.6	1.4	2.4	1.8	2.1	2.0	2.7	2.1	2.5	**31.81**	**0.0000**
11	Outer bract length	3.8	7.4	4.9	5.9	4.1	7.6	4.7	5.8	5.4	7.2	5.8	6.4	5.0	6.8	5.5	6.1	**12.24**	**0.0000**
12	Outer bract width	1.8	3.8	2.4	2.8	2.2	3.0	2.4	2.7	2.1	3.0	2.4	2.6	1.8	2.8	2.2	2.5	**17.13**	**0.0001**
13	No. of spikelets per cm	3	7	5	6	4	6	5	5	4	6	4	5	4	7	5	6	**3.99**	**0.0078**
14	No. of spikes per 4 cm	2	7	4	5	3	6	5	6	4	5	4	5	3	6	4	5	**6.51**	**0.0002**
–	Ratio character (calyx tube/calyx limb length)*	1.3	3.3	2.0	2.5	2.1	3.0	2.3	2.6	2.5	3.8	3.0	3.2	1.5	2.3	1.7	2.0	**272.51**	**0.0000**

No. number; *Min* minimum value; *Max* maximum value; *LQ* lower quartiles; *UQ* upper quartiles. Statistically significant values (*p* < 0.05) are given in bold

*Calyx limb is the wider part of the calyx (including calyx lobes and the upper scarious part of the calyx tube)

gradient S (Eppendorf, Hamburg, Germany) with the same amplification profile: denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 45 s; annealing for 45 s at 52 °C; extension at 72 °C for 1 min; and a final extension of 10 min at 72 °C.

PCR products were sequenced commercially with corresponding F primers by Macrogen Europe, Amsterdam, the Netherlands (http://dna.macrogen.com/eng/) via Sanger sequencing using 96-capillary 3730xl DNA analyser automated sequencer (Applied Biosystems, Inc. USA). Sequence chromatograms were edited manually using Chromas Lite 2.0.1 (Technelysium, Australia) and aligned manually using Muscle (Edgar 2004) in MEGA 5.04 (Tamura et al. 2011). All sequences generated in the present study were deposited in the GenBank (accession numbers MH607254-MH607363 (3′*rps*16-5′*trn*K) and MH607144-MH607253 (*rpl*32-*trn*L)).

Phylogenetic analysis and divergence time estimates

Sequences were readily alignable among all accessions in both plastid matrices, and the length of the concatenated matrix was 1520 bp (Online Resource 1). Based on the Akaike information criterion calculated in MEGA 5.04 (Tamura et al. 2011), the Hasegawa–Kishino–Yano (HKY) model of sequence evolutions had the best fit to the concatenated matrix. Gaps in the alignment were treated as missing data in phylogenetic analysis.

We used a Bayesian approach in BEAST version 1.7.2 (Drummond et al. 2012) for phylogenetic analysis because it does not require potentially misleading outgroups for rooting, and it may account for the genealogical uncertainty due to the stochastic nature of the coalescence process. Along with the HKY model of sequence evolution, we used four rate heterogeneity categories and a demographic model of constant population size as a tree prior for modelling changes in population size through time. Although interpretation of the population size under the constant population size model in BEAST analysis may be affected by insufficient sampling of some species, such as *G. besserianum* and *G. incanum*, we used this model to avoid over parameterisation. We used a strict clock to estimate divergence times in our phylogeny because it is the most appropriate model for intraspecific data (Brown and Yang 2011) and because we aimed at keeping the number of estimated parameters at a minimum. Molecular clock was calibrated with published substitution rates of 1.01×10^{-9} substitutions/site/year for synonymous sites of cpDNAs in seed plants (Graur and Li 2000). These values approximate the evolutionary rates of introns and non-coding spacers of organelle DNA (Chiang et al. 2009) and have been applied previously to other angiosperms (e.g. Molins et al. 2011; Stojanović et al. 2015; Aleksić et al. 2018). All parameters were sampled once every 1000 steps from 5 million MCMC steps. TRACER version 1.4.1 (Rambaut and Drummond 2007) was used to assess acceptable mixing, likelihood stationarity of the MCMC chain and adequate effective sample sizes for each parameter (≥ 200). TreeAnnotator 1.4.2 was used to construct a maximum clade credibility tree after discarding the initial 25% of the sampled parameters and trees as burn-in and to summarise the posterior distributions of nodes. Posterior probabilities (PP) ≥ 0.95 were considered as good support and those ranging from 0.85 to 0.94 as moderate support for recovered clades. Bayesian Inference (BI) tree was visualised and edited using FigTree 1.0 (Rambaut 2006).

Phylogeographic analyses

Genealogical relations among haplotypes detected in studied *Goniolimon* species were assessed by constructing median-joining (MJ) network (Bandelt et al. 1999) with NETWORK version 4.6.1.1 (http://www.fluxus-engineering.com/network_terms.htm). To take advantage of informative length mutations, we coded simple insertions/deletions (indels) as characters, after recording those longer than one base pair (bp) as single-bp indels. They were weighted as ten, while length mutations at microsatellite loci were also coded as characters and included in the analysis. Given the difference in evolutionary rates of microsatellites (Schlötterer 1998) and base substitutions for synonymous sites of cpDNAs in seed plants (Graur and Li 2000), length mutations at microsatellite loci were downweighted proportionally, as four. Parameter ε was kept at 0 to recover the minimum spanning network (Bandelt et al. 1999). Potential phylogeographic structure was determined by assessing the congruence of detected genealogically linked lineages and their extant spatial distribution.

Morphometric analyses

Reproductive organs (calyx and bracts) from each plant sample were positioned between the microscope slides and scanned at high resolution using standard PC tools and adjusted transparency scale. The majority of the quantitative morphological characters were measured with a Digimizer Image Analysis software (MedCalc Software, Belgium). The exceptions are characters, the number of spikelets per cm and the number of spikes per 4 cm which were assessed visually. Two characters, calyx tube and calyx limb length, were used only for the calculation of a ratio character and were not used in further analyses.

We used analysis of variance (ANOVA) to determine statistical significance of morphological characters which will be subsequently used in further analyses. Two-dimensional principal component analysis (PCA) was performed with all individuals assigned to populations of three species, *G.*

dalmaticum, *G. italicum* and *G. tataricum* s.l., in order to determine variability, distances and relatedness between populations of different species. On the next set of taxa, comprising *G. italicum* and *G. tataricum* s.l., we carried out three-dimensional PCA, cluster analysis using unweighted pair group method with arithmetic mean (UPGMA) and canonical discriminant analysis (CDA). Three-dimensional PCA was performed with population centroids of individual scores in order to determine the variability and position between populations of different species. UPGMA method, based on scores from PCA, was used to obtain a dendrogram in order to demonstrate relationships between populations. CDA was carried out with pre-defined groups indicated in previous analyses (three-dimensional PCA centroids and UPGMA) in order to delineate the most important characters that mostly contribute to differentiation of the studied groups of populations. Confidence polygons with a coefficient of 95% were also given in the CDA graph. All analyses were performed with STATISTICA 7.0 for Windows work package (StatSoft, 2008).

The qualitative characters used in our work were selected as the most informative ones from those available in the Flora Europaea (Pignatti 1972) and regional floras (Gajić 1972; Ančev 1982; Micevski and Matevski 1995) as well as based on personal observations. They comprised calyx tube indumentum, outer, middle and inner bract indumentum and calyx lobe shape. Along with the geographical range, they were used for defining the identification key for proposed *G. tataricum* subspecies (Table 3).

The identification key was based on quantitative and qualitative characters and distribution ranges of the taxa in question. Qualitative characters without antithesis in the identification key are given in italics and in square brackets.

Interquartile range was provided for quantitative characters with minimum and maximum values given in brackets.

Results

Molecular analyses

DNA regions and alignments

The aligned lengths of 3′*rps*16-5′*trn*K and *rpl*32-*trn*L intergenic spacers were 739 bp and 781 bp, respectively, with a total length of the concatenated plastid matrix of 1520 bp (Online Resource 2). The matrix comprised 51 variable positions (38 parsimony informative sites and 13 singletons), 10 insertions/deletions (indels) of variable length (one to 17 bp) and 11 microsatellites, of which ten comprised a mononucleotide motive and one a dinucleotide motive. (Characteristics of the two plastid regions are given in Table 4, and their annotation is given in Online Resource 1.)

