THE MOLECULAR PHYLOGENETIC ANALSIS OF SELECTED *GALANTHUS* SPECIES FROM NORTHWEST TURKEY

by

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BS. in Molecular Biology and Genetics, Boğaziçi University, 2000

Submitted to the Institute of Environmental Sciences in partial fulfillment of

the requirements for the degree of

Master of Science

in

Environmental Sciences

Boğaziçi University

2005

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ABSTRACT

The molecular phylogenetic relationship of the *Galanthus* taxa found in Northwest Turkey was investigated. *Galanthus plicatus* subsp. *byzantinus*, *Galanthus nivalis* and their potential hybrid *Galanthus* x*valentinei* nothosubsp. *subplicatus* were analyzed using ribosomal DNA (ITS) region and chloroplast introns (*trn*L(UAA) intron, intragenic spacer between *trn*L(UAA)-*trn*F(GAA)). The samples morphologically identified as *G. nivalis* and found in Turkey, did not genetically match G. *nivalis* recognized in Europe, they were identical to the samples defined as *G.* x*valentinei* nothosubsp. *subplicatus*. *G.* x*valentinei* nothosubsp. *subplicatus* samples showed no indications of recent hybridization or instability in any of the molecular markers examined. They were placed on a separate node indicating a genetically stable taxonomic unit, showing a range of morphological markers. The samples morphologically identified as *G. plicatus* subsp. *byzantinus* were on the nuclear DNA level very similar to *G. plicatus*. The chloroplast sequences, however, were very similar to those of *G.* x*valentinei* nothosubsp. *subplicatus.* This could be indicative of an ancient hybrid but currently a stable species. These results show that the presence of *G. nivalis* in Turkey seems highly unlikely, and that *G.* x*valentinei* nothosubsp. *subplicatus* should be defined as a separate stable taxonomic unit that requires recognition and further population ecology research.

ÖZET

Marmara bölgesinde bulunan üç *Galanthus* (Kardelen) türünün moleküler filogenetik incelemeleri yapıldı. *Galanthus plicatus* subsp. *byzantinus*, *Galanthus nivalis* ve morfolojik olarak hibritleri olarak kabul edilen *Galanthus* x*valentinei* nothosubsp. *subplicatus*'un çekirdekte bulunan ribosomal DNA (ITS) bölgesi ile kloroplast intronlarının (*trn*L(UAA) intronun ve *trn*L(UAA)-*trn*F(GAA) genleri arasındaki bölgenin) sekansları analiz edildi. Morfolojik olarak *G. nivalis* diye tanımlanan bitkiler genetik açıdan Avrupa'da kabul edilen *G. nivalis*'den farklı, ve *G.* x*valentinei* nothosubsp. *subplicatus* örnekleri ile aynı olduğu anlaşıldı. *G.* x*valentinei* nothosubsp. *subplicatus* örnekleri ise hiçbir moleküler belirteçte hibritleşme belirtileri veya değişkenlik göstermedi. Filogenetik analizle genetik olarak ayrı ve sabit bir tür olduğu anlaşıldı. Morfolojik olarak *G. plicatus* subsp. *byzantinus* olarak tanımlanan bitkiler nükleer DNA seviyesinde *G. plicatus*'a yakın durdu. Ama kloroplast sekansları, *G.* x*valentinei* nothosubsp. *subplicatus*'a çok benzer çıktı. Bu iki genom arasındaki uyumsuzluk eski bir hibrit olabileceği anlamına gelebilir. Bu sonuçlar, Türkiye'de *G. nivalis* bulunmadığına dair kanıtlar sunmakta ve *G.* x*valentinei* nothosubsp. *subplicatus*'un hibrit değil, ayrı bir taksonomik ünite halinde yeniden adlandırılmasını ve popülasyon incelemeleriyle birlikte yeni bir koruma stratejisinin oluşturulmasını gerektirmektedir.

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DNA	Deoxy-ribonucleic Acid
rDNA	Ribosomal Deoxy-ribonucleic Acid
tRNA	Transfer Ribonucleic Acid
ITS	Internal Transcribed Spacer
PCR	Polymerase Chain Reaction
dATP	2'-deoxyadenosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dCTP	2'-deoxyguanosine 5'-triphosphate
G+C	Guanine + Cytosine
bp	Base Pairs
pg	10^{-9} Kg
µL	10^{-6} L
mM	10^{-3} M
µM	10^{-6} M
U	Units
NJ	Neighbor Joining
MP	Maximum Parsimony
CI	Consistency Index
RI	Retention Index
2-D	Two Dimentional
CITES	Convention for the International Trade of Endangered Species

1. INTRODUCTION

This study aims to use molecular biology as methodological tools, to improve the conservation efforts of certain plant species. Identification and comparison of species at the DNA level, constructs a premise to clarify the status of each taxonomic unit. Molecular identifications of each species lead to better defined populations which in turn results in coherent population ecology studies. The detailed knowledge of each species' genetic identity, indirectly but inevitably, leads to the better protection and conservation of species.

1.1. Conservation Biology and Phylogenetics

Conservation biology is a multidisciplinary science that aims to protect biodiversity and prevent the extinction of populations. It proceeds through three main lines: the documentation of the variety of living organisms, understanding the causes and the consequences of the loss of biodiversity and developing methods to prevent such losses (Caro, 1998). The identification and documentation of biodiversity through systematics is the key factor for each conservation effort, where coherent strategies are built accordingly to save the existing taxons.

Systematics deals with the recognition of species (Beebee T. and Rowe G., 2004), and is made up of two interrelated disciplines, taxonomy and phylogeny. The field of taxonomy identifies and classifies organisms according to their morphological features. Phylogeny, on the other hand, is a discipline that investigates the evolutionary history and the relationships between the classified living organisms. Through phylogenetic analysis, ancestral relationships can be inferred and taxonomic statuses confirmed.

In the field of phylogeny, the reconstruction of ancestral relationships between organisms is done through the assembly of phylogenetic trees. Trees are built using quantified morphological features as data, and analytical methods such as distance matrices or discrete methods (maximum parsimony and maximum likelihood) as the construction tools.

Until recently, phylogeny was only constructed by assigning character states to anatomical and morphological characters. Nowadays, with the advance of molecular biology techniques, a more dedicated field of systematics, namely, molecular systematics has developed (Beebee T. and Rowe G., 2004). Taxa are re-defined using properties at the protein and DNA level and through molecular phylogenetics; molecular properties are quantified and compared. The enquiry into evolutionary relationships between different taxa has become more comprehensive and detailed through molecular analyses. Thus today, every conservation biology scheme that aims to preserve genetic diversity has to include a thorough molecular phylogenetic analysis as a beginning point.

1.2. Molecular Phylogenetics

Molecular Phylogenetics emerged during the 1960's, when protein contents were compared between closely related species using protein electrophoresis techniques (Hillis and Moritz, 1990; Berlocher, 1984; Buth, 1984). Allozymes were used as tools for analysis, and the differences in sizes and electrophoretic mobilities were compared. These analyses, however, were not detailed enough as the proteins that did match in electrophoretic mobility could be withholding differences at the gene level (Coyne, 1982).

This problem was overcome during the early 1980's, with the development of low cost, high yield molecular methods such as the polymerase chain reaction (PCR). Since then both animal and plant physiologists have been able to directly examine the DNA and amino acid sequences of taxa to construct phylogenetic inferences and re-define taxonomic statuses.

There are several different techniques that allow the analysis of DNA using PCR, for molecular phylogenetic analysis. Restriction Fragment Length Polymorphism (RFLP) is a method where parts of the DNA are cleaved with restriction enzymes and compared. Even though simple, it requires prior knowledge of the restriction enzymes that can be used on the DNA of the samples, which is costly with unidentified genomes. Another method, Random Amplified Polymorphic DNA (RAPD) overcomes this problem by using many random sequences as primers and compares possible polymorphisms (Williams et al., 1990). Although this is a useful and less costly tool for phylogenetic analysis, the

technique has poor repeatability (Gehrig et al. 1997). Still another approach analyzes the parts of DNA that are highly variable called mini- or micro-satellite sequences that provide unique fingerprints for individuals however is not widely used for interspecific analyses (Parker et al., 1998). Yet, the direct sequencing of parts of the genome, using sequences that differ at the differing taxonomic levels is presently, the most prominent, detailed and cost effective method of phylogenetic analysis.

1.3. Plant Phylogenetic Analysis

The era of plant molecular phylogenetic analysis began with the comparison of *rbc*L gene sequences (Chase et al., 1993). The *rbc*L gene is a chloroplast gene that encodes the large subunit of ribulose biphosphate carboxylase (Rubisco) in plants (Zurawski et al., 1981). As the product of this gene is an important photosynthetic protein, these analyses were first done to investigate photosynthetic pathways. Free primers were distributed to many labs and was able to initiate the sequencing of at least 500 angiosperm species' *rbc*L gene (Chase et al., 1993) which produced the first major study of plant DNA for phylogenetic inference.

Since then several genes and introns of the chloroplast, mitochondria and nuclear genome have been used for the analysis of plant phylogenies at different taxonomic levels. The nuclear genome in plant evolves nearly twice as fast as chloroplast DNA due to its biparental mode of inheritance (Wolfe et al., 1987), and certain regions are used to differentiate between taxons at the species level. In addition it contains information on possible hybridizations due to its bi-parental mode of inheritance. Chloroplast genome, on the other hand, points to parental lineages and the path of gene flow attributable to its uniparental mode of inheritance. Even though the mitochondria genome is also uniparentally inherited, it evolves nearly five times slower than nuclear DNA in plants (Wolfe et al., 1987) and undergoes frequent rearrangements (Palmer and Herbon., 1987), which makes it an unfavorable marker for phylogenetic inference in plants. For concrete phylogenetic deductions, chloroplast markers and nuclear markers should be analyzed together, both to identify interspecific hybridizations and to increase phylogenetic resolution (Baldwin et al., 1995).

1.3.1. Nuclear Phylogenetic Marker: Ribosomal DNA

A nuclear genetic marker that is widely used for phylogenetic analysis is the region that codes for the ribosomal subunits (the ribosomal multigene family). This region is present in the plant nuclear genome in tandem repeated copies which can range from a hundred to thousands of repeats per genome. These repeats can be found either on a single chromosome or on different chromosomal locations (Alvarez and Wendel, 2003). Each ribosomal DNA set consists of 18S-5.8S-26S nuclear rDNA genes with ITS (internal transcribed spacers) 1 and 2 in between (Figure 1.1). The ITS region has been a focus of phylogenetic studies for approximately 15 years.

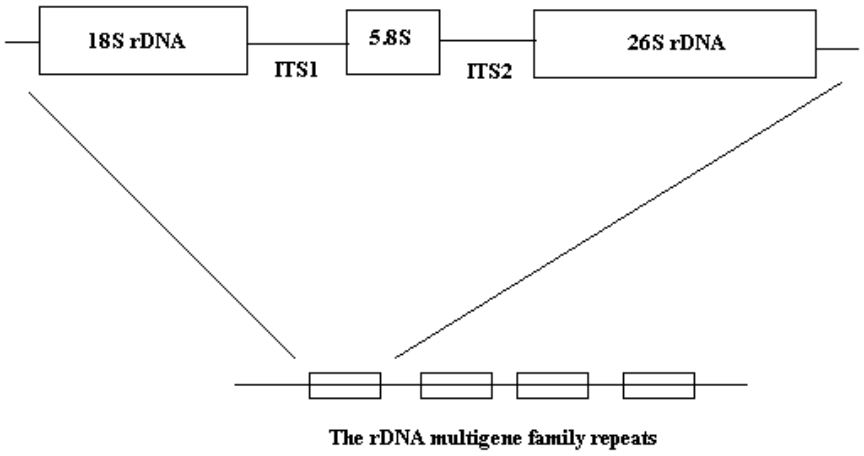


Figure 1.1. The schematic representation of the rDNA subunits and the multigene family.

All functional copies of the rDNA repeats are known to be uniform in the genome (Alvarez and Wendel, 2003). This uniformity is achieved by a phenomenon called concerted evolution, which occurs via unequal crossing over or rapid gene conversion (Arnheim, 1983; Elder and Turner, 1995; Zimmer et al., 1980). This mechanism allows any differences in one of the copies in the multigene family to homogenize so that the whole family contains the same sequence. There are cases, however, where some copies in the genome might not be identical. Such cases can happen in newly formed hybrid species

or polyploid plants, where concerted evolution might not have had enough time to take place. The multigene family, then, might consist both of paternal and maternal lineages or non-functional copies where the cloning of the different rDNA's would be necessary for analysis (Alvarez and Wendel, 2003).

Nonetheless the homogeneity of the rDNA per individual would not directly infer a pure species. The parental sequences causing different repeat types can combine together in time, forming chimeric sequences showing within populational differences (McDade, 1992). There can also be cases where concerted evolution might have taken place and one of these lineages or chimeric sequences has become dominant and homogenous in all of the multigene family (Alvarez and Wendel, 2003). Such possibilities suggest that concerted evolution would work at different evolutionary rates with differential operative strategies, so all of these possibilities should be carefully determined when these sequences are used for phylogenetic analysis.

In addition, taxa are faced with the possibility that some of the repeats in the family might have mutations in critical regions and are not translated leading to pseudogenes. If these sequences are not handled by concerted evolution and corrected, they could harbor many mutations and result in different rDNA copies corrupting phylogenetic analysis. These pseudogenes can be detected through G+C content, secondary structure, methylation processes (Bucker et al., 1997) and rate of substitutions in conserved regions.

1.3.1.1. ITS (internal transcribed spacer) region. The ITS region is easily accessible as universal primers have been designed that work on most plant and fungal DNA (White et al., 1990). The spacer ITS1 is located between the 3'end of 18S rDNA and the 5' of the 5.8S rDNA whereas ITS2 is located at the 3'end of 5.8S and the 5'end of 26SrDNA. Both of these regions are transcribed and spliced during the formation of ribosome.

Even though these spacers are not a part of the ribosome, they contribute to the formation of ribosome. Certain regions in the ITS1 spacer are critical for the assembly of the large and small ribosomal subunits in yeast (Musters et al., 1990; van Nues et al., 1994). Additionally deletions or point mutations in ITS2 region affect the maturation of the large 26S subunit (Sande et al., 1992).

Both of the ITS1 and ITS2 spacers in angiosperms consist of conserved motifs that play a crucial role in the production of ribosomal subunits in plants. For example, ITS2 has a GGU base triplet (Liu et al., 1994) that is linked with the formation of the secondary structure of the RNA. Such conserved secondary structural motifs positioned in specific regions of the helices are involved in the ITS2 excision processes (Mai and Coleman, 1997).

The functional roles of the ITS1 and ITS2 spacers in the production of the ribosome suggest that they are under evolutionary stress and will not harbor too many random mutations. This makes these regions ideal for interspecies differentiation, where they are expected to show uniformity over individuals from the same species.

1.3.1.2. 5.8S coding region. The 5.8S region, which is also amplified together with the ITS regions is around 163 base pairs long in angiosperms, and is located in between the two spacers (Jobes et al., 1997). This region is not widely used in low-level phylogenetic studies due to its high conservation at the species level, and its relatively short length (Troitsky et al., 1991; Suh et al., 1992).

1.3.2. Chloroplast Phylogenetic Marker: The tRNA Intragenic Spacers

The uniparental mode of inheritance of the chloroplast genome allows systematics to define parents of hybrids and the path of gene flow in speciation. The size of the chloroplast genome is around 150 kb and consists mostly of coding genes. It has four distinctive regions, a large single copy (LSC), 2 inverted repeats (IR) that are exact inverted copies of each other and a small single copy (SSC) (Curtis et al., 1984). The rate of evolution of the IR region, consisting mostly of rRNA genes, is approximately three times slower than the rest of the chloroplast DNA. The slow rate has been related to the possible processes that allow the two repeats to remain exactly the same (Wolfe et al., 1987).

