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Phylogeny, geographic distribution, and new taxonomic circumscription of the *Crocus reticulatus* species group (Iridaceae)

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Abstract: Recent phylogenetic analyses proved several infrageneric units within the genus *Crocus* to be para- or polyphyletic. In an attempt to arrive at a system of *Crocus* that closely reflects species relationships, we provide here phylogenetic, morphometric, geographic, and nomenclatorial data for the species of a narrower-defined, monophyletic *Crocus* series *Reticulati*. We sequenced the ETS and ITS regions of the nuclear ribosomal DNA in 9 *Reticulati* and 19 outgroup species. Three chloroplast loci (*trn*L–F, *rps*16–*trn*Q, *mat*K–*trn*K) were sequenced in the newly defined series *Reticulati* species and 1 outgroup. Data were analyzed with Bayesian and parsimony algorithms. The phylogenies resulted in 2 clearly separated, geographically defined species groups within the series *Reticulati*. The southern one comprises only the taxa from Turkey, while the species of the second group are distributed from Italy in the west through the areas north of the Black Sea to the Caucasus in the east. To arrive at monophyletic species we describe here *C. danubensis* sp. nov., *C. filis-maculatis* sp. nov., and *C. orphei* sp. nov. as new species, and we define *C. reticulatus* s.s. to comprise only the populations in the area north and east of the Black Sea.

Key words: *Crocus*, chloroplast loci, ETS, evolution, ITS, phylogeny, ribosomal DNA, systematics, taxonomy

1. 1. Introduction

The genus *Crocus* L. consists currently of about 160 recognized species (Mathew, 1982; Petersen et al., 2008; Kerndorff and Pasche, 2011; Kerndorff et al., 2011, 2013; Peruzzi and Carta, 2011; Ranđelović et al., 2012; Harpke et al., 2014) occurring from western Europe and northwestern Africa to western China, with the center of species diversity on the Balkan Peninsula and in Turkey. Many crocuses are known as popular ornamentals, and saffron, the dried styles of *C. sativus* L., is one of the world's most expensive spices. Molecular systematic studies (Petersen et al., 2008; Seberg and Petersen, 2009; Harpke et al., 2013) recently showed that several of the infrageneric taxonomic units of Mathew's (1982) revision of the genus are not monophyletic. Among them is series *Reticulati* B.Mathew of section *Nudiscapus* B.Mathew, with species occurring in the phylogenetic trees in clades intermingled with taxa of series *Biflori* B.Mathew and series *Speciosi* B.Mathew. Traditionally, within *C. reticulatus*, a species described from the Caucasus (Weber and Mohr, 1805), 2 subspecies are recognized on morphological grounds: subsp. *reticulatus* and subsp. *hittiticus* (T.Baytop & B.Mathew) B.Mathew (Mathew, 1982), occurring from Italy to southwestern Russia and Turkey. However, according to Mathew, subsp. *reticulatus* is highly heterogeneous, including 3 cytotypes with 2n = 10, 12, and 14 chromosomes. Under subsp. *reticulatus* Mathew (1982) subsumed as synonyms several taxa, such as, for instance, *C. micranthus* and *C. variegatus*. To arrive at a systematic treatment of *Crocus* that reflects natural relationships among taxa we are currently analyzing and circumscribing monophyletic units of the genus (Kerndorff et al., 2013; Harpke et al., 2014). Here we provide data for the *C. reticulatus* species group in its new definition based on 2 molecular data sets and morphological characters.

Molecular markers are able to provide higher numbers of qualitative characters for closely related taxa

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in comparison to morphology, which in addition often shows only quantitative variation. As series *Reticulati* species belong to a young group within *Crocus* (Harpke et al., 2013), fast-evolving nuclear markers are necessary to resolve taxon relationships. Therefore, we used sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA; Baldwin et al., 1995; Álvarez and Wendel, 2003) that had already proven useful in *Crocus* phylogenetics (Harpke et al., 2013, 2014), together with the external transcribed part (ETS) of the intergenic spacer, separating the 45S-rDNA tandem-repeat units. This marker is applied here for the first time in *Crocus* systematics. In addition to these nuclear loci, we also analyzed sequences of 3 chloroplast regions. One of these (*trn*L–F) is often used in plant phylogeography, as it is normally quite variable (e.g., Bänfer et al. 2006; Jakob and Blattner, 2006). PCR primers for the other 2 regions (*rps*16–*trn*Q and *mat*K–*trn*K) were newly designed for this study.

Harpke et al. (2013) analyzed diverse morphological characters traditionally used in *Crocus* taxonomy regarding their potential to discern monophyletic groups within the genus. They concluded that none of the morphological characters were unequivocally able to discern sections or series within the genus. Nevertheless, a combination of such characters might be indicative for smaller taxonomic units within *Crocus*. In an attempt to characterize series *Reticulati* species with respect to their morphological differences and similarities, we evaluated 9 morphological characters and analyzed them by univariate analysis methods.

With our study we want to answer the following questions: (*i*) What are the phylogenetic relationships among series *Reticulati* taxa? (*ii*) Based on these, which taxa do or do not belong to a monophyletic series *Reticulati*? (*iii*) Is it possible to arrive at a historical biogeography of the series? (*iv*) Can we define processes playing a role in species diversification in the series? Finally, (*v*) are there taxa that have to be newly described to arrive at a monophyletic species?

2. Materials and methods

2.1. Taxon sampling

We included 61 individuals representing 9 species of series *Reticulati* in its traditional circumscription (Mathew, 1982) and 12 species as outgroups derived from other closely related series of *Crocus* in our molecular data set. The taxa are listed in Table 1. To analyze species defined to belong to series *Reticulati* in its new circumscription, all together 101 individuals were screened for their morphology. Herbarium vouchers of all studied materials were deposited in the herbaria of the IPK Gatersleben (GAT), Pisa University (PI), or the University of Athens (ATHU).

2.2. Molecular methods

The extraction of genomic DNA and amplification of the nuclear rDNA ITS and chloroplast *trn*L–F region, consisting of the *trn*L gene with its intron and the intergenic spacer between the *trn*L and *trn*F genes, was conducted according to Harpke et al. (2013). To obtain marker regions with additional or higher variability in *Crocus*, we used contigs derived from the assembly of low-coverage next-generation sequencing (on the Illumina HiSeq platform) of 3 *Crocus* species to compare entire chloroplast genomes and 45S-rDNA sequences among the species. For the 3 most variable regions of the chloroplast, PCR primers were designed and tested. For 2 regions (*rps*16–*trn*Q and *mat*K–*trn*K) we obtained reliable PCR amplicons, which proved to be variable in a set of sequences from diverse *Crocus* species. The assembled 45S rDNA was used to infer the position and sequence of the ETS region for the 3 *Crocus* species. For this region, *Crocus*-specific PCR primers and nested sequencing primers were designed and used for further analyses (Table 2).

Amplifications of the marker regions were performed with 1.5 U Taq DNA polymerase (QIAGEN) in the supplied reaction buffer, 0.2 µM of each dNTP, 50 pmol of each primer (Table 2), Q-Solution (QIAGEN) with a final concentration of 20%, and about 20 ng of total DNA in a 50-µL reaction volume in a GeneAmp PCR System 9700 (PerkinElmer). The amplification consisted of 3 min of initial denaturation at 95 °C and 35 cycles of 30 s at 95 °C, 45 s at the primer-specific annealing temperature (Table 2), and 60 s at 70 °C, followed by a final extension for 8 min at 70 °C. PCR products were purified using NucleoFast 96 PCR plates or NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) following the manufacturer's protocol, and were eluted in 30 µL of water. In a few cases where more than one PCR fragment was obtained, a fragment of the right size was cut from an agarose gel, eluted, purified, and then subjected to sequencing. Both strands of the PCR products were directly sequenced with Applied Biosystems' BigDye Terminator technology on an ABI 3730xl automatic DNA sequencer using either the primers from PCR amplifications or, in the case of the ETS region, the nested sequencing primers ETS-SF and 18S_IGS-SR.

2.3. Phylogenetic analyses

Forward and reverse sequences were manually checked, edited where necessary, and combined in consensus sequences for each locus and individual. All newly obtained sequences were submitted to the EMBL nucleotide database. Sequence accession numbers for the studied individuals are given in Table S1 (supplementary online materials). Sequences from single loci were aligned using the CLUSTAL algorithm and alignments were adjusted manually. For each locus, the best-fitting model of DNA evolution was estimated in MrModeltest 2.3 (Nylander,

Table 1. Studied *Crocus* taxa and their origins.

Taxon	Origin
C. ancyrensis (Herb.) Maw	Turkey; LST-077, cultivated, origin: Kartal Geçidi, 1340 m, type region, GAT 25832
	Turkey; BATM-340, cultivated, origin: Yağdonduran Geçidi, 1720 m, GAT 29388
	Turkey; LST-114, cultivated, origin: Bakırlı yaylası, 1480 m, GAT 29387
	Turkey; RUDA-061, cultivated, origin: Topuz dağı Geçidi, 1535 m, GAT 25830
	Turkey; HKEP0927, S of Divriği, 1550 m, GAT 7146
C. angustifolius Weston	Ukraine; cultivated, origin: Crimea, Jalta, GAT 7236
C. danubensis Kernd. et al. sp. nov.	Serbia; HKEP1344, Danube valley near Negotin, 45 m, type location, GAT 23019 (type)
	Ukraine; Zubov-07, cultivated, origin: N of Manipol, GAT 25806
C. filis-maculatis Kernd. & Pasche sp. nov.	Turkey; HKEP1207, foothills of Taurus N of Adana, 800–900 m, type location, GAT 25833 (type)
	Turkey; HKEP1357, Anti-Taurus N of Burhaniye, 960 m, GAT 29389
C. hittiticus T.Baytop & B.Mathew	Turkey; Taurus mountains around Güzeloluk, 1380 m, GAT 7263
	Turkey; HKEP1112, Uzuncaburç, 1400 m, GAT 7466
C. micranthus Boiss.	Turkey; HKEP0919, Cilician gate, type location, GAT 7141
	Turkey; Batm-402Bc, cultivated, origin: Adana, Göksun-Develi, 1580 m, GAT 25831
C. orphei Karamplianis & Constantin sp. nov.	Greece; Falakro Mountain, 1470 m, (*Karamplianis & Tsiftsis 1839*), ATHU
	Greece; Falakro Mountain, 1210 m, (*Karamplianis & Tsiftsis 1843*, type), ATHU
C. reticulatus Stev. ex. Weber & Mohr	Russia; Banketov-09, cultivated, origin: Stavropol distr., Pjatigorsk, 550 m, GAT 25805
	Moldova; cultivated, Bessarabia, GAT 23084
	Russia; Northern Caucasus: Teberda State Biospheric Reservation, 2400 m , type region, GAT 23082
	Georgia; CMGG-026, cultivated, origin: near Devdoraki glacier, 1700 m, GAT 25805
C. variegatus Hoppe & Hornsch.	Italy; TCH-3508, cultivated, origin: Trieste, 250 m, type region, GAT 7264
	Italy, TCH-1014, cultivated, origin: Trieste, 400 m, type region, GAT 25809
	Italy; Abruzzo, Collarmele, 1425 m, voucher at PI
	Italy; HKEP1311, Abruzzo, Borgorose, 800 m, GAT 25809
	Italy; HKEP1315, Abruzzo, Barisciano, 1100 m, GAT 25808
	Slovenia; Golob-07, cultivated, near Šentilj, GAT 25803
	Hungary: Cultivated, 40 km east of Budapest, GAT 25807
	Bulgaria: Vrasta province, 500 m, GAT 25829
Other taxa of ser. *Reticulati* sensu Mathew	
C. abantensis Baytop & B.Mathew	Turkey; GAT 7235
C. cancellatus Herb.	Turkey; HKEP1028, GAT 7180
	Turkey; HKEP1033, GAT 7133
	Turkey; HKEP1354, GAT 29390
C. gargaricus Herb.	Turkey; GAT 7255
C. herbertii B.Mathew	Turkey; GAT 7396
C. hermoneus Kotschy ex Maw subsp. *palaestinus* Feinbrun	Jordan; IABH 7
C. lycius B.Mathew	Turkey; GAT 7181
C. pamphylicus B.Mathew	Turkey; GAT 7213
Outgroup taxa from other series	