Characters (point mutations and/or indels) synapomorphic for particular populations, species and lineages

Table 4 Characteristics of plastid loci used in this study

Locus	Aligned length (in bp)	Variable sites (PIS/S)	Indels	SSRs
*rpl*32-*trn*L	781	34 (28/6)	9	4
3′*rps*16-5′*trn*K	739	17 (10/7)	1	7
Matrix	1520	52 (38/13)	10	11

PIS parsimony informative sites; *S* singletons; *indels* insertions/deletions; *SSRs* microsatellites

Table 3 Qualitative and quantitative morphological characters used for an identification key for *Goniolimon tataricum* subspecies (*G. tataricum* subsp. *tataricum*, *G. tataricum* subsp. *croaticum*, *G. tataricum* subsp. *italicum* and *G. tataricum* subsp. *graecum*)

| Characters	*G. tataricum* subsp. *tataricum*	*G. tataricum* subsp. *croaticum*	*G. tataricum* subsp. *italicum*	*G. tataricum* subsp. *graecum*
Calyx tube indumentum	Sparsely and densely hirsute	Sparsely hirsute up to half of its length	Densely hirsute up to rib apices	Mostly densely hirsute up to rib apices
Calyx lobe shape	Subulate	Subulate	Subulate	Rounded
Outer, middle and inner bract indumentum	Sparsely and densely pubescent	Sparsely pubescent, rarely glabrous	Always densely pubescent	Usually densely pubescent
Calyx length (mm)	(5.9–)7.3–8.1(–9.2)	(7.9–)8.7–9.4(–10.0)	(7.9–)8.4–9.0(–10.2)	(6.2–)6.8–7.4(–7.7)
Calyx lobes length (mm)	(1.1–)1.4–1.7(–2.2)	(1.9–)2.4–2.5(–2.9)	(1.2–)1.4–1.6(–1.9)	(0.8–)0.9–1.0(–1.2)
Inner bract length (mm)	(4.0–)5.4–6.2(–7.4)	(5.8–)6.5–7.1(–7.4)	(5.1–)5.5–6.2(–7.2)	(4.9–)5.5–6.0(–6.6)
Middle cusp of inner bract length (mm)	(1.3–)2.1–2.5(–3.4)	(2.3–)2.6–2.8(–3.0)	(1.9–)2.1–2.6(–3.1)	(2.0–)2.1–2.2(–2.8)
Cusp of middle bract length (mm)	(0.9–)1.6–2.0(–2.8)	(2.0–)2.1–2.5(–2.7)	(1.7–)2.0–2.6(–3.1)	(1.5–)1.7–2.0(–2.4)
Calyx tube length/Calyx lobes length (mm)	2–2½	1½–2	2–2½	3–3½

comprising populations of a single or several species have been observed in one or both plastid regions (Online Resource 3). Since molecular data may be informative in taxonomic work (Filipowicz et al. 2012), and non-standard barcoding fragments may be useful for distinction of closely related species in the taxonomic context (Seberg and Petersen 2009; Korotkova et al. 2011; González-Gutiérrez et al. 2013; Aleksić et al. 2018), these characters were considered as molecular diagnostic characters which may be used as barcodes for the distinction of closely related taxa at the molecular level (CBOL Plant Working Group 2009).

Phylogenetic analysis and divergence time estimates

The number of individuals used for phylogenetic analysis was downscaled to 42 by retaining only one out of several individuals that had an identical sequence. That way, the representation of abundant very short branches which do not add information was avoided, and the resolution in the internal nodes was improved (e.g. Jones et al. 2017). The names of excluded individuals were listed in tip labels after the name of the individual used for the analyses (Fig. 2).

A maximum clade credibility tree from the BEAST analysis (BI tree, Fig. 2) revealed two non-supported clades, each comprising individuals belonging to several species. Only five subclades recovered in these clades were well supported, and three of them comprised individuals belonging to more than one species. Although species relations remained unresolved by our phylogenetic analysis, we were able to draw some conclusions based on these well-supported subclades. *Goniolimon tataricum* individuals from populations distributed in the Carpathians (AP) and adjacent southward parts of the central Balkans, i.e. in the regions around rivers Morava and Vardar (BR, GE, HI, KA, OP, RU and SI), comprised the first well-supported subclade. *G. incanum* from southern Bulgaria (KR) was the first diverging lineage in the second well-supported subclade which comprised also "*G. graecum*" from north-eastern Greece (SE) and *G. tataricum* from the Struma River valley in western Bulgaria (BO, BL, TI and RP). The third well-supported subclade comprised a single species endemic to central Greece, *G. heldreichii* (KL), while the fourth comprised *G. besserianum* individuals from the western Black Sea coast (JU, GD and BT) and adjacent *G. tataricum* individuals from the valley of the Nišava River in eastern Serbia (CI) and nearby region in western Bulgaria
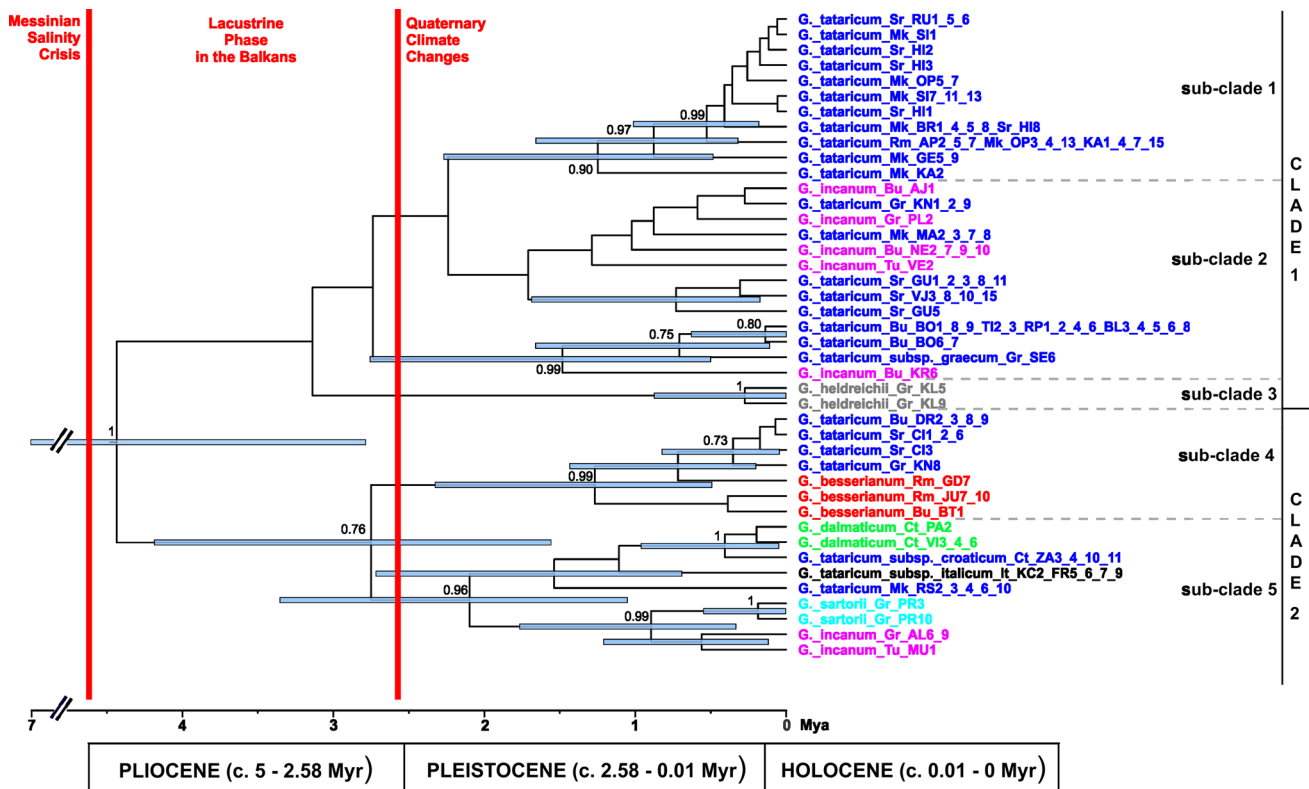
Fig. 2 Chronogram of *Goniolimon* taxa calibrated with published substitution rates of 1.01×10^{-9} substitutions/site/year for synonymous sites of plastid DNA in seed plants. Numbers at nodes refer to posterior probabilities. Blue bars indicate the 95% highest posterior probability of the node age estimate. Individuals having an identical sequence are listed in tip labels after the name of the individual used in the analyses. Geological epochs are given according to the International Commission on Stratigraphy

(DR), and a single individual from population KN from northern Greece. The fifth subclade was the most heterogeneous because it comprised individuals belonging to six taxa, *G. dalmaticum* (PA and VI), *G. incanum* (AL and MU), *G. sartorii* (PR), individuals from a single population of *G. tataricum* (RS) and *G. italicum* (KC and FR) and "*G. croaticum*" (ZA). Some of these lineages were well supported, which comprised *G. dalmaticum* and   "*G. croaticum*" and lineage comprising *G. sartorii* and *G. incanum*.