Chloroplast coding genes evolve slowly and are generally used for family or higher-level taxonomic investigations. Coding genes such as *rbc*L (Gielly and Taberlet, 1994) found in the LSC, or *mat*K located within *trn*K exon in the LSC (Wolfe et al., 1991) or

*atp*B are tools used in such analysis. Non-coding regions of the chloroplast, however, tend to evolve more rapidly and can be used for relations below the family level (Gielly and Taberlet, 1994).

1.3.2.1. The *trn*L(UAA) intron and *trn*L(UAA) 3'exon -*trn*F(GAA) intragenic spacer.

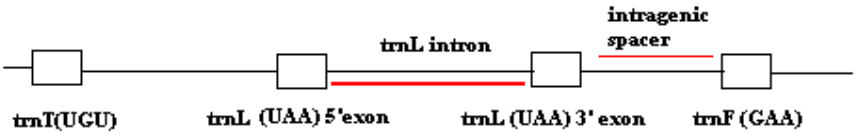


Figure 1.2. The schematic representation of tRNA genes and the non-coding regions. Taken from Taberlet et al. (1991).

The highly conserved chloroplast tRNA coding genes are present in the LSC and are spaced out in concession (Figure 1.2) (Taberlet et al., 1991). There are intragenic spacers between the different *trn* genes that can be amplified using universal primers designed by Taberlet et al. (1991) and used for phylogenetic inference. These intragenic spacers, which can evolve up to 11 times faster than *rbc*L gene, are widely used for intrafamilial and intrageneric level of phylogenetic research (Gielly and Taberlet, 1994). The *trn*L intron is a Group I intron, which is self-splicing and evolves approximately 1.6 times slower than the intragenic spacer (Gielly and Taberlet, 1994).

1.4. *Galanthus* Species and Molecular Phylogenetics

Galanthus L. is the Latin genus name for the widely cultivated snowdrop plants. The *Galanthus* genera belongs to the multicellular and eukaryotic *Plantae* kingdom, under the class *Angiospermae* (angiosperms, flowering plants), order *Asparagles* that are monocots, and the family *Amaryllidaceae*. There are 19 recognized *Galanthus* species occurring in many natural habitats in Europe and the Middle East (Davis, 1999; Davis et al., 2001; Zonneveld et al., 2003), 16 of which are present in Turkey. They reside in woodlands and forests, in cool environments with plenty of water (Davis et al., 2001).

The molecular karyotypic properties of all *Galanthus* species except polyploids are similar, having 2n =24 chromosomes (Sveshnikova, 1965). The nuclear DNA content of the different diploid *Galanthus* species, analyzed through flow cytometry, is between 50pg-90pg (Zonneveld et al., 2003). The molecular phylogenetic analysis of most of the *Galanthus* species have been recently done by Lledo et al. (2004), however, some species that grow in Turkey have not yet been added to the analysis.

There are three taxonomic groups that reside in Northwest Turkey, which have not yet been analyzed,. The phylogenetic relationships have been defined on the morphological level but have not yet been dwelled into at the molecular level.

On the Asian side of Istanbul, down to the province of Bursa there are swarms of *Galanthus plicatus* subsp. *byzantinus* (Figure 1.3). Even though it is registered in literature as occurring in Northwest Turkey, on both sides of the Bosphorous (Bishop et al., 2001), it is not clear if there are any pure individuals residing on the European side. The plants that are collected on the European side of the Bosphorous are mostly described as hybrids. Expectedly, *G. plicatus* subsp. *byzantinus* populations have been listed as under threat due to hybridization with *G.nivalis* in the western Istanbul area (Anonymous, 1999). The plants flower around January to April at altitudes between 100-300m (Bishop et al., 2001; Davis et al., 1999).

Figure 1.3. *G. plicatus* subsp. *byzantinus*.
(Photograph taken from the Royal Horticultural Society website:
www.rhs.org.uk/events/londonshows/londonfebruary2002.asp)

Galanthus x*valentinei* nothosubsp. *subplicatus* populations, are found on the European side of the Bosphorous. *G.* x*valentinei* is the general name given to hybrids of *Galanthus plicatus* and *Galanthus nivalis* (Davis et al., 2001) and the prefix 'notho-' indicates that it is a hybrid. *G.* x*valentinei* nothosubsp. *subplicatus* is the name specifically given for the hybrid between *G. nivalis* and *G. plicatus* subsp. *byzantinus* (Davis et al., 2001). *G.* x*valentinei* nothosubsp. *subplicatus* is probably the only naturally occurring hybrid of the *Galanthus* genera (Davis et al., 2001). The morphological markers of the individuals of the hybrid resemble both parents in variable degrees, even though all individuals are always separable from either parent (Davis et al., 2001). This hybrid is endemic to the province of Istanbul, but is also thought to spread to the provinces of Edirne, Kirklareli, and Tekirdağ (Davis et al., 2001). The plants flower between January-March at altitudes 30-150m (Davis, 2002). The taxon is not under any danger, however is listed in CITES Appendix II, for threats that can occur with uncontrolled trade[1].

Figure 1.4. *G. nivalis*. (Photograph taken from the following website: http://www.f-lohmueller.de/botanic/lily/Amaryllidaceae/Galanthus/Galanthus015.htm

[1] CITES: Convention on International Trade in Endangered Species of Wild Fauna and Flora, adopted in Washington in 1973

The European side of the Bosphorous also encloses populations of *G. nivalis* (Figure 1.4). The species is found all over Europe and has also been observed in Turkey, however only in the European side of the Bosphorous, and not in Asia Minor (Davis, 1999) (Table 1.1). The individuals that have been previously described as *G. nivalis* in Turkey are presently described as the hybrid *G.* x*valentinei* nothosubsp. *subplicatus,* hence the occurrence *of G. nivalis* in Turkey is unclear. The plants flower from January to May, at altitudes 100-1400m (Davis 1999). The biggest threat to the species in Turkey is habitat loss and hybridization with *G. plicatus* subsp. *byzantinus* (Anonymous, 1999).

Table 1.1. Distribution and status of *G.nivalis* in Europe. Taken from EURO+Med Plant Database http://www.s2you.com/euromed/

COUNTRY	STATUS	COUNTRY	STATUS
Austria	*	Switzerland	NT
Liechtenstein	*	Netherlands	*
Belgium	*	Spain	*?
Luxembourg	*	Hungary	*
Bosnia-Herzegovina	CR-VU	Italy	*
Great Britain	*	Moldova	R
Bulgaria	CR-VU	Poland	*
Czech Republic	VU	Romania	*
Croatia	*	Sicily	*
Denmark	*	Slovakia	NT
Estonia	*	Slovenia	*
France	*	Serbia & Montenegro	*
Germany	VU	Sweden	*
Greece	R	***Turkey***	***VU***
Ireland	*	Ukraine	R

Abbreviations: CR: critically endangered, VU: vulnerable, NT: near threatened, R: rare, * : present, *?: doubt about presence.

2. THESIS OBJECTIVE

The aim of this thesis is to define unclear taxonomic statuses using a molecular phylogenetic approach. *G. plicatus* subsp. *byzantinus*, *G.* x*valentinei* nothosubsp *subplicatus* and *G. nivalis* that reside in North-west Turkey have been identified and classified morphologically, however need to be confirmed at the molecular level. Particularly, the hybrid classification of *G.* x*valentinei* nothosubsp. *subplicatus* has been under question.

The nuclear phylogenetic analysis, which will also reveal possible hybridization between species will be done through the ribosomal ITS1 and ITS2 regions. Chloroplast markers, *trnL*(UAA) intron and the intragenic spacer between *trnL*(UAA) and *trnF* (GAA) genes, on the other hand, will be used to define the maternal parent of the possible hybrid *G.* x*valentinei* nothosubsp. *subplicatus* and to define the path of gene flow.

The accurate taxonomic identification and phylogenetic analysis of species would allow more precise conservation efforts. *Galanthus* species in Turkey are listed as threatened. *G.* x*valentinei* nothosubsp. *subplicatus* due to its range should be classified, maybe as vulnerable, if found to be a pure species. These analyses would either confirm the current statuses of conservation or would lead to a re-classification and a re-definition of the taxonomic units and lead to new conservation strategies.

3. MATERIALS AND METHODS

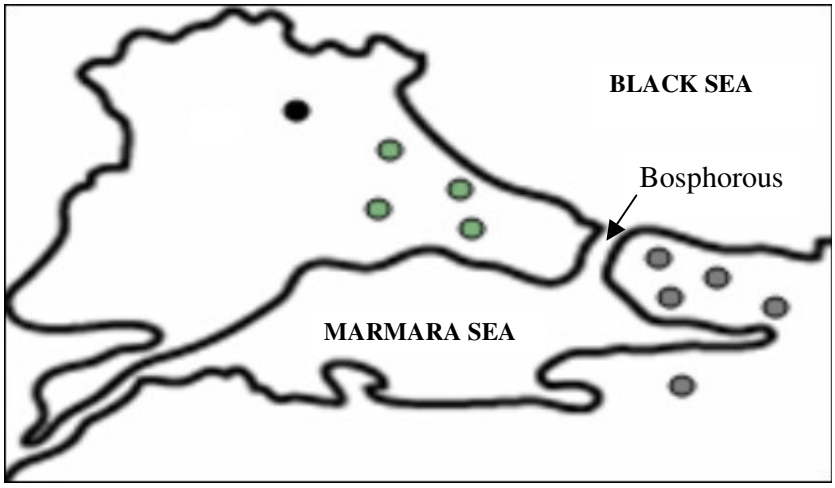


Figure 3.1 Map of approximate locations of sample collection in Northwest Turkey.

3.1. Plant Samples

The plant samples were collected from selected locations in both Anatolian Marmara and European Marmara regions of Turkey (Figure 3.1, Table 3.1). *G. plicatus* subsp. *byzantinus* were collected from 5 different sites in the Anatolian region of the Marmara. *G.* x*valentinei* nothosubsp. *byzantinus* was collected from 4 different sites in the European side of the Marmara. The *G. nivalis* in Turkey sample was collected further west from a town called Soğucak. Another *G. nivalis* sample and the outgroup species sample, *Sternbergia Lutea*, was kindly provided by the Botanical Gardens of Istanbul University, Department of Botany.

The samples were collected at the end of February, at the time they were flowering. They were identified according to their morphological features, and collected together with their bulbs. 10 bulbs per location were obtained and planted. Leaves of the plants were used as material for molecular analysis.

Table 3.1 Geographical coordinates of collected samples.

Sample	Taxanomic Nomenclature	Coordinates(N)	Coordinates(E)	Altitude(m)
1	*G. plicatus* subsp. *byzantinus*	N40 53' 11,3"	E29 41' 12,0"	493m
2	*G. plicatus* subsp *byzantinus*	N41 04' 09,2"	E29 20' 15,8"	18m
3	*G. plicatus* subsp *byzantinus*	N40 56' 36.9"	E29 15' 25,7"	444m
4	*G. plicatus* subsp *byzantinus*	N41 03' 58.7"	E29 20' 55.4"	20m
5	*G. plicatus* subsp *byzantinus*	N40 35' 54,7"	E29 16' 13,1"	86m
6	*G.* x*valentinei* nothosubsp *subplicatus*	N41 10' 52,2"	E28 24' 12,5"	51m
7	*G.* x*valentinei* nothosubsp *subplicatus*	N41 11' 10,5"	E28 24' 46,2"	32m
8	*G.* x*valentinei* nothosubsp *subplicatus*	N41 08' 03,8"	E28 27' 53,3"	NA*
9	*G.* x*valentinei* nothosubsp *subplicatus*	N41 22' 58,4"	E 28 11' 42,5"	227m
10	*G. nivalis (TR)*	N41 38' 53,2"	E27 39' 43,6"	300m
11	*G. nivalis (TR)*	NA*	NA*	NA*

*NA: not available

3.2. DNA Extraction and Molecular Analysis

Fresh leaf cells were lyzed using freeze fracturing; leaves were frozen in liquid nitrogen, powdered and preserved in –80 °C until further analysis. DNA from frozen powdered tissue was extracted using Qiagen Plant DNA Extraction Minikit.

3.2.1. PCR Amplification of Nuclear Ribosomal ITS Region

The amplification of the ribosomal intragenic spacer region (ITS1, ITS2 and 5.8S rDNA) was performed using the universal primers taken from White et al. (1990).

Forward primer **ITS5** 5'-GGAAGTAAAAGTCGTAACAAGG-3'
Reverse primer **ITS4** 5'-TCCTCCGCTTATTGATATGC-3'

The PCR reaction mix in 100 µL contained 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2mM of each dNTP (dATP, dCTP, dGTP and dTTP); 1 µM of each primer, 200 ng of DNA and 5U of *Taq* Polymerase (Promega). The amplification sequence had an initial denaturation step at 94 °C for 2 min 30 s. This step was followed by 30 cycles of the following reactions, denaturation at 95 °C for 30 s, annealing at 52 °C for 1 min 30 s, and an

elongation at 72 °C for 3 min. After these cycles, a final elongation step was included which kept the samples at 72 °C for 7 min. The obtained PCR products were purified using PCR Product Purification Kit (Progmega), following its procedures.

3.2.2. PCR Amplification of the Chloroplast Introns

The two non-coding regions of the chloroplast DNA, *trnL*(UAA) intron and the spacer between *trnL*(UAA) 3' and *trnF*(GAA) 5' were amplified using universal primers designed from conserved chloroplast tRNA gene sequences (Taberlet et al., 1991)

The *trnL* (UAA) intron was amplified using the following primers.

Forward primer **c**	5'-CGAAATCGGTAGACGCTACG-3'
Reverse primer **d**	5'-GGGGATAGAGGGACTTGAAC-3'

The intergenic spacer between the *trnL* (UAA) 3'exon and *trnF*(GAA) was amplified using the following primer pair.

Forward primer **e**	5'-GGTTCAAGTCCCTCTATCCC-3'
Reverse primer **f**	5'-ATTTGAACTGGTGACACGAG-3'

The PCR reaction was carried out using the same protocol as previously used in ITS amplification. The annealing temperature was similarly 52°C.

3.2.3. Gel Electrophoresis

The PCR products were run on 2% Agarose gels prepared in 1X TAE (Tris-acetate-EDTA) buffer. For the detection of bands under ultraviolet light, 2.5 µL of ethidium bromide was added to the gel. 10 µL of PCR products were loaded on the gel, mixed with 2 µL 6X loading dye (Promega). The products were run at 200V, for 30 min in an electrophoresis tank containing 1X TAE.

3.2.4. Sequencing of the PCR Products

Sequencing reactions were carried out in the Genetic Laboratories in Acıbadem Hospital, by Ms. Fulya Taylan. The reaction was done using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, USA). 3 µL of purified PCR products were added to the reaction mix containing 5 pmol of primer, 8 µL of sequencing reagent premix added to a total volume of 20 µL. The samples were sequenced with both forward and reverse primers separately. The cycle sequencing was performed in ABI 9700 Thermocycler (Applied Biosystems) with 25 cycles of 95 °C for 20 sec., 50 °C for 15 sec. and 60 °C for 60 sec. After cycle sequencing, the unbound dyes were removed by DyeEx 2.0 Dye removal Kit (Qiagen). The purified products were analyzed on the ABI 3100 Genetic Analyzer (Applied Biosystems).

3.3. Statistical Analysis

The chromatograms obtained from sequencing reactions were edited through 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 Clustal W computer program (Thompson et al., 1994).

The phylogenetic analyses of the sequences were executed on MEGA Version 2.1 (Kumar et al., 2001). The phylogenetic trees were constructed using the Neighbor-Joining (NJ) method (Saitou et al., 1987). Tamura Nei (Tamura and Nei, 1993) distances were used as the reference distances between the sequences examined, and used in the construction of the NJ trees. Tamura Nei method of distance calculation accounts for the substitution rate differences between nucleotides and the possible inequality of nucleotide frequencies.

NJ method is fast and provides branch lengths; however, it uses distances rather than discrete characters (Soltis et al., 2003). Hence, all analyses were repeated using max-mini branch and bound search of Maximum Parsimony (MP). The consensus phylogenetic tree of all possible parsimony trees was produced and inferred.