Table 1. (Continued).

Series *Aleppici* B.Mathew	
C. aleppicus Baker	Jordan; IABH 18357
Series *Biflori* B.Mathew	
C. biflorus Mill.	Italy; Abruzzo, PI
Series *Flavi* B.Mathew	
C. adanensis T.Baytop & B.Mathew	Turkey; GAT 7148
C. graveolens Boiss. & Reut. ex Boiss.	Turkey; GAT 7457
Series *Interexti* B.Mathew	
C. fleischeri J.Gay	Turkey, GAT 7139
Series *Laevigati* B.Mathew	
C. laevigatus Bory & Chaub subsp. *pumilis* Rukšāns	Greece; CR12-022, GAT 23019
C. tournefortii J.Gay	Greece; GAT 7202
Series *Speciosi* B.Mathew	
C. pulchellus Herb.	Greece; GAT 29391
C. speciosus M.Bieb.	Turkey; GAT 19558
Unplaced	
C. nerimaniae Yüzb.	Turkey; GAT 7378
C. yataganensis Kernd. & Pasche	Turkey; GAT 7380
C. almehensis C.Brickel & B.Mathew	Iran; TARI 69170

Table 2. Analyzed genome regions.

Region	Primer name	Primer sequence (5'-3')	Amplicon size in bp	PCR annealing temperature
ITS	ITSA [1]	GGA AGG AGA AGT CGT AAC AAG G	~750	54 °C
	ITSB [1]	CTT TTC CTC CGC TTA TTG ATA TG		
	18s F [2,3]	ACC GAT TGA ATG GTC CGG TGA AGT GTT CG		59 °C
	26s R [2,3]	CTG AGG ACG CTT CTC CAG ACT ACA ATT CG		
ETS	18S_IGS_rev	GTT CAT ACT TAC ACA TGC ATG G	~600	54 °C
	ETS_fw	GCA CGT GAG TGG TTT TGG		
	18S_IGS-SR	TTA CAC ATG CAT GGC TTA ATC		
	ETS-SF	GTG AGT GGT TTT GGA TCT		
*trn*L^{UAA}–*trn*F^{GAA}	Cp07 [4]	GGA AAT GGG GAT ATG GCG	~720	54 °C
	trnFr [4]	AAA ATC GTG AGG GTT CAA GTC		
	c [2,5]	CGA AAT CGG TAG ACG CTA CG		56 °C
	f [2,5]	ATT TGA ACT GGT GAC ACG AG		
*rps*16–*trn*Q^{UUG}	rpS16f	CAG GAA CAG AAC AAA CTA TGT CG	~750	56 °C
	trnQr	GGT CCC GTT ACT CGG AGG TTC G		
*mat*K–*trn*K^{UUU}	matKf	CAT TTC CAC TTG AAC CAT AAG CAG G	~800	56 °C
	trnKr	AGA CCA CGA CTG ATC CTG AAA GG		

[1]Blattner (1999); [2]used for *C. orphei*, [3]Gruenstaeudl et al. (2009); [4]Jakob and Blattner (2006); [5]Taberlet et al. (1991).

2004). The sequences of nuclear loci and chloroplast loci were both concatenated, resulting in 2 data sets. The nuclear data were subjected to phylogenetic analyses using Bayesian phylogenetic inference (BI) with MrBayes 3.2 (Ronquist et al., 2012) and maximum parsimony (MP) with PAUP* 4b10 (Swofford, 2002). For the chloroplast data we calculated a Bayesian tree and also a haplotype network using TCS (Clement et al., 2000). The data set of the nuclear loci consisted of sequences from all individuals included in the analysis (Table 1), while for the chloroplast data set only sequences from individuals belonging to the newly defined series *Reticulati* s.s. plus 1 outgroup were analyzed.

For BI, 2 times 4 chains were run for 3 million generations under the appropriate models of sequence evolution (nuclear data set: GTR+Gamma+I; chloroplast data set: F81+Gamma), sampling a tree every 1000 generations. Converging log-likelihoods, potential scale reduction factors for each parameter, and inspection of tabulated model parameters in MrBayes suggested that stationarity had been reached in all analyses. The first 25% of trees of each run were discarded as burn-in. Two independent runs of BI analysis were performed to confirm that separate analyses converged on the same result. In each of the 2 analyses, the same topology and similar posterior probabilities (pp) of nodal support resulted.

For the MP analysis we used the heuristic search algorithm in PAUP* with TBR branch swapping, MULTREES on, and 100 random sequence additions. The phylogenetic trees were summarized as a strict consensus tree. Branch support was evaluated by bootstrap analysis using 1000 data resamples with the same settings as before, except that no random sequence additions were performed. Consistency (CI) and retention (RI) indices were calculated in PAUP*.

To obtain a chloroplast haplotype genealogy for the species of series *Reticulati* s.s., insertions/deletions (indels) in the alignment that likely originated from single mutational events were reduced to single alignment positions. This shortened alignment was subjected to statistical parsimony analysis in TCS where gaps are treated as a fifth character state.

2.4. Morphological and morphometric analyses

Nine characters of 101 individuals were measured from herbarium vouchers resembling *C. reticulatus* (in the new species circumscription: *C. danubensis*, *C. micranthus*, *C. orphei*, *C. reticulatus*, *C. variegatus*; see Section 3 and Table 3). For multiple pairwise comparisons of continuous quantitative characters (scored from a to f in Table 3), the Kruskal–Wallis test for independent samples was used (with Bonferroni correction for multiple comparisons). Other qualitative characters were simply checked for constancy in all the 101 individuals mentioned above and in 45 individuals belonging to clearly distinct taxa, such as *C. ancyrensis*, *C. angustifolius*, *C. filis-maculatis*, and *C. hittiticus*.

2.5. Karyological analysis

For a chromosome analysis of typical *C. reticulatus* from Moldova (Table 1), squash preparations were made from root tips of individuals temporarily cultivated in pots and harvested in early spring in the Botanic Garden of the University of Pisa, according to the following procedure: pretreatment in 0.4% colchicine solution for 4 h was followed by Carnoy fixing for 45 min and hydrolysis in 1 N HCl for 9 min at 60 °C. Staining was conducted with leuco-basic fuchsin for 3 h. At least 10 chromosome plates were counted in order to establish the chromosome number.

3. Results

3.1 Nuclear rDNA analyses

The length of the ETS region varies from 524 to 526 bp for series *Reticulati* s.s. species and has a size of up to 529 bp in the outgroup taxa. In contrast, length differences in the

Table 3. Comparison of morphological features among *C. danubensis* (n = 22), *C. micranthus* (n = 12), *C. reticulatus* (n = 16), *C. variegatus* (n = 35), and *C. orphei* (n = 16). Quantitative numerical values are expressed as (minimum)percentile 10–percentile 90(maximum) in mm. Quantitative morphological characters are coded by letters (see also Table 4).

	C. danubensis	*C. micranthus*	*C. reticulatus*	*C. variegatus*	*C. orphei*
Dominant flower color	White to soft lilac	White to soft lilac	Lilac	Lilac	Lilac
Throat color	White to pale yellow	Yellow	Yellow	Yellow	White to pale yellow
Corm fibers width (mm) [**a**]	(0.21)0.23–0.44	(0.23)0.24–0.43(0.48)	(0.1)0.11–0.16(0.18)	(0.16)0.21–0.43(0.6)	(0.28)0.29–0.39(0.40)
Filament length (mm) [**b**]	(5)5.4–6.3(6.5)	(4)4.4–5(6)	(4)4.6–7(7.7)	(2.5)3.7–6(7)	5–7
Anther length (mm) [**c**]	(6)7–9.4(9.7)	(8)8.4–9(9.5)	(7.5)8.5–14(15)	(6)7.5–10.7(13)	(10)10.5–12.2(14.5)
Style length (mm) [**d**]	(4.4)5–9.4(10.6)	(5)5.4–6	5–7.5(9.5)	(4.4)5–9.5(10.6)	(6)7–11.5(12)
Outer segments length (mm) [**e**]	(20.9)22–26.4(27.6)	(20)21.5–24.9(25)	23–29(33)	(18)21–30(35)	(20)22–30(35)
Outer segments width (mm) [**f**]	(4)5.4–7.7(9)	5–7	(8)8.5–10(11)	(4)6–10(11.5)	(7)8–10.5(15)

ITS data set are up to 10 times higher (621–672 bp), partly caused by 3 variable microsatellite repeat motifs. Sequence differences of the ETS region are therefore mainly caused by base substitutions instead of indels. The average genetic diversity based on pairwise Jukes–Cantor distances is 13% higher within the ETS data set in comparison to the ITS data set.