Divergence time estimates are given only for strongly and moderately supported nodes (Fig. 2). The initial split to two clades was dated to 4.55 Myr (2.77–6.44 Myr). The divergence of subclade 5 coincides with the onset of the Quaternary because it was dated to 2.19 Myr (1.09–3.37 Myr). The divergence of the remaining well-supported subclades occurred during the Quaternary, with subclade 2 diverging prior to subclade 4 (1.58 Myr (0.49–2.77 Myr) vs. 1.29 Myr (0.49–2.28 Myr)) and subclade 1 being the most recently diverging lineage (0.89 Myr (0.30–1.68 Myr)). The split of

G. dalmaticum and "*G. croaticum*" was dated to 0.49 Myr (0.00–0.99 Myr).

Phylogeographic analysis

All 42 haplotypes found in individuals belonging to seven *Goniolimon* species were organised into five genealogically linked lineages recovered in MJ network (Fig. 3) which were more or less concordant with well-supported subclades in the BI tree. They were labelled A-E, and their spatial distribution is presented in Fig. 4.

The most heterogeneous lineage A was concordant with the subclade 5. A single haplotype, h39, found in *G. italicum*, was in the centre of this lineage, and it gave rise through a series of mutations to haplotypes organised into four sublineages, of which three corresponded to different species, namely *G. tataricum* from north-western North Macedonia (RS), *G. sartorii* (local endemic to southern Greece) and *G. incanum* (populations AL from north-eastern
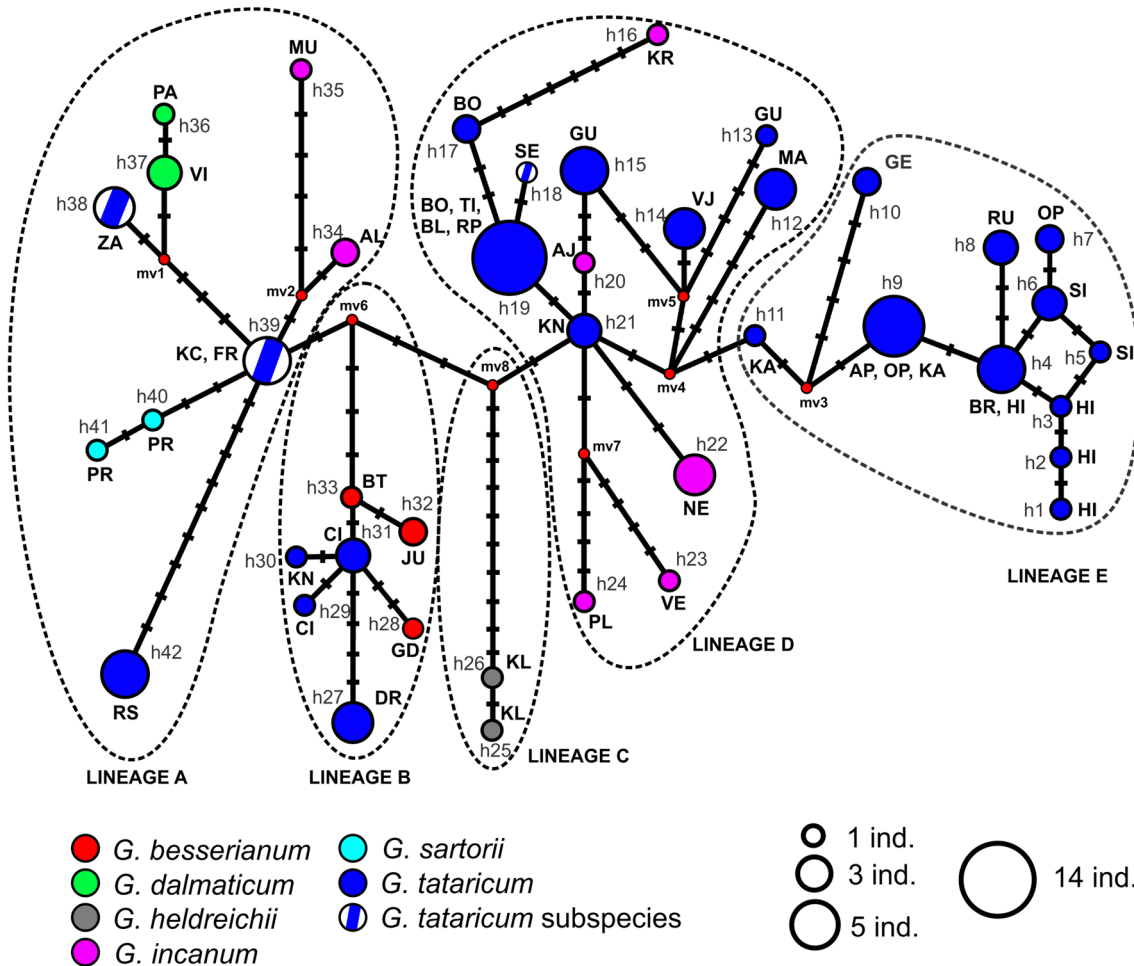
Fig. 3 Median-joining network of haplotypes (h1–h42) found in 110 individuals of seven *Goniolimon* taxa. The size of the circles is proportional to the number of individuals. Small black circles indicate ancestral hypothetical haplotypes (mv). Each short solid line represents one mutational step. Acronyms of populations are given in Table 1. Spatial distribution of detected lineages is presented in Fig. 4
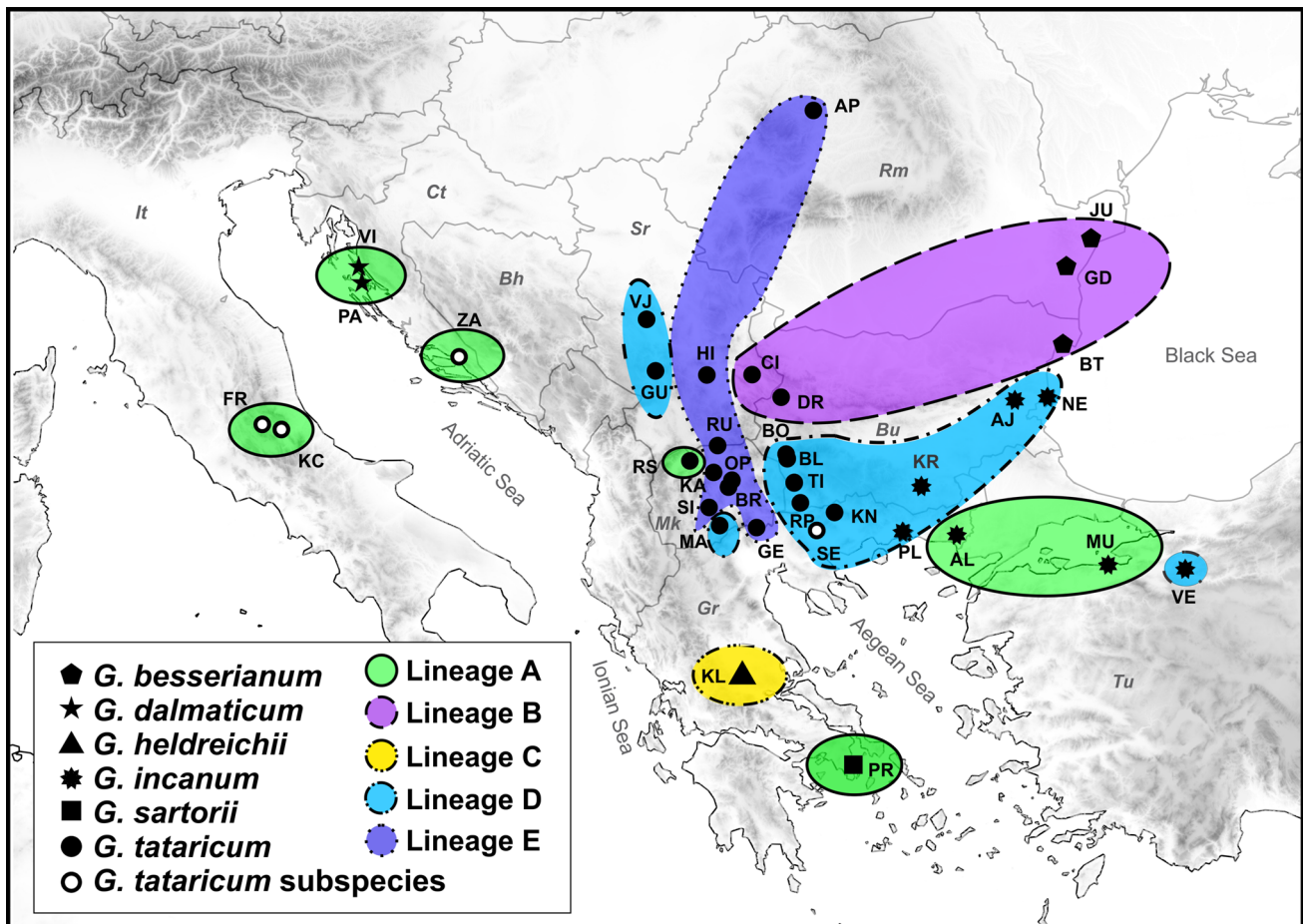
Fig. 4 Spatial distribution of genealogically linked lineages of *Goniolimon* taxa detected in Median-joining network presented in Fig. 3. Acronyms of populations are given in Table 1

Greece and MU from north-western Turkey). The fourth sublineage comprised haplotypes detected in *G. dalmaticum* (VI and PA) found at the Adriatic Sea coast and "*G. croaticum*" from the continental part of Croatia (ZA).