Bootstrapping (Felsenstein, 1985) was applied 1000 times to both methods of tree construction. Missing data or gaps in the aligned sequences were pair wise deleted for analysis. The corresponding sequences of an outgroup species, *Sternbergia Lutea*, were used for rooting all phylogenetic trees.

Table 3.2. The GENBANK[2] accession numbers of *Galanthus* species ITS region and chloroplast intron sequences.

Nuclear rDNA sequences (ITS1-5.8S-ITS2)

Accession Number	Taxa
AY101304	*G. plicatus*
AY101294	*G. alpinus*
AY101295	*G. cilicicus*
AY101296	*G. elwesii*
AY101297	*G. fosteri*
AY101298	*G. gracilis*
AY101299	*G. ikariae*
AY101300	*G. krassnovii*
AY101301	*G. lagodechianus*
AY101302	*G. nivalis*
AY101303	*G. platyphyllus*
AY101305	*G. regiane-olgae*
AY101306	*G. regiane-olgae subsp vernalis*
AY101307	*G. transcaucasius*
AY101308	*G.woronowii*

Chloroplast DNA sequences

Accession Number	Taxa
AY357136	*G. nivalis* (*trn*L inton and *trn*L-*trn*F spacer)
AF104730	*G. plicatus* (*trn*L-*trn*F spacer)
AF104799	*G. plicatus* (*trn*L intron)

[2] GENBANK is the genetic database that contains publicly available DNA sequences on the internet. (www.ncbi.nlm.nih.gov/)

3.4. Comparison with Other *Galanthus* Species

The taxons analyzed were compared with other *Galanthus* species, in order to relate these species to the rest of the *Galanthus* genera. Certain *Galanthus* species' ITS region sequences were available on GenBank database (Table 2.2). There were only two *trn*L(UAA) intron and *trn*L(UAA)-*trn*F(GAA) intragenic spacer region sequences belonging to *Galanthus* species, on Genbank.

For the comparison with other species, the taxons morphologically defined as *G.* x*valentinei* nothosubsp. *subplicatus*, *G. nivalis (TR)* and *G. plicatus* subsp. *byzantinus* were each represented by consensus sequences. These consensus sequences were produced via the sequences obtained from the samples collected from different locations, morphologically identified as belonging to the same taxon (Appendix I and II). The sequences were highly similar and only insertions/deletions that were explicitly due to sequencing error were eliminated. Appendix III contains all of the *Galanthus* series sequences analyzed in the thesis.

3.5. Checking for the Functionality of the ITS region

The G+C content of the ITS region was analyzed for possible pseudogene content. Additionally ITS1, 5.8S, and ITS2 sequences were screened separately to check for functionality.

3.5.1. Angiosperm 5.8S Specific Site

The universal primers that were used to amplify rDNA region are prone to amplifying contaminant DNA as they have been derived from fungi (White et al., 1990). Therefore the sequences were first screened for any fungal or algal contamination. The 5.8S rDNA coding sequence was determined, using instructions of Jobes and Thien (1997) and the conserved 14 base pair region that differentiates between flowering plants and fungi/algae was analyzed.

3.5.2. ITS1 Functionality

The conserved angiosperm motif in ITS1, GGCRY- (4 to 7n) -GYGYCAAGGAA (Liu and Schardl, 1994) was analyzed in order to check for the functionality of the region. This region is expected to harbor random mutations in the case of pseudogenes.

3.5.3. ITS2 Functionality: Through the analysis of Secondary mRNA Structure

The secondary structures of the consensus ITS2 sequences were analyzed to check for the functionality of these sequences. The secondary structure predictions were done using MFOLD[3] (Zucker et al., 1999). The thermodynamically optimal and suboptimal folding were computed at T= 20ºC and at T= 37ºC. Both temperatures yielded similar structures. The structures with the highest loop free energy at T= 20ºC were selected for comparison.

[3] MFOLD is available on http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html.

4. RESULTS AND DISCUSSION

4.1. The Phylogenetic Analysis of *Galanthus* Species through Nuclear ITS Region

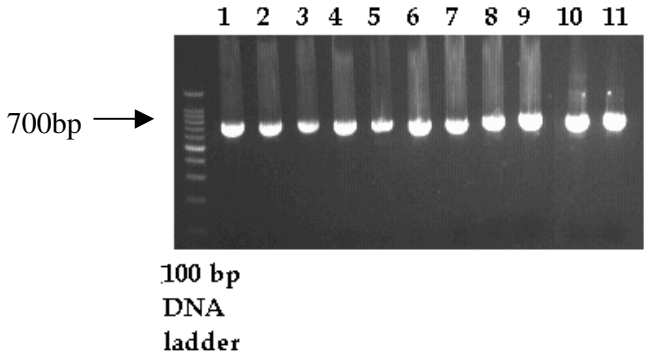


Figure 4.1. The gel electrophoresis of ITS region PCR products of samples.

The PCR products of the nuclear ITS region obtained from each of the 11 samples yielded a clear single band on electrophoresis gel. All samples had single bands that were representative of products of similar sizes, with similar electrophoretic mobility (Figure 4.1). The sequencing of these products revealed 650-652 base pairs per sample, with similar low range G+C values (Table 4.1). The chromatograms obtained during sequencing had low level of background noise and did not show any explicit heterogeneity or polymorphisms in any region of the samples (Figure 4.2).

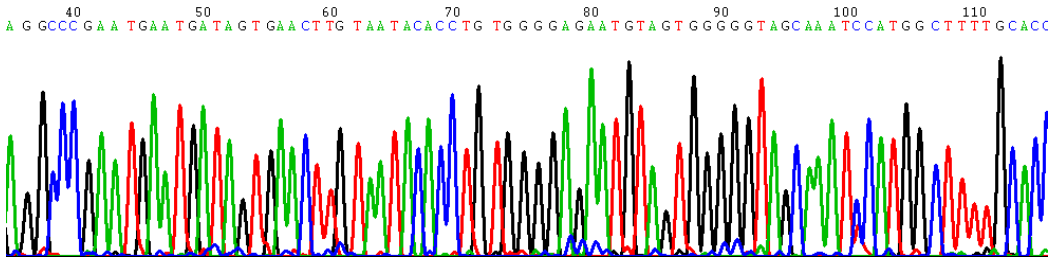


Figure 4.2. A section of the sequencing chromatogram of the ITS1 region of sample 11.

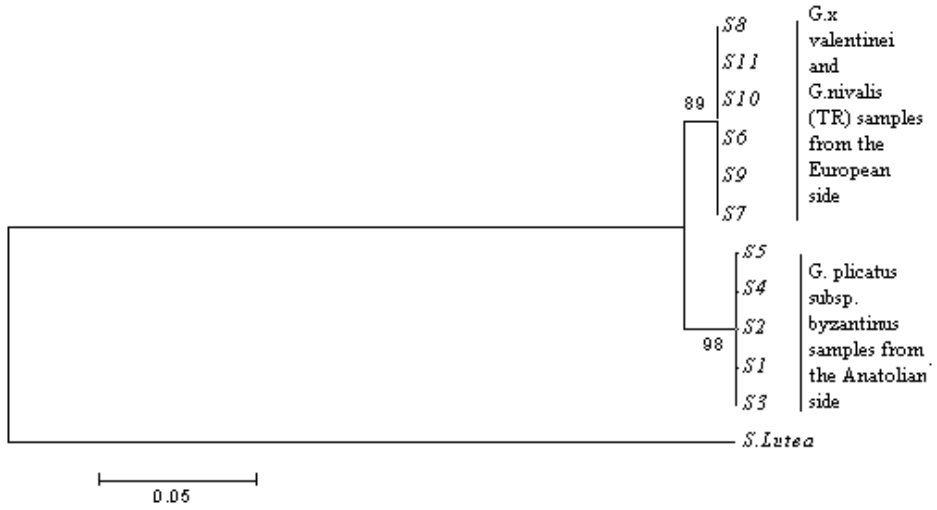
Table 4.1 The DNA content of ITS1-5.8S-ITS2 region of the samples.

		All Regions
Sample	**Taxanomic Identification**	*G+C content (%)*
S1	*Galanthus plicatus* subsp. *byzantinus*	44.09
S2	*Galanthus plicatus* subsp *byzantinus*	44.09
S3	*Galanthus plicatus* subsp *byzantinus*	44.17
S4	*Galanthus plicatus* subsp *byzantinus*	44
S5	*Galanthus plicatus* subsp *byzantinus*	44
S6	*Galanthus*x *valentinei* nothosubsp *subplicatus*	43.69
S7	*Galanthus*x *valentinei* nothosubsp *subplicatus*	43.69
S8	*Galanthus*x *valentinei* nothosubsp *subplicatus*	43.69
S9	*Galanthus*x *valentinei* nothosubsp *subplicatus*	43.69
S10	*Galanthus nivalis (TR)*	43.69
S11	*Galanthus nivalis (TR)*	43.69

The 5.8S rDNA coding sequence was determined, using instructions of Jobes and Thien (1997) and the conserved 14 base pair region that differentiates between flowering plants and fungi/algae was analyzed. The conserved region, which consists of an EcoRI site (**TTC**) in fungi and algae and none (**TCC**) in angiosperms, confirmed that all material obtained belonged to angiosperms. BLAST[4] sequence analysis software, revealed high similarity of these sequences to *Galanthus* species' rDNA regions, further confirming that these sequences belong to *Galanthus* species.

The sequences were compared using Tamura Nei distances and NJ method, which placed samples 1-5 and 6-11 grouping together, with very low distances within groups (Figure 4.3). The distances within samples of each node pointed out the similarity of sequences between these samples. In addition, the tree also reflected the geographical positioning of these samples, with samples 1-5 being collected from the Anatolian side of the Bosphorous, whereas the samples 6-11 collected from the European side.

[4] BLAST: Basic Local Alignment Search Tool is found on the GENBANK database website.



*Numbers indicate bootstrap values and branch lengths indicate distance. *S.Lutea* is used to root the tree.

Figure 4.3. The phylogenetic tree of the *Galanthus* samples constructed using rDNA (ITS1-5.8S-ITS2) sequences.

As the within group samples had very similar sequences supported by calculated close to zero distances, for further analysis one representative sequence, a consensus sequence was constructed from the different samples of each morphologically defined taxonomic group. *G.* x*valentinei* nothosubsp *subplicatus* and *G. nivalis(TR)* samples had identical sequences in spite of the intronic regions included, hence a single consensus sequence was done for both taxonomic units and will be referred to as *G.xvalntinei/niv(TR). G. plicatus* subsp. *byzantinus* samples' consensus sequence will be referred to as *G. plicatus sb.*

4.1.1 Functionality of the ITS Region

Ribosomal genes belong to a multi gene family, which evolve by concerted evolution, leading to inter-genic homogenization of each sequence (Baldwin et al., 1995). However, there is a possibility that some copies might degenerate into pseudogenes, and become inactive. These paralogous copies would accumulate mutations that can differ intraspecifically and would be misleading in phylogenetic analyses, when compared with other species. To eliminate such a possibility the samples' ITS regions were screened for functionality.

Primarily, there were no divergences in the sequences of different samples identified as the same taxon, suggesting that these sequences are preserved throughout the taxonomic unit. The G+C content of sequences can also be an indicator of functionality as very low G+C compositions can suggest pseudogenes. The ITS1 and ITS2 sequences obtained from *G.* x*valentinei* nothosubsp. *subplicatus/G.niv(TR)* and *G.plicatus* subsp. *byzantinus* had lower G+C content, 45.31 and 44.0 percent respectively, than the proposed angiosperm range (50%-75%) by Baldwin et al. (1995). However all other Galanthus species obtained from GENBANK also similarly had low G+C contents (43%-47%).

Additionally, for further analysis of the functionality of the region, ITS1 and ITS2 sequences were examined separately. The conserved angiosperm motif in ITS1, GGCRY-(4 to 7n) -GYGYCAAGGAA (Liu and Schardl, 1994) was found with one substitution at position 149 (C→T). However, this substitution was also present in all other *Galanthus* species examined (Figure 4.4).

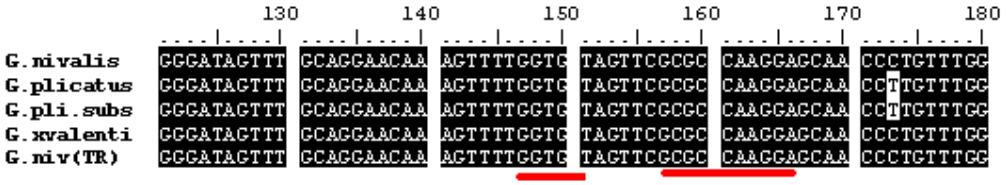


Figure 4.4. The conserved angiosperm 21 base pair core motif (underlined) of ITS1 in selected *Galanthus* species.

The analysis of ITS2 sequences was performed via predicted secondary structures. MFOLD program revealed around 9 optimal and suboptimal secondary folds for both sequences and the fold with the highest loop free energy was selected for comparison. The hypothetical secondary structures of *Gx.val/niv*(TR) and *G.plicatus* subsp. *byzantinus* were similar in structure, yielding 7 helices (Figure 4.5; Figure 4.6). The universal juxtaposition (U●U) in helix II was observed in both structures (Mai et al., 1997). The conserved GGU triplet in position 143-145 was also present, however at the 5' base of helix III and not the apex. The base differences between *Gx.val/niv(TR)* and *G.plicatus* subsp. *byzantinus*, showed that the former had correctional substitutions when compared to the latter.

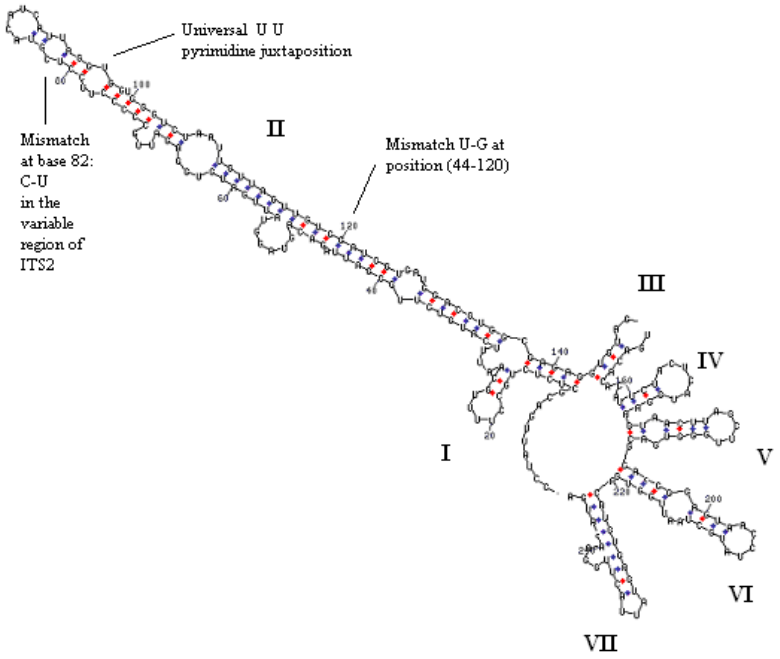


Figure 4.5. Predicted secondary structure of ITS2 mRNA of *G. plicatus* subsp. *byzantinus.*(dG = -91.1)

