

**IDENTIFICATION OF VARIATIONS IN SOME *Crocus* L. TAXA FROM
BOLU BY ANALYSES OF rDNA ITS AND cpDNA *trnL*-F REGIONS
SEQUENCES**

by

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**THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
THE ABANT İZZET BAYSAL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE
IN
THE DEPARTMENT OF BIOLOGY**

JANUARY 2015

Approval of the Graduate School of Natural and Applied Sciences.

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ABSTRACT

IDENTIFICATION OF VARIATIONS IN SOME *Crocus* L. TAXA FROM BOLU BY ANALYSES OF rDNA ITS AND cpDNA *trnL*-F REGIONS SEQUENCES

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January 2015, 115 pages

In this preliminary study, taxonomic boundaries of *Crocus abantensis* T. Baytop & B. Mathew, *C. ancyrensis* (Herb.) Maw, *C. biflorus* Mill. subsp. *pulchricolor* (Herb.) B. Mathew, *C. olivieri* J. Gay subsp. *olivieri*, *C. speciosus* M. Bieb. subsp. *speciosus* and *C. x paulineae* Pasche & Kerndorff sampled from Bolu province were determined by using the base sequences of rDNA ITS and cpDNA *trnL*-F regions. The genetic status of the hybrid taxon, *C. x paulineae* was examined and its parents were determined in terms of molecular data. Existence of some other possible hybrids taxa between *C. abantensis* and *C. olivieri* subsp. *olivieri* were also investigated based on these molecular data in the studied area.

Additionally, this study was carried out to determine the experimental protocols being involved in a phylogeographical research on *Crocus* taxa in Turkey.

In molecular analyses, gDNA isolated from the samples, and then ITS and *trnL*-F regions were amplified using the ITS-4, ITS-5A, *trnL*-e, and *trnL*-f primers in a PCR apparatus. After editing of base sequences by using CodonCode Aligner program, the multiple alignments of the sequences were made with the CLUSTAL W packed program. Consequently, all the phylogenetic trees based on the sequences were created by using the PAUP* 4.0b10 program. The phylogenetic trees of ITS and *trnL*-F produced slightly different results containing more or less similar clades. This result was interpreted that it occurred because of having short, 230-300 bp sequences of *trnL*-F in regard to ITS base sequences (680-700 bp). Therefore, the trees based on the ITS sequences were mostly considered during the interpretations of phylogenies of taxa.

The results of distance and character based methods showed a quite positive congruencies between the phylogenetic trees such as parsimony, bootstrap, UPGMA, and NJ analyses. It was found that there were no differences between the groups of taxa obtained from this study and the groups of taxa obtained from the phenetic analyses by some other researchers. Finally, genetic status of the *C. x paulineae* taxon supported to be a hybrid of *C. abantensis* x *C. ancyrensis* species in terms of ITS and *trnL*-F analyses. Presence of other hybrid taxa between *C. abantensis* and *C. olivieri* subsp. *olivieri* could not be phylogenetically found in the research area.

Keywords: *Crocus*, ITS, *trnL*-F, PAUP, phylogeny, systematics, Bolu, Turkey

ÖZET

BOLU'DA BULUNAN BAZI *Crocus* L. (Çiğdem) TAKSONLARININ rDNA ITS VE cpDNA *trnL*-F BÖLGELERİNİN DİZİ ANALİZLERİYLE ÇEŞİTLİLİĞİNİN TANIMLANMASI

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Ocak 2015, 115 sayfa

Bu ön çalışmada Bolu ilinden örneklenen *Crocus abantensis* T. Baytop & B. Mathew, *C. ancyrensis* (Herb.) Maw, *C. biflorus* Mill. subsp. *pulchricolor* (Herb.) B. Mathew, *C. olivieri* J. Gay subsp. *olivieri*, *C. speciosus* M. Bieb. subsp. *speciosus* ve *C.x paulineae* Pasche & Kerndorff taksonlarının rDNA'nın ITS ve cpDNA'nın *trnL*-F bölgelerinin baz sekansları kullanılarak taksonomik sınırları belirlenmiştir. *C. x paulineae* melez taksonunun genetik durumu incelenerek moleküler veriler göre ebeveynleri belirlenmiştir. Çalışma bölgesinde *C. abantensis* ve *C. olivieri* subsp. *olivieri* arasındaki bazı olası melezlerin varlığı da moleküler verilere dayanarak araştırılmıştır.

Ek olarak bu çalışma Türkiye'deki *Crocus* taksonlarının üzerinde yapılacak bir filocoğrafi çalışmada kullanılacak deneysel protokolün belirlenmesi için yapılmıştır.