The tree obtained by BI (Figure 1) shows that *Crocus* series *Reticulati* s.l. (i.e. sensu Mathew, 1982) species occur in different clades of the tree, partly interspersed with species belonging to other series. What we treat as series *Reticulati* s.s. is divided into 2 geographically clearly defined groups with species either confined to Turkey or occurring widespread between Italy and the Caucasus region except Turkey. The branch support for the clade is low (0.85 pp) but both geographical groups are highly supported (1 pp). Within the Turkish clade *C. filis-maculatis* is sister to the remainder of the taxa, while the widespread species group is further divided into a clade consisting of taxa from Greece and the Black Sea area (northeastern clade) and a clade consisting of taxa mainly from the Balkan Peninsula, Italy, and Hungary together with a geographic outlier obtained from Ukraine.

The MP analysis resulted in 60 equally parsimonious trees of 712 steps length (CI = 0.564, RI = 0.793) with a topology of the strict consensus tree (Figure S1, supplementary online materials) completely compatible with the tree resulting from BI. The only differences regard a basal polytomy within the clade of Turkish series *Reticulati* taxa that is resolved in BI, although partly with low support values, and the position of *C. abantensis* in a polytomy in BI versus its sister group relationship with the series *Biflori* and *Reticulati* taxa in MP.

3.2. Chloroplast DNA analyses

The sequence lengths of the 3 chloroplast regions analyzed for this study are 672–677 bp for *trn*L–F (13 variable positions), 609–622 bp for *rps*16–*trn*Q (14 variable positions), and 624–631 bp for *mat*K–*trn*K (14 variable positions) within series *Reticulati* s.s. Concatenation of the 3 loci for series *Reticulati* s.s. species plus *C. graveolens* as an outgroup taxon resulted in an alignment length of 1981 bp (1943 bp without outgroup), which was used for BI. Shortening of gaps for the statistical parsimony analysis in TCS for the series *Reticulati* s.s. species resulted in an alignment of 1917 bp. TCS calculated a 95% connection limit of 19 steps for this alignment and inferred 23 chloroplast haplotypes occurring in the series *Reticulati* s.s. individuals.

Comparable to the results of the nuclear data set, a clear differentiation between chloroplast haplotypes occurring in and outside of Turkey is visible in the chloroplast haplotype network (Figure 2) and the BI tree (Figure S2, supplementary online materials), although resolution of the tree is otherwise lower. Both groups are separated by 8 mutational steps in the network, for which no haplotypes were detected in the analyzed individuals. The chloroplast haplotypes at internal positions in the network, which normally represent the oldest alleles within a genealogy, occur for the northwestern group in Greece (Figures 2 and 3), indicating 2 colonization events for the Black Sea area and 1 migration route through the Balkan Peninsula towards Italy. The chloroplast haplotype of a peculiar *C. danubensis* population from Ukraine (Zubov07) falls within this latter group, suggesting another independent colonization of the Black Sea area, probably from the Pannonian Basin. Within the Turkish part of the network no central haplotype was found. Three groups can be discerned, 2 of them from southern Turkey and 1 occurring widespread in central and northwestern Turkey (Figures 2 and 3). No clear pattern of possible dispersion routes is obvious for the Turkish series *Reticulati* taxa.

3.3. Morphological and morphometric analyses

Among the populations included within series *Reticulati* s.s., some could be clearly discerned due to qualitative morphological features, such as yellow flowers in *C. ancyrensis* and *C. angustifolius* (the former also without external stripes), filaments with a peculiar dark spot in *C. filis-maculatis*, blackish-maroon anthers in *C. hittiticus*, and a white to pale-yellow throat in *C. danubensis* and *C. orphei* (Table 3; Figures 4a and 4b). Within the material superficially resembling typical *C. reticulatus* sensu Mathew, at least 4 entities could be distinguished by morphometric univariate analysis. Typical *C. reticulatus* (from the locus classicus region) differs from all of them by its narrower corm fibers (Table 4). The most distinctive species is *C. micranthus*, which is also marked by pale- to light-lilac flowers, similar to *C. danubensis* (Table 3). All species are discerned by at least one morphological parameter according to our statistical analysis, with the exception of *C. danubensis* versus *C. variegatus* (Table 4). However, these 2 species are easily distinguished by their throat colors (Table 3; Figure 4a).

3.4. Karyological analysis

The sampled population of *C. reticulatus* from Moldova proved to be diploid, with 2n = 12 chromosomes (Figure S3, supplementary online materials). Chromosome sizes range between 4 and 6 μm.

4. Discussion

4.1. New nuclear and chloroplast marker regions for *Crocus* systematics

For closely related species within the series of *Crocus*, resolution of phylogenetic trees was somewhat low when multiple chloroplast genes (Seberg and Petersen, 2009) or the nuclear rDNA ITS regions (Harpke et al., 2013) were used as markers. This led researchers to use DNA

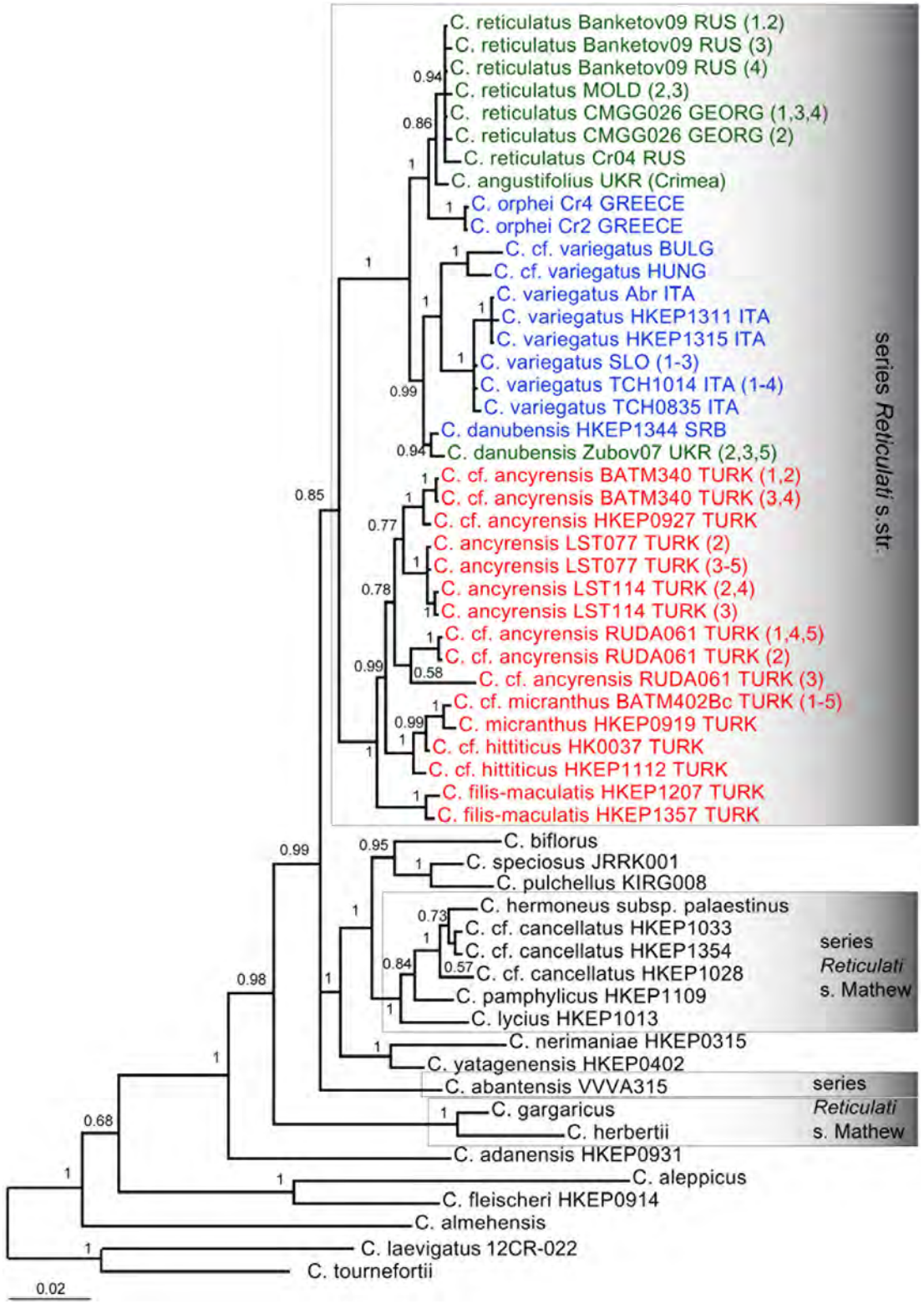

Figure 1. Phylogenetic tree obtained by Bayesian phylogenetic inference of the combined sequences of the nuclear rDNA ETS and ITS regions. Numbers along branches give posterior probabilities. Species names are followed by their collection number (Table 1) and geographical origin (BULG = Bulgaria, GEOR = Georgia, HUNG = Hungary, ITA = Italy, MOLD = Moldova, RUS = Russia, SLO = Slovenia, SRB = Serbia, TURK = Turkey, UKR = Ukraine). Numbers in brackets indicate the individuals sharing identical sequences. Codes after species names of series *Reticulati* s.s. correspond to the results of the analysis of chloroplast haplotypes and their geographical distribution as shown in Figures 2 and 3.