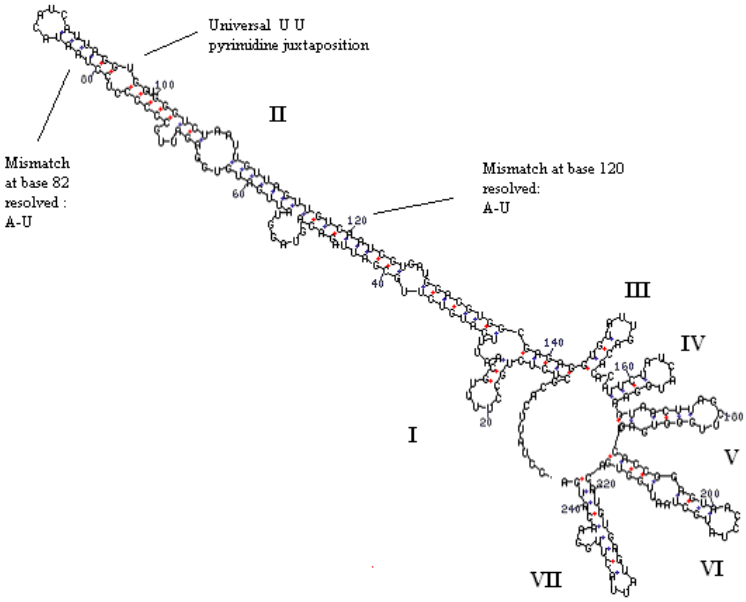
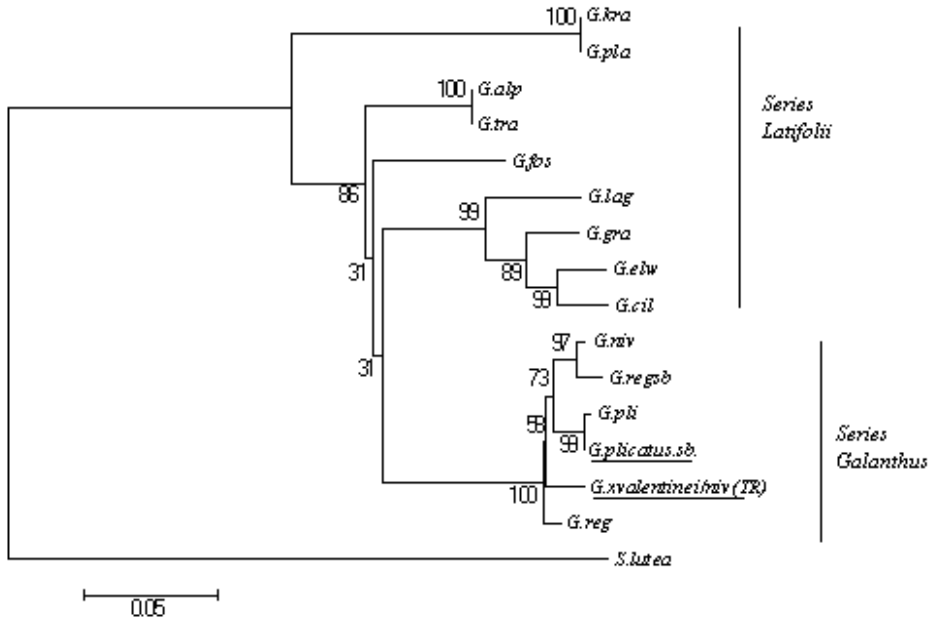


Figure 4.6. Predicted secondary structure of ITS2 mRNA of *G.* x*valentinei* nothosubsp. *subplicatus.* (dG = -94.3)

The detailed analyses of the conservation patterns in ITS1 and ITS2 regions and the lack of heterogeneity of sequences between different samples strongly suggest that the sequences analyzed are functional sequences and do not contain pseudogenes. Therefore, reliable phylogenetic inferences can be obtained via the use of these sequences.

4.1.2. Phylogenetic Analysis



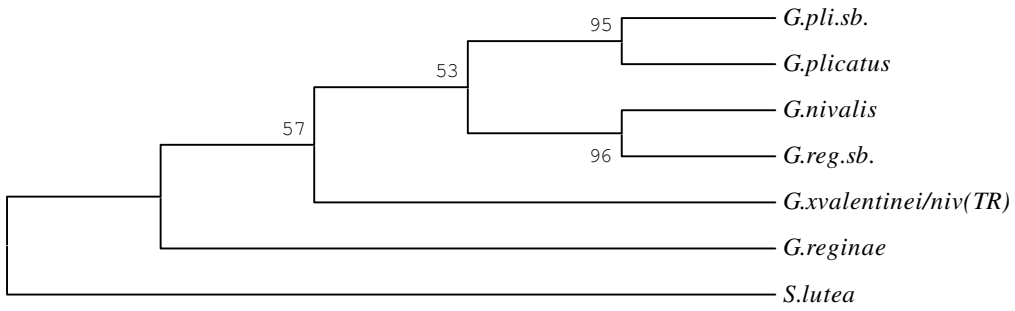
*Numbers indicate bootstrap values and branch lengths indicate distance. *S. Lutea* is used to root the tree.

Figure 4.7. The phylogenetic tree of *Galanthus* species constructed using rDNA region (ITS1-5.8S-ITS2) data.

The ITS region, including ITS1, ITS2 and 5.8S coding sequences, were compared between all available *Galanthus* species on Genbank, including *G.* x*valentinei/niv(TR)* and *G.plicatus subsp. byzantinus* using Tamura Nei distances and NJ (Figure 4.7). The Turkish samples clustered together with the *Galanthus* series described by Davis (1999) in agreement with morphological data. *G. plicatus* and its subspecies *G. plicatus* subsp. *byzantinus* grouped together with high bootstrap value. *G.nivalis* and *G. reginae* subsp. *vernalis* also grouped together in agreement with morphological data, suggesting closer relatedness of *G.reginae* subsp. *vernalis* to *G.nivalis* than *G.reginae* (Davis et al., 2001).

G.xvalentinei/niv(TR) remained on a separate branch. The *Galanthus* series species separated from the rest of the *Galanthus* species with 100 percent bootstrap value.

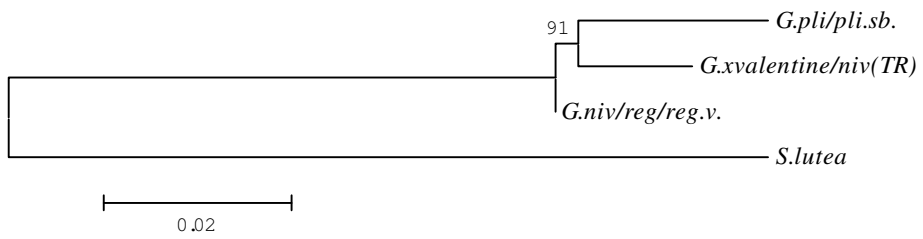
Similarly, the detailed analysis of the variable intronic regions ITS1 and ITS2 of the *Galanthus* series, using Maximum Parsimony branch and bound method (tree length = 197 steps, CI = 0.97, RI = 0.75, number of sites = 455, repeated 1000 times) showed *G. plicatus* grouping together with its subspecies *G. plicatus* subsp.*byzantinus* (Figure 4.8). *G.*x*valentinei/nivalis (TR)* was placed again on a separate branch, not clustering with either *G. plicatus* or *G. nivalis*.



*Numbers indicate bootstrap values. *S. Lutea* is used to root the tree.

Figure 4.8. The consensus MP tree of the *Galanthus* series, ITS1 and ITS2 sequences.

The phylogenetic analysis using NJ with Tamura Nei distances, of the highly conserved 5.8S rDNA region showed that *G.nivalis*, *G. reginae* and *G. reginae* subsp. *vernalis* shared identical sequences. *G. plicatus* and its subspecies *G. plicatus* subsp. *byzantinus* also had identical sequences, both similarities referring to the slow rate of evolution of this region. In the constructed phylogenetic tree, *G.*x *valentinei/niv(TR)*, which had its own unique sequence, was placed on the same node as *G. plicatus* and its subspecies, indicating closer relatedness to *G. plicatus* with respect to *G. nivalis* and its close relatives (Figure 4.9).



* Numbers indicate bootstrap values and branch lengths indicate distance. *S. Lutea* is used to root the tree.

Figure 4.9. The phylogenetic tree of the *Galanthus* series including the Turkish samples with 5.8S rDNA sequences.

In summary, nuclear ITS data confirmed that *G.plicatus* subsp.*byzantinus* is a subspecies of *G. plicatus*, by the clustering of the two taxa in all phylogenetic trees. *G.*x*valentinei* nothosubsp. *subplicatus* and *G. nivalis(TR)* samples had identical sequences of the ITS region, indicating that these samples belong to the same taxonomic unit. The unique sequence of *G.xvalentinei/niv(TR)* when compared to other closely related species, suggests that it can be classified as a separate taxonomic unit. The phylogenetic trees also suggest that *G.xvalentinei/niv(TR)* shows closer relatedness to *G. plicatus* than to *G. nivalis.*

4.2. Phylogenetic Analysis of the *Galanthus* Species through Chloroplast Introns

The PCR products of the samples' chloroplast *trn*L(UAA) intron and the spacer between *trn*L(UAA)-*trn*F(GAA) genes showed clear bands with similar sizes and electrophoretic mobility (Figure 4.10). The chromatograms obtained from sequencing of these samples also had low level of background noise.

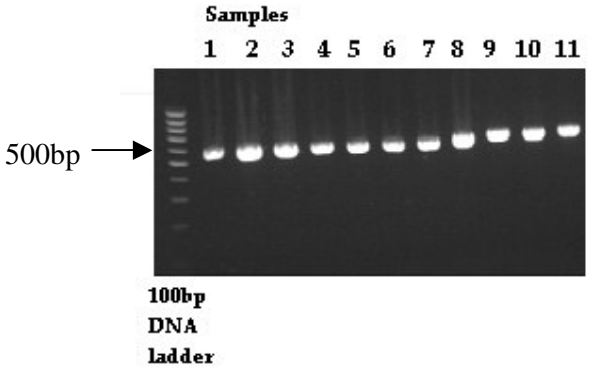


Figure 4.10. The gel electrophoresis of *trn*L(UAA) intron PCR products of the samples.

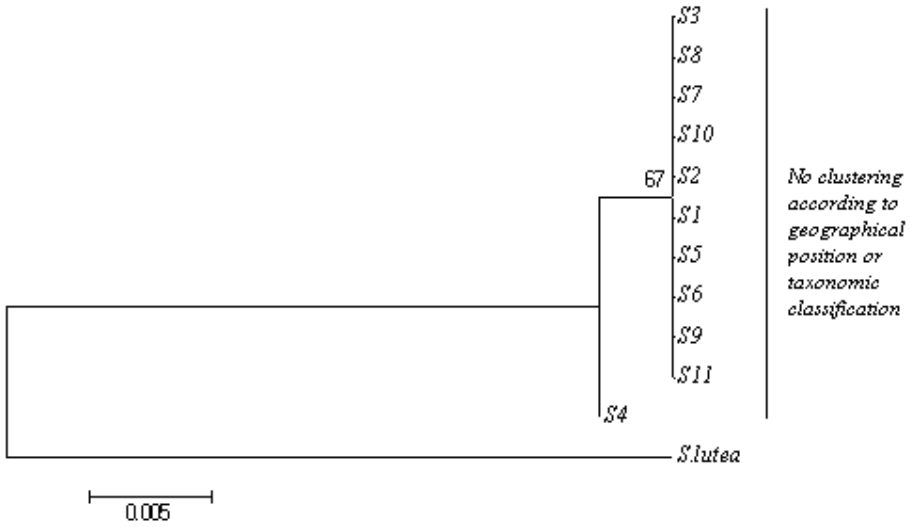
The intragenic spacer between *trn*L(UAA) 3' exon and *trn*F(GAA) 5' of the different samples had lengths between 350-353 bases with low G+C content in all samples (Table 4.2). In addition, the group I intron, *trn*L(UAA), was found to be 466 bases long and having higher G+C contents, most likely due to its functional property.

Table 4.2. The G+C content of the samples' chloroplast intron DNA.

Sample	**Taxanomic Identification**	**trnL(UAA) intron** *G+C content (%)*	**trnL(UAA)-trnF(GAA) spacer** *G+C content (%)*
S1	*Galanthus plicatus* subsp. *byzantinus*	34.55	28.77
S2	*Galanthus plicatus* subsp *byzantinus*	34.55	28.77
S3	*Galanthus plicatus* subsp *byzantinus*	34.55	28.77
S4	*Galanthus plicatus* subsp *byzantinus*	34.55	28.57
S5	*Galanthus plicatus* subsp *byzantinus*	34.55	28.57
S6	*Galanthus*x *valentinei* nothosubsp *subplicatus*	34.12	28.77
S7	*Galanthus*x *valentinei* nothosubsp *subplicatus*	34.12	28.69
S8	*Galanthus*x *valentinei* nothosubsp *subplicatus*	34.12	28.49
S9	*Galanthus*x *valentinei* nothosubsp *subplicatus*	34.12	28.41
S10	*Galanthus nivalis (TR)*	34.33	28.77
S11	*Galanthus nivalis (TR)*	34.33	28.77

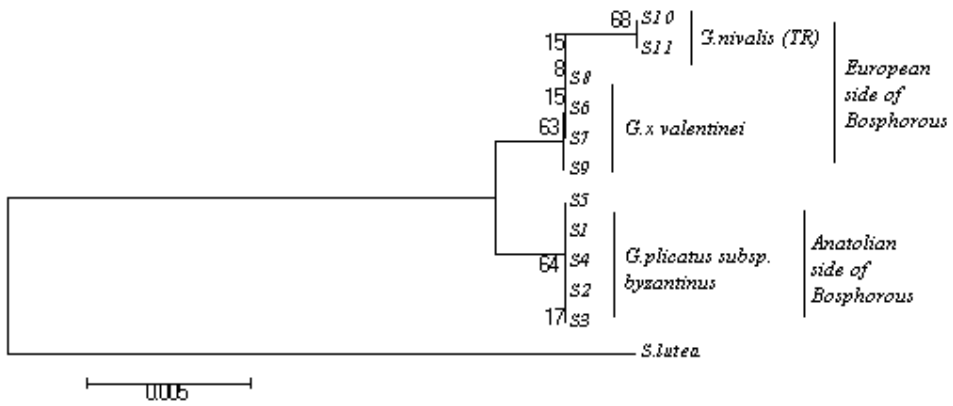
The detailed analysis of the chloroplast sequences showed that even though these sequences are introns, they are highly conserved between closely related taxonomic units. The spacer between the chloroplast *trn*L(UAA) and *trn*F(GAA) genes were found to have accumulated very few random point mutations differing between the samples of the same taxon, unlike the uniformity observed with the nuclear ITS sequences. However, overall there was high similarity between all samples examined, which did not show any clustering according to geography or taxonomic classification (Figure 4.11).

The phylogenetic analysis using Tamura Nei distances and NJ method of the group I intron *trn*L(UAA) showed, similar to ITS results, Anatolian *G. plicatus* subsp. *byzantinus* samples grouping together, and samples from the European side, *G.* x*valentinei* nothosubsp. *subplicatus* and *G. nivalis(TR)* clustering together, but in different nodes, projecting the geographical separation (Figure 4.12). In contrast to the ITS results, *G. x valentinei* nothosubsp. *subplicatus* and *G.nivalis (TR)* did not have identical sequences as they differed consistently by one base out of the 466 compared. However, all nodes had low bootstrap value (63-68%), pointing out the high similarity of the different sequences.



*Numbers indicate bootstrap values and branch lengths indicate distance. *S. Lutea* is used to root the tree.

Figure 4.11. The phylogenetic tree of *Galanthus* samples using chloroplast intragenic spacer between *trn*L(UAA) and *trn*F(GAA) genes.

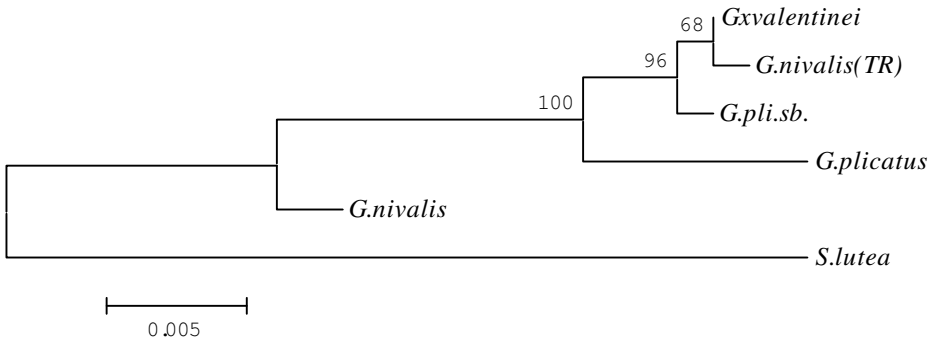


*Numbers indicate bootstrap values and branch lengths indicate distance. *S. Lutea* is used to root the tree.