Moleküler analizlerde gDNA materyallerden izole edilerek ITS-4, ITS-5A, *trnL*-e, and *trnL*-f primerlerinin kullanılmasıyla ITS ve *trnL*-F bölgeleri bir PCR cihazında çoğaltılmıştır. CodenCode Aligner programı kullanılarak baz sekanslarının düzenlenmesinden sonra sekansların çoklu hizalanması CLUSTAL W programı ile yapılmıştır. Sonuçta PAUP* 4.0b10 programı kullanılarak sekanslara dayanan filogenetik ağaçlar oluşturulmuştur. Aşağı yukarı benzer kladları içeren ITS ve *trnL*-F filogenetik ağaçları çok az farklı olan sonuçlar ortaya koymuştur. Bu sonucun ITS sekanslarına (680-700 bp) nazaran 230-300 bp'lik *trnL*-F baz sekanslarının kısa olmasından ortaya çıktığı yorumlanmıştır. Bu nedenle taksonların filojenilerinin yorumlanması sırasında çoğu kez ITS sekanslarına dayanmış ağaçlar dikkate alınmıştır.

Parsimoni, bootstrap, UPGMA ve NJ analizleri gibifilogenetik ağaçlar arasında uzaklık ve karakter temelli metot sonuçları oldukça olumlu uyumluluklar göstermiştir. Diğer bazı araştırmacılar tarafından elde edilmiş fenetik analizlerdeki takson grupları ile bu çalışmadan elde edilmiş takson grupları arasında farklılıkların olmadığı bulunmuştur. Son olarak ITS ve *trnL*-F analizlerine göre *C. x paulineae* taksonunun genetik durumu *C. abantensis* x *C. ancyrensis*'in bir melezi olduğunu desteklemiştir. Araştırma alanında *C. abantensis* ve *C. olivieri* subsp. *olivieri* arasında diğer melez taksonların varlığı filogenetik olarak bulunamamıştır.

Anahtar Kelimeler: *Crocus*, ITS, *trnL*-F, PAUP, Filojeni, sistematik, Bolu, Türkiye

To My Father, Şükrü ÇEVİK

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Prof. Dr. M. Tekin BABAÇ, who has never spared his guidance, assistance and support. This study can not be carried out without his pioneering.

I owe thanks to my co-supervisor, Assist. Prof. Dr. Emel Uslu, for her support and willingness to answering questions and solving problems.

I would like to thank to Assist. Prof. Dr. Fatih Coşkun for his technical support in completion in this thesis.

I would like to thank to M.Sc. Engin Kumru, M.Sc. Berk Püral, M.Sc. Gözde Damla Ertuğrul, M.Sc. Meriç Tanyeri, M.Sc. Ozan Yılmaz, Ahmet Erden, Derya İspir, Sinan Çevik for their support and also friendship.

I also would like to thanks my brother, Ferhat Çevik, for his constant support and encouragement.

Lastly, I also would like to thanks my family and to my friend, Eray Duru, for their constant support and encouragement.

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ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphisms
bp	BasePair
cDNA	Complementary DNA
cpDNA	Chloroplast DNA
ddNTP	Dideoxyribonucleoside Triphosphate
dH₂O	Distilled Water
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxyribonucleoside Triphosphate
ETOH	Ethyl Alcohol
ETS	External Transcribed Spacer
gDNA	Genomic DNA
IGS	Intergenic Spacer
ITS	Internal Transcribed Spacers
mat K	Maturase K gene
mtDNA	Mitochondrial DNA
NJ	Neighbour Joining
NOR	Nucleolar Organizer Region
nrDNA	Nuclear Ribosomal DNA
PAUP*	Phylogenetic Analysis Using Parsimony and Other Methods
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic Acid
rpm	Rotations Per Minute
Taq	<i>Thermus aquaticus</i>
TBE	Tris-Boric Acid-EDTA
TE	Tris-EDTA
T_m	Melting Temperature
UPGMA	Unweighted Pair-Group Method of Averages

1. INTRODUCTION

Turkey has a very rich flora. Geographical location of Turkey, effect of various climatic factors, topographical and geological structure, and availability of various soil types may be listed among the reasons of this results. Turkey takes place at a location where three different phytogeographical regions, such as Irano-Turanian, Mediterranean, and Euro-Siberian (Euxine Province), meet each other (Figure 1). The composition of populations in Anatolia by introduction of Irano-Turanian elements from east and southeast, Mediterranean elements from south and west, and Euro-Siberian elements from North of Turkey had been the most significant reason of this diversity (Davis, 1971).