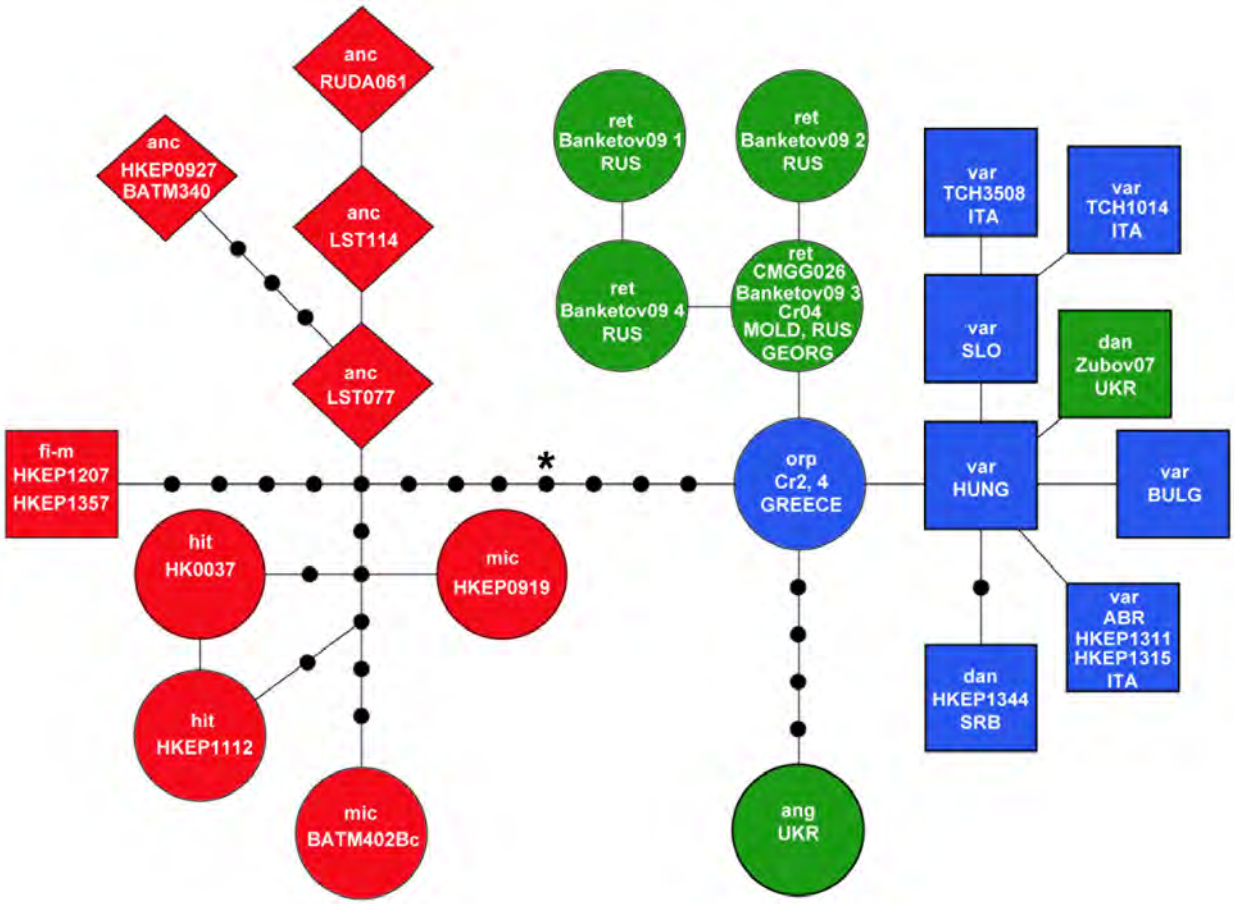

Figure 2. Chloroplast haplotype genealogy for the newly circumscribed *Crocus* series *Reticulati* derived from concatenated sequences of the *trn*L–F, *rps*16–*trn*Q, and *mat*K–*trn*K regions of the chloroplast genome. Black dots depict missing haplotypes, i.e. alleles not found in the analyzed individuals. The asterisk indicates the position of the root of the network. Geographical distribution of the chloroplast haplotypes is provided in Figure 3. The codes of haplotypes include the abbreviations for the species names (anc = *C. ancyrensis*, ang = *C. angustifolius*, dan = *C. danubensis*, hit = *C. hittiticus*, fi-m = *C. filis-maculatis*, mic = *C. micranthus*, orp = *C. orphei*, ret = *C. reticulatus*, and var = *C. variegatus*) followed by their collection number (see also Table 1) and, for samples outside of Turkey, by the country of origin (abbreviations according to Figure 1).

fingerprint methods also for interspecific analyses in *Crocus* (Erol et al., 2014; Larsen et al., 2014). There are examples proving that such methods might increase phylogenetic resolution (e.g., Pleines and Blattner, 2008) although due to their anonymous nature we deem them as poor markers in systematics (Pleines et al., 2009). The main problem we see is the basically noncumulative nature of anonymous markers, i.e. in contrast to DNA sequences they are not readily stored in open databases that are extended by every newly submitted sequence (Blattner and Friesen, 2006). To overcome such restrictions, we combined here sequences of the rDNA ETS and ITS regions. This data set vastly increased phylogenetic resolution in comparison to ITS- or chloroplast-based phylogenetic trees. Sequence differences in the ETS region of *Crocus* are mainly caused by substitutions instead of indels, which makes alignment much easier in comparison to the ITS region. Thus, ETS will probably also be useful above the series level in *Crocus*.

The newly applied chloroplast marker regions allowed discerning 6 (*rps*16–*trn*Q) and 7 (*mat*K–*trn*K) additional chloroplast haplotypes in comparison to the *trn*L–F data set. This proves that starting out from sequences of the entire chloroplast chromosome for few species within a genus helps to identify potentially variable regions that can be used in phylogenetic and phylogeographic analyses. As observed elsewhere, our chloroplast analysis also gained from the higher resolution resulting from a genealogical analysis approach in comparison to tree-based analysis, when differences among taxa were small to moderate (Posada and Crandall, 2001; Jakob and Blattner, 2006).

4.2. New circumscription of series *Reticulati*

Mathew (1982) erected series *Reticulati* to harbor crocuses with fibrous-reticulate corm tunics, which are otherwise

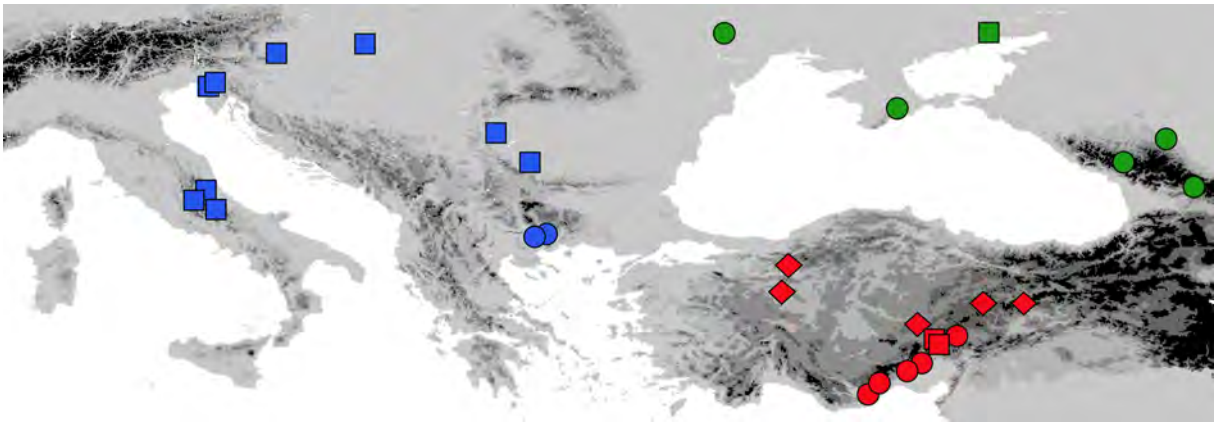

Figure 3. Distribution map of the analyzed *Crocus* series *Reticulati* populations. Symbols and colors refer to the phylogenetic groups obtained by analyses of nuclear (Figure 1) and chloroplast loci (Figure 2).

also morphologically relatively heterogeneous and thus difficult to place systematically. Our phylogenetic analysis of the ETS and ITS regions of nuclear rDNA clearly separated 4 species groups belonging to Mathew's series *Reticulati* (series *Reticulati* s.s., *C. gargaricus* and *C. herbertii*, *C. abantensis*, and *C. cancellatus* and its relatives; Figure 1). We also included in the analysis a few species belonging to series *Biflori* and *Speciosi* to illustrate that series *Reticulati*, in its traditional circumscription, is polyphyletic (Figure 1). This result is in accord with the earlier studies of Petersen et al. (2008) and Harpke et al. (2013). To overcome polyphyly, we define here series *Reticulati* in a much narrower sense including only *C. ancyrensis*, *C. angustifolius*, *C. danubensis*, *C. hittiticus*, *C. micranthus*, *C. orphei*, *C. reticulatus*, and *C. variegatus* (indicated in Table 1 and Figure 1), while the excluded taxa will be dealt with elsewhere. This clade obtains only a support value of 0.85 pp in this analysis, although in an extended analysis including many more species from outside series *Reticulati* support increases to 1 pp (not shown). Species of the newly defined series *Reticulati* are now all spring-flowering and have a bracteole, trifid styles, and reticulate corm tunics. The characters separating them from the closely related taxa with a reticulate corm tunic, which formerly belonged to this series, are (*i*) the presence of a bracteole (missing in *C. gargaricus*), (*ii*) the trifid styles (multifid in *C. cancellatus* and *C. hermoneus*) and, except for *C. ancyrensis*, (*iii*) the presence of an intense brownish-violet striping of the perigone (never brownish in *C. cancellatus*). Investigations of the corm tunic of the former series *Reticulati* member *C. sieheanus* Hort. ex B.L.Burt., which is phylogenetically allied to *C. danfordiae* Vis, revealed that it is not fibrous-reticulate at all (the tunics have very small parallel bands). Distinction of *C. abantensis* is easy because this phylogenetically very distinct species has clear blue unstriped flowers. The taxa of the 'sieberi group' (*C. cvijici* Košanin, *C. dalmaticus* Vis., *C. jablanicensis* Randjel. & V.Randjel., *C. robertianus* C.Brickell, *C. rujanensis* Randjel. & D.A.Hill, *C. sieberi* Gay and its subspecies, and *C. veluchensis* Herb.) belong phylogenetically to section *Crocus* but possess a fibrous-reticulate corm tunic and have no prophyll (Harpke et al. 2013). They are still not evaluated and require a thorough analysis of morphological parameters.

According to the results of our phylogenetic analysis, *C. reticulatus* sensu Mathew, having one of the largest distribution areas in the genus, cannot be kept as a single species with 2 subspecies. Instead, new species have to be defined and some old names, put into synonymy by Mathew, must be revived. Concerning the width of the tunic fibers, it is clear that only plants from Georgia, Moldavia, and Russia conform to the typical *C. reticulatus*. Species closely related to *C. reticulatus* are *C. angustifolius* and *C. orphei* from Greece. The populations from Bulgaria, Hungary, Italy, and Slovenia form a highly supported clade, for which the name *C. variegatus*, used by Hoppe and Hornschuch (1818) for plants from the Italian/Slovenian Karst formation, is already available. The populations from Serbia/Ukraine represent a species new to science, *C. danubensis*. The latter taxon and *C. variegatus* collectively are sisters to the clade including *C. reticulatus*, while all the populations from Turkey form a third distinct lineage. Within this latter group 4 distinct clades can be recognized. The first corresponds to individuals morphologically resembling *C. hittiticus*. Although the 2 investigated *C.* cf. *hittiticus* populations were found to be paraphyletic, they are molecularly (chloroplast and nuclear markers) and morphologically (blackish-maroon anthers) clearly differentiated from their closest relatives. The second clade corresponds to *C. micranthus*, described by Boissier (1859) for plants from Cilicia (Cilician gate) and formerly considered a synonym of *C. reticulatus* (Mathew, 1982).