Figure 4.12. The phylogenetic tree of *Galanthus* samples with chloroplast trnL(UAA) intron data.

4.2.1. Phylogenetic Analysis of the Chloroplast Introns

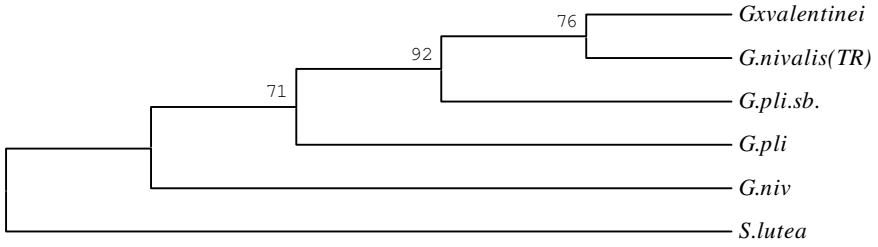
For each morphologically defined taxonomic unit a consensus sequence was made, from the different samples collected from selected regions. There were only two species' sequences that were available on GenBank, *G. plicatus* and *G. nivalis* that could be used for these comparisons. The NJ tree constructed from both introns added together showed the Turkish samples clustering together, sharing a node with *G. plicatus* with high bootstrap value (Figure 4.13).



* Numbers indicate bootstrap values and branch lengths indicate distance. *S. Lutea* is used to root the tree.

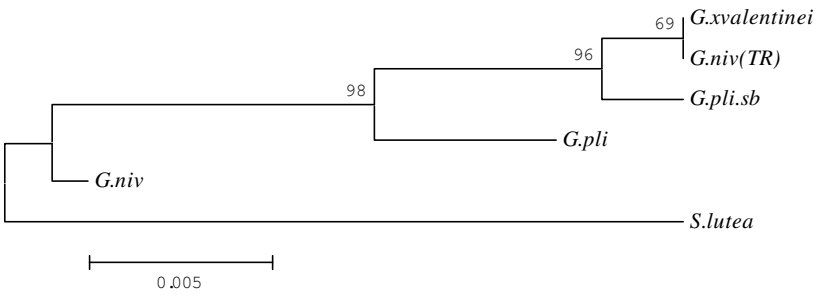
Figure 4.13. The phylogenetic tree of the *Galanthus* series using chloroplast *trn*L(UAA) data and the spacer between *trn*L(UAA) 3'exon and *trn*F(GAA).

Consequently, the comparison of the functional, group I intron, *trn*L(UAA) sequences alone also showed the Turkish samples sharing a node with *G. plicatus* and not with *G. nivalis,* in trees constructed both from Maximum Parsimony branch and bound method. (tree length = 313 steps, CI = 0.99, RI = 0.83, number of sites = 466) and NJ method using Tamura Nei distances (Figure 4.14; Figure 4.15 respectively).



* Numbers indicate bootstrap values. *S. Lutea* is used to root the tree

Figure 4.14. The MP consensus parsimony tree of the *Galanthus* series with *trn*L(UAA) Group I intron.



* Numbers indicate bootstrap values and branch lengths indicate distance. *S. Lutea* is used to root the tree

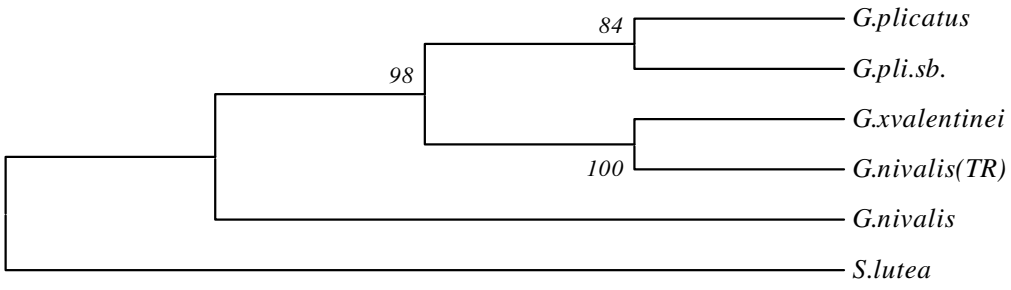
Figure 4.15. The phylogenetic tree of the *Galanthus* series using chloroplast trnL(UAA) intron data.

These analyses show that even though the chloroplast introns are highly conserved between close species, they are also phylogenetically informative. In addition, as chloroplast DNA is maternally inherited in angiosperms, it can reflect the path of gene flow. In the trees obtained, all Turkish samples clustered closely together reflecting their

close geographical proximities. Besides, all samples examined seem to be closely related to *G. plicatus* and not *G. nivalis.* These data are in concordance with the conserved nuclear 5.8S rDNA data, which showed that *G. plicatus* subsp. *byzantinus*, *G.* x*valentinei* nothosubsp. *subplicatus* and *G. nivalis(TR)* samples are closer to *G. plicatus* than to *G. nivalis*. However, *G.plicatus* subsp. *byzantinus* surprisingly had closer relatedness to *G.* x*valentinei* nothosubsp. *subplicatus* than to *G. plicatus* as in the nuclear data.

A consistent difference of one base was observed between *G.* x*valentinei* nothosubsp. *subplicatus* and *G. nivalis(TR) trn*L(UAA) intron samples, however, between all other taxonomic groups examined there were at least 7 base differences observed (e.g. between *G. plicatus* and *G. plicatus* subsp. *byzantinus*). Together with the findings of identical ITS and trnL(UAA)-trnF(GAA) spacer sequences, with a single base pair difference in the trnL(UAA) intron, it can strongly be inferred that *G.* x*valentinei* nothosubsp. *subplicatus* and *G. nivalis(TR)* belong to the same taxonomic unit.

4.3. The Combined Analysis of Nuclear and Chloroplast Markers



* Numbers indicate bootstrap values. *S. Lutea* is used to root the tree.

Figure 4.16. The MP consensus tree of the chloroplast introns and nuclear ITS regions analyzed together.

Adding the sequences obtained from both genomes and increasing the number of parsimony informative sites in a single MP branch and bound analysis (number of sites =1491; number of parsimony informative sites= 30, tree length 259, CI= 0.98, RI= 0.85) again showed *G.* x*valentinei* nothosubsp. *subplicatus* samples relating closer to *G. plicatus* than *G. nivalis* (Figure 4.16). *G. plicatus* is naturally found in Anatolian Turkey, Romania and Crimea (Davis et al., 2001) which reflects on and explains the close phylogeny observed with the analyzed Turkish samples.

The two dimensional representations of Tamura Nei distances of the Turkish samples with respect to *G. plicatus* and *G. nivalis* were constructed (Figure 4.17; Figure 4.18). Both graphs revealed that *G.* x*valentinei* nothosubsp. *subplicatus* has similar chloroplast DNA distances as *G. plicatus* subsp. *byzantinus* to both *G. plicatus* and *G. nivalis*. This reflects the slow evolution of these sequences, the close geographical positioning of the samples and hence the gene flow.

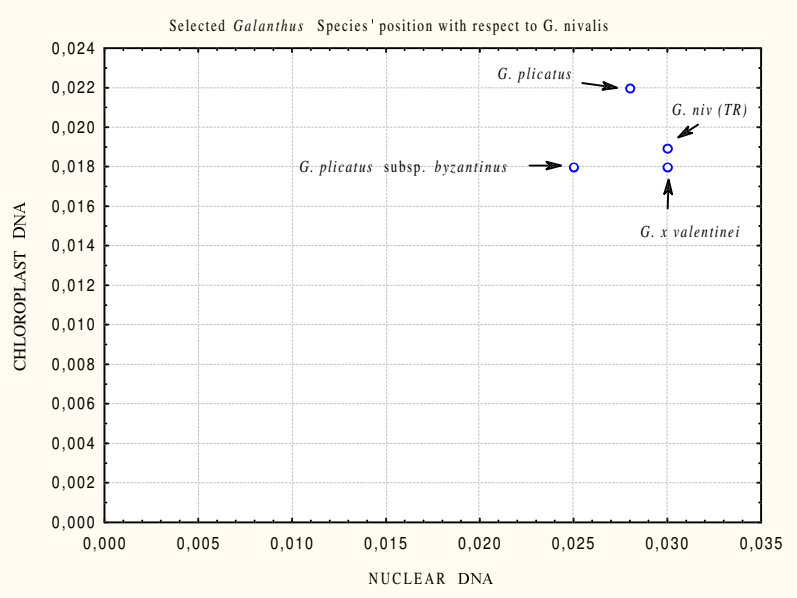


Figure 4.17. 2-D representation of chloroplast and nuclear DNA distances with respect to *G. nivalis*.

Furthermore these graphs show *G.* x*valentinei* nothosubsp. *subplicatus* standing equidistant to both *G. plicatus* and *G. nivalis* when comparing the nuclear sequences. This slightly contradicts the phylogenetic trees obtained using these distances in previous chapters, as they were showing closer relatedness to *G. plicatus* rather than *G. nivalis*. However, these 2-dimensional presentations only reflect distances and not the evolutionary quality of the differences hence, it can be inferred that *G.* x*valentinei* nothosubsp. *subplicatus/ G. nivalis (TR)* has its own unique nuclear ITS region sequence, with similar number of substitutions compared to both *G. plicatus* and *G. nivalis*. Only the phylogenetic trees infer that these substitutions are evolutionarily more related to *G. plicatus*. Additionally, nuclear distances showed *G. plicatus* subsp. *byzantinus* standing much closer to *G. plicatus* than to *G. nivalis*.

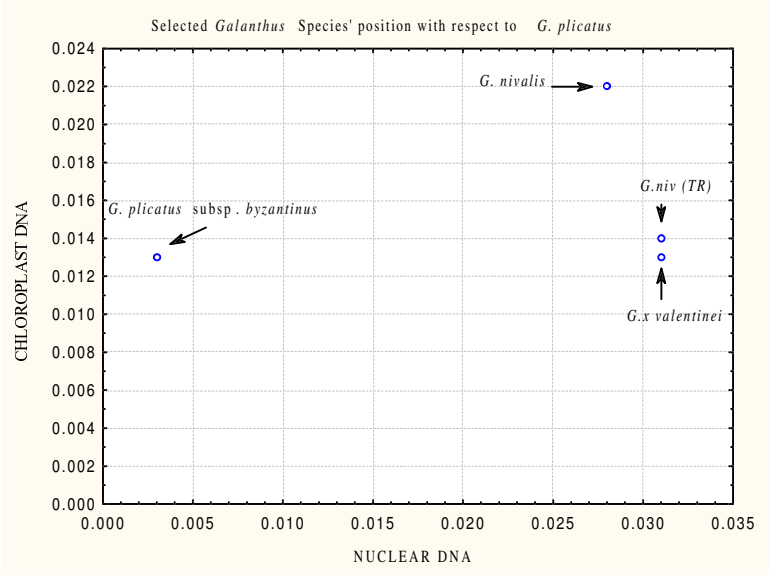
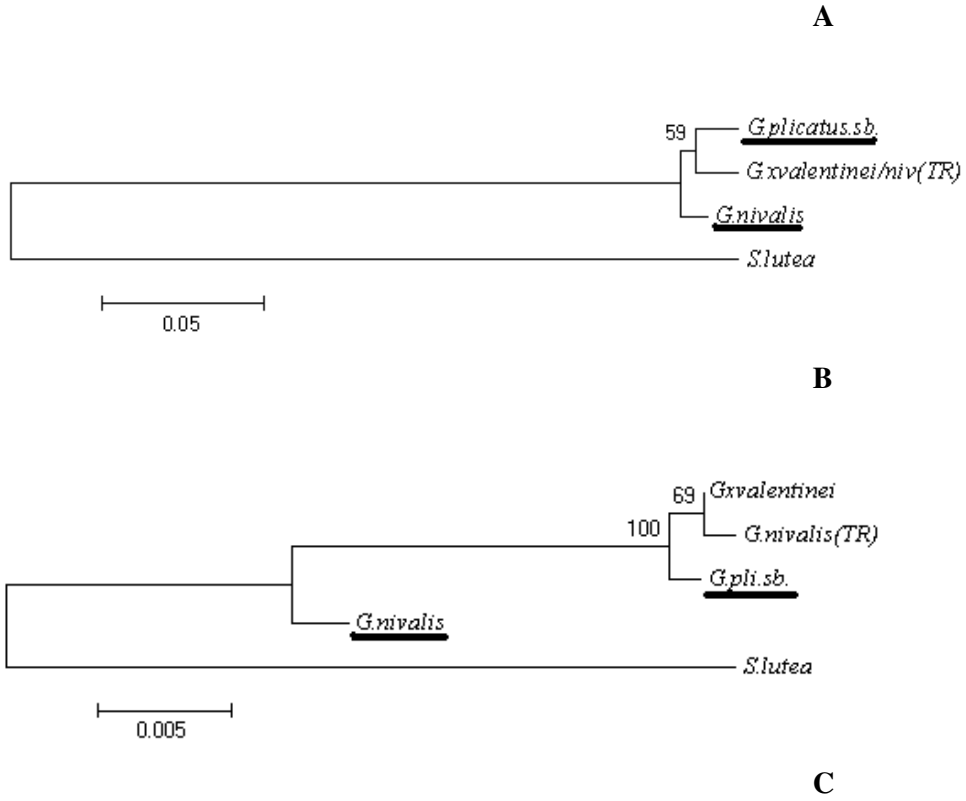


Figure 4.18. 2-D representation of chloroplast and nuclear DNA distances with respect to *G. plicatus*.

4.4. Is *G.* x*valentinei* nothosubsp. *subplicatus* a Hybrid?

There is literature identifying *G.* x*valentinei* nothosubsp. *subplicatus* as a hybrid swarm (Davis et al., 2001; Brickell, 1984; Webb, 1974). Morphologically it has been described as showing traits from both *G. nivalis* and *G. plicatus* subsp. *byzantinus*, and garden hybrids produced from these two species have similar features (Davis et al., 2001).

However, the genomic markers analyzed in this study show no indications of the *G.* x*valentinei* nothosubsp. *subplicatus* samples belonging to a hybrid taxa. ITS sequence repeats are identical via concerted evolution and would show heterogeneity with recent hybridization events (Baldwin et al., 1995). All ITS sequences belonging to different samples of *G.* x*valentinei* nothosubsp. *subplicatus* are homogeneous, monophyletic, and most importantly, identical. Hence the sequences, which are also unique when compared to other close species, are suggestive of a stable species.



*Tamura Nei distances with NJ. Numbers indicate bootstrap values and branch length indicate distance.

Figure 4.19. The phylogenetic trees of potential parents (underlined) with *G.* x*valentinei* nothosubsp. *subplicatus* (A: Both nuclear and chloroplast DNA data; B: only nuclear DNA data; C: only chloroplast nuclear data.).

Concerted evolution can act fast and lead to the preservation of either parent's sequences or a chimeric sequence of both parents, which would lead to homogenized sequences (Baldwin et al., 1995). However, this does not seem to be the case with *G.* x*valentinei* nothosubsp. *subplicatus* since there are no indications of a hybrid sequence or high similarity to either putative parent. Yet, another possibility would be that hybrid species would also show incongruence between chloroplast and nuclear data (Baldwin et al., 1995). Again, both genomic analyses show similar results (Figure 4.19).