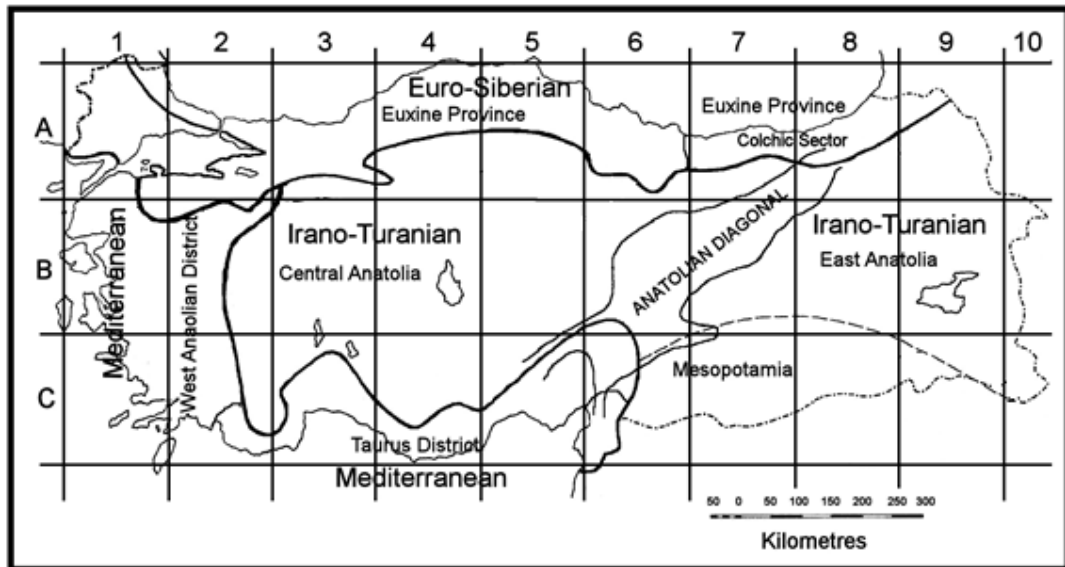


Figure 1: Phytogeographical regions of Turkey and the Anatolian Diagonal (modified from Davis, 1971)

Turkey is among the richest countries of the world in respect of endemic plants (Özhatay, and Kültür, 2006; Güner et al., 2000). While the endemism rate is in between 30-35% in Turkey, it is being specified as 20% in Iran and Greece, and 21% in Europe (Haspolat, 2011). The number of Turkish endemic taxa determined as per regions of plant geography had been recorded as 1220 unit for Irano-Turanian, as 1050 units for Mediterranean, and as 300 units for Euro-Siberian. It is not known by which plantgeography element these 500 units of taxa -which are other than these- are covered (Tan, 2010; Ekim et al., 2000; Kence, 1992). Turkey has a surface area which is one-fifteenth of the total surface of European countries. But while 2,750 of total 12,000 units of fern and flowering plants are endemic in Europe, more than 3,000 of 12,000 plant species existing in our country are endemic as being peculiar to Turkey (Özhatay and Kültür, 2006).

1.1 Classification of Plants

The classification of plants continues since Aristotle (2500 BC). Developments in taxonomic studies can be divided into 4 different phases observed in various locations in the world: Exploratory or pioneer, consolidation or systematics, experimental or biosystematics, and encyclopaedic or holotaxonomic phases (Singh, 2010). Even if these phases coincide with each other, they include differences from each other in respect of traced material, method, and techniques. By obtaining much information relevant to variation from the herbarium records and land surveys, biosystematics phase –following the systematic phase which stands out by the preparation of flora and monographs- is very remarkable by the significant leaping of taxonomic studies(Singh, 2010). Transplant, population, hybridization, and

chromosome studies made during this phase had caused the rise of visions which are very similar to modern studies of today(Davis, and Heywood, 1963; Singh, 2010). Thus in the encyclopaedic phase, it is observed that gigantic data and information accumulated by the addition of ultrastructural data, such as pollens, data relevant to secondary chemical compounds containing by plants (due to chemotaxonomic studies), data relevant to proteins and nucleic acids to old gathered data and information relevant to plants(Davis, and Heywood, 1963; Stuessy, 2009; Singh, 2010). Again in this phase, gathering of this information through the modern computers which arise in parallel to technological developments (due to data bases), its analysis and synthesizing of these analyses in meaningful manners are being performed rapidly(Abbott et al., 1985; Stuessy, 2009; Singh, 2010; Simpson, 2010). Thus it is observed that it is now reached to a saturation point in the phenetic classification of plants (Judd et al., 2002; Singh, 2010), and that there exists a burst in studies relevant to phyletic (cladistics) classifications made based on the evolutionary history of plants (Judd et al., 2002; Singh, 2010). Today, allozymes and isozyme studies made relevant to proteins, and phenetic and phyletic (cladistics) studies relevant to data, information obtained from studies relevant to genes on DNA and RNA and/or relevant to some locations had caused the rise of a new classification methodology (Judd et al., 2002; Stuessy, 2009; Singh, 2010; Simpson, 2010). But today the group of these studies, in which cladistic classifications relevant to DNA and RNA macro molecules are being made, is called “molecular systematic”(Judd et al., 2002; Stuessy 2009; Singh, 2010; Simpson 2010).