Lineage B was concordant with the subclade 4 and comprised haplotypes belonging to two species, *G. besserianum* (eastern Romania—JU and GD—and north-eastern Bulgaria—BT) and *G. tataricum* from eastern Serbia (CI), western Bulgaria (DR) and a single individual from population KN from northern Greece.

Lineage C, which was concordant with the subclade 3 in the BI tree, comprised two genealogically close haplotypes, h25 and h26, belonging to a single species, *G. heldreichii*, which is local endemic to Greece. They evolved from a hypothetical haplotype mv8 via 10 and 12 mutation steps, respectively.

A heterogeneous lineage D comprised individuals from particular populations belonging to two species, *G. tataricum* and *G. incanum*, grouped together into one non-supported subclade in the BI tree. However, one sublineage of

this lineage, comprising *G. tataricum* from the western part of Bulgaria (populations BO, BL, TI and RP), "*G. graecum*" (north-eastern Greece—SE) and one individual of *G. incanum* from southern Bulgaria (population KR), was concordant with the subclade 2. All haplotypes belonging to this lineage evolved via a series of mutations from two genealogically close haplotypes, h21 (currently found in *G. tataricum* individuals from northern Greece—KN) and a hypothetical haplotype mv4, both positioned at the backbone of the MJ network. These haplotypes were, in addition, included into a reticulate structure comprising three additional haplotypes.

Lineage E comprised haplotypes found in individuals from populations of a single species, *G. tataricum*, which were grouped into the well-supported subclade 1 in the BI tree. Given the positioning of genealogically linked haplotypes in MJ network and their current spatial distribution, it appears that the ancestors of this lineage gave rise to currently distinct individuals of *G. tataricum* from North Macedonia (population GE), on the one hand, and to individuals currently found in Romania (AP) and

regions around rivers Morava and Vardar (BR, HI, KA, OP, RU and SI). Through a series of mutations, the most frequent and widely distributed haplotype from the latter group (h9) gave rise to several other haplotypes with a more or less localised distribution (e.g. h8 from RU, h1–h3 from HI). The observed pattern (star-like structure) reflects past radiation in terms of limited gene flow (Richards et al. 1998), while reticulations at the backbone of the MJ network are indicative of past hybridisation among individuals belonging to different populations (Avise 2000).

Morphometric analyses

ANOVA revealed that all 14 examined morphometric characters and one ratio character contributed towards the statistically significant differences between populations/groups ($p < 0.05$). The ratio character (calyx tube/calyx limb length) had the highest F value (Table 2).

The first three principal components of the two-dimensional PCA (Fig. 5a, Online Resource 4a) explained 66.37% of the total variability (38.30%, 15.44% and 12.63%, respectively). Populations of *G. dalmaticum* (VI and PA) were clearly separated from those representing *G. tataricum* s.l. and *G. italicum* along the second principal component. Characters that mostly contributed to the separation of these two groups were: calyx length, inner bract length, middle
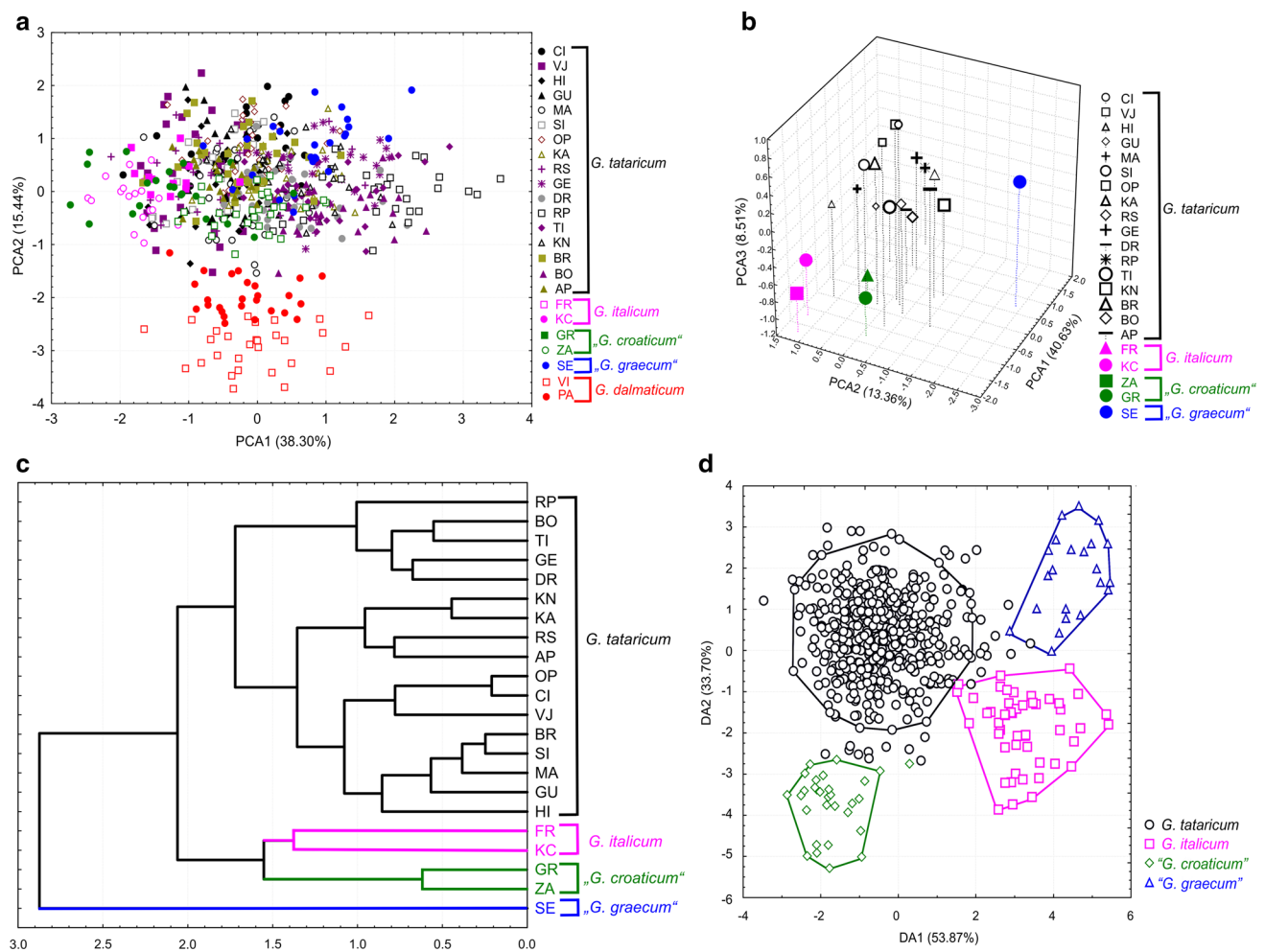
Fig. 5 Results of multivariate statistical analyses of examined *Goniolimon* taxa based on the set of 14 quantitative and one ratio character. Abbreviations of populations correspond to those given in Table 1. **a** two-dimensional PCA in 641 individuals from 24 populations of *G. dalmaticum*, *G. italicum* and *G. tataricum* s.l., **b** three-dimensional PCA of population centroids in 586 individuals from 22 populations of *G. italicum* and *G. tataricum* s.l., **c** unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of *G. italicum* and *G. tataricum* s.l., **d** CDA with four groups pre-defined in previous statistically analyses (three-dimensional PCA and UPGMA). Individuals of *G. tataricum* are labelled with empty black circles, "*G. graecum*" with blue triangles, *G. italicum* with pink squares and "*G. croaticum*" with green rhombus

cusp of inner bract length, outer cusp of inner bract length, middle bract length and cusp of middle bract length.