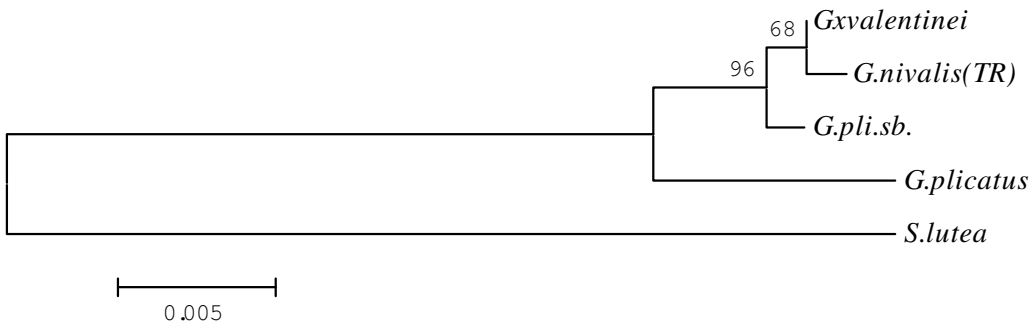
Total nuclear DNA content show *G. nivalis* having an average of 72.2 pg, whereas *G. plicatus*, *G. plicatus* subsp. *byzantinus* and *G.* x*valentinei* nothosubsp. *subplicatus* have on average 55.4 pg, 57.9 pg and 54.4 pg (Zonneveld et al., 2003). A garden hybrid between *G. nivalis* and *G. plicatus* subsp. *byzantinus* shows an expected intermediate value of 64.6 pg and not 54.4 pg (Zonneveld et al., 2003). These data are in congruence with the data analyzed in this thesis, identifying both *G.* x*valentinei* nothosubsp. *subplicatus* and *G. plicatus* subsp. *byzantinus* as having similar nuclear content with *G. plicatus* and not with *G. nivalis*. The lack of *G. nivalis* in close geographical proximity to *G.* x*valentinei* nothosubsp. *subplicatus* populations also supports against the hybrid theory. Even though morphologically, individuals show variations of both "parents", there are currently no molecular data that can support it.

4.5. Could *G. plicatus* subsp. *byzantinus* be a Hybrid?

*Tamura Nei distances with NJ. Numbers indicate bootstrap values and branch length indicate distance.

Figure 4.20. The phylogenetic tree of *G. plicatus* subsp. *byzantinus* with *G.* x*valentinei* nothosubsp. *subplicatus* and *G. plicatus* using the ITS region.

The molecular DNA sequences obtained from populations of *G. plicatus* subsp. *byzantinus* show incongruent chloroplast and nuclear data (Figures 4.18, 4.20 and 4.21). Nuclear ITS sequences places *G. plicatus* subsp. *byzantinus* in close relatedness with *G. plicatus*, whereas chloroplast introns suggest very close association to *G.* x*valentinei* nothosubsp. *subplicatus* and not *G. plicatus*.



*Tamura Nei distances with NJ. Numbers indicate bootstrap values and branch length indicate distance.

Figure 4.21. The phylogenetic tree of *G. plicatus* subsp. *byzantinus* with *G.* x*valentinei* nothosubsp. *subplicatus* and *G. plicatus* using chloroplast introns.

Contrasting nuclear and chloroplast data suggest that there could be different levels of introgression and that the species under question could be an ancient hybrid. If *G.* x*valentinei* nothosubsp. *subplicatus* is defined as a stable taxonomic unit, then we can infer that *G. plicatus* subsp. *byzantinus* could be an ancient hybrid between *G. plicatus* and *G.* x*valentinei* nothosubsp. *subplicatus.* The different populations of *G. plicatus* subsp. *byzantinus* in Marmara had similar functional ITS sequences that is not representative of a recent hybrid swarm. Hence, it can only be concluded that *G. plicatus* subsp. *byzantinus* could be an ancient hybrid, that has stabilized into an established taxonomic unit.

G. plicatus is found in the western and central part of the Black Sea region in the North of Turkey (Bishop M., et al., 2001). Even though today, these species do not overlap, the spatial location of the three species adds weight to an ancient hybridization hypothesis (Figure 4.22). However, actual *G. plicatus* samples from Black Sea region

should be collected and the molecular markers analyzed, in order to make a thorough conclusion. The *G. plicatus* sequences that have been included in this analysis are from GENBANK, with unknown origin.

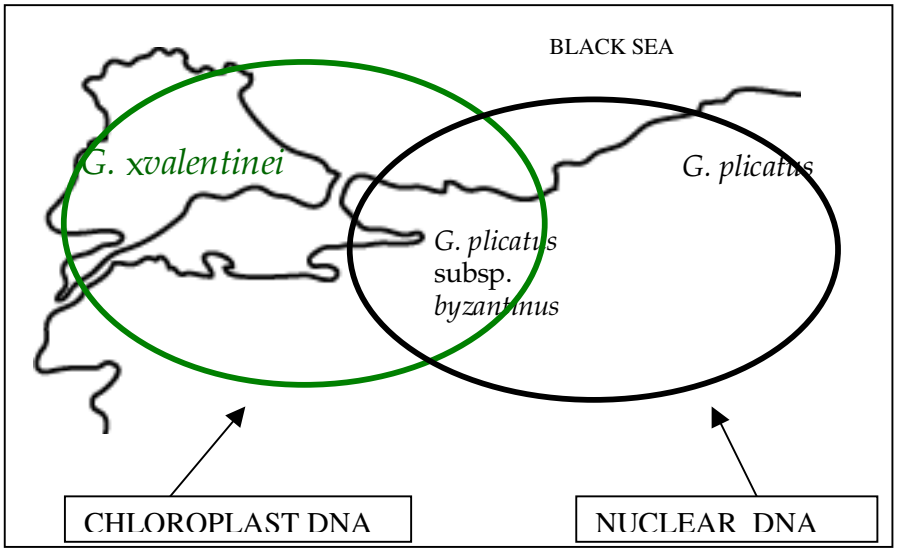


Figure 4.22. The map of distribution of *G. plicatus* subsp. *byzantinus, G.* x*valentinei* nothosubsp. *subplicatus* and *G. plicatus* in Turkey. The circles represent the DNA regions that are close to identical between species.

5. CONCLUSIONS

The samples morphologically identified as *G. nivalis* found in Turkey [abbreviated as *G. nivalis(TR)* in the thesis], were not genetically identical to the *G. nivalis* recognized in Europe. More samples from different locations close to the border should be analyzed, but as of yet, it can be suggested that there is no *G. nivalis* present in Turkey-in-Europe.

Samples taken from the European side of the Bosphorous and morphologically identified as *G. nivalis* seems to belong to the same taxonomic unit as *G.* x*valentinei* nothosubsp. *subplicatus*. All samples had identical nuclear ITS sequences and highly similar chloroplast introns. The phylogenetic analyses of the chloroplast sequences revealed closer relatedness of *G.* x*valentinei* nothosubsp. *subplicatus* and *G. nivalis(TR)* to *G. plicatus* and *G. plicatus* subsp. *byzantinus* than to *G. nivalis*. The phylogenetic analysis of the nuclear ITS sequences placed this taxonomic group onto a separate branch in the clade representing the *Galanthus* series. These results suggest that *G.* x*valentinei* nothosubsp. *subplicatus*/*G. nivalis(TR)* samples are of the same taxonomic unit.

G. x*valentinei* nothosubsp. *subplicatus* has been morphologically defined as a hybrid. However, there were no indications of recent hybridization or instability in any of the molecular markers examined. All markers were consistent and showed unique sequences. It seems that *G.* x*valentinei* nothosubsp. *subplicatus* is a genetically stable taxonomic unit however showing a range of morphological markers.

The samples morphologically identified as *G. plicatus* subsp. *byzantinus* are on the nuclear DNA level very similar to *G. plicatus*. The chloroplast sequences, however, are very similar to those of *G.* x*valentinei* nothosubsp. *subplicatus.* This inconsistency could be indicative of an ancient hybrid species, however more molecular analyses need to be done.

Both *G. nivalis* and *G. plicatus* subsp. *byzantinus* have been described as being under threat of hybridization in Turkey-in-Europe (Anonymous, 1999). These data suggest that there are probably no pure *G. plicatus* subsp. *byzantinus* or *G. nivalis* in the European side of the Bosphorous. *G. plicatus* subsp. *byzantinus* samples can only be found in the Asian side of the Bosphorous. And there are probably no *G. nivalis* in Turkey. These data should be incorporated into plant conservation databases.

The plants that are present in Turkey-in-Europe seem to be a stable taxonomic unit with a unique genome. Population ecology studies need to be conducted for re-naming and re-defining the conservation status of these plants. *G.* x*valentinei* nothosubsp. *subplicatus* is listed as near threatened, however if the range of this plant is only limited within the Turkish borders (less than 20.000 km^2), it might be listed as vulnerable and endemic, which would lead to changes in the current conservation policies. These data need to be matched up with population ecology studies, immediately, to be effective in the preservation of these *Galanthus* species.

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Appendix A. Construction of the consensus ITS region sequences.
Samples 1-5: *G. plicatus* subsp. *byzantinus*, 6-9: *G.*x*valentinei* nothosubsp. *subplicatus* and 10-11: *G. nivalis(TR)*

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55
S-01            TTGAGGCCCG AATGAATGAT AGCGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG
S-02            TTGAGGCCCG AATGAATGAT AGCGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG
S-03            TTGAGGCCCG AATGAATGAT AGCGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG
S-04            TTGAGGCCCG AATGAATGAT AGCGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG
S-05            TTGAGGCCCG AATGAATGAT AGCGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG
G.pli.sb.       TTGAGGCCCG AATGAATGAT AGCGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG

S-06            TTGAGGCCCG AATGAATGAT AGTGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG
S-07            TTGAGGCCCG AATGAATGAT AGTGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG
S-08            TTGAGGCCCG AATGAATGAT AGTGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG
S-09            TTGAGGCCCG AATGAATGAT AGTGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG
G.xval/n(TR)    TTGAGGCCCG AATGAATGAT AGTGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG
S-10            TTGAGGCCCG AATGAATGAT AGTGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG
S-11            TTGAGGCCCG AATGAATGAT AGTGAACTTG TAATACACCT GTGGGGAGAA TGTAGTGGGG


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
S-01            GTAGCAAATC CATGGCTTTT GCACCTTATG GTGCCCTTGT CATCGTCACC TTGCATGTTG
S-02            GTAGCAAATC CATGGCTTTT GCACCTTATG GTGCCCTTGT CATCGTCACC TTGCATGTTG
S-03            GTAGCAAATC CATGGCTTTT GCACCTTATG GTGCCCTTGT CATCGTCACC TTGCATGTTG
S-04            GTAGCAAATC CATGGCTTTT GCACCTTATG GTGCCCTTGT CATCGTCACC TTGCATGTTG
S-05            GTAGCAAATC CATGGCTTTT GCACCTTATG GTGCCCTTGT CATCGTCACC TTGCATGTTG
G.pli.sb        GTAGCAAATC CATGGCTTTT GCACCTTATG GTGCCCTTGT CATCGTCACC TTGCATGTTG

S-06            GTAGCAAATC CATGGCTTTT GCACCTTATG GTACCCTTGT CATTGTCACC TTGCATGTTG
S-07            GTAGCAAATC CATGGCTTTT GCACCTTATG GTACCCTTGT CATTGTCACC TTGCATGTTG
S-08            GTAGCAAATC CATGGCTTTT GCACCTTATG GTACCCTTGT CATTGTCACC TTGCATGTTG
S-09            GTAGCAAATC CATGGCTTTT GCACCTTATG GTACCCTTGT CATTGTCACC TTGCATGTTG
G.xval/n(TR)    GTAGCAAATC CATGGCTTTT GCACCTTATG GTACCCTTGT CATTGTCACC TTGCATGTTG
S-10            GTAGCAAATC CATGGCTTTT GCACCTTATG GTACCCTTGT CATTGTCACC TTGCATGTTG
S-11            GTAGCAAATC CATGGCTTTT GCACCTTATG GTACCCTTGT CATTGTCACC TTGCATGTTG


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
S-01            TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCTTGTT
S-02            TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCTTGTT
S-03            TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCTTGTT
S-04            TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCTTGTT
S-05            TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCTTGTT
G.pli.sb        TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCTTGTT

S-06            TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCCTGTT
S-07            TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCCTGTT
S-08            TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCCTGTT
S-09            TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCCTGTT
G.xval/n(TR)    TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCCTGTT
S-10            TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCCTGTT
S-11            TGTGGGATAG TTTGCAGGAA CAAAGTTTTG GTGTAGTTCG CGCCAAGGAG CAACCCTGTT
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
S-01            TGGATTGAGT AGTATGTGAC AAGTATTATA TTGTAGTATG TGGCATGATC TCGATATATT
S-02            TGGATTGAGT AGTATGTGAC AAGTATTATA TTGTAGTATG TGGCATGATC TCGATATATT
S-03            TGGATTGAGT AGTATGTGAC AAGTATTATA TTGTAGTATG TGGCATGATC TCGATATATT
S-04            TGGATTGAGT AGTATGTGAC AAGTATTATA TTGTAGTATG TGGCATGATC TCGATATATT
S-05            TGGATTGAGT AGTATGTGAC AAGTATTATA TTGTAGTATG TGGCATGATC TCGATATATT
G.pli.sb        TGGATTGAGT AGTATGTGAC AAGTATTATA TTGTAGTATG TGGCATGATC TCGATATATT

S-06            TGGATTGAGT AGTGTGTGAC AAGTATTATA TTATAGTACG TGGCATGATC TCGATATATT
S-07            TGGATTGAGT AGTGTGTGAC AAGTATTATA TTATAGTACG TGGCATGATC TCGATATATT
S-08            TGGATTGAGT AGTGTGTGAC AAGTATTATA TTATAGTACG TGGCATGATC TCGATATATT
S-09            TGGATTGAGT AGTGTGTGAC AAGTATTATA TTATAGTACG TGGCATGATC TCGATATATT
G.xval/n(TR)    TGGATTGAGT AGTGTGTGAC AAGTATTATA TTATAGTACG TGGCATGATC TCGATATATT
S-10            TGGATTGAGT AGTGTGTGAC AAGTATTATA TTATAGTACG TGGCATGATC TCGATATATT
S-11            TGGATTGAGT AGTGTGTGAC AAGTATTATA TTATAGTACG TGGCATGATC TCGATATATT


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   245        255        265        275        285        295
S-01            AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA
S-02            AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA
S-03            AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA
S-04            AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA
S-05            AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA
G.pli.sb        AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA

S-06            AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA
S-07            AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA
S-08            AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA
S-09            AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA
G.xval/n(TR)    AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA
S-10            AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA
S-11            AACTTGCATG ACTCTTGGCA ACGAATATCT TGGCTCTGGC ATTGATGAAG AATGTAGCGA


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345        355
S-01            AATATGTTAT TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT
S-02            AATATGTTAT TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT
S-03            AATATGTTAT TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT
S-04            AATATGTTAT TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT
S-05            AATATGTTAT TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT
G.pli.sb        AATATGTTAT TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT

S-06            AATATGTTAC TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT
S-07            AATATGTTAC TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT
S-08            AATATGTTAC TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT
S-09            AATATGTTAC TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT
G.xval/n(TR)    AATATGTTAC TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT
S-10            AATATGTTAC TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT
S-11            AATATGTTAC TTGGTGTGAA TTGCAGAATC TTGTGAACCA TCGAGTCTTT GAATGCAAGT
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   365        375        385        395        405        415
S-01            TGCGCTCAAG GTTATTAGAC TAAGGGCACT CTTGCCTGGG CATCACGCCT ATTGACGCTC
S-02            TGCGCTCAAG GTTATTAGAC TAAGGGCACT CTTGCCTGGG CATCACGCCT ATTGACGCTC
S-03            TGCGCTCAAG GTTATTAGAC TAAGGGCACT CTTGCCTGGG CATCACGCCT ATTGACGCTC
S-04            TGCGCTCAAG GTTATTAGAC TAAGGGCACT CTTGCCTGGG CATCACGCCT ATTGACGCTC
S-05            TGCGCTCAAG GTTATTAGAC TAAGGGCACT CTTGCCTGGG CATCACGCCT ATTGACGCTC
G.pli.sb        TGCGCTCAAG GTTATTAGAC TAAGGGCACT CTTGCCTGGG CATCACGCCT ATTGACGCTC