1.2 Molecular Systematics

In recent years, use of molecular and morphological data have accelerated in the classification of plant species (Mummenhoff et al., 1997; Graham et al., 2000; Judd et al., 2002; Liu et al., 2002; Simpson, 2010). In higher plants, nuclear (core) and cytoplasm (mitochondria and chloroplasts) have their own genetic materials responsible for self inheritance. These genetic materials differs from each other in repair and replication models. Core genome sexually reproducing a linear structure, while the chloroplast and mitochondrial genomes are circular in structure and they reproduce asexually (Judd et al., 2002; Simpson, 2010).

In molecular systematic studies, nuclear and organellar genomes can be used as data sources. Mitochondrial DNA (mtDNA) shows a quite variable structure. Therefore, nuclear (nr) and chloroplast (cp) DNAs are used more frequently in plant systematics. Today, it is known that molecular studies make an important contribution to the solution of systematic problems (Judd et al., 2002; Simpson, 2010). One of the methods used for this purpose in nrDNA is located on the ITS (Internal Transcribed Spacers) region (Baldwin and Markos, 1998). In recent years, this region has become a frequently used area in present day's modern systematic studies (Froslev et al., 2005; Fior et al., 2006). With this type of study, the ITS regions of taxa reproduced, base polymorphism can be determined by looking at the degree of relationship between taxa. Anatomical, karyological, morphological and cytological data used to support the traditional taxonomic methods, as well as using molecular data is currently contributing to the solution of systematic problems. DNA-DNA hybridization, protein markers and PCR-based methods are used in molecular systematics. Recently, PCR-based techniques have been used in more systematic studies.

These are special techniques such as RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphisms) and microsatellite DNA. Region of ETS (External Transcribed Spacer), IGS (intergenic Spacer) and ITS (Internal Transcribed Spacers) which is located on the genomic DNA, and region of *matK*, *trnT-trnL* that located on cpDNA are replicated by using PCR methods, and then making DNA sequence analysis of these regions are used extensively in systematic studies.

1.2.1 DNA Types used in Molecular Systematics

1.2.1.1 Chloroplast Genome

DNA of chloroplast (cpDNA) is a closed, circular molecule such as mtDNA. cpDNA also matches itself as semi-conserved, and it is inherited by the maternal. Chloroplast genome of flowering plants had been conserved at high degree with a length of 120-217 kilobases. Chloroplast DNA evolves slower than nuclear genome which contains more non-coding regions (Soltis et al., 1997; Pillay, and Mazzella, 1997). In many species the genome covers small and large unique strand regions, and these split from each other by a couple of reverse iteration (Doebley, and Blanton, 1987). Thereasons of using chloroplast DNA in plant phylogenetic studies can be given as fallow: Structural stability of cpDNA, its haploid (n) nature, its uniparental transfer which occurs in general, and its non-recombinant nature.

1.2.1.2 Nuclear Genome

The size of nuclear genome varies among organisms. And the source of this variation is duplication, deletion, polyploidy, and mutation of genes. Genetic drift is among the other factors causing variation. This condition indicates that nuclear genome may provide information in phylogenetic respect. Number of chromosomes and variations in karyotype are very significant data for the examination of the development of plants (Zhang, and Sang, 1999).

1.2.1.3 Relationship Between rDNA and ITS Regions

rDNA regions in genomic DNA consists of multiple gene structures, and consecutive sequenced iterative strands. Iterations of rDNA is settled at NOR (Nucleolar Organizer Region) regions of genomic DNA, and they consist of genes coding 18S small subunit, 5.8S, and 28S large subunit rDNAs (Baldwin et al., 1995). ITS regions are settled within these rDNA iterations which are available in the genomic DNA. These regions are being transcribed by the subunits of rDNA, and it consists of two parts (ITS1 and ITS2) separating the conserved regions (18S, 5.8S and 28S) (Baldwin et al., 1995). These ITS regions are able to be easily amplified through PCR studies by using universal primaries (ITS2, ITS3, ITS4 and ITS5) which can be connected to rDNA gene regions (White et al., 1990).

While it had been determined that the deletions occurring at specific regions of ITS-1 (18S-5.8S) prevent the maturation of small and large subunits of rDNAs, it had been observed that specific deletions and point mutations at the ITS-2 (5.8S -26S) region prevent the development of large subunit of rDNA. This is an evidence of the fact that these regions are significant for rDNAs even if they do not face translation (Baldwin, 1995).