Further analysis carried out with exclusion of *G. dalmaticum* revealed clear separation of "*G. croaticum*", "*G. graecum*" and *G. italicum* from *G. tataricum* (three-dimensional PCA centroids, Fig. 5b and UPGMA, Fig. 5c). The first three principal components of the three-dimensional PCA centroids (Fig. 5b, Online Resource 4b) explained 62.50% of the total variability (40.63%, 13.63% and 8.51%, respectively). In three-dimensional PCA centroid analysis, the characters that mostly contributed to the separation of these groups along first three principal components were: calyx tube/calyx limb length, inner bract width, calyx lobe length and number of spikes per 4 cm. In UPGMA dendrogram, three clades were obtained, and "*G. croaticum*" and *G. italicum* were positioned within the same, second clade. Morphometric characters that mostly contributed to separation between groups "*G. croaticum*" and *G. italicum*, calyx lobe length and calyx tube/calyx limb length are presented in Fig. 6.

Following the outcomes of the three-dimensional PCA centroid (Fig. 5b) and UPGMA (Fig. 5c), we carried out CDA with four pre-defined groups, namely "*G. croaticum*", "*G. graecum*", *G. tataricum* and *G. italicum* (Fig. 5d, Online Resource 4c–e). The per cent of the discrimination explained by the first three discriminant axes were 53.87%, 33.70% and 12.43%, respectively (100% in total). CDA showed four clearly separated groups: *G. tataricum*, *G. italicum*, "*G. graecum*" and "*G. croaticum*". The characters that mainly contributed to the separation of the groups along the first two axes were calyx lobes length, calyx length, middle bract length and cusp of middle bract length.

Discussion

Taxonomic considerations on *Goniolimon taxa*

Based on our morphometric analysis, thorough examination of qualitative characters and personal observations during the comparative study of all specimens, we defined a new taxonomic and chorological approach for the investigated plants, which distinguishes four subspecies within *Goniolimon tataricum*. As mentioned earlier, Buzurović et al. (2016) pointed out to the misidentification of *G. tataricum* as *G. dalmaticum* in the continental part of Dalmatia (Croatia) (Nikolić 2015; Bogdanović 2015). In this region, *G. tataricum* s.l. inhabits xerophilous pastures and rocky grounds in hilly regions at 170–350 m a. s. l., while *G. dalmaticum* grows on halophilous sites in the coastal eumediterranean zone. In this paper, we demonstrate that individuals from the continental Dalmatia should be treated as a new subspecies, *G. tataricum* subsp. *croaticum* (see Taxonomic treatment). They are characterised by long calyx lobes and one

synapomorphic molecular character (nucleotide character G in position 163 in *rpl*32-*trn*L region, Online Resource 3, see Taxonomic treatment) which, however, was found in some individuals from one out the two studied populations of this taxon. Thus, further studies that include additional specimens from the GR population should be used to confirm the utility of this diagnostic molecular character.

We circumscribe here another new subspecies, *G. tataricum* subsp. *graecum*, represented by individuals found in surroundings of the settlement Serres (SE) in the north-eastern Greece (Fig. 5). Individuals belonging to this subspecies are characterised by calyx lobes that are much shorter than those found in other representatives of *G. tataricum*. Furthermore, one point mutation (nucleotide character C at position 693 in *rpl*32-*trn*L region, Online Resource 3) was recorded in a single individual from this site that was used for molecular analyses. However, the value of this molecular character for barcoding in the taxonomic context (CBOL Plant Working Group 2009) should be confirmed in further studies that include additional specimens.

Finally, we reduced the taxonomic status of *G. italicum* to a lower subspecific rank (*G. tataricum* subsp. *italicum*) and rejected diagnostic arguments used by Tammaro et al. (1982) for the species description. Thus, our analysis showed that longer middle vs. lateral cusps of the inner bract is a common feature in both *G. italicum* and *G. tataricum*, and that both taxa are found in similar habitats (xerophilous pastures and rocky grounds in hilly regions). In addition, Tammaro et al. (1982) pointed to the large disjunction between the Apennine Peninsula and the westernmost part of the distribution range of *G. tataricum* known at that time (Transylvania and Thracia). However, Tammaro et al. (1982) neglected Pančić's (1856) findings of *Statice tatarica* L. (= *G. tataricum*) in the Balkans, which were confirmed more than 100 years later (Ranđelović and Stamenković 1986). In comparison with *G. tataricum* subsp. *tataricum*, *G. tataricum* subsp. *italicum* is characterised by a longer calyx, calyx ribs and cusp of the middle bract (see Taxonomic treatment and Fig. 6).

Evolutionary history of the studied *Goniolimon* taxa

The presented clarification of *Goniolimon* infrageneric taxonomy is essential for better understanding of the evolutionary history of *Goniolimon* taxa in south-eastern Europe and the Apennines. For example, the existence of *G. tataricum* subsp. *italicum* in the Apennines raises the question regarding the occurrence of *G. tataricum* across the Adriatic Sea. Over the past decades, numerous studies of plant species demonstrated that such amphi-Adriatic distribution of taxa was established mainly during the Messinian Salinity Crisis (MSC, 5.96–5.33 Myr) (Frajman and Schneeweiss 2009; Kučera et al. 2010; Surina
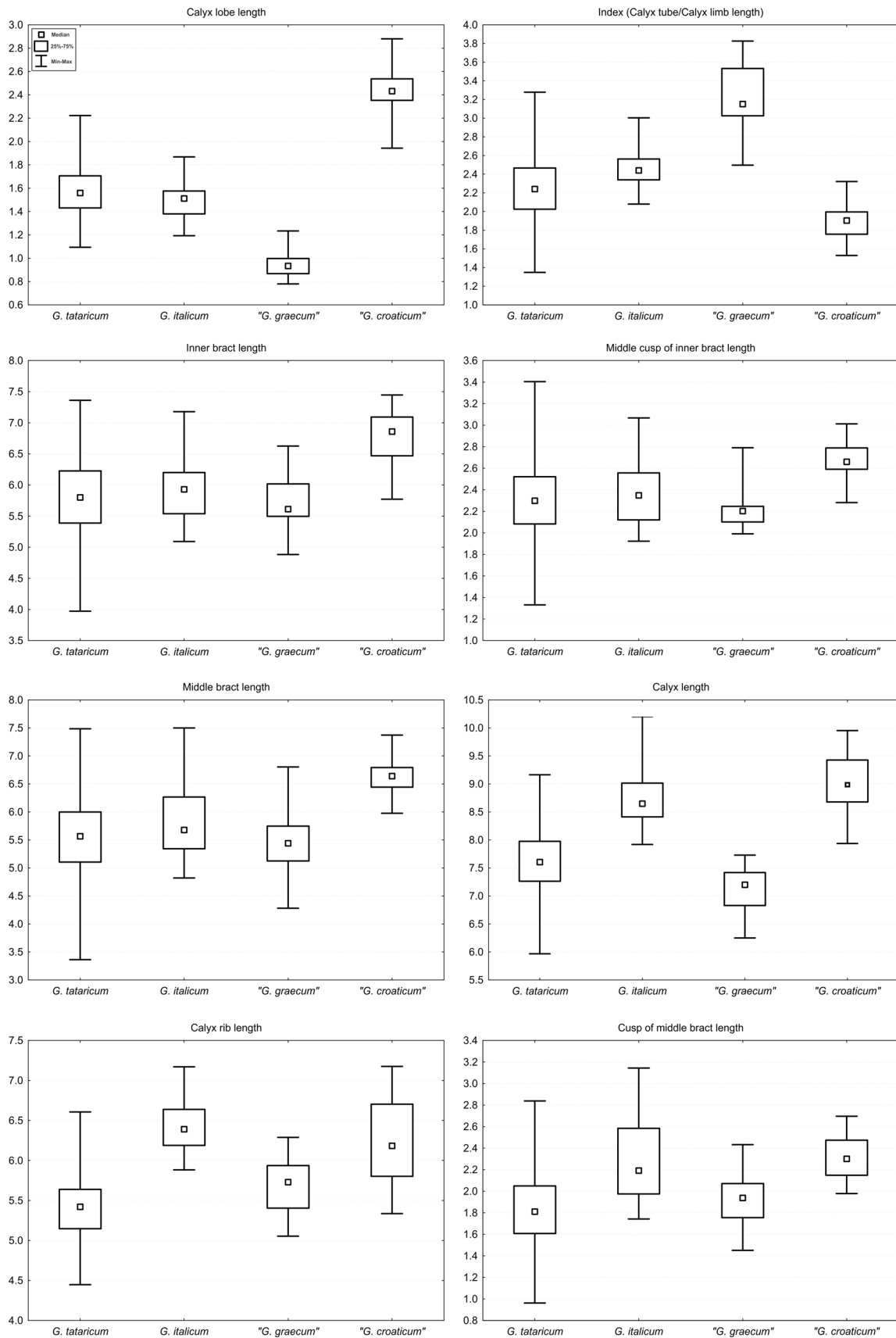
Fig. 6 Variability of selected morphological characters in *Goniolimon tataricum*, *G. italicum*. "*G. croaticum*" and "*G. graecum*"