S-06            TGTGCTCAAG GTTATTAGGC TAAGGGCACT CCTGCCTGGG CATCACACCT ATTCACGCTC
S-07            TGTGCTCAAG GTTATTAGGC TAAGGGCACT CCTGCCTGGG CATCACACCT ATTCACGCTC
S-08            TGTGCTCAAG GTTATTAGGC TAAGGGCACT CCTGCCTGGG CATCACACCT ATTCACGCTC
S-09            TGTGCTCAAG GTTATTAGGC TAAGGGCACT CCTGCCTGGG CATCACACCT ATTCACGCTC
G.xval/n(TR)    TGTGCTCAAG GTTATTAGGC TAAGGGCACT CCTGCCTGGG CATCACACCT ATTCACGCTC
S-10            TGTGCTCAAG GTTATTAGGC TAAGGGCACT CCTGCCTGGG CATCACACCT ATTCACGCTC
S-11            TGTGCTCAAG GTTATTAGGC TAAGGGCACT CCTGCCTGGG CATCACACCT ATTCACGCTC


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   425        435        445        455        465        475
S-01            TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC
S-02            TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC
S-03            TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC
S-04            TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC
S-05            TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC
G.pli.sb        TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC

S-06            TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC
S-07            TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC
S-08            TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC
S-09            TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC
G.xval/n(TR)    TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC
S-10            TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC
S-11            TCTGCCTTTT GCAATTTCAT GTCTTGCGAT TAGACAGTAG GTATTGATGT GGAGATTGCC


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   485        495        505        515        525        535
S-01            CCCTTCCTCG TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCGATC GTGATGGACG
S-02            CCCTTCCTCG TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCGATC GTGATGGACG
S-03            CCCTTCCTCG TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCGATC GTGATGGACG
S-04            CCCTTCCTCG TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCGATC GTGATGGACG
S-05            CCCTTCCTCG TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCGATC GTGATGGACG
G.pli.sb        CCCTTCCTCG TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCGATC GTGATGGACG

S-06            CCCCTCCTAA TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCAATC GTGATGGACG
S-07            CCCCTCCTAA TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCAATC GTGATGGACG
S-08            CCCCTCCTAA TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCAATC GTGATGGACG
S-09            CCCCTCCTAA TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCAATC GTGATGGACG
G.xval/n(TR)    CCCCTCCTAA TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCAATC GTGATGGACG
S-10            CCCCTCCTAA TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCAATC GTGATGGACG
S-11            CCCCTCCTAA TACATCATTA GGTGGTGGGT CTAATTGTTA GTTGTCAATC GTGATGGACG
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
S-01            TGGCGAGAGG TGTAGTTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA
S-02            TGGCGAGAGG TGTAGTTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA
S-03            TGGCGAGAGG TGTAGTTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA
S-04            TGGCGAGAGG TGTA-TTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA
S-05            TGGCGAGAGG TGTA-TTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA
G.pli.sb        TGGCGAGAGG TGTAGTTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA

S-06            TGGCGAGAGG TGTA-TTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA
S-07            TGGCGAGAGG TGTA-TTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA
S-08            TGGCGAGAGG TGTA-TTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA
S-09            TGGCGAGAGG TGTA-TTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA
G.xval/n(TR)    TGGCGAGAGG TGTA-TTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA
S-10            TGGCGAGAGG TGTA-TTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA
S-11            TGGCGAGAGG TGTA-TTGAC ACACATTCTA TCATGGAAGT AACTTAGCTT GGGTGACGCA
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                ....|....| ....|....| ....|....| ....|....| ....|....| .
                   605        615        625        635        645
S-01            CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A
S-02            CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A
S-03            CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A
S-04            CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A
S-05            CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A
G.pli.sb        CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A

S-06            CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A
S-07            CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A
S-08            CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A
S-09            CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A
G.xval/n(TR)    CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A
S-10            CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A
S-11            CCGGAGTAAC CTATGCTAAT TGGTGACATG TGAGTATTAC TTGGAACATG A
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Appendix II. Construction of consensus trnL(UAA) intron and trnL(UAA) 3'exon-trnF(GAA) intragenic spacer sequences. Samples 1-5: *G. plicatus* subsp. *byzantinus*, 6-9: *G.*x*valentinei* nothosubsp. *subplicatus* and 10-11: *G. nivalis(TR)*

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55
S1              TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
S2              TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
S3              TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
S4              TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
S5              TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
G.pli.sb.       TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
S6              TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
S7              TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
S8              TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
S9              TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
Gxval.          TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
S10             TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
S11             TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
G.niv(TR)       TGGGAACTTC CAAATTCAGA GAAACCCTGG AACTAAAAAT GGGCAATCCT GAGCCAAATC
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65        75         85         95        105        115
S1              TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
S2              TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
S3              TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
S4              TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
S5              TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
G.pli.sb.       TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
S6              TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
S7              TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
S8              TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
S9              TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
Gxval.          TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
S10             TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
S11             TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
G.niv(TR)       TTTATTTTTA GAAAAACAAG GGTTTGAAAA ACTAGAAAGG GATAGGTGCA GAGACTCAAT
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
S1              GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
S2              GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
S3              GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
S4              GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
S5              GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
G.pli.sb.       GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
S6              GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
S7              GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
S8              GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
S9              GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
Gxval.          GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
S10             GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
S11             GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
G.niv(TR)       GGAAGCTGTT CTAACGAATG GAGTTGACTA CGTTGCGTCG GTAACTGGCT ATCGAAATTA
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
S1              AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
S2              AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
S3              AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
S4              AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
S5              AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
G.pli.sb.       AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
S6              AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
S7              AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
S8              AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
S9              AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
Gxval           AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
S10             AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
S11             AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT
G.niv(TR)       AAGTAAAGAA TGACCTATAT ATCTAATACG CACGTATACA TACTGGCATA TCAAACGATT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   245        255        265        275        285        295
S1              AATCGAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCGG
S2              AATCGAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCGG
S3              AATCGAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCGG
S4              AATCGAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCGG
S5              AATCGAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCGG
G.pli.sb.       AATCGAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCGG
S6              AATCTAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCAG
S7              AATCTAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCAG
S8              AATCTAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCAG
S9              AATCTAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCAG
Gxval.          AATCTAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCAG
S10             AATCTAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCAG
S11             AATCTAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCAG
G.niv(TR)       AATCTAGAAA CGAATCCATA CCGAATCCAT ATATATATAC GCAATATATT TTAAATTCAG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345        355
S1              AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
S2              AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
S3              AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
S4              AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
S5              AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
G.pli.sb.       AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
S6              AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
S7              AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
S8              AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
S9              AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
Gxval           AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
S10             AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
S11             AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
G.niv(TR)       AGTTATTGTG GATCTATTCT AATCGAAGTT AAAGGAATAA TCAAATATTC AGTGATCAAA
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   365        375        385        395        405        415
S1              TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
S2              TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
S3              TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
S4              TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
S5              TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
G.pli.sb.       TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
S6              TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
S7              TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
S8              TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
S9              TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
Gxval           TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
S10             TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
S11             TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA
G.niv(TR)       TCATTCATTC CAGAGTTTGA TAGACCATTT TTTCAAAAAT GATTAATCGG ACGAGAATAA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   425        435        445        455        465        475
S1              AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAACAATG AAATTTTTAA CTTCTTAACT
S2              AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAACAATG AAAT--TTAA CTTCTTAACT
S3              AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAACAATG AAATTTTTAA CTTCTTAACT
S4              AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAACAATG AAATTTTTAA CTTCTTAACT
S5              AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAACAATG AAATTTTTAA CTTCTTAACT
G.pli.sb.       AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAACAATG AAATTTTTAA CTTCTTAACT
S6              AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAACAATG AAATTTTTAA CTTCTTAACT
S7              AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAACAATG AAATTTTTAA CTTCTTAACT
S8              AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAACAATG AAATTTTTAA ATTCTTAACT
S9              AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAACAATG AAATTTTTAA CTTTTTAACT
Gxval           AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAACAATG AAATTTTTAA CTTCTTAACT
S10             AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAGCAATG AAATTTTTAA CTTCTTAACT
S11             AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAGCAATG AAATTTTTAA CTTCTTAACT
G.niv(TR)       AGAGAGAGTC CCATTCTGCA TGTCAATACC GACAGCAATG AAATTTTTAA CTTCTTAACT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   485        495        505        515        525        535
S1              ATTTATCTTA TTTTTTTTT- CATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
S2              ATTTATCTTA TTTTTTTTT- CATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
S3              ATTTATCTTA TTTTTTTTT- CATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
S4              ATTTATCTTA TTTTTTTTC- -ATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
S5              ATTTATCTTA TTTTTTTTT- -ATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
G.pli.sb.       ATTTATCTTA TTTTTTTTT- CATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
S6              ATTTATCTTA TTTTTTTTT- CATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
S7              ATTTATCTTA TTTTTTTTTT CATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
S8              ATTTATCTTA TTTTTTTTT- CATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
S9              ATTTATCTTA TTTTTTTTTT CATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
Gxval           ATTTATCTTA TTTTTTTTT- CATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
S10             ATTTATCTTA TTTTTTTTT- CATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
S11             ATTTATCTTA TTTTTTTTT- CATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
G.niv(TR)       ATTTATCTTA TTTTTTTTT- CATAAGCGGT TCAAATAAAA TTCAATATTT TTCTCATTCA
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
S1              TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
S2              TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
S3              TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
S4              TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
S5              TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
G.pli.sb.       TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
S6              TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
S7              TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
S8              TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
S9              TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
Gxval           TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
S10             TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
S11             TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT
G.niv(TR)       TTCTACTCTT TAACAAATGG ATCCAAACAT AAATCTTTTG ATCTTATACC AATTTGGTTT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645        655
S1              GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
S2              GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
S3              GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
S4              GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
S5              GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
G.pli.sb.       GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
S6              GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
S7              GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
S8              GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
S9              GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
Gxval           GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
S10             GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
S11             GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT
G.niv(TR)       GAATAGATAT GATACCCGTA CAAATGACCA TATATGGCCA TGGAATTCCT ATTATTGAAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   665        675        685        695        705        715
S1              CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
S2              CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
S3              CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
S4              CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTAAAAATCT
S5              CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
G.pli.sb.       CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
S6              CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
S7              CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
S8              CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
S9              CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
Gxval           CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
S10             CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
S11             CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
G.niv(TR)       CATTCACAGC CCATATCATT ATCCTTACAT TCACAAAGAA AGTCTTCTTT GTGAAAATCT
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   725        735        745        755        765        775
S1              AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
S2              AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
S3              AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
S4              AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
S5              AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
G.pli.sb.       AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
S6              AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
S7              AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
S8              AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
S9              AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
Gxval           AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
S10             AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
S11             AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
G.niv(TR)       AATAAATTAG GGGACTAGGT CAAAATTTTT AATACTTTTT TGAGTCTATT TCATTTACAT
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                ....|....| ....|....| ..
                   785        795
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S2              AGATACAAAT ACTCTACTAG GA
S3              AGATACAAAT ACTCTACTAG GA
S4              AGATACAAAT ACTCTACTAG GA
S5              AGATACAAAT ACTCTACTAG GA
G.pli.sb.       AGATACAAAT ACTCTACTAG GA
S6              AGATACAAAT ACTCTACTAG GA
S7              AGATACAAAT ACTCTACTAG GA
S8              AGATACAAAT ACTCTACTAG GA
S9              AGATACAAAT ACTCTACTAG GA
Gxval           AGATACAAAT ACTCTACTAG GA
S10             AGATACAAAT ACTCTACTAG GA
S11             AGATACAAAT ACTCTACTAG GA
G.niv(TR)       AGATACAAAT ACTCTACTAG GA
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Appendix III. DNA Sequences of the species of *Galanthus* series used in phylogenetic analysis.

trnL(UAA)intron
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          10         20         30         40         50         60         70         80         90         100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
G.nivalis
TGGGAACTTCCAAATTCAGAGAAACCCTGGAACTAAAAATGGGCAATCCTGAGCCAAATCTTTATTTTTAGAAAAACAAGGGTTTGAAAAACTAGAA~~~
G.plicatus
TGGGAACTTCCAAATTCAGAGAAACCCTGGAACTAAAAATGGGCAATCCTGAGCCAAATCTTTATTTTTAGAAAAACAAGGGTTTGAAAAACTAGAA~~~
G.pli.sb.
TGGGAACTTCCAAATTCAGAGAAACCCTGGAACTAAAAATGGGCAATCCTGAGCCAAATCTTTATTTTTAGAAAAACAAGGGTTTGAAAAACTAGAA~~~
G.xvalentinei
TGGGAACTTCCAAATTCAGAGAAACCCTGGAACTAAAAATGGGCAATCCTGAGCCAAATCTTTATTTTTAGAAAAACAAGGGTTTGAAAAACTAGAA~~~
G.nivalis(TR)
TGGGAACTTCCAAATTCAGAGAAACCCTGGAACTAAAAATGGGCAATCCTGAGCCAAATCTTTATTTTTAGAAAAACAAGGGTTTGAAAAACTAGAA~~~
S.lutea
TGGGAACTTCCAAATTCAGAGAAACCCTGGAACTAAAAATGGGCAATCCTGAGCCAAATCTTTATTTTTA~~~~~~~~~~GGGTTTGAAAAACTGGAATAA