ITS region provides significant ease in the identification of species compared to morphological data, and it is being preferred in phylogenetic studies (Cerbah et al., 1998).

Small subunit rDNA is a highly conserved region. It is frequently used for reconstruction of phylogenetic trees at higher taxonomic levels (Baldwin, 1992). Large subunit rDNA is longer compared to small subunit rDNA, and it shows more variation in respect to base content (Baldwin, 1992).

As the ITS and IGS regions of rDNA iterations indicate high degree of variation, they are being used in solving taxonomic problems encountered in studies in between types, in studies made at the level of species and in population studies (Freeman, and Herron, 1999). But due to longer structural part of IGS (4–5kb) regions compared to ITS regions and due to difficulty in the analysis of strands, ITS regions are mostly being used in phylogenetic studies (Freeman, and Herron, 1999).

1.2.2 Some of the Methods used in Molecular Systematics

1.2.2.1 RFLP (Restriction Fragment Length Polymorphism)

RFLP, DNA markers are among the first discovered marker (Saiki et al., 1998). RFLP of genomic DNA with restriction enzymes which cut region 4-6 nucleotides. Thus, DNA fragments of different lengths are formed (Angiolillo et al., 1999). The resulting DNA fragments are separated by size on electrophoresis. Even a single nucleotide in the cutting zone caused by mutations, variations in length of the restriction fragment is determined (Jones et al., 1997).

Southern blotting with an appropriate membranetransferred DNA is hybridized with probes labeled with biotinylation. Hybrids, is monitored byautoradiography technique. Thus, specific to species and varieties of plants restriction bands are determined. In comparison, if you have a difference in the length of their DNA, polymorphism is obtained.

The most critical step of this method is the choice of the appropriate probe. As a probe, it is often used DNA fragments 200-2000 bp in length. The most important feature of the probe is the presence of few copies in the genome (Yıldırım, and Kandemir, 2001).

RFLP in the research, Phylogeography, hybrid zones, and angiosperm related studies, could be used safely (Remi et al., 2001; Motomi et al., 2000). In addition, a RFLP markers previously mapped subsequently be used to determine relative species.

RFLP markers can also be used in the determination of heterozygous individuals (Rosa et al., 2003). The use of radioisotope probes and techniques to take a long time, are the most important disadvantage of RFLP analysis. Another disadvantage is that large quantities of pure DNA are used. Laboratory and material costs to be more restricts the use of RFLP (Yıldırım, and Kandemir, 2001). In addition, based on these markers on the maps, commonly seen large gaps (Erlich, 1989).

1.2.2.2 RAPD (Randomly Amplified Polymorphic DNA)

With the discovery of PCR initially to amplify specific regions of DNA base sequence of the known fields, some studies were performed using primer pairs. This technique is independently developed by two different groups of scientists.

Welsh and McClelland, this technique AP-PCR (Arbitrary Primed-PCR) gave the name. In principle two techniques are the same (Welsh, and McClelland, 1990).

The RAPD assay is based on the amplification of target genomic DNA with single arbitrary chosen primers with nucleotide in length of about 10 bp, the G+C content of which is between 30 % and 70 % (Williams et al., 1990).

Different from other PCR methods, is the use of a single primary. Primer on the complementary template DNA, which binds to two sites close to each other in the intervening DNA replicates.

The amplified DNA sequences by electrophoresis, separated on agarose or polyacrylamide gel, stained with ethidium bromide or silver staining (Staub et al., 1996; Babalola, 2003). Amplification band in primary attachment point by looking at the presence or absence of polymorphism is detected (Rafalski, and Tingey, 1993). Displacement of a single nucleotide in the primer, may well lead to a completely different in RAPD method. This is indicative of the sensitivity of the system. To get good results in the method, it is important to choose an appropriate primer.

The major disadvantage of RAPD method, because it is the dominant markers heterozygotes is difficult to diagnose (Tingey, and Tufa, 1993; Bardakcı, 2001). RAPD markers; genome mapping, gene tagging, genetic fingerprinting to identify, clone identification and can be used in phylogenetic studies (Babaoğlu et al., 2004).

1.2.2.3 AFLP (Amplified Fragment Length Polymorphism)

It is a technique developed by Zabeau and Vos in 1993 for the molecular studies. AFLP (Amplified Fragment Length Polymorphism) technique was developed to overcome the disadvantages of RAPD technique (Yıldırım, and Kandemir, 2001).

AFLP is based on selective amplification that is, cut with the restriction enzymes of DNA fragments. AFLP is a combination of PCR-RFLP technique. Primers may be sequence-specific or random (Saiki et al., 1998).