et al. 2014) when the shallow northern part of the Adriatic Sea was desiccated, and the western part of the Balkan Peninsula was connected by land with the middle/northern Apennine Peninsula (Popov et al. 2006; Rouchy and Caruso 2006). Migrations of plant taxa were also possible during Pleistocene glacials (Frajman et al. 2009; Surina et al. 2014; Đurović et al. 2017; Frajman and Schönswetter 2017), and they were usually directed towards the Apennines (Rešetnik et al. 2016; Chumova et al. 2017) and sporadically in the opposite direction (Dobeš et al. 2013; Chumova et al. 2017). We demonstrate and discuss below that the MSC most likely have played a role in the current amphi-Adriatic distribution of *G. tataricum* as well and that such inferences can be drawn only from phylogeographic analysis (Schaal et al. 1998; Avise 2000) which can accommodate properly processes such as retention of ancestral polymorphisms, multifurcations, interspecific hybridisation and introgression that characterise the evolutionary history of studied taxa and are known to hamper phylogenetic inference (Purvis and Garland 1993; Lewis et al. 2005; Maddison and Knowles 2006; Joly et al. 2009).

Although inferences concerning the age and the centre of origin of *G. tataricum* are indeed speculative at this point due to the lack of studies providing such data, this species may have evolved somewhere in Asia rather than in the Balkan Peninsula, possibly 5.9 Myr. (Note: this node was not supported in the chronogram of Volkova et al. 2017 based on ITS data.) In case that *G. tataricum* emerged outside of the Balkan Peninsula prior to the MSC, the occurrence and the spread of an ancient *G. tataricum* lineage throughout southeastern Europe and the Apennine Peninsula may indeed have been facilitated by the forthcoming MSC. We hypothesise that the descendants of this ancient lineage are the taxa whose extant individuals harbour genealogically related haplotypes positioned on the one (left) side of our MJ network (Fig. 3), i.e. two local endemic species (*G. dalmaticum* and *G. sartorii*), two subspecies of *G. tataricum* (*G. t.* subsp. *croaticum* and *G. t.* subsp. *italicum*) and individuals of *G. tataricum* from a single site in central Balkans (RS). This is supported by the facts that: (i) these taxa are currently found at localised and rather distant sites scattered throughout the Balkans and the Apennine Peninsula, suggesting that such a wide current distribution reflects ancient expansion throughout a large area followed by fragmentation in terms of limited gene flow (Schaal et al. 1998; Avise 2000), and (ii) sufficient time has passed since the fragmentation of these ancient populations to enable accumulation of mutations, resulting in the occurrence of new species and subspecies (Rieseberg and Willis 2007). The observed spatial distribution of extant genealogically linked haplotypes belonging to this ancient lineage may thus be explained by the past east–west migrations in the Mediterranean Basin (Quézel 1978; Lledó et al. 2005; Hardion et al. 2016), followed by fragmentation and speciation which was facilitated by the geo-historic processes in the Balkan Peninsula.

MSC generally coincided with the lacustrine phase in the Balkans (Krstić et al. 2012) which was triggered by the onset of desiccation of the Pannonian Sea (c. 5.8 Myr, Magyar et al. 1999; Harzhauser and Mandić 2008) and was finished during the Pliocene (3.4–2.8 Myr, Suc 1984). This lacustrine phase played an important role during the evolutionary history of the Balkan's taxa (Stojanović et al. 2015; Aleksić et al. 2018), and it may have facilitated speciation in *Goniolimon* as well. This is possible because the initial split in our tree to two clades (Fig. 2), of which one comprised the above-mentioned ancient lineage that gave rise to several taxa including *G. tataricum* subsp. *italicum*, was dated to 4.55 Myr (2.77–6.44 Myr). On the other hand, the assumed turbulent dynamics of populations of the ancient *G. tataricum* lineage at that time may also account for the hypothesised retention of ancestral polymorphisms in *G. tataricum* subsp. *italicum*. It is well known that ancestral and presumably most abundant haplotypes are usually retained in populations experiencing frequent demographic changes (expansions, founder effects and bottlenecks, Schaal et al. 1998; Avise 2000). Furthermore, predominant vegetative propagation, which is also common in range edge, isolated and small-sized populations (Beatty et al. 2008), and which has been observed in extant Italian *Goniolimon* populations (Petriccione and Marrone 2013), also favours retention of prevalent haplotypes. Therefore, it is very likely that the polymorphisms currently present in *G. tataricum* subsp. *italicum* indeed represent ancestral polymorphisms that were retained in populations of this taxon.

It is worth mentioning that the sites on the opposite sides of the Adriatic Sea, inhabited today by two subspecies of *G. tataricum*, *G. t.* subsp. *italicum* and *G. t.* subsp. *croaticum*, were connected by land during the MSC (Popov et al. 2006; Rouchy and Caruso 2006). Assuming that the MSC indeed accounts for the current amphi-Adriatic distribution of *G. tataricum*, this would suggest that the independent evolution of ancient populations of *G. tataricum* at the opposite sides of the Adriatic Sea that became isolated after the MSC, proceeded at similar evolutionary rates, and resulted in the occurrence of two *G. tataricum* subspecies. However, the role of the MSC during the evolutionary history of *G. tataricum* was not indicated in our phylogenetic analysis which supported much later (Pleistocene) evolution of *G. tataricum* subsp. *italicum* (Fig. 2). Although expansions of plant taxa from the Balkans to the Apennines were possible during Pleistocene glacials as well (Frajman et al. 2009; Surina et al. 2014; Rešetnik et al. 2016; Chumova et al. 2017; Đurović et al. 2017; Frajman and Schönswetter 2017), the outcomes of our phylogenetic analysis have to be taken with caution due to the retention of ancestral polymorphisms and other phenomena known to hamper phylogenetic inference

(e.g. Purvis and Garland 1993; Lewis et al. 2005; Maddison and Knowles 2006; Joly et al. 2009). Furthermore, given the positioning of haplotypes found in *G. tataricum* subsp. *italicum* in our MJ network (Fig. 3) that gave rise to several taxa found in the Balkans, the Pleistocene (c. 1.5 Myr) migration of an ancient *G. tataricum* lineage from the Balkans to the Apennines would have to be followed by subsequent migrations in the opposite direction and exceptionally fast speciation in the Balkans resulting in the occurrence of two new species and one subspecies.

G. tataricum individuals from a single site in the central Balkans, RS, may represent a unique and distinct entity among all the studied individuals of this species in this region. Haplotype h42 found in these individuals belongs to the ancient *G. tataricum* lineage A and is characterised by a rather high number of synapomorphic molecular characters (Online Resource 3). However, individuals from RS are morphologically indistinguishable from sympatric and abundant *G. tataricum* individuals from the central Balkans (Fig. 5) whose haplotypes do not belong to the ancient lineage A, but to lineages D and E (Fig. 3). This would suggest that the introgression of the cytoplasm from genetically distinct *G. tataricum* lineages (arriving to the Balkans after the initial expansion of an ancient lineage throughout this region, see later) into individuals belonging to the ancient lineage (already present in the Balkans) took place in the past. Such processes were rather common in glacial refugia during Pleistocene ice ages, especially in terms of uneven species abundance (Rieseberg 1995), and were observed also in other plant species in this region (e.g. Aleksić et al. 2018). Interspecific hybridisation and introgression, indicated by polyphyly in phylogenetic trees (e.g. Lledó et al. 2005; Volkova et al. 2017) and by nesting of haplotypes belonging to one species among those found in other species in haplotype networks (Schaal et al. 1998; Avise 2000), were observed among *G. tataricum* and species used for molecular analyses only, *G. besserianum* and *G. incanum*. However, further studies, with high-resolution molecular markers from different genomes and samples from the entire distribution range of these species, are required for revealing their evolutionary unfolding.

