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Molecular phylogeny of *Galanthus* (Amaryllidaceae) of Anatolia inferred from multiple nuclear and chloroplast DNA regions

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Abstract: Anatolia hosts 14 of the 22 taxa of genus *Galanthus* L. The phylogenetic relationships of *Galanthus* species were explored using DNA sequence data from both nuclear and chloroplast DNA of 70 specimens covering all subdivisions of *Galanthus* present in Turkey. Our data showed that species of the same geography clustered together phylogenetically, in contrast to the presently recognized taxonomy based on morphological characters. The 5.8S rRNA sequence data by itself is sufficiently informative for constructing phylogenetic trees. Our data confirm the identity of *Galanthus koenenianus* as a new taxon, well separated from *G. alpinus*; both are clustered within subseries *Viridifolii*, rather than within subseries *Glaucaefolii* in contrast to previous suggestions, but in accordance with their geographical occurrence. *G. trojanus* also forms a distinct branch within subseries *Glaucaefolii*. *G. krasnovii* diverged out of all other *Galanthus* taxa as a separate branch in all phylogenetic trees. Samples collected based on morphological characters as *G. nivalis* were clearly different from *G. nivalis* based on the sequence data in GenBank. Sequencing data also revealed that the *G. xvalentinei* from locus classicus (ISTF; SYZB 3008) significantly differed from all other *Galanthus* specimens distributed in Thrace known as *G. xvalentinei*.

Key words: *Galanthus*, Amaryllidaceae, molecular phylogeny, nrDNA ITS, cpDNA *trn*L-F

1. Introduction

Galanthus L., a member of the family Amaryllidaceae, is a genus of bulbous monocotyledons occurring naturally in Europe, Asia Minor, and the Near East (Davis, 1999). In addition to 19 recognized species, the genus comprises 22 taxa including subspecies and varieties (Bishop et al., 2001). The history of infrageneric classification in *Galanthus* dates back to Linnaeus, who had described the first species, *G. nivalis* L.; following the identification of this most familiar and widespread snowdrop species in Europe, several new species, varieties, and garden-worthy variants were named throughout the 19th and 20th centuries. This rapid increase in names is closely paralleled with the attempts to classify *Galanthus* into an ordered system. The works of Kemularia-Nathadze (1947), Stern (1956), and Davis (1999) can be cited among the most notable taxonomic studies on *Galanthus*. Stern (1956) divided *Galanthus* into 3 series according to their leaf vernation: section *Nivales* Beck, section *Plicati* Beck, and section *Latifolii* Stern. Later, Davis (1999), taking into consideration their geographic distribution as well as leaf vernation, merged series *Nivalis* and *Plicati* into series *Galanthus* and divided series *Latifolii* into 2 subseries: *Glaucaefolii* (Kem.-Nath) A.P.Davis and *Viridifolii* (Kem.-Nath) A.P.Davis.

Turkey can be considered as one of the centers of species diversity for *Galanthus*, just like Greece and the neighboring countries of the Balkans and the Caucasus. Among 14 taxa (and 1 hybrid) present in Turkey, 6 of them are endemic: *G. plicatus* M.Bieb. subsp. *byzantinus* (Baker) D.A.Webb, *G. cilicicus* Baker, *G. elwesii* Hook. f. var. *monostictus* P.D.Sell, *G. koenenianus* Lobin, C.D.Brickell & A.P.Davis, *G. trojanus* A.P.Davis & N. Özhatay, and *G. xvalentinei* Beck nothosubsp. *subplicatus* (N.Zeybek) A.P.Davis. The first review on Anatolian species was written by Brickell (1984) in his treatment of the *Flora of Turkey*, where 8 species and 3 subspecies were recognized. Later, Zeybek (1988) produced a new review in which 14 new subspecies were described for 7 species, giving a total of 24 taxa. This study was followed by a work on the *Galanthus* of Turkey (Zeybek and Sauer, 1995), which included an updated account of 8 species, 15 subspecies, and 2 varieties. Indeed, several species known to be

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present in Turkey, such as *G. alpinus* Sosn. var. *alpinus*, *G. koenenianus*, *G. peshmenii* A.P.Davis & C.D.Brickell, and *G. krasnovii* A.P.Khokhr., were excluded in that study. In 2001, Davis and Özhatay described a new species of *Galanthus* from West Anatolia, named as *G. trojanus* A.P.Davis and N.*Özhatay*. On morphological ground, this species has been described as closely allied to *G. nivalis* L. and *G. rizehensis* Stern. However, the affinity of *G. trojanus* remained uncertain (Davis et al., 2001; Davis and Özhatay, 2001). Another unresolved point in the systematics of Anatolian *Galanthus* species includes the taxonomic groups present mainly in the Marmara region. *G. xvalentinei* nothosubsp. *subplicatus*, which grows on the European side of the Bosphorus, is considered as a natural hybrid between *G. nivalis*, a species found all over Europe, and *G. plicatus* subsp. *byzantinus* (Davis et al., 2001). The individuals that have been previously described as *G. nivalis* were questioned and later revisions concluded that they were not *G. nivalis* but *G. xvalentinei* nothosubsp. *subplicatus* (Yüzbaşıoğlu, 2010, thesis, İstanbul University). Further work is needed to clarify whether "pure" *G. nivalis* occurs in Turkey (Davis et al., 2001).

In our study, we applied a molecular phylogenetic approach in order to establish the taxonomical relationship among *Galanthus* species that grow in Turkey and to provide clarification regarding taxonomic issues. For this purpose, 2 different commonly used genetic markers, nuclear and chloroplast, were applied. The first one, internally transcribed spacer regions (ITS1 and ITS2) of nuclear ribosomal DNA, became widely used at the genus level or below genus level comparisons (Alvarez and Wendel, 2003). ITS1 and ITS2 separate the 3 rRNA genes and are cleaved from the precursor transcripts during the formation of the mature rRNAs and it was found that they have a definite role in the processing of nuclear ribosomal RNAs (Musters et al., 1990; van Nues et al., 1994; Mai and Coleman, 1996). This selection pressure gives ITS regions the characteristic of being relatively conserved. Their sequences, nevertheless, diverge more than rRNA subunits (26S, 18S, and 5.8S) and are sufficiently variable to resolve phylogenetic relationships. Its relatively fast evolutionary rate and its easy amplification using universal primers (Baldwin et al., 1995) made the ITS region one of the most frequently used sequences in plant phylogenetic studies involving closely related taxa (Soltis and Soltis, 1998). The second molecular marker is from the chloroplast genome: the *trn*L(UAA) intron and the noncoding spacer between the *trn*L(UAA) and the *trn*F(GAA) genes. Comparison of nuclear DNA-based phylogenies with maternally inherited chloroplast sequence-based reconstruction is recommended, particularly when reticulate evolution (characterized by occasional hybridization) is expected in a group (Soltis and Soltis, 1998). Therefore, a number of markers located on the LSC region of the chloroplast genome, such as the *rbc*L gene, the noncoding *trn*L–F spacer, and the *trn*L(UAA) intron, the last 2 of which lie between highly conserved tRNA genes, are preferred extensively in phylogenetic studies. Especially *trn*L–F sequence information has been used successfully to resolve generic, and in some cases even species level, relationships in angiosperms (Garcia-Jacas et al., 2001; Koch et al., 2001; Muthama Muasya et al., 2002). In general, noncoding regions of the chloroplast DNA tend to evolve more rapidly than coding regions due to the accumulation of insertions/deletions, making them very useful for comparisons below the family level (Gielly and Taberlet, 1994).

2. Materials and methods

2.1. Plant materials

In total, 15 different taxa (including *Galanthus nivalis*) of *Galanthus* L. species, collected from 37 different locations, were studied. We used *Sternbergia lutea* (L.) Ker-Gawl. ex Sprengel as the outer group in phylogenetic tree constructions (Lledo et al., 2004). *Sternbergia lutea* was kindly provided by the Botanical Garden of İstanbul University. The geographical locations from where the samples were collected for this work are listed in the Table, along with the accession numbers of the sequences submitted to GenBank. All plant samples were collected at the time they were flowering. At least one bulb per location was obtained and leaves of the plants were used as material for molecular analysis. We also included the sequence data for *G. nivalis* from GenBank (FN663919 and AY357136, for nuclear ITS and chloroplast *trn*L-F sequences, respectively).