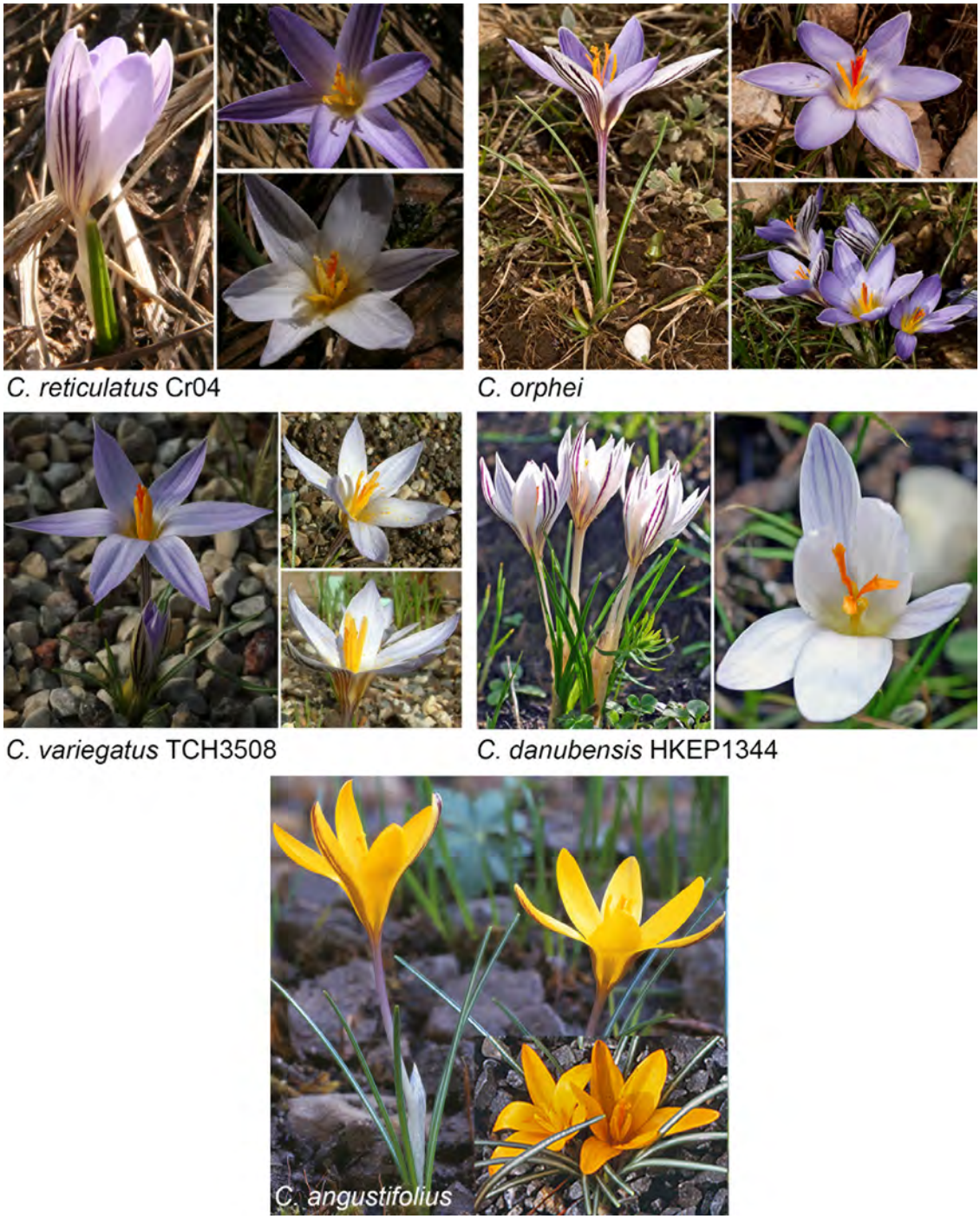

Figure 4. Photographs of above-surface parts of the species of *Crocus* series *Reticulati*.

The third corresponds to *C. ancyrensis*, a species already considered distinct by Mathew (1982). The individuals belonging to this latter species are, however, quite diverse regarding their DNA sequences and might consist of up to 4 different taxa. The fourth lineage is described here as a species new to science: *C. filis-maculatis*.

4.3. Geographic distribution and speciation processes

We base the analysis of biogeography mainly on the results of chloroplast analysis, as these mostly maternally inherited markers are generally transmitted by seeds in angiosperms and often provide clearer geographic structuring in comparison to biparentally inherited

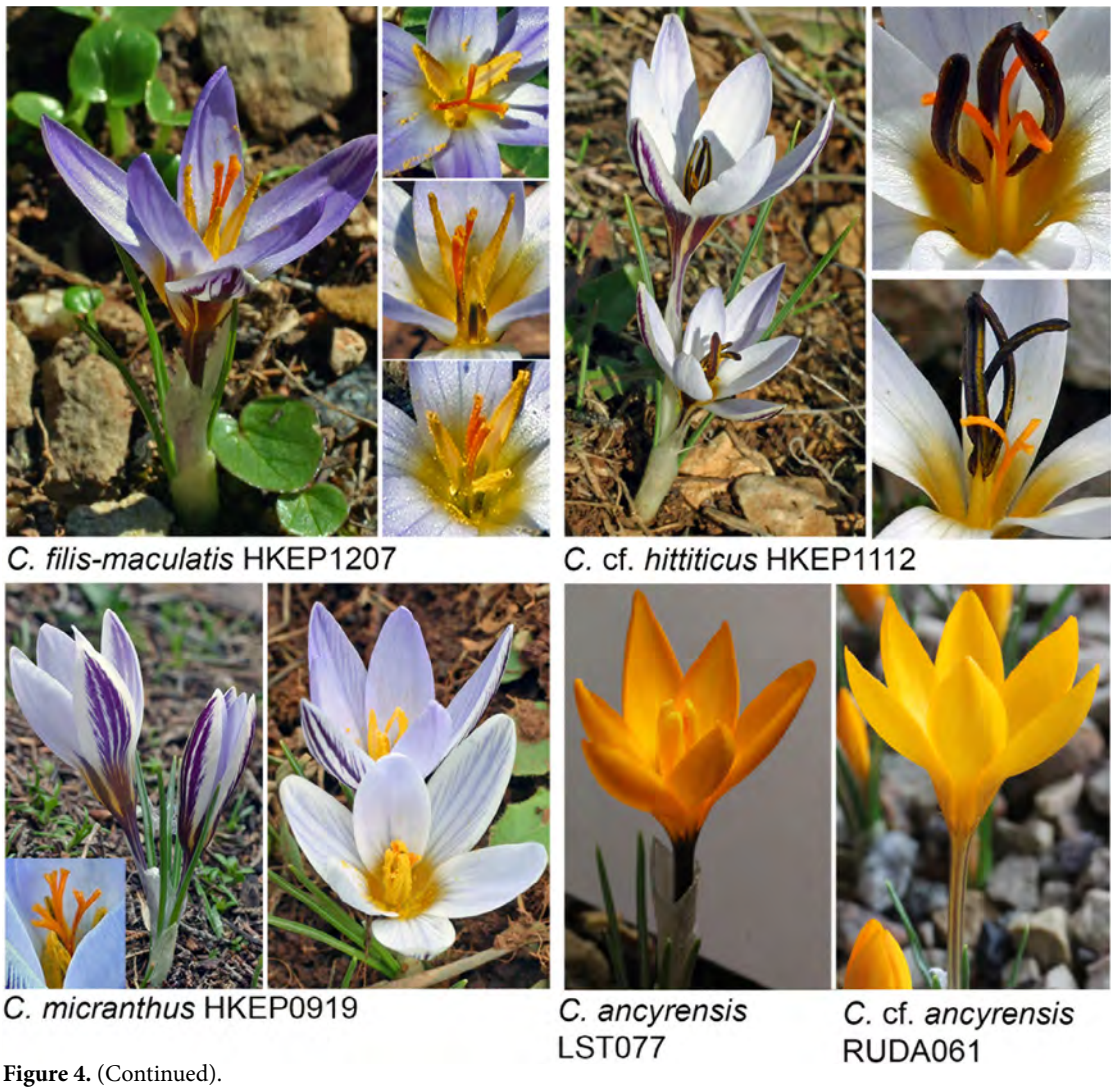

Figure 4. (Continued).

Table 4. Pairwise comparisons (Kruskal–Wallis with Bonferroni correction) of quantitative morphological features among taxa seemingly close to *C. reticulatus*. Each character is coded as a letter (see Table 3). Only those characters showing differences significant at the 0.01 level are reported. a = corm fibers width, b = filament length, c = anther length, d = style length, e = outer segments length, f = outer segments width, n.s. = not significant.

	C. danubensis	*C. micranthus*	*C. reticulatus*	*C. variegatus*
C. micranthus	d	-		
C. reticulatus	a/c/f	a/f	-	
C. variegatus	n.s.	f	a	-
C. orphei	c/d/f	c/d/f	a	c

nuclear markers, which have a gene flow component through pollen transmission. The chloroplast analysis is, however, completely compatible with the results obtained from nuclear markers, although the geographic resolution, at least for the non-Turkish taxon group, is slightly higher in the chloroplast data set. Chloroplast haplotype genealogies are regularly used in infraspecific phylogeographic analyses (Posada and Crandall, 2001)

but also proved useful to illustrate haplotype relationships at higher taxonomic levels (Bänfer et al., 2006; Jakob and Blattner, 2006; Kiefer et al., 2009; Gurushidze et al., 2010). Here a clear split is visible in nuclear and chloroplast data, separating a clade of taxa occurring in the west and northeast of the distribution area from the clade of Turkish taxa. The chloroplast data allow us to put forward a possible migration scenario for the non-Turkish species, starting out of northeastern Greece with migration through the Balkan Peninsula and Pannonian Basin towards Italy in the west and 2 lineages colonizing the area north and east of the Black Sea, possibly through the Danube valley as a migration pathway and/or via long-distance dispersals. However, different chloroplast haplotypes occur in Ukrainian and Serbian *C. danubensis* populations. To distinguish here between incomplete lineage sorting and chloroplast capture, 2 possible mechanisms explaining this pattern, a thorough sampling of populations along the Danube valley and the steppes between Serbia and Ukraine would be necessary.

No such biogeographic scenario is currently possible for the Turkish taxa. Extended population sampling seems necessary for this kind of analysis within Turkey. It is currently also not possible to determine where the series *Reticulati* originated, because no basal taxon or chloroplast haplotype was found connecting the Turkish and the northwestern group. However, considering that these groups are distributed east and west of the Aegean Sea, this area might be a likely candidate.