Advantages of AFLP; polymorphism is more, it is faster than RFLP, using the minimum primary test plurality marker is created, and highly suitable for fingerprint analysis. The disadvantages of the technique; pure and high molecular weight DNA is needed, and is not economical.

1.2.2.4 Minisatellites (SSR) and Microsatellites (VNTR)

Microsatellites, short repeated sequences are present in the genomes of all eukaryotes and usually consists of 1-6 bp. Obey Mendel's rules, ensure that microsatellite have been sought for molecular markers (Okogbenin, and Fregene, 2006).

Mini-satellite generally comprises 9-100 base pairs (Ergül, 2000). VNTR are used in forensic identification of criminals.

1.2.2.5 ISSR (Inter Simple Sequence Repeats)

ISSR technique, 5' and 3' end of the amplified short repeated DNA sequences as primers, using PCR products, after separation by size on gel electrophoresis, detection of DNA on the gel contains. As Primer, this is secured by 2-4 different or the same nucleotide, in a simple repetitive DNA sequence used.

ISSR (Inter-Simple Sequence Repeats) technique, which is almost identical with the cost of RAPD, is quite reliable according to RAPD, and repeatability rate is high. This technique as genetic diversity, classification, find molecular markers and evolutionary studies are used (Fang, and Roose, 1997).

To the difference in the annealing temperature of the primers, and does not meet the similar sized bands complicate the use of the technique (Welsh, and McClelland, 1990).

1.2.2.6 ITS Region and Its General Specifications

ITS regions of DNA piece of the core consists of two variable regions which are not coded. These gaps called as ITS-1 and ITS-2 are located in between the conserved regions of 18S, 5.8S and 26S. ITS-1 region is located in between small subunit (SSU) and 5.8S subunit, and ITS-2 region is located in between large subunit (LSU) and 5.8S. ITS-1 and ITS-2 have a length of about 300 bp. and the 5.8S subunit has a length of 164 bp (Baldwin, 1992). There exist conserved strands on both sides of ITS region. Thus, they are being characterized as universal primary.

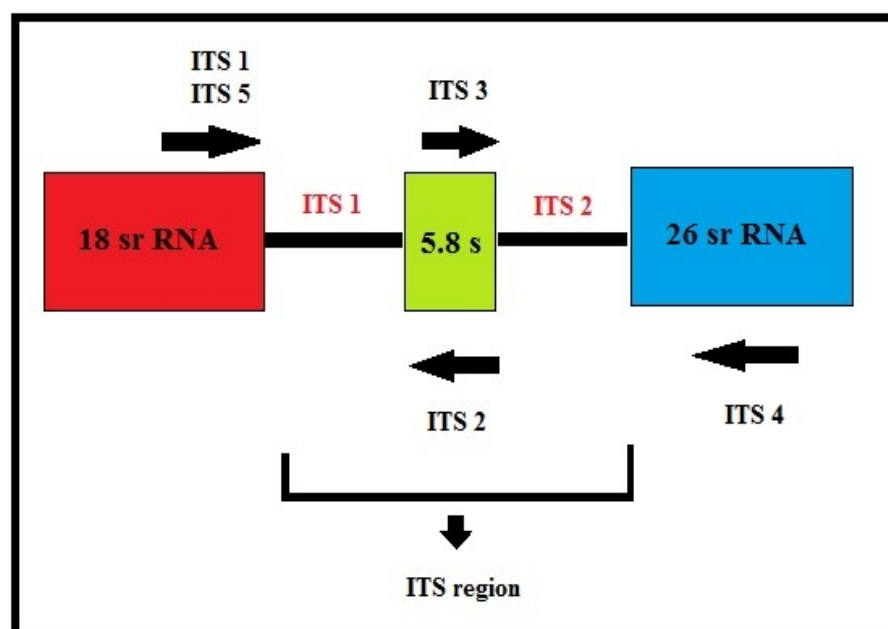


Figure 2. ITS Region (Modified from White et al., 1990)

The general specifications of ITS regions are listed as follows(Baldwin et al., 1995; Çebi Kılıçoğlu, and Özkoç, 2008):

- 1) Having a suitable size for phylogenetic analysis (600-700 bp). The regions can be duplicated as required by using the modified forms of these primers.
- 2) They have high degree of copies on genomic DNA. It had been determined that these regions show more variation compared to conserved rDNA gene regions.
- 3) As it exists among conserved regions, it had been determined that it is used as a universal primary for the duplication and sequencing of UTS region.
- 4) Data provided by ITS1 and ITS2 in phylogenetic aspect is at various levels. In the analyses relevant to these regions, ITS1 data provide more phylogenetic solution, and its nucleotide content is more variant at a rate of about 29% compared to ITS2.
- 5) In phylogenetic analyses at the level of types and species, DNA contents provide explanatory data.
- 6) It plays a role in the formation of mature 18S, 5,8S and 28S subunits of rDNA.