2.2. DNA extraction, PCR amplification, and sequencing

For each sample, fresh leaves were processed in liquid nitrogen and preserved at –80 °C. DNA from frozen powdered tissue was extracted using the QIAGEN Plant DNA Extraction Mini Kit, following the manufacturer's instructions. The amplification of the nuclear rRNA ITS region was performed using the universal primers ITS4 and ITS5, designed by White et al. (1990). Chloroplast markers were amplified using the universal primers C and D to amplify the *trn*L(UAA) intron and primers E and F to amplify the intergenic spacer between the chloroplast *trn*L(UAA)3' exon and the *trn*F(GAA) gene designed from conserved chloroplast tRNA gene sequences (Taberlet et al., 1991). The same PCR temperature profile was used for both nuclear and cpDNA amplifications. The PCR reaction mix, in 100 µL, contained 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µM of each primer, 200 ng of DNA, and 1.25 U of Taq polymerase (Go Taq Flexi DNA Polymerase, Promega) in the supplier's enzyme buffer. PCR cycles were as follows: 2 min 30 s at 94 °C for initial denaturation, then 30 cycles of 30 s at 95 °C, 1 min 30 s at 52 °C for annealing,

Table. List of specimens included in this study with voucher and location information, along with GenBank accession numbers.

Synopsis of the genus[a]	Species name	Origin	Voucher*	GenBank accession number		
				ITS1+5.8S+ITS2	trnL(UAA)	trnL-F
Subseries GLAUCAEFOLII	*G. peshmenii*	Antalya	SYZB2851	GU329668	GU329567	GU329625
	G. peshmenii	Antalya	SYZB2854	-	GU329568	GU329622
	G. peshmenii	Antalya	SYZB2856	GU329669	GU329569	GU329623
	G. peshmenii	Antalya	SYZB2861	GU329670	GU329570	GU329624
	G. cilicicus	Mersin	SYZB2863	GU329653	GU329561	GU329592
	G. cilicicus	Mersin	SYZB2865	GU329654	GU329562	GU329593
	G. elwesii var. *monostictus*	Antalya	SYZB2183	GU329657	GU329539	GU329604
	G. elwesii var. *monostictus*	Mersin	SYZB3042a	GU329656	GU329540	GU329605
	G. elwesii var. *monostictus*	Antalya	SYZB3052	GU329655	GU329538	GU329606
	G. elwesii var. *monostictus*	İzmir	SYZB2440	GU329688	GU329537	GU329595
	G. elwesii var. *elwesii*	Afyon	SYZB3033	-	-	GU329594
	G. elwesii var. *elwesii*	Konya	SYZB3040	-	-	GU329596
	G. elwesii var. *elwesii*	Eskişehir	SYZB3057	GU329687	GU329536	GU329597
	G. elwesii var. *elwesii*	Isparta	SYZB3035	GU329680	GU329529	GU329603
	G. elwesii var. *elwesii*	Bolu	SYZB3062	GU329681	GU329530	GU329598
	G. elwesii var. *elwesii*	Ankara	SYZB3065	GU329682	GU329531	GU329599
	G. elwesii var. *elwesii*	Eskişehir	SYZB3078	GU329683	GU329532	GU329600
	G. elwesii var. *elwesii*	Isparta	SYZB3085	GU329684	GU329533	-
	G. elwesii var. *elwesii*	Isparta	SYZB3087	GU329685	GU329534	GU329601
	G. elwesii var. *elwesii*	Karaman	SYZB3092	GU329686	GU329535	GU329602
	G. gracilis	Tekirdağ	SYZB2600	GU329662	GU329543	GU329609
	G. gracilis	Bursa	SYZB3017	GU329661	GU329547	GU329610
	G. gracilis	Balıkesir	SYZB3021	GU329658	GU329548	GU329611
	G. gracilis	Kütahya	SYZB3022	GU329659	GU329545	GU329612
	G. gracilis	Manisa	SYZB3026	GU329663	GU329544	GU329614
	G. gracilis	İzmir	SYZB 3030	GU329660	GU329546	GU329613
	G. alpinus var. *alpinus*	Rize	SYZB2895	GU329649	GU329559	GU329591
	G. alpinus var. *alpinus*	Rize	SYZB2868	GU329651	GU329556	GU329587
	G. alpinus var. *alpinus*	Rize	SYZB2870	GU329650	GU329557	GU329588
	G. alpinus var. *alpinus*	Rize	SYZB2894	-	GU329558	GU329589
	G. alpinus var. *alpinus*	Rize	SYZB2919	GU329652	GU329560	GU329590
	G. koenenianus	Trabzon	SYZB3112	GU329691	GU329549	GU329615
	G. rizehensis	Trabzon	SYZB2867	GU329702	GU329577	GU329635
	G. rizehensis	Trabzon	SYZB2901	GU329699	GU329579	GU329634
	G. rizehensis	Trabzon	SYZB2891	GU329703	GU329578	-
	G. rizehensis	Giresun	SYZB2884	GU329704	-	GU329645

Table. (Continued).

Subseries VIRIDIFOLII	*G. rizehensis*	Artvin	SYZB2915	GU329701	GU329581	GU329638
	G. rizehensis	Ordu	SYZB2903	GU329700	GU329580	GU329636
	G. rizehensis	Rize	SYZB2896	-	GU329586	GU329646
	G. woronowii	Ordu	SYZB2905	GU329677	GU329585	GU329643
	G. woronowii	Artvin	SYZB2873	-	GU329582	GU329641
	G. woronowii	Artvin	SYZB2878	GU329676	GU329583	GU329642
	G. woronowii	Artvin	SYZB2882	GU329678	GU329584	GU329644
	G. fosteri	Amasya	SYZB2908	GU329690	GU329542	GU329608
	G. fosteri	Kayseri	SYZB3101	GU329689	GU329541	GU329607
	G. krasnovii	Artvin	SYZB2925	GU329692	GU329550	GU329616
Series GALANTHUS	*G. nivalis* × *xvalentinei*	İstanbul	SYZB2589	GU329664	GU329563	GU329617
	G. nivalis × *xvalentinei*	İstanbul	SYZB2590	GU329665	GU329564	GU329618
	G. nivalis × *xvalentinei*	İstanbul	SYZB2591	GU329666	GU329565	GU329619
	G. nivalis × *xvalentinei*	İstanbul	SYZB2592	-	GU329566	GU329620
	G. nivalis × *xvalentinei*	Edirne	SYZB2597	GU329667	-	GU329621
	G. nivalis × *xvalentinei*	Kırklareli	SYZB2405	-	-	-
	G. xvalentinei	İstanbul	SYZB3008	GU329679	GU329555	GU329647
	G. xvalentinei	İstanbul	SYZB2401	-	-	-
	G. xvalentinei	İstanbul	SYZB2085	-	-	-
	G. xvalentinei	İstanbul	SYZB2403	-	-	-
	G. plicatus subsp. *plicatus*	Sinop	SYZB2615	GU329696	GU329576	GU329633
	G. plicatus subsp. *plicatus*	Sinop	SYZB2618	GU329697	GU329575	GU329632
	G. plicatus subsp. *plicatus*	Bolu	SYZB3059	GU329698	GU329574	GU329631
	G. plicatus subsp. *byzantinus*	İzmit	SYZB3009	GU329693	GU329572	GU329628
	G. plicatus subsp. *byzantinus*	İzmit	SYZB3010	GU329695	GU329573	GU329630
	G. plicatus subsp. *byzantinus*	Kocaeli	SYZB2397	-	-	-
	G. plicatus subsp. *byzantinus*	İstanbul	SYZB2394	-	-	-
	G. plicatus subsp. *byzantinus*	İstanbul	SYZB2088	-	-	-
	G. plicatus subsp. *byzantinus*	Yalova	SYZB2094	-	-	-
	G. plicatus subsp. *byzantinus*	Bursa	SYZB3013	GU329671	GU329552	GU329627
	G. plicatus subsp. *byzantinus*	Bilecik	SYZB3016	GU329672	GU329551	GU329626
	G. trojanus	Balıkesir	SYZB2424	GU329673	-	-
	G. trojanus	Çanakkale	SYZB3018	GU329674	GU329553	GU329639
	G. trojanus	Balıkesir	SYZB3019	GU329675	GU329554	GU329640

[a]Following Bishop et al. (2001).
*Vouchers stored at ISTF and at ISTE.

and 3 min at 72 °C for extension, followed by a final 7 min extension at 72 °C. After each amplification process, PCR products were purified by using the Wizard SV PCR Clean-Up System (Promega) following the supplier's instructions. The sequencing reactions were done using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences). Sequencing reactions were carried out using the same primers of PCR amplification. Each PCR product was sequenced twice using the forward and the reverse primers separately. The cycle sequencing was done on an ABI 9700 Thermocycler (Applied Biosystems). The final products were analyzed on the ABI 3100 Genetic Analyzer (Applied Biosystems).