Within the genus *Crocus*, speciation seems often to be connected with changes in chromosome numbers among geographically adjacent species (Harpke et al., 2013, 2014; Schneider et al., 2013). In series *Reticulati* this mechanism seems to be less important at the species level, although the 3 main clades resulting from our phylogenetic analysis seem differentiated by chromosome numbers. The Turkish species are characterized by 2n = 10 (Brighton et al., 1973; Özhatay, 2002), although for *C. filis-maculatis* the karyotype is still unknown. The mainly northeastern clade with *C. angustifolius*, *C. reticulatus*, and *C. orphei* is marked by 2n = 12 (Brighton et al., 1973; Karamplianis et al., 2013; this study), while the mainly northwestern group represented by *C. variegatus* and *C. danubensis* has 2n = 14 chromosomes (Chichiriccò et al., 1981; Mitic, 2001). It is noteworthy to mention that 2n = 12 with up to 5 B chromosomes was also reported from the Balkans (Brighton et al., 1973; Lovka, 1995), but the taxonomic identity of these populations has to be carefully checked. Taxa of the Turkish group are all occurring at comparably high elevations (800–1700 m), often in isolated mountain stocks. Thus, geographic isolation might be one of the main reasons for the generally high species diversity of *Crocus* in Turkey and could also contribute to species diversity in the Turkish series *Reticulati* taxa. In contrast, within *C. variegatus*, the Italian populations from Abruzzo are geographically and also in the molecular data sets well separated, although the morphological analysis did not reveal clear differences.

4.4. Taxonomic remarks

Here we attempt to recognize independently evolving metapopulation lineages different from other such lineages within series *Reticulati* as species (De Queiroz, 2007). To define such species we follow evidence from morphology, phylogeny, and, where known, the existence of crossing barriers to come up with monophyletic taxonomic units. Using these criteria we include the following 9 species as belonging to series *Reticulati*. Three of them are described here as new species, while for 2 we resume older names regarded as synonyms by Mathew (1982). In particular, *C. ancyrensis* (2n = 10), *C. angustifolius* (2n = 12), both with yellow flowers, and *C. hittiticus* (2n = 10), with blackish-maroon anthers, were already previously recognized as independent taxa, although the latter was treated at the subspecific rank by Mathew (1982). However, the rank of *C. hittiticus* as a species is justified, as strong crossing barriers to closely related taxa seem to exist, which is evident by the lack of observed (fertile) hybrids even in cultivation. We restrict the application of the name *C. reticulatus* to plants with narrow corm fibers and 2n = 12 occurring in the Caucasus, Moldavia, and Russia. *Crocus micranthus* (2n = 10) is characterized by rather small flowers, mainly white/lilac (rarely deeper colored) and heavily striped brownish-violet on the outside of the outer perigone segments, while *C. variegatus* (2n = 14) has wider fibers and occurs from Italy to the western and northwestern Balkans. *Crocus danubensis* (2n = 14), with disjunct populations in the Serbian and Ukrainian steppes, is characterized by a white to pale-yellow throat. However, it is likely that *C. danubensis* is also distributed in other countries, especially along the Danube. *Crocus filis-maculatis*, endemic to Turkey, has larger, more bluish-violet flowers, often buff-colored on the outside of the outer perigone segments, peaky at top and heavily striped or speckled violet-brown, and filaments with a dark spot. *Crocus orphei* (2n = 12), earlier reported as *C. reticulatus* (Karamplianis et al., 2013), has larger anthers and a different chromosome number with respect to *C. variegatus*. In addition, this taxon has very thin leaves, which are the narrowest observed in the whole series. Unique also are the bracts and bracteoles, which have a green tinge at their apex. The perigone segment shape, as defined by the length-to-width ratio, is at 1:2–1:3.2 generally very low. This is remarkable compared to the otherwise mostly narrow and pointed segments of its relatives with perigone segment ratios far higher than 1:3. Accordingly, the newly circumscribed series *Reticulati* contains currently the 9 species described below. However, molecular analyses indicate the presence of additional taxa

in the groups to which we refer here as *C.* cf. *ancyrensis* and *C.* cf. *hittiticus*, and maybe even *C.* cf. *micranthus* and *C.* cf. *variegatus*. We refrain from describing such new taxa here, because not for all of the species from Turkey were the types seen or type locations visited, or we had only a few specimens for the morphological investigations (*C.* cf. *variegatus* HUNG and BULG). For the series *Reticulati* species described below, we provide photographs of the above-surface parts of the plants in Figure 4 and of corm tunics in Figure 5.

Crocus ser. ***Reticulati*** B.Mathew, The Crocus: 61 (1982)

Type species: *C. reticulatus* Steven ex Weber & Mohr

1) ***C. ancyrensis*** (Herb.) Maw, Gard. n.s. 16: 528 (1881) ≡ *C. reticulatus* var. *ancyrensis* Herb., J. Hort. Soc. London 2: 279 (1847)

Described from Turkey (Angora).

Type: Not designated.

Distribution: Central and northern Turkey (Mathew, 1982).

Figure 5. Corm tunics of the species of *Crocus* series *Reticulati*.

2) ***C. angustifolius*** Weston, Univ. Bot. 2: 238 (1771)

Described from cultivated material of unspecified origin.

Type: Not designated.

Distribution: Crimea, Ukraine, and Armenia.

3) ***C. danubensis*** Kerndorff, Pasche, N.Randjelovic & V.Randjelovic sp. nov.

It is distinct from *C. reticulatus* by wider corm fibers; from *C. variegatus* by white to pale-yellow (not yellow) throat; from *C. orphei* by pale-lilac flowers, smaller anthers, and wider leaves.

Corm 0.7–1.3–1.6 cm in diameter. Corm tunic of thick (0.21–0.36–0.44 mm) reticulate fibers. Cataphylls 4, silvery to brownish at tips when fresh, yellowish-brown when dry. Leaves 3–3.4–6 (n = 30) largely reaching the flower at anthesis, dark green, 1–1.9–2.6 (3) mm (n = 21) wide, glabrous, 1 rib underneath on both sides of the leaf. Width of the white stripe <1/3 to 1/3 of the leaf diameter. Throat white to very pale-yellow. Perigone tube white, always violet-striped, around 7 cm long at anthesis. Outer perigone segments between 21 and 27 mm but usually 24 mm long (n = 17), between 4 and 9 mm but usually 6.5 mm broad (n = 17). Inner perigone segments between 19 and 26 mm but usually 22 mm long. Outer and inner segments significantly pointed at top. Inside and outside of all segments white or very faintly lilac. The outside of the outer segments with predominantly 3 brownish-violet stripes, rarely up to 5 stripes. The accompanying smaller stripes insignificant, not or very rarely connecting the 3 main ones. Prophyll absent. Bract and bracteole present, skinny, silvery with a tinge of brown. Filaments slightly yellow, 5–5.9–6.5 mm long. Anthers 7.8–8.8–9.5 mm long (n = 17), yellow; connective colorless to slightly yellow, pollen yellow. Styles are orange, divided into 3 branches, which are between 4.4 and 10.6 mm but usually 7.7 mm long (n = 17), significantly widened or trumpet-shaped at apex. The styles are mostly overtopping the stamen (94%) between 1.5 and 7.6 mm, on average 4 mm (n = 17). The rest is shorter than stamen. Capsules and seeds not seen. Chromosome number 2n = 14.

Type (holotype): Serbia, near Negotin, 44.2°N, 22.6°E, 45 m, 11 March 2013, *HKEP1344*, (GAT 23019!).

Distribution: Pannonian Basin, Ukraine.

4) ***C. filis-maculatis*** Kerndorff & Pasche sp. nov.

It is distinct from all other taxa by peculiarly dark-spotted anthers.

Corm globose, about 10 mm in diameter. Tunics coarsely reticulate. Neck extension normally less than 3 mm long, sometimes textured up to 1–2 cm. Cataphylls 2–3, silvery-white to brownish at the apex. Leaves 2–5.2–8 (n = 34) mostly much longer than flowers at anthesis, dark green, 1–1.5 mm in diameter wide, glabrous, very rarely ciliate, 1 or 2 ribs underneath on each side of the leaf. Width of white stripe approximately 1/3 of leaf width. Throat deep yellow, prominent, glabrous, perigone tube whitish to violet. Outer perigone segments between 14 and 25 mm but usually 21 mm long (n = 31), between 4 and 8 mm but mostly 5.4 mm wide (n = 25). Inner perigone segments between 14 and 24 mm but usually 19 mm long (n = 31) and between 4 and 9 mm (frequently 5.5 mm) wide (n = 25). Inside, all perigone segments are faintly to deeply lilac-blue, sometimes with darker blue veins. The outsides of the outer segments are mostly more or less intensely striped with a prominent stripe in the middle, rarely speckled on a plain lilac or buff-colored ground. Outside coloring of the inner segments is mostly uniformly soft lilac-blue without markings. Prophyll absent. Bract and bracteole present, silvery-white. Filaments 2–4.5 mm, on average 3.3 mm long (n = 32), yellowish with an irregular more or less intensive violet spot on their outsides, glabrous; anthers elongate arrow-shaped becoming thinner towards the apex, 7–11 mm, on average 8.6 mm long (n = 32), yellow, connective white, pollen yellow. Styles are orange, divided into 3 branches, which are not or only very slightly widened towards the apex; branches 3.5–8 mm and on average 5.3 mm long, scabrid to papillate. The styles are mostly shorter than to equal to the stamens (83%), less frequently longer (17%, n = 30). Chromosome number unknown.

Type (holotype): Turkey, Anti-Taurus, Province Adana, hills north of Adana towards the Taurus mountains, 800–1000 m, 17 March 2012, *HKEP 1207*, (GAT 25833!).

Distribution: Turkey, Anti-Taurus, Adana Province, very local in the hillsides north of Adana, in clearings of open pine forests, together with *Pinus nigra* subsp. *pallasiana*, *Rubus*, grasses, and others. *Crocus filis-maculatis* grows only on calcareous formations.

5) ***C. hittiticus*** T.Baytop & B.Mathew, Kew Bull. 30: 244 (1975)

≡ *C. reticulatus* subsp. *hittiticus* (T.Baytop & B.Mathew) B.Mathew, The Crocus: 72 (1982)

Described from Turkey (Mersin).

Type (holotype): Turkey, Mersin prov., Silifke to Gülnar, Kandil gorge, 750 m, 7 March 1973, T. Baytop 23976 (ISTE).

Distribution: Southern Turkey (Mathew, 1982).