ITS1 and ITS2 are two different copy regions existing within conserved ribosomal genes (18S, 5.8S and 28S), and they are very useful in systematic aspect in comparing the closely related taxa (Baldwin et al., 1995; Malyshev and Kartel, 1997; Goel et al., 2002; Şahin, 2011). Thus, this region had started to be used in the evolutionary classification of angiosperms since 1990s.

The reasons of use of ITS region in the phylogenetic studies can be specified as follows;

- It is of a length which can provide reliable information in phylogenetic classifications (600–700 bp).
- They include much more number of copies.
- They are close to rDNA gene regions extremely conserved at taxonomic levels within types and species.
- It provides more extensive information at type and species level in phylogenetic studies.
- ITS regions show more rapid variation compared to rDNA gene regions.
- When the ITS1 and ITS2 regions are compared, ITS1 data provide more robust results compared to ITS2 in phylogenetic studies.
- Universal primers are used for the amplification and sequencing of ITS region (Baldwin et al., 1995).
- In the classification of species, ITS region sequences provide better results compared morphological data (Eddie et al., 2003; Cerbah et al., 1998).

1.2.2.7trnL-F Region

DNA based cladistic analyses assist the classification of species which cannot be resolved morphologically (Fior et al., 2006). Chloroplast DNA (cpDNA) strand varieties extensively search the angiosperm and interspecific relations in between other plants. Thus, it is being used in phylogenetic assessment. But the low evolutionary rate of these molecules is limiting at intraspecific degree (Taberlet et al., 1991; Türkteş et al., 2012). One of the regions which is not coded is cpDNA parts; t-RNA (*trnT-trnF*) region generally operates due to being the phylogenetic relation address under the family in the widest sense.

Gap in between the genes is in between *trnL* (UAA) 3'exon and *trnF* (GAA) gene (Taberlat et al., 1991; Gielly, and Taberlat, 1996; Gielly, and Taberlat, 1994; Liu et al., 2005). *trnL* gene includes two conserved exons. These are divided by one group of introns. This region has a high replacement rate. Thus, it is being used in phylogenetic analyses in between species (Yang, and Pak, 2006).

The nucleotide number of *trnL-trnF* region in monocots and dicots varies nearly in between 120-350 bp, and these regions are easily being duplicated and sequenced (Taberlet et al., 1991).

1.3 Genus *Crocus* L.

Iridaceae family, which the *Crocus* L. type belongs, covers 90 genera and about 1,800 species in the world, and it is mainly spread at southern hemisphere (Ali, and Mathew, 2000). The family is typically being characterized by isobilateral equal leaves, epigynous flower state and three stamen flowers (Rudall, 1994). Iridaceae family taxa are also being used in medicine and pharmaceutics due to their significant chemical compounds as well as being grown as ornamental plants at parks and gardens due to their attractive flowers (Baytop, 1984; Schmidt et al., 2007; Bathaie, and Mousavi, 2010).

The name of the type *Crocus* had been derived from the Latin word “crocatu” which means saffron yellow. While the Greek name of saffron is “krokos”, it is “zafaran” in Arabic which also means yellow (Carter, 2011). And its Turkish name is “çiğdem”.

Crocus is perennial, and it is a plant having underground corm. The corms of *Crocus* may be round, egg-shaped or flat, and it is very rich in respect of starch content. The corms are annual, and while the old corms languish, the new corms occur in between the phases of flowering and fructification. The new corm develops from the axillary buds at the top of the old corm (Mathew, 1982). As the leaves of *Crocus* type vary in between species in respect of number, size, structure and color, they are significant characteristics used in classification. Scapus exists on each of the flowers of *Crocus*. Scapus is not seen on the soil until fruiting time (Erol, 2004). The structure of scapus lifts up the capsule in this phase and grows rapidly. There exists paper like leaves coming out from various points of scapus. These are profiles, bracts and bracteoles. According to Bowles (1952) the profile at the lower section is located on basal spathe (at the base of scapus), and it is adjacent to the newly grown corm.

General flower formula of *Crocus* is $P_{3+3} A_{3G} (3)$, but it can sometimes have flower sections with different numbers. Despite the size, form and colors of the segments are attractive; the color may vary a lot among the species such as white, yellow, blue and purple. Thus, their use as taxonomic characteristic may not be significant (Mathew, 1982).

Crocus types mostly grow by themselves at forestlands or meadowlands and heathlands. They mostly prefer semi shadow – bright locations and mild climates.