2.3. Statistical analysis of the sequencing data

Sequences were edited manually using the BioEdit Sequence Alignment Editor (Hall, 1999). Ambiguous bases were corrected using the corresponding base of the sequence that was obtained by the reverse primer. Multiple sequences were aligned using ClustalW, with default parameters (Thompson et al., 1994), and consensus sequences were created for each species or subspecies.

ITS sequences required a higher level of manual adjustment for alignment than chloroplast sequences due to the moderate level of background noise at the 5' end of the ITS1 and at the 3' end of ITS2 (about 60 nucleotides in total). Therefore, this region was not included in the alignment constructed for phylogenetic analysis. Finally an alignment of 541 bp in length was produced.

The sequence data of chloroplast markers were combined in order to obtain a more useful and larger dataset at the intraspecific level. An alignment of 852 bp in length was produced by the combination of the *trn*L(UAA) intron sequence with that of *trn*L-F.

The phylogenetic analyses of the sequences were performed by using MEGA Version 5.0 (Tamura et al., 2011). The strict consensus trees were constructed using the minimum evolution (ME), maximum likelihood (ML), and maximum parsimony (MP) methods (Rzhetsky and Nei, 1993; Saitou and Nei, 1987; Saitou, 1988). Kimura-2 parameter (Kimura, 1980) and p-distance (Nei and Kumar, 2000) methods were used as the reference distances between the sequences examined. Bootstrapping (BS) (Felsenstein, 1985) was applied 1000 times to all 3 methods of tree construction. Missing data or gaps in the aligned sequences were pairwise deleted for ME analysis. However, all the positions were used in MP and ML analysis. The corresponding sequences of an outgroup species, *Sternbergia lutea*, were chosen for rooting all phylogenetic trees (Lledo et al., 2004). Throughout this manuscript we only present the ME trees.

3. Results

3.1. Analysis of ITS region

The alignment of 541 positions included 301 variable characters (55.6%), of which 117 (21.6%) were parsimony-informative. In the phylogenetic analysis based on ITS data, *Galanthus* species were divided into 3 distinct clades in accordance with earlier observations based on morphology. These 3 clades are series *Galanthus* and, within the series *Latifolii*, subseries *Viridifolii* and subseries *Glaucaefolii* (Figure 1).

Series *Galanthus* formed a monophyletic group with a strongly supporting BS value of 100%. Two subspecies of *G. plicatus* (*G. plicatus* subsp. *byzantinus* and *G. plicatus* subsp. *plicatus*) grouped together. The second branch of this clade contained samples of *G. xvalentinei*, which closely resembles *G. nivalis* in morphological characters. However, due to the questionable status of *G. nivalis* in Turkey, we referred to these samples as *G. nivalis* × *xvalentinei* in all figures. The third branch contained *G. nivalis*, the sequence of which was taken from GenBank with no geographical information.

Subseries *Glaucaefolii* revealed monophyly, except for *G. alpinus* and *G. koenenianus*. *G. alpinus* and *G. koenenianus* were grouped together with subseries *Viridifolii*, with which their geographical distribution overlapped. Moreover, in our analysis, *G. trojanus*, reported earlier as a species of uncertain affinity (Bishop et al., 2001), was clustered within the clade for subseries *Glaucaefolii*, yet as a separate branch in the ME tree. The separation of *G. trojanus* from the other species of *Glaucaefolii* was also evident in ML analysis (not shown). Finally, *G. krasnovii* diverged out of all 3 clades on a separate branch.

3.1.1. Analysis through 5.8S rRNA gene

When analyzing the ITS data, the relatively high variability of the 5.8S rRNA gene led us to examine this region by itself. The 5.8S rDNA coding sequence contains a conserved 14 bp motif located approximately 74 nucleotides into the 5.8S rRNA gene. This motif, involved in intramolecular base pairing to form part of a stem required for proper functioning of the ribosomal RNA, was shown to be highly conserved among angiosperms both in length and in nucleotide sequence (Jobes and Thien, 1997). On the other hand, we observed 2 closely related but distinct 14 bp sequence patterns in the 5.8S rRNA among *Galanthus* species (Figure 2). One of the sequence motif clusters subseries *Glaucaefolii*, except for *G. alpinus* and *G. koenenianus*, and the other sequence motif clusters subseries *Viridifolii* and series *Galanthus*. The classification of *G. alpinus* and *G. koenenianus* outside *Glaucaefolii* is consistent with our ITS data (Figure 1). In this highly conserved sequence motif, *G. fosteri* Baker, belonging to series *Viridifolii*, diverged from the whole set with one base difference.

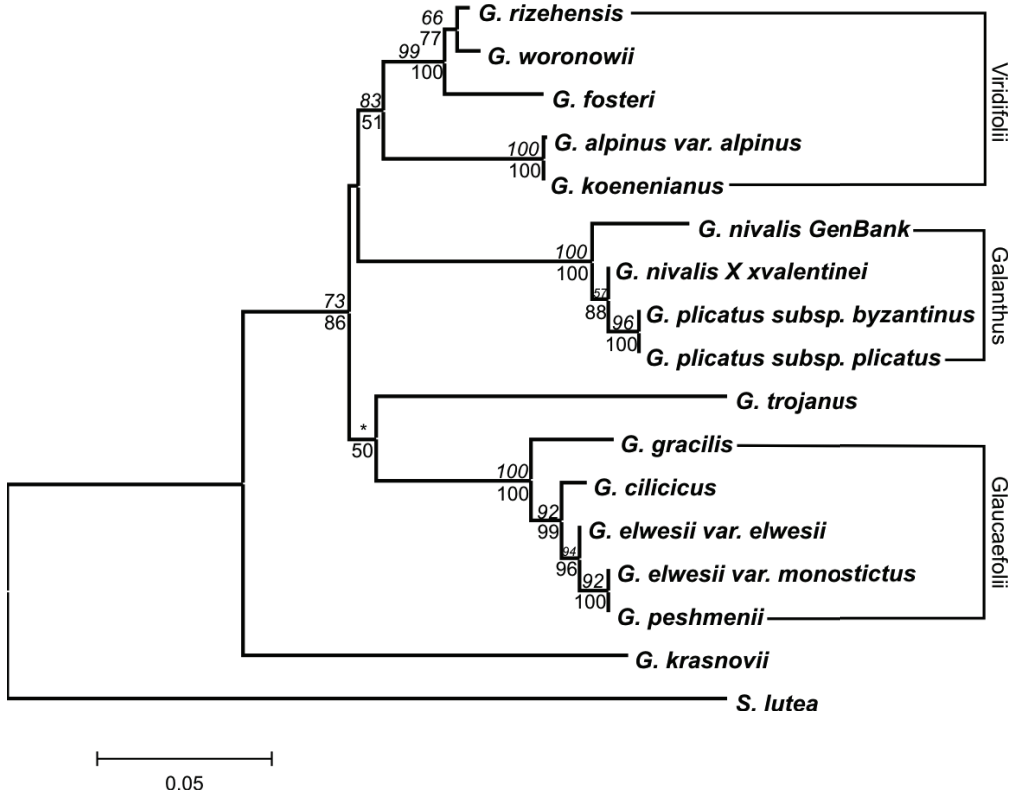

Figure 1. Minimum evolution tree based on nuclear rRNA ITS sequence data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown below the branches. The evolutionary distances were computed using the Kimura-2 parameter method in MEGA5. Bootstrap values calculated from a maximum likelihood tree generated from the same data are also presented (upper numbers in italics) except for (*), where the topology does not match for the branch of *G. trojanus*.