6) ***C. micranthus*** Boiss., Diagn. Pl. Orient., 2(4): 95 (1859)

Described from Cilicia (Turkey).

Type (lectotype, designated here): Cilicia, s.d., *Aucher-Eloy-Herbier d'Orient n. 2127* (G-BOIS 00330331!, isolectotypes in G 00380823! and K!).

Distribution: At present only known from southern Turkey, Cilicia, at the Cilician Gate and on the mountains in its vicinity.

7) ***C. orphei*** Karamplianis & Constantin. sp. nov.

It is distinct from *C. reticulatus* by wider corm fibers; from *C. variegatus* by white to pale-yellow (not yellow) throat; from *C. danubensis* by lilac flowers, larger anthers, and narrower leaves.

Corm subglobose, 15–24 mm in diameter. Corm tunic coarsely reticulate fibrous splitting into a disc with radial fibers basally and forming a neck with flaccid and strong fibers apically, fibers 0.28–0.34–0.40 mm wide. Leaves 4–5, 0.5–1 mm broad, green, sparsely ciliate at margins; white stripe 1/3 to rarely larger than 1/3. Two ribs on both sides underneath the leaves. Cataphylls 3–4, white, often with brownish tinge at apex, not persistent. Prophyll absent. Bracts and bracteoles equal in length, white, greenish at apex. Outer perigone segments 22–22.5–35 mm long, 8–9.8–15 mm broad. Inner perigone segments 18–32 mm long, 9–17 mm wide. Segment shape ovate, length-to-width ratio 2.0–3.2. Inside all segments uniformly lilac. Outside of outer segments mostly buff-colored with 3–5 prominent brown-violet stripes not connected by smaller ones. Outside of inner segments concolorous deep lilac, sometimes having a median longitudinal stripe or spotted in basal part of the center of the segments deep violet to maroon. Throat white to pale-yellow, glabrous or slightly papillate. Perigone tube striped throughout its length. Filaments 5–5.9–7 mm long, yellow. Connective pale yellow, sometimes blackish marginally, 0.5–0.6 mm broad. Anthers yellow 10–11.6–14.5 mm long. Capsule 15–18 × 7–10(–14) mm, ellipsoid; each chamber 7–8 mm wide. Seeds brownish 3–3.5 × 1.5–2.5 mm, with a prominent raphe reaching half of seed length. Caruncle slightly distinct and flattened, c. 0.5 × 1 mm, pale-colored; chalaza yellowish, rough, with ridges, testa brownish, smooth, and papillate. Chromosome number 2n = 12.

Type (holotype): Greece, Macedonia, Mt. Falakro, in openings and margins of mixed *Fagus* and *Pinus* forest, 1210 m, 30 March 2010, *Th. Karamplianis & S. Tsiftsis 1843* (ATHU!).

Distribution: Northeastern Greece; so far only known from Mt. Falakro.

8) ***C. reticulatus*** Steven ex Weber & Mohr, Beitr. Naturk. 1: 45 (1805)

Described from Caucasus.

Type (lectotype, designated here): Habitat ad lineam Caucasicam, s.d., (L!).

Epitype (in support of the above designated lectotype): Northern Caucasus, near Teberda, 2400 m, 19 May 2011, M. Schnittler (GAT 23082!). The only original material available is lacking a corm. For this reason, we deem it opportune to designate a complete epitype in support of the above-designated lectotype.

Distribution: Moldavia, southwestern Russia, Caucasus.

9) ***C. variegatus*** Hoppe & Hornsch., Tageb. Reis. Adriat.: 187 (1818)

Described from the area between Basovizza (Italy) and Lipica (Slovenia).

Lectotype (designated here): Karst, s.d., *Hoppe* (JE 00009839!).

Distribution: Italy, western and northwestern Balkans.

Identification key to *Crocus* series *Reticulati*

1 Perigone segments yellow 2
1* Perigone segments not yellow 3
2 Perigone segments not striped ..... *C. ancyrensis*
2* Outer perigone segments with heavy brownish-violet stripes on the adaxial surface ..... *C. angustifolius*
3 Anthers blackish-maroon ..... *C. hittiticus*
3* Anthers yellow, rarely with a blackish margin of connective 4
4 Filaments darkly spotted ..... *C. filis-maculatis*
4* Filaments not as above (concolorous) 5
5 Corm with fibers (0.1)0.11–0.16(0.18) mm wide ..... *C. reticulatus*
5* Corm with fibers (0.16)0.22–0.42(0.6) mm wide 6
6 Anthers (8)8.4–9(9.5) mm long, outer segments 5–7 mm wide ..... *C. micranthus*
6* Anthers (10)10.5–12.2(14.5) mm long, outer segments (7)8–10.5(15) mm wide, leaves ≤1.2 mm wide ..... *C. orphei*
6** Anthers (6)7.5–10.7(13) mm long, outer segments (4)6–10(11.5) mm wide, leaves >1.2 mm wide 7
7 Throat yellow ..... *C. variegatus*
7* Throat white to pale-yellow ..... *C. danubensis*

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Table S1. DNA sequence accession numbers of individuals used in this study.

Taxon	Voucher	Individual no.	ETS	ITS	*rps*16–*trn*Q	*mat*K–*trn*K	*trn*L–F
C. ancyrensis (Herb.) Maw	LST-077, GAT 25832	2	LM993421	LM993458	-	-	-
		3	LM993422	LM993459	LM993533	LM993475	LM993590
		4	LM993422	LM993459	LM993534	LM993476	LM993591
		5	LM993422	LM993459	LM993535	LM993477	LM993592
	BATM-340, GAT29388	1	LM993380	LM993436	LM993527	LM993469	LM993585
		2	LM993380	LM993436	LM993528	LM993470	LM993586
		3	LM993381	LM993437	LM993529	LM993471	LM993587
		4	LM993381	LM993437	LM993530	LM993472	LM993588
	LST-114, GAT 29387	1	-	-	LM993536	LM993478	LM993593
		2	LM993423	LM993460	LM993537	LM993479	LM993594
		3	LM993424	LM993461	LM993538	LM993480	LM993595
		4	LM993423	LM993460	LM993539	LM993481	LM993596
		5	LM993423	LM993460	LM993540	LM993482	LM993597
	RUDA-061, GAT 25830	1	LM993425	LM993462	LM993541	LM993483	LM993598
		2	LM993426	LM993463	LM993542	LM993484	LM993599
		3	LM993427	LM993464	-	-	-
		4	LM993425	LM993462	-	-	-
		5	LM993425	LM993425	-	-	-
	HKEP0927, GAT 7146		LM993406	HE663987	LM993532	LM993474	HE663987
C. angustifolius Weston	GAT 7236		LM993385	HE801186	LM993531	LM993473	LM993589
C. danubensis Kernd. et al. sp. nov.	HKEP1344, GAT 23019		LM993416	LM993453	LM993552	LM993494	LM993608
	Zubov-07, GAT 25806	3	LM993431	LM993467	LM993557	LM993499	LM993613
		5	LM993431	LM993467	LM993558	LM993500	LM993614
C. filis-maculatis Kernd. & Pasche sp. nov.	HKEP1207, GAT 25833		LM993413	LM993450	LM993566	LM993508	LM993622
	HKEP1357, GAT 29389		LM993418	LM993455	LM993567	LM993509	LM993623
C. hittiticus T.Baytop & B.Mathew	HK0037, GAT 7263		LM993401	HE663967	LM993568	LM993510	HE864172
	HKEP1112, GAT 7466		LM993412	HE664011	LM993569	LM993511	HE864205
C. micranthus Boiss.	HKEP0919, GAT 7141		LM993405	HE801072	LM993548	LM993490	HE864190
	Batm-402Bc, GAT 25831	1	LM993382	LM993438	LM993543	LM993485	LM993600
		2	LM993382	LM993438	LM993544	LM993486	LM993601
		3	LM993382	LM993438	LM993545	LM993487	LM993602
		4	LM993382	LM993438	LM993546	LM993488	LM993603
		5	LM993382	LM993438	LM993547	LM993489	LM993604
C. orphei Karamplianis & Constantin sp. nov.	Karamplianis & Tsiftsis 1839		LM993388	LM993440	LM993550	LM993492	LM993606
	Karamplianis & Tsiftsis 1843		LM993387	LM993439	LM993551	LM993493	LM993607
C. reticulatus Stev. ex. Weber & Mohr	Banketov-09, GAT 25805	1	LM993377	LM993433	LM993570	LM993512	LM993624
		2	LM993377	LM993433	LM993571	LM993513	LM993625
		3	LM993378	LM993434	LM993572	LM993514	LM993626
		4	LM993379	LM993435	LM993573	LM993515	LM993627
		5	-	-	LM993574	LM993516	LM993628

Table S1. (Continued).

		1	-	-	LM993554	LM993496	LM993610
	GAT 23084	2	LM993390	LM993442	LM993555	LM993497	LM993611
		3	LM993390	LM993442	LM993556	LM993498	LM993612
	GAT 23082		LM993400	LM993448	LM993579	LM993521	LM993633
		1	LM993398,	LM993446,	LM993575	LM993517	LM993629
	CMGG-026, GAT	2	LM993399	LM993447	LM993576	LM993518	LM993630
	25805	3	LM993398	LM993446	LM993577	LM993519	LM993631
		4	LM993398	LM993446	LM993578	LM993520	LM993632
C. variegatus Hoppe & Hornsch.	TCH-3508, GAT 7264		LM993429	LM993466	LM993584	LM993526	LM993638
		1	LM993428	LM993465	LM993580	LM993522	LM993634
	TCH-1014, GAT 25809	2	LM993428	LM993465	LM993581	LM993523	LM993635
		3	LM993428	LM993465	LM993582	LM993524	LM993636
		4	LM993428	LM993465	LM993583	LM993525	LM993637
	Collarmele		LM993391	LM993443	LM993559	LM993501	LM993615
	HKEP1311, GAT 25809		LM993414	LM993451	LM993560	LM993502	LM993616
	HKEP1315, GAT 25808		LM993415	LM993452	LM993561	LM993503	LM993617
		1	LM993392	LM993444	LM993562	LM993504	LM993618
	Golob-07, GAT 25803	2	LM993392	LM993444	LM993563	LM993505	LM993619
		3	LM993392	LM993444	LM993564	LM993506	LM993620
		4	-	-	LM993565	LM993507	LM993621
	GAT 25807		LM993389	LM993441	LM993549	LM993491	LM993605
	GAT 25829		LM993432	LM993468			
Outgroup taxa							
C. abantensis Baytop & B.Mathew	GAT 7235		LM993430	HE664019			
C. cancellatus Herb.	HKEP1028, GAT 7180		LM993409	HE663998			
	HKEP1033, GAT 7133		LM993410	HE664001			
	HKEP1354, GAT 29390		LM993417	LM993454			
C. gargaricus Herb.	GAT 7255		LM993393	HE801138			
C. herbertii B.Mathew	GAT 7396		LM993394	HE801151			
C. hermoneus Kotschy ex Maw subsp. *palaestinus* Feinbrun	IABH 7		LM993395	HE864268			
C. lycius B.Mathew	GAT 7181		LM993408	HE663993			
C. pamphylicus B.Mathew	GAT 7213		LM993411	LM993449			
C. aleppicus Baker	IABH 18357		LM993383	HE801175			
C. biflorus Mill.	Abruzzo		LM993386	HE801121			
C. adanensis T.Baytop & B.Mathew	GAT 7148		LM993407	HE663988			
C. graveolens Boiss. & Reut. ex Boiss.	GAT 7457		-	-	LN606700	LN606699	HE664010
C. fleischeri J.Gay	GAT 7139		LM993403	HE663983			

Table S1. (Continued).