The observed phylogeographic structure of studied *Goniolimon* taxa in south-eastern Europe is rather specific, because genealogically close haplotypes belonging to the ancient *G. tataricum* lineage are scattered throughout a rather large part of the Balkan Peninsula (and also in the Apennines), while another group of genealogically closely related haplotypes (lineages *D* and *E*) is found in the centre of this region (Fig. 4). Such a pattern reflects successive waves of past expansions of genetically distinct lineages throughout the same region with older lineages having more widespread distribution than younger lineages when

dispersal is limited (Schaal et al. 1998; Avise 2000). As already mentioned, the first wave of expansion corresponds to that of the ancient *G. tataricum* lineage, most likely during the MSC, and the second to the subsequent expansion of another genetically distinct lineage. This would be possible if *G. tataricum* was continuously present in a nearby refugium, for instance, in the region north of the Black Sea (Franzke et al. 2004) or in the southern Urals (Kamelin 1998; Velichko 1999), which may have served as a source for successive migrations/expansions throughout south-eastern Europe at the end of the Pliocene/beginning of the Pleistocene or later, during Pleistocene interglacials. Multiple migrations from the east (i.e. from glacial refugia north and maybe also south of the Black Sea) towards the Balkans during the Pleistocene have been recorded in *Gymnospermium,* which has more or less similar distribution range as *G. tataricum* (Barina et al. 2017).

Our molecular data, however, cannot enable further inferences regarding the second wave of colonisation of the Balkan Peninsula by *G. tataricum*. Since all haplotypes belonging to lineages D and E evolved from a few genealogically close haplotypes found at the backbone of the MJ network (h21, h9 and two hypothetical haplotypes, Fig. 3), discovered mainly in a small and localised area (central North Macedonia and nearby north-eastern Greece), it is possible that the common ancestor of both lineages arrived to the Balkans in the past and that diversification of the partial lineage D (well-supported subclade 2, Fig. 2; onset of diversification dated to 1.58 Myr (0.49–2.77 Myr), Fig. 2) predated that of the lineage E (well-supported subclade 1, Fig. 2; onset of diversification dated to 0.89 Myr (0.30–1.68 Myr), Fig. 2). Consequently, diversification of these lineages may just represent sudden, localised and time-separated bursts of a single ancient lineage that arrived to the Balkans in the past, maybe with the onset of the Quaternary, and persisted in this region over a long time. This is possible because Quaternary climate changes have had a limited impact on taxa in the Balkans, and caused only altitudinal migrations of up to several hundred metres in response to periodic shifts of long cold and shorter warm periods (Hewitt 1996). However, we cannot exclude the possibility that the two *G. tataricum* lineages, *D* and *E*, expanded independently throughout the Balkans from the putative nearby eastward glacial refugium, especially if this species was continuously present in such a refugium.

In conclusion, we have improved the infrageneric taxonomy of the genus *Goniolimon*, clarified numerous ambiguities related to the distribution of its taxa in south-eastern Europe and the Apennines, and demonstrated that the evolutionary history of *G. tataricum* in this region was as complex as that reported for other steppe species and genera (e.g. Franzke et al. 2004; Hurka et al. 2012; Seregin

et al. 2015; Friesen et al. 2016; Volkova et al. 2017). However, given the refugial character of the Balkan Peninsula (Hewitt 1996; Médail and Diadema 2009) as well as the fact that populations of *G. tataricum* in this region represent marginal populations of a species having a wider range of distribution, their evolutionary history was somewhat specific, and characterised by two or more waves of colonisation from nearby refugial source populations, and frequent fragmentations, expansions, secondary contacts, diversification, speciation and intra- and interspecific hybridisation. Although certain aspects regarding the evolutionary history of *G. tataricum* and related taxa remained unresolved, our study contributes to a better understanding of the history of understudied steppe species. Further phylogeographic and phylogenetic studies, with improved taxon sampling and use of high-resolution molecular markers from additional genomic resources, are needed to resolve issues related to the age and the centre of origin of the genus *Goniolimon* and the evolutionary history of its taxa.

Taxonomic treatment

Goniolimon tataricum (L.) Boiss. **subsp.** *graecum* Buzurović, **subsp. nov.**—HOLOTYPE: Grčka, Centralna Makedonija, Serres, 164 m a. s. l., 41.10008°N, 23.53718°E, kserofilne livade, neogeni sedimenti, 20 Jun 2015, *U. Buzurović, S. Bogdanović, I. Rešetnik* and *I. Ljubičić* (holotype: BEOU 45784!; isotypes: BEO 53261!, ZAGR 44842!) (Online Resource 5).

Description: Perennial plant, height 15–40 cm, with robust taproot. Stems leafless, sparsely puberulent, ribbed at the base and narrowly to broadly winged in the upper part. Basal leaves in rosette, 8–20 × 1–1.5 cm, lanceolate to spathulate, glabrous, papillose at the margin, entire or slightly undulate, sparsely whitish punctate, with acuminate or mucronate apices. Spikes with 4–6 spikelets per cm; spikelets 1-2(3)-flowered. Bracts usually densely pubescent. Outer bract (5.4–)5.8–6.4(–7.2) × (2.1–)2.4–2.6(–3.0) mm. Middle bract (4.3–)5.1–5.7(–6.8) × (1.4–)1.6–1.8(–2.2) mm; cusp of middle bract (1.4–)1.8–2.1(–2.4) mm long. Inner bract (4.9–)5.5–6.0(–6.6) × (2.4–)2.6–2.8(–3.4) mm, 3-cuspidate, the middle cusp longer than lateral; middle cusp (2.0–)2.1–2.2(–2.8) mm long, lateral cusp (1.2–)1.3–1.5(–1.6) mm long. Calyx (6.2–)6.8–7.2(–7.7) mm long, with 5 ribs mostly reaching the base of the calyx lobes; calyx tube mostly densely hirsute up to rib apices. Calyx ribs (5.1–)5.4–5.9(–6.3) mm long; calyx lobes (0.8–)0.9–1.0(–1.2) mm long, very short and rounded. Calyx tube 3–3½ times longer than calyx limb. Corolla pale pink.

Phenology: Flowering from June to July.

Distribution: Stenoendemic taxon found in the surroundings of the settlement Serres in NE Greece.

Habitat and ecology: Inhabits xerophilous pastures in hilly regions on limestone, at 200 m a. s. l.

Additional specimens examined: GREECE: Serres, sub. *Goniolimon dalmaticum*, *K.H.* and *F. Rechinger 2211* (W); Serres, sub. *Goniolimon dalmaticum* var. *besserianum*, *K.H.* and *F. Rechinger 2210* (W); Macedonia *orientalis*, Serrai (Seres), in collibus calc, 8 Jun 1936, sub. *Goniolimon dalmaticum* (C.Presl) Rchb., *K.H.* and *F. Rechinger 9181b* (BM); Macedonia *orientalis*, Serrai (Seres), in collibus calc, 8 Jun 1936, sub. *Goniolimon dalmaticum* (C.Presl) Rchb. var. *serresianum* Rchb.f., *K.H.* and *F. Rechinger 9181a* (BM).

Goniolimon tataricum (L.) Boiss. **subsp.** *croaticum* Buzurović & Bogdanović, **subsp. nov.**—HOLOTYPE: Hrvatska, okrug Šestanovac, Zadvarje, 207 m a. s. l., kserofilne livade, krečnjak, 43.43103°N, 16.88793°E, 12 Jul 2015, *U. Buzurović, S. Bogdanović* (holotype: BEO 82734!; isotypes: BEOU 45789!, ZAGR 38864!) (Online Resource 6).

Description: Perennial plant, height 15-35 cm, with robust taproot. Stems leafless, glabrous, ribbed at the base and narrowly winged in the upper part. Basal leaves in rosette, 4–15 × 1–1.5 cm, lanceolate to spathulate, glabrous, entire or slightly undulate, whitish punctate, with acuminate or mucronate apices. Spikes with (4–)5–6(–7) spikelets per cm; spikelets 1-2-flowered. Bracts sparsely pubescent, rarely glabrous. Outer bract (5.0–)5.5–6.1(–6.8) × (1.8–)2.2–2.5(–2.8) mm. Middle bract (6.0–)6.4–6.8(–7.4) × (1.3–)1.6–1.8(–2.1) mm, hairy in the central part and cusps; cusp of the middle bract (2.0–)2.1–2.5(–2.7) mm long. Inner bract (5.8–)6.5–7.1(–7.4) × (2.1–)2.5–2.8(–3.0) mm, 3-cuspidate, hairy in the central part and cusps; the middle cusp longer than lateral; middle cusp (2.3–)2.6–2.8(–3.0) mm long, lateral cusp (1.6–)1.7–1.9(–2.1) mm long. Calyx (7.9–)8.7–9.4(–10.0) mm long, with 5 ribs reaching the base of the calyx lobes; calyx tube sparsely hirsute up to half of its length. Calyx rib (5.3–)5.8–6.7(–7.2) mm long, calyx lobes (1.9–)2.4–2.5(–2.9) mm long, subulate. Calyx tube 1½–2 times longer than calyx limb. Corolla pale to dark pink.