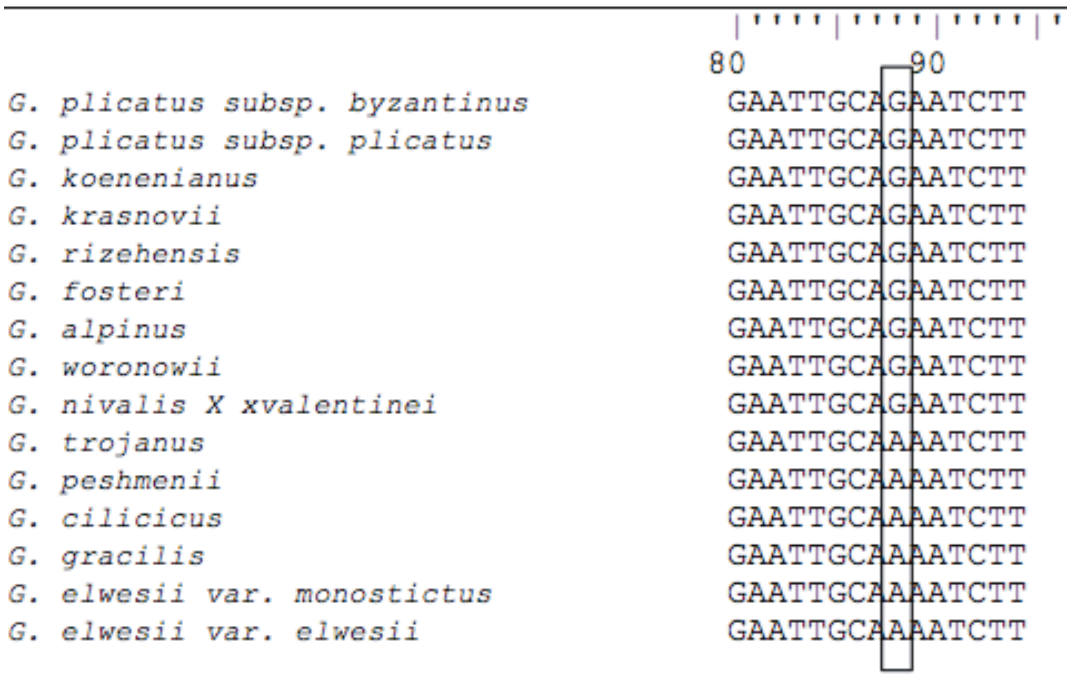

Figure 2. Sequencing chromatogram of the 14 bp conserved motif in the 5.8S rRNA gene.

The alignment of 171 positions in the 5.8S rRNA gene included 52 variable characters (30.4%) and 32 (18.7%) were parsimony-informative. The phylogenetic trees constructed using the complete 5.8S rRNA data confirms the ITS data (Figure 3). The distinction between series *Galanthus*, subseries *Glaucaefolii*, and subseries *Viridifolii* was well distinguished and their monophyly was confirmed. As in the ITS-based trees, *G. alpinus* and *G. koenenianus* were grouped along with subseries *Viridifolii*, and *G. trojanus* grouped together with subseries *Glaucaefolii*. Furthermore, the separation of *G. trojanus* from the other members of *Glaucaefolii* as a separate branch was more evident in the 5.8S rRNA data (BS of 50% in ITS versus 84% in 5.8S rRNA data). *G. krasnovii* diverged from the whole set as in the ITS trees. We concluded that 5.8S rRNA sequences can be used alone for phylogenetic analysis of the *Galanthus* species.

3.2. Analysis of chloroplast introns

In contrast to the nuclear rRNA ITS region, chloroplast sequences exhibited some variation at intraspecies level. The number of these single nucleotide polymorphisms was higher in the *trn*L(UAA) intron than the *trn*L-F region. *G. elwesii* Hook. f. var. *elwesii* showed the highest number of nucleotide variation in both chloroplast markers in line with the observation that *G. elwesii* var. *elwesii* showed the highest level of morphological variation among the *Galanthus* taxa of Anatolia.

The sequence alignment of the intergenic spacer regions included 381 bases, 38 (9.9%) of which were variable characters and 13 (3.4%) were parsimony-informative. On the other hand, the alignment of the *trn*L(UAA) intron sequences included 471 positions, resulting in 117 (24.4%) characters, of which 15 (3.1%) were informative. The relatively low number of parsimony-informative sites for each individual chloroplast marker resulted in low resolution phylogenetic trees with decreased BS values (not shown). Therefore, in order to improve the accuracy, both alignments were combined: 151 (17.2%) variable characters, of which 17 (1.9%) were parsimony-informative sites, were obtained in the final dataset of 852 bases. Even though the combined chloroplast data still had a low number of parsimony-informative sites, the phylogenetic trees constructed using chloroplast sequences were rather consistent with the ITS data (Figure 4).

The combined chloroplast data confirm the earlier classification of major series of *Galanthus* species as monophyletic groups: series *Galanthus*, subseries *Viridifolii* and subseries *Glaucaefolii* with the exception of *G. alpinus*, *G. gracilis* Čelak., and *G. fosteri*. *G. alpinus* grouped together with subseries *Viridifolii*, in accordance with the phylogenetic trees obtained from ITS data. *G. gracilis*, on the other hand, clustered on the same node with series *Galanthus*, and *G. fosteri* grouped together with subseries *Glaucaefolii* in contrast to ITS data (see Section 4). However, the BS value for *G. fosteri* was low.

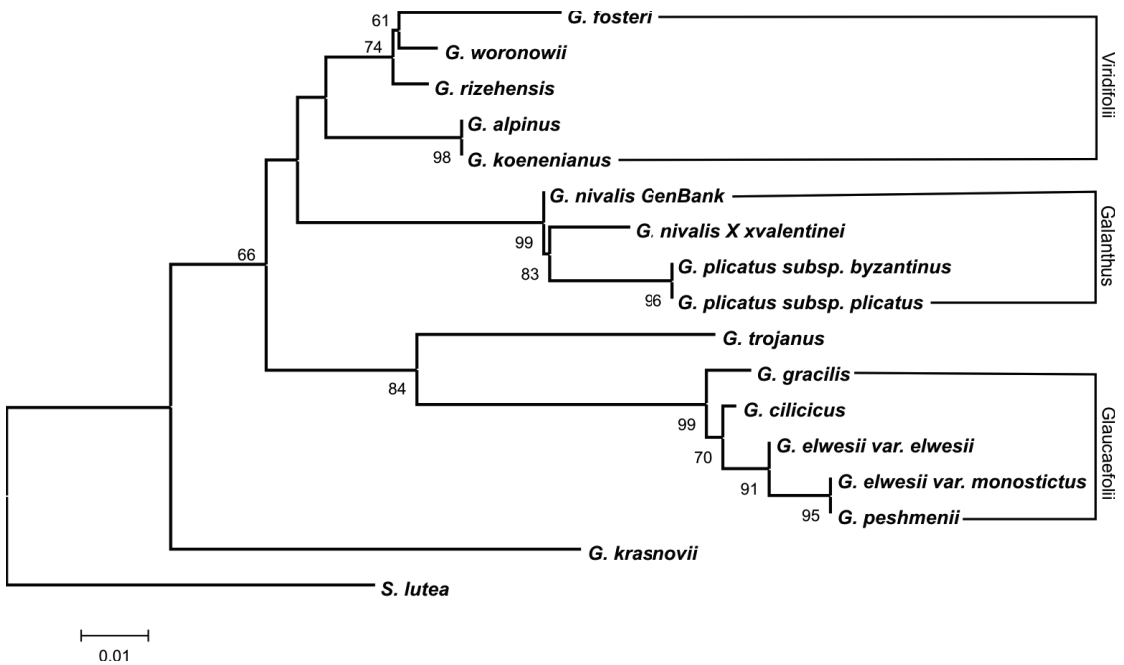

Figure 3. Minimum evolution tree based on 5.8S rRNA sequence data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Kimura-2 parameter method in MEGA5.