         110        120        130        140        150        160        170        180        190        200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
G.nivalis
~~~~~~~~~~AGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGGAGTTGACTACGTTGCGTTGGTAACTGGCTATCGAAATTAAAGTAAAG
G.plicatus
~~~~~~~~~~AGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGGAGTTGACTACGTTGCGTCGGTAACTGGCTATCAAAATTAAAGTAAAG
G.pli.sb.
~~~~~~~~~~AGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGGAGTTGACTACGTTGCGTCGGTAACTGGCTATCGAAATTAAAGTAAAG
G.xvalentinei
~~~~~~~~~~AGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGGAGTTGACTACGTTGCGTCGGTAACTGGCTATCGAAATTAAAGTAAAG
G.nivalis(TR)
~~~~~~~~~~AGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGGAGTTGACTACGTTGCGTCGGTAACTGGCTATCGAAATTAAAGTAAAG
S.lutea
AAAATAAAAAGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACGAATGGAGTTGACTACGTTGCGTTGGTAACAGGCTATCGAAATTAAAG~AAAG
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        210       220       230       240       250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
G.nivalis
AATGACCTATATATCTAATACGCACGTATACATACTGGCATATCAAACGATTAATCGCGAAACGAATCCATACCGAATCCATATATATATACGCAATATA
G.plicatus
A-TGACCTATATATCTAATACGCACGTATACATACTGGCATATCAAACGATTAATCGCGAAACGAATCCATACCGAATCCATATATATATACGCAATATA
G.pli.sb.
AATGACCTATATATCTAATACGCACGTATACATACTGGCATATCAAACGATTAATCGAGAAACGAATCCATACCGAATCCATATATATATACGCAATATA
G.xvalentinei
AATGACCTATATATCTAATACGCACGTATACATACTGGCATATCAAACGATTAATCTAGAAACGAATCCATACCGAATCCATATATATATACGCAATATA
G.nivalis(TR)
AATGACCTATATATCTAATACGCACGTATACATACTGGCATATCAAACGATTAATCTAGAAACGAATCCATACCGAATCCATATATATATACGCAATATA
S.lutea
AATGACCTATATATCTAATACGCACGTATACATACTGGCATATCAAACGATTAATCGCGAAACGAATCCATACCAAATCCATATATATATATGCAATATA
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        310       320       330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
G.nivalis
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G.plicatus
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G.pli.sb.
TTTTAAATTCGGAGTTATTGTGGATCTATTCTAATCGAAGTTAAAGGAATAATCAAATATTCAGTGATCAAATCATTCATTCCAGAGTTTGATAGACCAT
G.xvalentinei
TTTTAAATTCAGAGTTATTGTGGATCTATTCTAATCGAAGTTAAAGGAATAATCAAATATTCAGTGATCAAATCATTCATTCCAGAGTTTGATAGACCAT
G.nivalis(TR)
TTTTAAATTCAGAGTTATTGTGGATCTATTCTAATCGAAGTTAAAGGAATAATCAAATATTCAGTGATCAAATCATTCATTCCAGAGTTTGATAGACCAT
S.lutea
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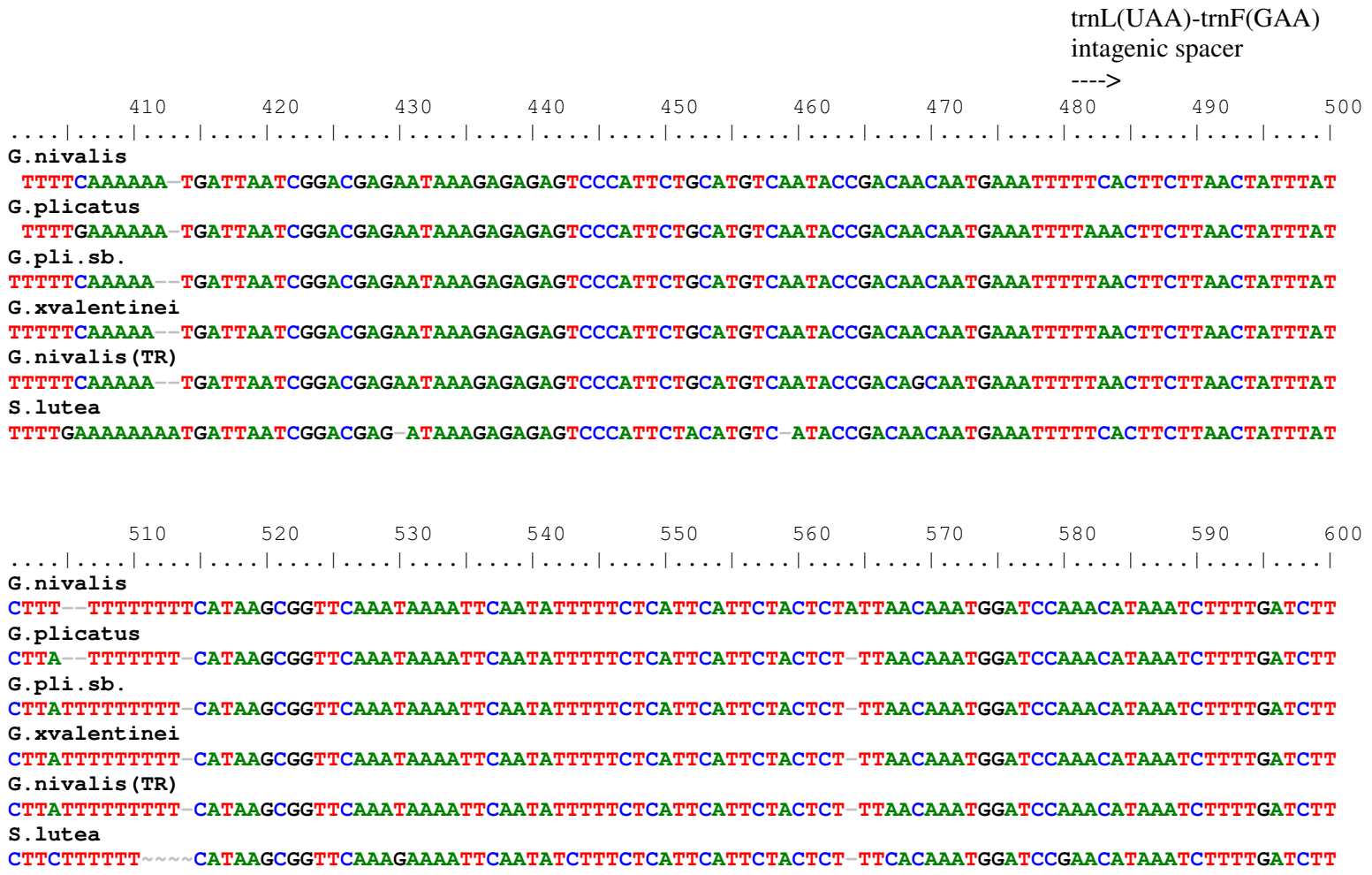
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G.nivalis
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G.plicatus
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G.pli.sb.
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G.xvalentinei
TTTTTCAAAAA--TGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTGCATGTCAATACCGACAACAATGAAATTTTTAACTTCTTAACTATTTAT
G.nivalis(TR)
TTTTTCAAAAA--TGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTGCATGTCAATACCGACAGCAATGAAATTTTTAACTTCTTAACTATTTAT
S.lutea
TTTTGAAAAAAAATGATTAATCGGACGAG-ATAAAGAGAGAGTCCCATTCTACATGTC-ATACCGACAACAATGAAATTTTTCACTTCTTAACTATTTAT
510 520 530 540 550 560 570 580 590 600
G.nivalis
CTTT--TTTTTTTTCATAAGCGGTTCAAATAAAATTCAATATTTTTCTCATTCATTCTACTCTATTAACAAATGGATCCAAACATAAATCTTTTGATCTT
G.plicatus
CTTA--TTTTTTT-CATAAGCGGTTCAAATAAAATTCAATATTTTTCTCATTCATTCTACTCT-TTAACAAATGGATCCAAACATAAATCTTTTGATCTT
G.pli.sb.
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G.xvalentinei
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G.nivalis(TR)
CTTATTTTTTTTT-CATAAGCGGTTCAAATAAAATTCAATATTTTTCTCATTCATTCTACTCT-TTAACAAATGGATCCAAACATAAATCTTTTGATCTT
S.lutea
CTTCTTTTTT~~~~CATAAGCGGTTCAAAGAAAATTCAATATCTTTCTCATTCATTCTACTCT-TTCACAAATGGATCCGAACATAAATCTTTTGATCTT

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        610       620       630       640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
G.nivalis
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G.plicatus
ATACCAATTTGGTTTGAATAGATATGATACCCGTACAAATGACCATATATGGCCATGGAATTCCTATTATTGAATCATTCACAGCCCATATCATTATCCT
G.pli.sb.
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G.xvalentinei
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G.nivalis(TR)
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S.lutea
ATACCAATTTGGTTTGAATAGATATGATACCCGTACAAATGAATATATATGGTCATGGAATTCCCATTATTGAATCATTCACAGTCCATAGCATTATCCT


        710       720       730       740       750       760       770       780       790       800
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
G.nivalis
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G.plicatus
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G.pli.sb.
TACATTCACAAAGAAAGTCTTCTTTGTGAAAATCTAATAAATTAGGGGACTAGGTCAAAATTTTTA-ATACTTTT--TTGAGTCT-ATTTC-ATTTACAT
G.xvalentinei
TACATTCACAAAGAAAGTCTTCTTTGTGAAAATCTAATAAATTAGGGGACTAGGTCAAAATTTTTA-ATACTTTT--TTGAGTCT-ATTTC-ATTTACAT
G.nivalis(TR)
TACATTCACAAAGAAAGTCTTCTTTGTGAAAATCTAATAAATTAGGGGACTAGGTCAAAATTTTTA-ATACTTTT--TTGAGTCT-ATTTC-ATTTACAT
S.lutea
TACATTCACAAAGAATGTCTTCTTTTTAAAAATCTAAGAAATTTGGGGACTAGGTCAAAATTTTTA-ATACTTTT-TTTTAGTCT-ATTTA-ATTTACAT
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ITS1
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        810       820       830       840       850       860       870       880       890       900
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
G.nivalis
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G.plicatus
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G.xvalentinei
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G.nivalis(TR)
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A-GATACAAATACTCTACTAGGATGATGCGGGGGGAATCGGCCCGAACGGACGATCGTGAACCAGTTGAGCACTGGTAAGGAGCG-GTAAGTGGGGCGGC
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        910       920       930       940       950       960       970       980       990      1000
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
G.nivalis
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G.plicatus
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G.pli.sb.
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G.xvalentinei
AAAT-CCATGGCTTTTGC-ACCTTATGGTACCCTTGTCATTGTCACCTTGCATGTTGTGTGGGATAGTTTGCAGGAACAAAGTTTTGGTGTAGTTCGCGC
G.nivalis(TR)
AAAT-CCATGGCTTTTGC-ACCTTATGGTACCCTTGTCATTGTCACCTTGCATGTTGTGTGGGATAGTTTGCAGGAACAAAGTTTTGGTGTAGTTCGCGC
S.lutea
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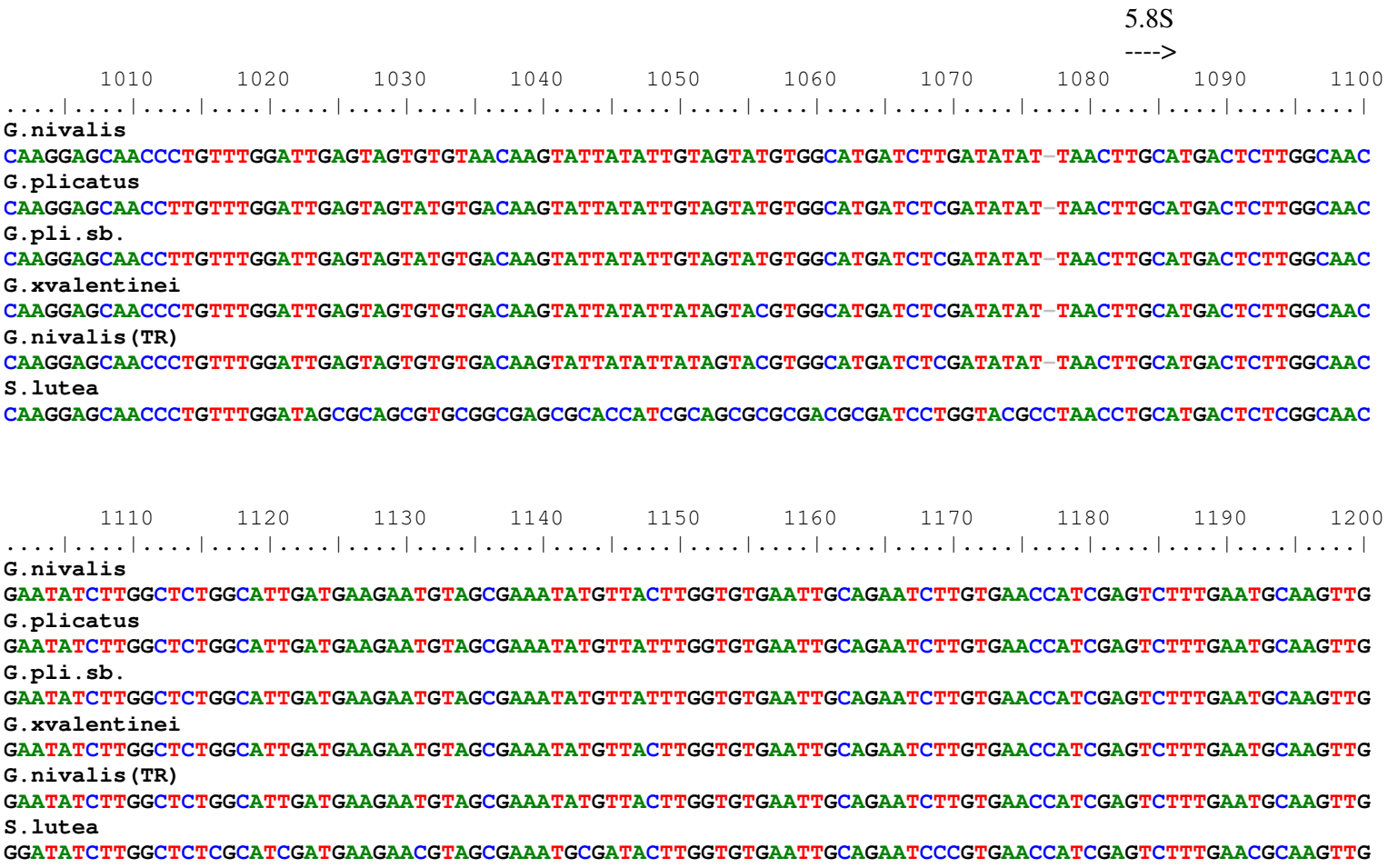
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G.nivalis
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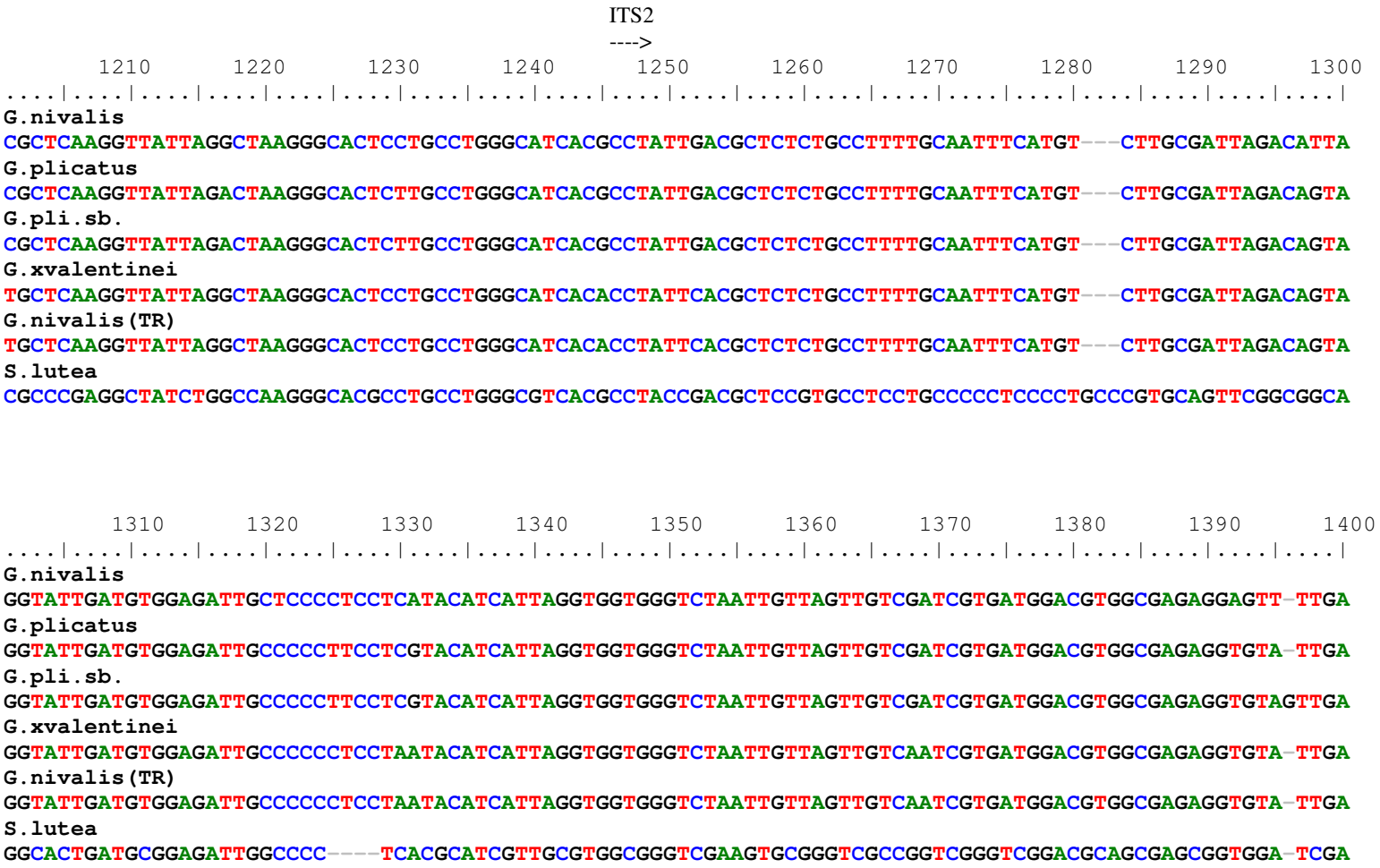
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