C. laevigatus Bory & Chaub subsp. *pumilis* Rukšāns	CR12-022, GAT 23019	LM993396	LM993445
C. tournefortii J.Gay	GAT 7202	LM993397	HE801123
C. pulchellus Herb.	GAT 29391	LM993420	LM993457
C. speciosus M.Bieb.	GAT 19558	LM993419	LM993456
C. nerimaniae Yüzb.	GAT 7378	LM993402	HE663977
C. yataganensis Kernd. & Pasche	GAT 7380	LM993403	HE663978
C. almehensis C.Brickel & B.Mathew	TARI 69170	LM993384	HE801162

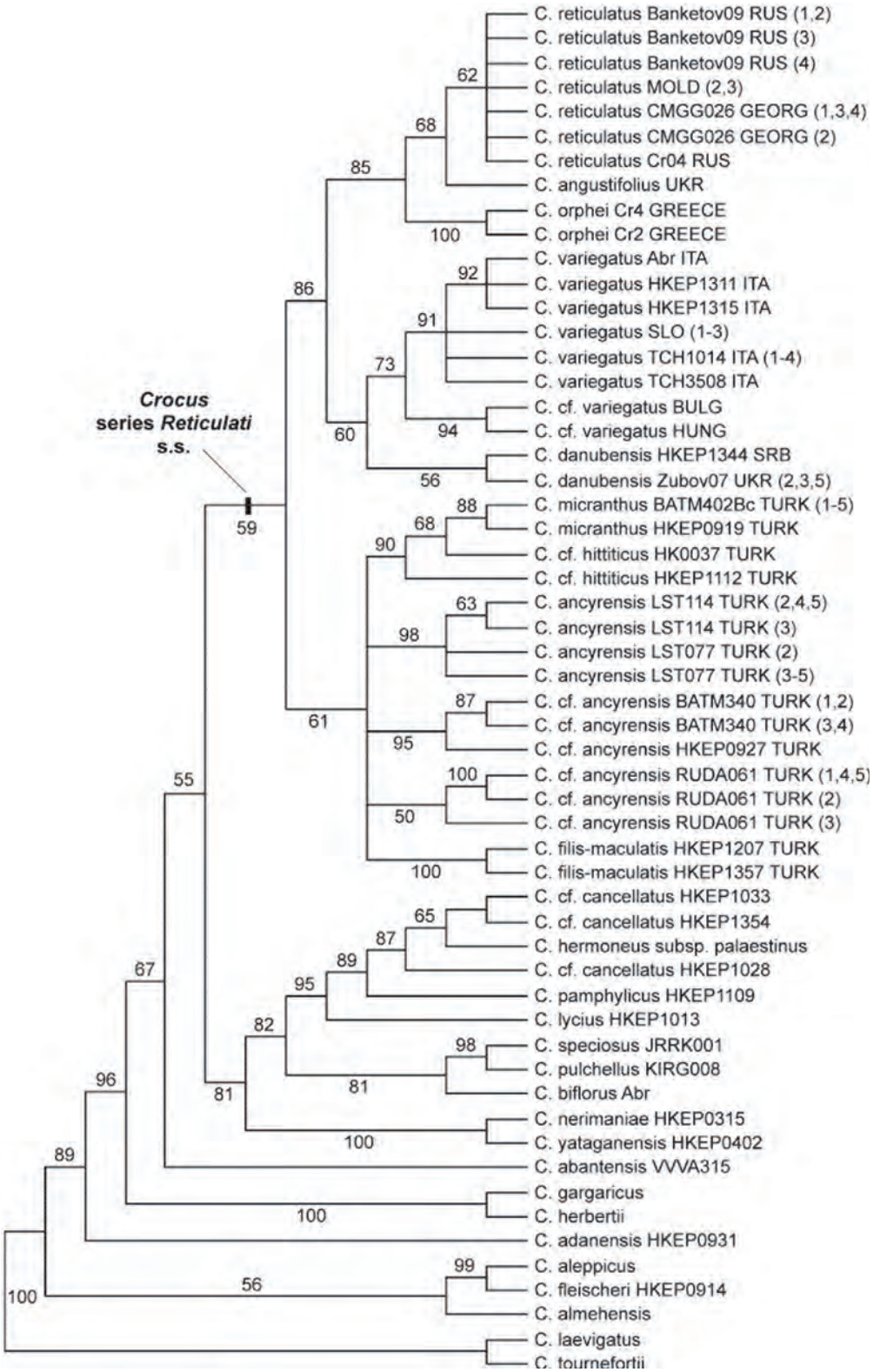

Figure S1. Maximum parsimony strict consensus tree of 60 equally parsimonious trees based on the nuclear data set.

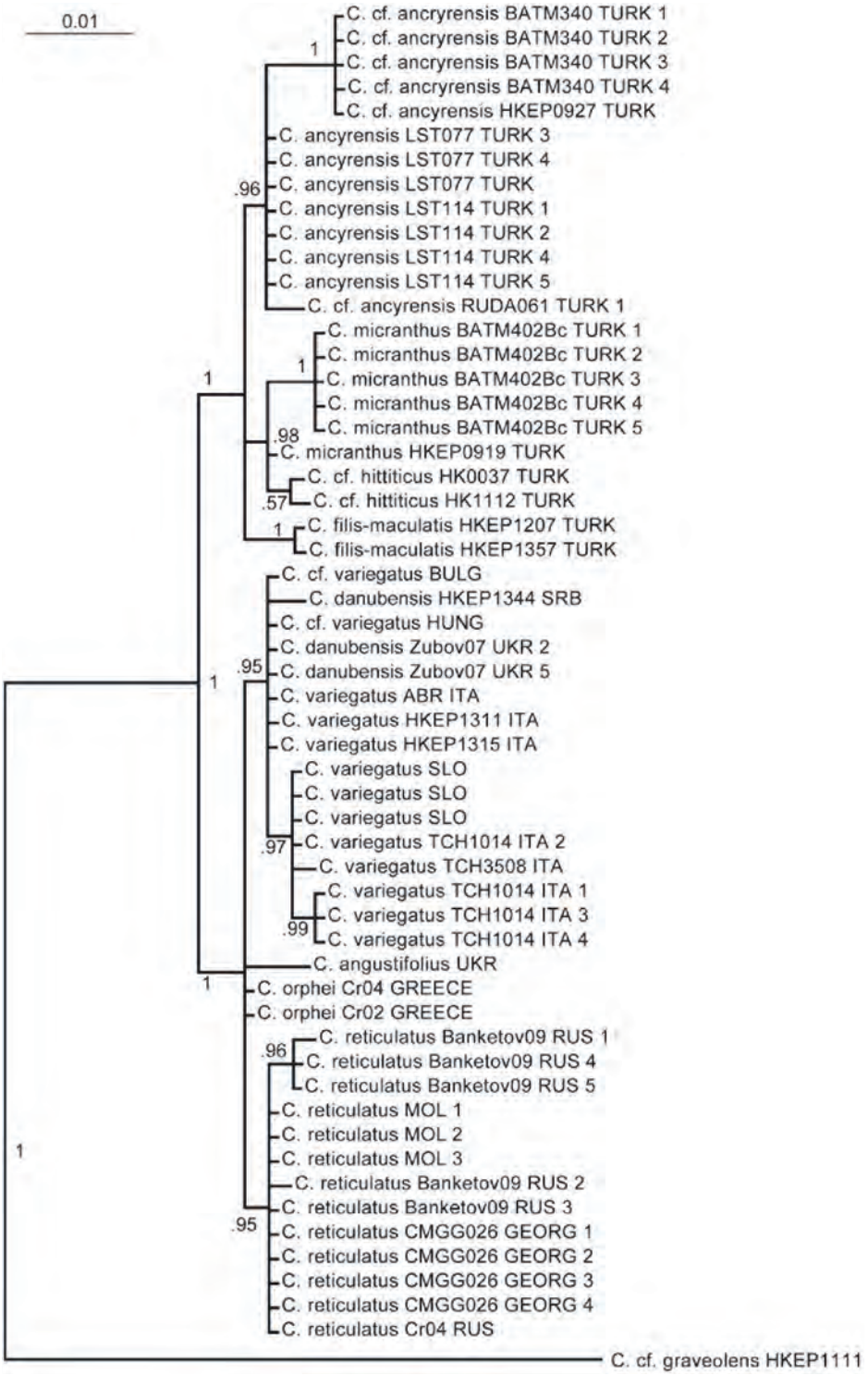

Figure S2. Phylogenetic tree obtained by Bayesian phylogenetic inference of the combined sequences of 3 chloroplast regions.

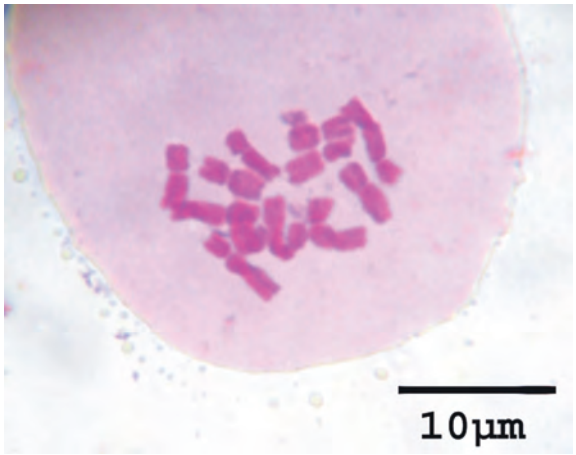

Figure S3. Mitotic metaphase plate of *Crocus reticulatus* s.s.