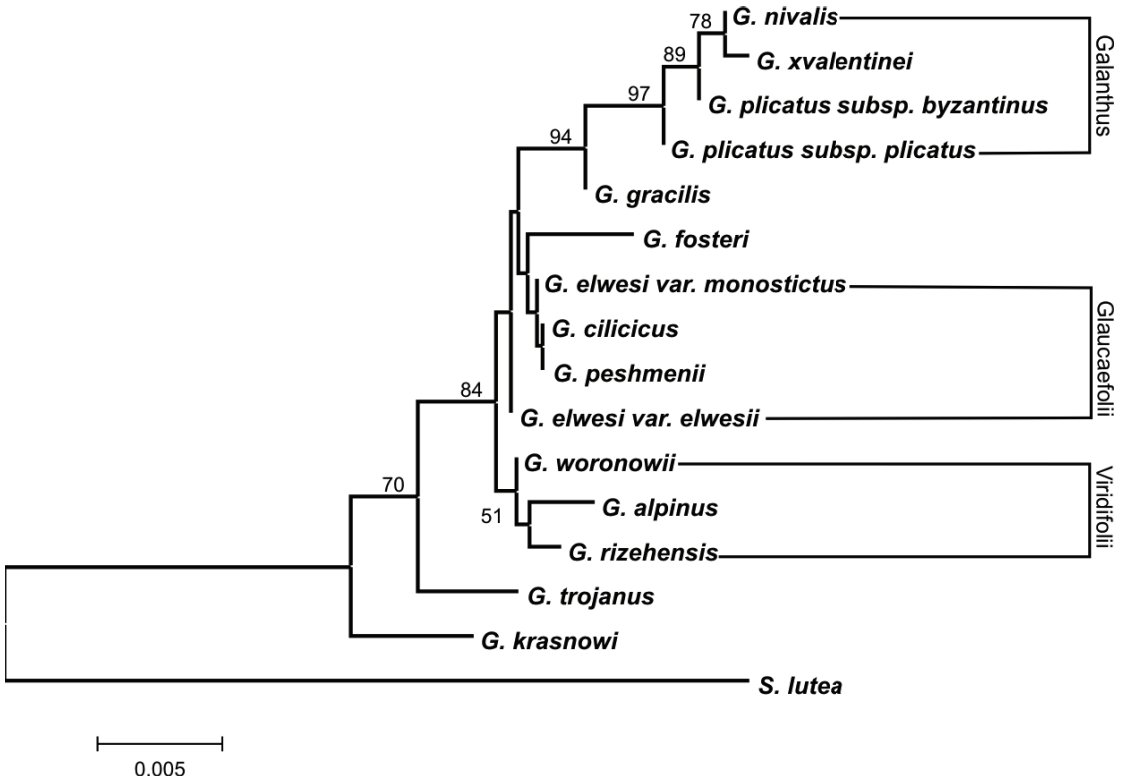

Figure 4. Minimum evolution tree based on combined *trn*L(UAA) and *trn*L-F sequence data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Kimura-2 parameter method in MEGA5. *G. nivalis* samples include SYZB2405-2589-2590-2591-2597, whereas G. *xvalentinei* is SYZB3008.

3.2.1. Chloroplast imprints

Intraspecies variation level was quite high in chloroplast markers, particularly for the *trn*L(UAA) intron. This means that both regions [*trn*L(UAA) and *trn*L-F] are open to point mutations and any argument based solely on these regions will not be a definite reflection of genetic relationships. However, apart from being able to represent the phylogenetic relationships when supported with nuclear and morphological data, these uniparentally inherited markers can also be used as genetic imprint in some of the species, for their very mutation types. For most of the cases, these regions represent insertions and deletions that occur after a duplication event. These sequences can be used as molecular signatures for their carrier species.

One such molecular signature is an insertion/deletion of 11 nucleotides. An 11-nucleotide insertion is present only in *G. rizehensis trn*L-F sequences (Figure 5). This 11 nucleotide insertion/deletion signature was used to differentiate *G. woronowii* Losinsk. from *G. rizehensis* species at a North Anatolian collection site where 2 species share the same location (Rize, Derepazarı). Specimen 2896 collected from this location was initially identified as *G. woronowii* based on its leaf pattern, although it showed similarities to *G. rizehensis* in terms of coloration. Similarly, specimen 2884 from Giresun exhibited characters of both G. *rizehensis* and G. *woronowii*, making it difficult to verify its identity. The presence of the 11 nucleotide insertion in their *trn*L-F sequences led to the conclusion for their identity as *G. rizehensis* (Figure 6).

Another molecular signature, in the form of insertions/deletions, was also present in the *G. koenenianus trn*L(UAA) intron. We observed a 70-nucleotide deletion, unique to this species (Figure 7).

4. Discussion

One important observation from the present work is that phylogenetic trees based on both nuclear and chloroplast data correlated well with the actual geographical distribution pattern of *Galanthus* species, rather than their recognized morphological classifications (Figure 8).

4.1. Series *Latifolii*

Subseries *Viridifolii* species, all collected from Northeast Anatolia (Trabzon, Rize, Artvin, and Amasya), clustered on the same node and formed a monophyletic group in all phylogenetic trees. In particular, the case of *G. alpinus*

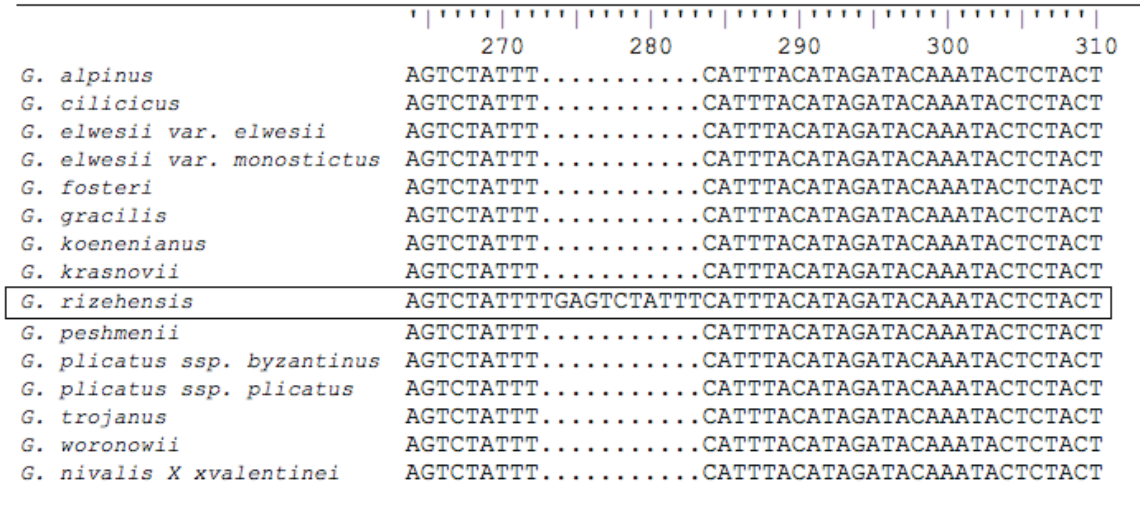

Figure 5. Section from the sequencing chromatogram of *Galanthus trn*L-F region. *G. rizehensis* has a unique insertion site of 11 nucleotides.

Figure 6. Section from the sequencing chromatogram of *G. woronowii* and *G. rizehensis trn*L(UAA) intron.