Phenology: Flowering from June to July.

Molecular diagnostic characters: Nucleotide character "G" in positions 163 in *rpl*32-*trn*L. Plastid sequence that describe individuals of *G. tataricum* subsp. *croaticum* is available in GenBank under accession numbers MH607203–MH607206.

Distribution: Local endemic of the continental part of Dalmatia in Croatia, restricted to district Šestanovac (settlements Zadvarje and Grabovac).

Habitat and ecology: Inhabits xerophilous pastures and grasslands (alliance *Ostryo-Carpinion orientalis*) and rocky grounds (alliance *Saturejon subspicatae*) in hilly regions on limestone, at 170–350 m a. s. l.

Additional specimens examined: CROATIA: Zadvarje, ispod vidikovca, 173 m a. s. l., krečnjak, UTM–XJ51, 43.429291°N, 16.88800°E, 12 Jul 2015, sub. *Goniolimon tataricum, U. Buzurović, S. Bogdanović s.n.* (BEO); okrug Šestanovac, Grabovac, 327 m a. s. l., krečnjak, UTM–XJ61, 43.431075°N, 16.980925°E, sub. *Goniolimon tataricum, U. Buzurović, S. Bogdanović 82735* (BEO); Dalmatia, Grabovac, limestone, 327 m a. s. l., UTM–XJ61, 12 Jul 2015, *U. Buzurović, S. Bogdanović 39895* (ZAGR); Grabovac, sub. *Goniolimon dalmaticum, Grubišić 6174* (ZA).

Goniolimon tataricum (L.) Boiss. **subsp. *italicum*** (Tammaro, Pignatti & G.Frizzi) Buzurović, **comb. et stat. nov.** ≡ *Goniolimon italicum* Tammaro, Pignatti & G.Frizzi, Webbia 36(1):39. 1982, emend. Buzurović

Description: Perennial plant, height 7-25 cm, with robust taproot. Stems leafless, densely puberulent, ribbed at the base and narrowly winged in the upper part. Basal leaves in rosette, 3–10×0.7–1.5 cm, lanceolate to spathulate, glabrous, papillose at the margin, entire or slightly undulate, whitish punctate, with acuminate or mucronate apices. Spikes with 4–6 spikelets per cm; spikelets 1-2-flowered. Bracts always densely pubescent. Outer bract (4.1–)4.7–5.8(–7.6)×(2.2–)2.4–2.7(–3.0) mm. Middle bract (4.8–)5.3–6.3(–7.5)×(1.3–)1.6–1.9(–2.3) mm; cusp of middle bract (1.7–)2.0–2.6(–3.1) mm long. Inner bract (5.1–)5.5–6.2(–7.2)×(2.1–)2.4–2.6(–3.1) mm, 3-cuspidate, the middle cusp longer than lateral; middle cusp (1.9–)2.1–2.6(–3.1) mm long, lateral cusp (1.1–)1.5–1.8(–2.2) mm long. Calyx (7.9–)8.4–9.0(–10.2) mm long, with 5 ribs mostly reaching the base of the calyx lobes; calyx tube densely hirsute up to rib apices. Calyx rib (5.9–)6.2–6.6(–7.2) mm long, calyx lobes (1.2–)1.4–1.6(–1.9) mm long, subulate. Calyx tube 2–2½ times longer than calyx limb. Corolla pale pink.

Phenology: Flowering from June to July.

Distribution: Local endemic of a restricted region of Abruzzo, province of L'Aquila in Italy (Tammaro et al. 1982; Petriccione and Marrone 2013; Morretti et al. 2015).

Habitat and ecology: Inhabits xerophilous pastures and rocky grounds in hilly regions on limestone, at 350–950 m a. s. l.

Additional specimens examined: ITALY: Abruzzo, Capestrano, Collelungo, 381, kserofilne livade, krečnjak, UTM–UG98, 42.28144°N, 13.77228°E, 11 Jul 2015, sub.

Goniolimon italicum, U. Buzurović, S. Bogdanović 45792 (BEOU), *53273* (BEO), *39888* (ZAGR); Abruzzo, Capestrano, zona arqueologica, 42.28333°N, 13.76667°E, 376 m a. s. l., 5 Jul 2002, *C. Aedo* et al. *699221* (MA); Abruzzo, Ocre, Fossa Raganesca, 800 m a. s. l., kserofilne livade, krečnjak, UTM–UG78, 42.30133°N, 13.47421°E, 11 Jul 2015, sub. *Goniolimon italicum, U. Buzurović, S. Bogdanović 45791* (BEOU), *53274* (BEO); Abruzzo, San Panfilo presso Ocre, prati aridi, 3 Oct 1981, 2*n* = 32, isotypus, *F. Tammaro 013365* (CAT); L'Aquila, Ocre, Fossa del Campanaro, in graminosis aridis, solo calcareo, 800 m a. s. l., 14 Oct 1980., isotypus *F. Tammaro 002425* (FI).

Identification key for *Goniolimon tataricum* subspecies

1a. Calyx lobes longer than 1.2 mm, subulate; calyx tube up to 2½ times longer than calyx limb 2

1b. Calyx lobes up to 1.2 mm ((0.8–)0.9–1.0(–1.2)), very short and rounded; calyx tube 3–3½ times longer than calyx limb; [*bracts usually densely pubescent; calyx tube mostly densely hirsute up to rib apices*]; known only from NE Greece (settlement Serres) .... ***G. tataricum* subsp. *graecum***

2a. Calyx lobes 1.2–2.0 mm; calyx tube 2–2½ times longer than calyx limb 3

2b. Calyx lobes longer than 2.0 mm ((1.9–)2.4–2.5(–2.9) mm long); calyx tube 1½–2 times longer than calyx limb; [*bracts sparsely pubescent, rarely glabrous; calyx tube sparsely hirsute up to half of its length*]; distribution only in continental part of Dalmatia in Croatia .... ***G. tataricum* subsp. *croaticum***

3a. Calyx (7.9–)8.4–9.0(–10.2) mm long; cusp of middle bract (1.7–)2.0–2.6(–3.1) mm long; calyx ribs (5.9–)6.2–6.6(–7.2) mm long; [*bracts always densely pubescent; calyx tube densely hirsute up to rib apices*]; distribution only in Italy .... ***G. tataricum* subsp. *italicum***

3b. Calyx (5.9–)7.3–8.1(–9.2) mm long; cusp of middle bract (0.9–)1.6–2.0(–2.8) mm long; calyx ribs (4.2–)5.2–5.7(–6.8) mm long; [*bracts sparsely to densely pubescent; calyx tube sparsely to densely hirsute*] .... ***G. tataricum* subsp. *tataricum***

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Information on Electronic Supplementary Material

Online Resource 1. Annotation of *rpl*32-*trn*L and 3′*rps*16-5′*trn*K plastid intergenic spacers used in the study and delineation of haplotypes detected in studied *Goniolimon* taxa.

Online Resource 2. Aligned matrix of two plastid intergenic spacers (3′rps16-5′trnK and rpl32-trnL) used in the study.

Online Resource 3. Positions of synapomorphic characters in two plastid regions in studied *Goniolimon* taxa.

Online Resource 4. a Eigenvalues for two-dimensional PCA by species *Goniolimon dalmaticum*, *G. italicum* and *G. tataricum* s.l. presented in Fig. 5a, **b** eigenvalues for three-dimensional PCA by species *G. italicum* and *G. tataricum* s.l. presented in Fig. 5b, **c** classification matrix for discriminant analysis by group presented in Fig. 5d, **d** standardised coefficients for canonical variables of CDA presented in Fig. 5d; characters that mostly contributed to the separation along particular axes are given in bold, **e** total canonical structure for canonical variables of CDA presented in Fig. 5d.

Online Resource 5. Holotype of *Goniolimon tataricum* (L.) Boiss. subsp. *graecum* Buzurović, subsp. nov. deposited in the Herbarium of the University of Belgrade (BEOU).

Online Resource 6. Holotype of *Goniolimon tataricum* (L.) Boiss. subsp. *croaticum* Buzurović & Bogdanović subsp. nov. deposited in the Herbarium of the Natural History Museum in Belgrade (BEO).

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