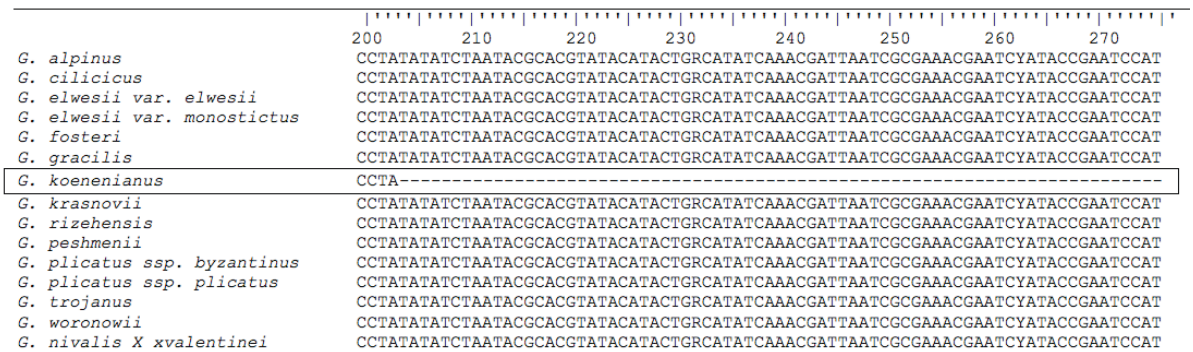

Figure 7. Section from the sequencing chromatogram of *Galanthus trn*L(UAA) intron. *G. koenenianus* has a unique deletion site of 70 nucleotides.

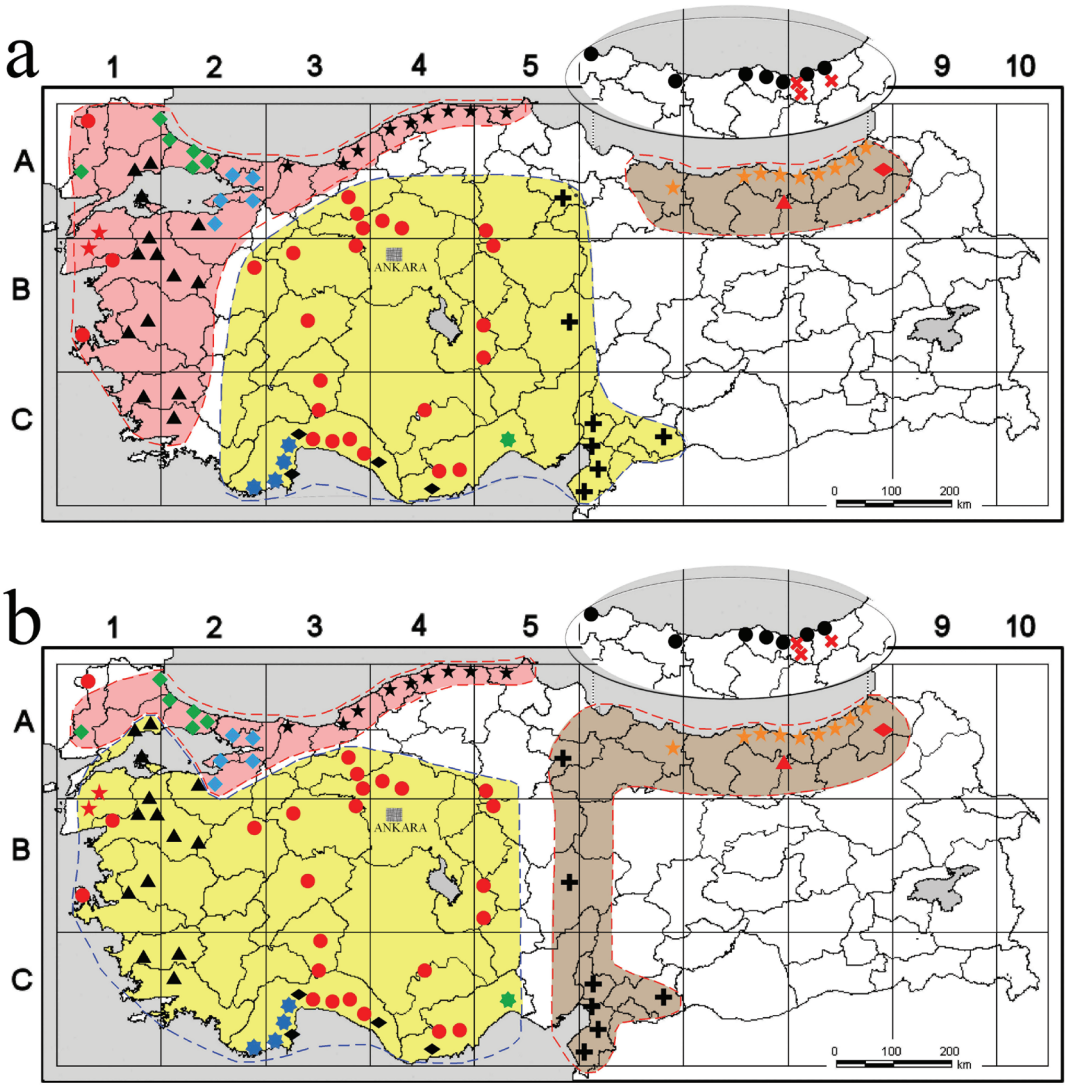

Figure 8. Maps showing the collection sites for the *Galanthus* samples used in this study. (✖) *G. alpinus* var. *alpinus*, (◆) *G. krasnovii*, (●) *G. elwesii* var. *elwesii*, (▲) *G. koenenianus*, (★) *G. trojanus*, (✷) *G. peshmenii*, (◆) *G. plicatus* subsp. *byzantinus*, (✷) *G. cilicicus*, (★) *G. woronowii*, (◆) *G. xvalentinei*, (★) *G. plicatus* subsp. *plicatus*, (✚) *G. fosteri*, (◆) *G. elwesii* var. *monostictus*, (●) *G. rizehensis*, (▲) *G. gracilis*. Colored areas represent the clustering pattern of nuclear-based (a) and chloroplast-based (b) phylogenetic trees shown in Figures 1 and 4: series *Galanthus* (pink), subseries *Viridifolii* (blue), subseries *Glaucaefolii* (yellow).

and *G. koenenianus* makes a good example supporting the argument of geographical proximity as the predictive factor of genetic similarity rather than the morphological characters (Friesen, 2006). According to morphological analyses, subseries *Glaucaefolii* includes the species *G. gracilis*, *G. cilicicus*, *G. peshmenii*, *G. elwesii*, *G. alpinus*, *G. angustifolius* (not present in Turkey), and *G. koenenianus*. However, in all ITS-based phylogenetic methods used in our study, *G. alpinus* and *G. koenenianus* always clustered together with *G. fosteri*, *G. woronowii*, and *G. rizehensis*, all belonging to subseries *Viridifolii* (series *Latifolii*), contradicting earlier reports that placed these 2 species under subseries *Glaucaefolii* (Davis, 1999; Bishop et al., 2001). It seems that *G. alpinus* and *G. koenenianus* cluster together with their geographic neighbors (Figure 8). This grouping is, in fact, strongly supported by the molecular data of Lledo et al. (2004). In their work, *G. alpinus* clustered on the same branch with *G. woronowii* and *G. fosteri*.

Alternatively, the placement of *G. koenenianus* as a separate branch on chloroplast-based phylogenetic trees, outside of their geographical neighbors, contradicts the

nuclear ITS data (data not shown). However, the presence of a 70-nucleotide deletion in the chloroplast *trn*L(UAA) intron, which is unique to *G. koenenianus*, might strongly distort the phylogenetic analysis. Therefore, we excluded *G. koenenianus* from the phylogenetic analysis of the chloroplast data (Figure 4). Indeed, the presence of this 70-nucleotide deletion in *G. koenenianus* can be used as a signature to identify this taxon as a separate species and differentiate it from *G. alpinus*.

Both in this work and in that of Lledo et al. (2004), *G. elwesii* var. *elwesii*, *G. gracilis*, *G. cilicicus*, *G. peshmenii*, and *G. elwesii* var. *monostictus* samples form a monophyletic group according to their rRNA ITS sequences. However, there are minor differences between ITS- and chloroplast-based phylogenetic trees. We observed that nuclear markers are less sensitive to the disruption of reproductive isolation than chloroplast markers. In fact, this observation is supported by a recent study where it was computationally shown that phylogenetic trees built from nuclear markers are more robust (Bakış et al., 2013).

We speculated that common ecological niches and shared geographical conditions (such as climate, pollination vectors, etc.) may enable the gene flow between species of the same geography. This gene flow may be much more apparent on the chloroplast genome.

The species in which we observed differences between nuclear- and chloroplast-based phylogenetic trees were *G. elwesii* var. *elwesii*, *G.gracilis*, and *G. fosteri*. All 3 taxa have a wide distribution range, both in terms of altitude and geographical position all the way from the northern to the southern coasts of Turkey.

G. elwesii var. *elwesii* is geographically the most widespread taxon of all, found in West Anatolia from the south to the north all the way to Edirne. Although phylogenetic trees based on ITS data clearly cluster *G. elwesii* var. *elwesii* within the series *Glaucaefolii*, when chloroplast data are used it forms a separate branch, though placed nearest to *Glaucaefolii*. Although the BS values in chloroplast-based analysis are not strong enough for a conclusion, this pattern is also confirmed in MP and ML trees (data not shown). Additionally, *G. elwesii* var. *elwesii* contains the highest number of nucleotide variation in both chloroplast markers studied (Taşçı, 2008, MSc thesis, Boğaziçi University). Considering the fact that this species also represents the highest level of morphological variation (Yüzbaşıoğlu, 2012, PhD thesis, İstanbul University), it is possible to correlate the high rate of chloroplast variability with the range of geographical distribution of *G. elwesii* var. *elwesii* in contact with other *Galanthus* taxa.

G. gracilis is also one of the most widespread species of all Anatolian *Galanthus* taxa. The samples were collected in West Anatolia from south to north (Manisa, Kütahya, İzmir, Balıkesir, Bursa), and one sample was even collected from Northwest Turkey (Tekirdağ). Even though ITS data relate *G. gracilis* to subseries *Glaucaefolii*, when chloroplast data are used, *G. gracilis* clustered together with series *Galanthus*, although it was the most distant species of this group. In support of its extended distribution range, *G. gracilis* was the second species having the highest rate of variation in both chloroplast markers after *G. elwesii* var. *elwesii* (Taşçı, 2008, MSc thesis, Boğaziçi University). Indeed, *G. gracilis* shares the same habitat with *G. plicatus* subsp. *byzantinus* in Bursa (they got as close to each other as 70 km –a bird's view– without encountering any physical barriers) and with *G. xvalentinei* in Thrace.

G. fosteri is the third taxon in which ITS and chloroplast data do not fully support each other. When analyzed using ITS data, it was grouped within subseries *Viridifolii*; however, when the chloroplast data were considered it formed a separate branch, nearest to *Glaucaefolii*. In fact, *G. fosteri* spread throughout a narrow corridor extending from North to South Anatolia all the way to Hatay. In the north, it neighbors *Viridifolii* species, yet in the south, it comes in close contact with *Glaucaefolii* species. Even though the phylogenetic trees based on chloroplast data have low BS values in all methods used, a stronger effect of geographical proximity on chloroplast-based phylogenetic analysis is clearly observed (Figure 8).

G. krasnovii, which was reported earlier to belong to subseries *Viridifolii* and identified as the most morphologically distinct member of the genus *Galanthus* (Davis, 1999), diverged out of all 3 clades on a separate branch.

4.2. Series *Galanthus*

The members of series *Galanthus*, i.e. *G. nivalis*, *G. plicatus* s.l., and *G. xvalentinei* nothosubsp. *subplicatus*, formed a well-supported monophyletic clade both in terms of nuclear and chloroplast markers.

4.2.1. The case of *G. plicatus* s.l.

The first case involved 2 subspecies of *G. plicatus*: *G. plicatus* subsp. *byzantinus* and *G. plicatus* subsp. *plicatus*. *G. plicatus* subsp. *byzantinus* is present in Anatolia, along the Asian side of the Bosphorus and extending to the eastern coast of the Marmara Sea; *G. plicatus* subsp. *plicatus*, on the other hand, grows throughout the western parts of the North Anatolian coast (Figure 9). These subspecies clustered together with high branch support for the nuclear marker. Taking into consideration the fact that they are the unique *Galanthus* throughout the İstanbul-Sinop line, their clustering pattern was significant.

One of the key morphological features for the discrimination of subsp. *plicatus* from subsp. *byzantinus* was the pigmentation pattern of the inner perianth segments. *G. plicatus* subsp. *plicatus* is considered to have with one green mark at the apex, whereas *G. plicatus* subsp. *byzantinus* shows both apical and basal coloration

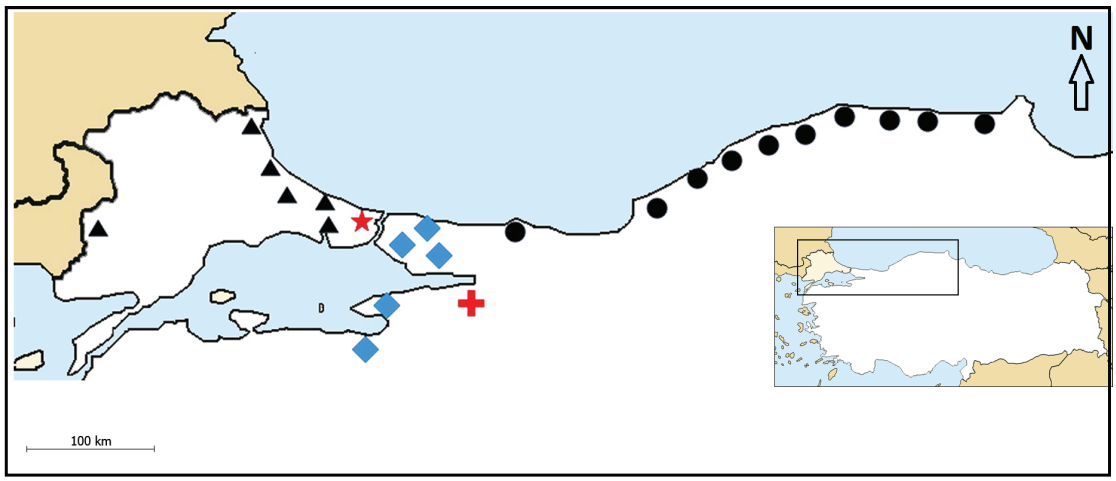

Figure 9. Map showing the collection sites for the series *Galanthus* samples. (▲) *G. xvalentinei*, (★) SYZB 3008, (◆) *G. plicatus* subsp. *byzantinus*, (●) *G. plicatus* subsp. *plicatus*, (✚) SYZB 3009-3010.

or occasionally a unique united pigmentation. Although the degree of morphological variation had been stated to be low at the intrapopulation level both for *G. plicatus* subsp. *byzantinus* and *G. plicatus* subsp. *plicatus*, the apical pigmentation phenotype typical for *G. plicatus* subsp. *plicatus* was also observed among a few *G. plicatus* subsp. *byzantinus* specimens (3009 and 3010) that shared the same locality with the former in İzmit and nearby. It can be noted that our phylogenetic analysis of northwestern Marmara species clustered these 2 specimens together with *G. plicatus* subsp. *plicatus* specimens as well (Figure 10). In line with our observations, the atypical distribution of coloration pattern was also reported among *G. plicatus* subsp. *plicatus* populations in Russia, where some *G. plicatus* subsp. *plicatus* individuals with apical and basal colorations typical of *G. byzantinus* were observed (Artjushenko, 1967). This suggests that the pigmentation phenotypes may not be an appropriate marker for discrimination of these 2 closely related subspecies and they may have evolved independently several times within distinct populations.

The fact that *G. plicatus* subsp. *byzantinus* samples, some of which are morphologically different in terms of apical and basal coloration, showed no differentiation in nuclear and chloroplast sequence level suggests that pigmentation phenotypes represent only phenetic variations within the same population.

4.2.2. The case of *G. xvalentinei* nothosubsp. *subplicatus*

The second case involved *G. xvalentinei* nothosubsp. *subplicatus*. *G. xvalentinei* nothosubsp. *subplicatus* is the correct name for all hybrids between *G. nivalis* and *G. plicatus* subsp. *byzantinus* (Bishop et al., 2001). *G. nivalis* occurs throughout West and Central Europe, and is thought to reach its southeastern border of distribution in northwestern Turkey. However, some populations located in Thrace and recognized previously as *G. nivalis* were recently identified as *G.* x*valentinei* nothosubsp. *subplicatus* (Davis et al., 2001). Our analysis with ITS and chloroplast data did not provide a precise distinction between *G. xvalentinei* and specimens identified initially as *G. nivalis*. However, both taxa differed clearly from the *G. nivalis* sequences of GenBank (Figures 1 and 3). Additional studies are needed to compare the pattern of morphological variations between distinct taxa, to construct a better classification of this group, and finally to make clear the occurrence of *G. nivalis* in Turkey.

One important observation from the molecular phylogenetic data was the positioning of *G. xvalentinei* 3008: this sample, which was collected from Kurt Kemeri in Belgrad Forest, belongs to the very population based on which the type specimen of *G. xvalentinei* was identified (Davis et al., 2001). ITS data indicated that this sample diverged from all other *G. xvalentinei* samples of Thrace and clustered on the same branch with *G. plicatus* s.l. (Figure 10). We further analyzed 3008 and observed that this sample has an identical *matK* sequence with the other *G. plicatus* samples 3009 and 3010 (İrem Ünlü, unpublished), confirming the ITS data.

According to our field observations, the above-mentioned population of *G. xvalentinei* located in Kurt Kemeri showed hybrid characters between *G. nivalis* and *G. plicatus* subsp. *byzantinus*, in terms of the leaf shape and the variations in its inner tepal coloration (Figure 11). *G. nivalis* is known to have applanate vernation along with apical coloration in its inner tepal segment, whereas *G. plicatus* subsp. *byzantinus* represents explicative vernation and both apical and basal type coloration in its inner tepal segment. Explicative vernation was well distin-

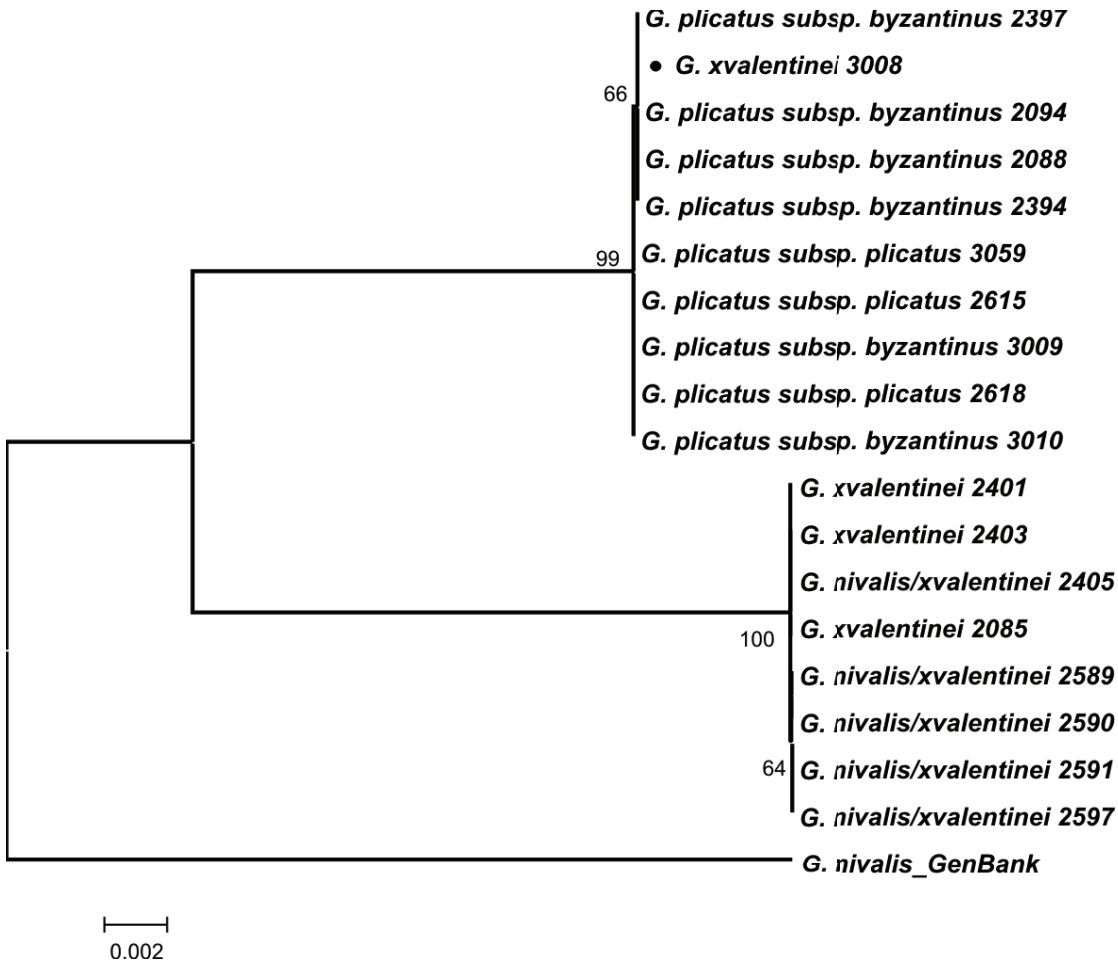

Figure 10. Minimum evolution tree based on nuclear ITS sequence data of *G. plicatus* subspecies (Çelen, 2005, MSc thesis, Boğaziçi University). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Kimura-2 parameter method in MEGA5.

guished in some of the specimens, and solely applanate in some others. There were even individuals with no basal-type coloration or some representing a particular type of pigmentation where the apical and basal colorations were merged. The edges of the leaves were slightly curved inwards. The curving of the edges was not symmetric on the 2 sides of the leaves. Moreover, when we compared the *G. xvalentinei* samples collected from İstanbul, Kırklareli, and Edirne, we observed that the atypical coloration of the inner tepal segment as well as the variations in the leaf shape became gradually rarer from east to west, as we move farther from Belgrad Forest. Similar leaf and color variations in the inner tepal segment were also reported by Davis et al. (2001) for *G. xvalentinei* samples collected between Belgrad Forest and Çatalca. Individuals with linear leaves as well as ones with very narrow oblanceolate leaves were observed within the same population located nearby Belgrad Forest.

In overall terms, the structural units were much more developed in *G. xvalentinei* samples of Belgrad Forest when compared with their western counterparts; an example of this was the leaf width extending up to 1.4 cm. The variation level decreased from east to west, starting from Çatalca: the leaves turned to be linear and narrow, and dominant coloration in the tepal segment turned to be apical; basal type coloration was rare.

Furthermore, the population of Kurt Kemeri, from where the *G. xvalentinei* sample 3008 was collected, also represented the apical coloration pattern in the inner tepal segment; this morphological feature overlaps well with that of *G. plicatus* subsp. *plicatus*, the main distribution range of which is the western parts of North Anatolia. Indeed, our field research showed that the population of Belgrad was the only one representing explicative vernation (*byzantinus*-type) with inward leaf curvings (*plicatus*-type) (Figure 11).

Figure 11. Image of *Galanthus xvalentinei* (SYZB 3008). a- variation in inner segment markings, b- habitus. Scale bar: a = 1 cm, b = 5 cm.

In summary, the clustering pattern of the type specimen for *G. xvalentinei*, which also complicates the ambiguous presence of *G. nivalis* in Turkey, urges the need for the reexamination of the morphological characters used to define *G. xvalentinei*. The fact that the whole genus *Galanthus* represents a high level of morphological variation but rather a low number of key features sufficient for taxonomical identification may complicate the clear status of certain species, particularly that of the series *Galanthus*.

It is useful as well as necessary to integrate criteria based on morphological features with molecular methods while evaluating taxonomic status of any species. In this work, previous assumptions concerning the taxonomy of *Galanthus* L. were in general terms confirmed by phylogenetic analysis results, but the study also showed some important discrepancies: phylogenetic relationship was more consistent with the geographical proximity between species than previously estimated. We concluded that a molecular approach is a prerequisite to catch the slight nuance between phenetic variations representing simple similarities due to shared habitat and those being the evidence of different origins.

Acknowledgements

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