They prefer rich, humid soils with well drainage, sandy, and including rotten leaves and 2% organic substance (Haspolat, 2011). Clayed soils or only clay decrease the development of root, and the corms have a dark tunic color which is not preferred. The precipitation shall be good in respect of their water requirements. 100 mm precipitation for basic fertilization enables availability of nitrogen at the root

region of the plant. The plants are resistant against cold. This genus prefers a pH value of 6-7 (Yücel, 2002; Benschop, 1993). Its leaves grow along with the flowers or after the flowers, and they continue to grow after flowering (Vurdu, and Çiçek, 1992).

Crocus is one of the three genera of the Ixioideae Rchb. ex Klatt subfamily of Iridaceae (Arber, 1925). The other two genera which it is closely related are *Romulea* Maratti and *Gladiolus* L. But in the recently performed taxonomic studies, these genera had been included in the subfamily of Crocoideae Burnett (Goldblatt et al., 2002).

Various *Crocus* species are encountered in Turkey as spread in each region. Moreover, as well as changing as per species, it also exists at heights in between *Crocus* species 20 mand 3250 m (Davis and Hedge, 1984).

The genus *Crocus* consists currently of about 100 recognized species (Petersen et al., 2008; Kerndorff, and Pasche, 2011; Kerndorff et al., 2011, 2012; Peruzzi, and Carta, 2011) occurring from Western Europe and Northwestern Africa to Western China with the center of species diversity in the Anatolian and the Balkan Peninsulas.

The number of taxa rapidly decreases beyond these regions. For instance, only 4 species are being known at Iberian Peninsula and 3 around Caspian Sea (Erol, 2004). Balkans and Turkey cover most of the *Crocus* taxa which are identified until now (Tutin et al., 1980; Mathew, 1982). 72 *Crocus* taxa in total naturally grow in our country (Mathew, 1984; Güner et al., 2000). Forty taxa in total, as being 19 of these species and 21 of their subspecies, are endemic for Turkey. Due to the number of taxon existing in the country, it had been reported that the homeland of *Crocus* can

be accepted as Turkey (Özyurt, and Odabaş, 1987; Harpke et al., 2013; Harpke et al., 2014). Southwest of Turkey is the center where the kinds of *Crocus* exist the most (Erol et al., 2011; Şık et al., 2008; Vurdu et al., 2004; Kravkaz et al., 2006).

Crocus consists a significant type in the world as well as our country in respect of its economical characteristics. Some *Crocus* types are being used as dye, medication and perfume since 1,600BC (Brighton et al., 1980). Despite this plant, which had an economic importance in the past in respect of spice, dye and medication, had been first grown in provinces such as Bolu, Tokat, Şanlıurfa, Adana, İzmir in our country, it had lost its importance in time, and its plantation areas had decreased and remained limited by the lands of a few producers in Safranbolu, Karabük province (İpek et al., 2009). In some of the regions of Anatolia, the corms of *Crocus* are being added as flavor inside rice or milk puddings as well as being consumed as uncooked (Ertuğ, 2000).

In the flora of Turkey, 5 taxa such as *Crocus abantensis* T. Baytop and Mathew, *C. ancyrensis* (Herbert) Maw, *C. biflorus* Miller subsp. *pulchricolor* (Herbert) Mathew, *C. olivieri* Gay subsp. *olivieri* and *C. speciosus* Bieb. subsp. *speciosus* had been reported within the borders of Bolu Province in Turkey.

Additionally, *C. x paulineae* Pasche & Kerndorff being the hybridization of *C. ancyrensis* and *C. abantensis* taxa had been added to these taxa. Only *C. speciosus* subsp. *speciosus* among these taxa blooms in autumn. Others bloom in spring (Mathew, 1984).

1.4 *Crocus* Taxa in the Study Area

According to recent data (Güner et al.,2000), Turkey houses about 10.500 flowering taxa. At least 8 or 9 taxa may probably exist in Bolunaturally.

The field of study covers two endemic species as being yellow flowered Ankara *Crocus* (*C. ancyrensis*) with a dispersion area starting from Aladağlar -being the location of natural dispersion of *Crocus* taxa at the province of Bolu- towards Ankara and nearly ending at northwest of Bolu, and blue flowered Abant *Crocus* (*C. abantensis*) with a narrow natural dispersion area only at Abant Mountains nearby the Abant Lake. These two species exist at limited size populations on Abant Mountains, and along with others as adjacent or as nested.

The hybrid species named as *C. x paulineae*, constituted by these two endemic species, comprises a new endemic type peculiar to Abant Lake and its surrounding at Bolu.

Even if it had been reported (Kanoğlu, 2011) that the *C. x paulineae* taxon is exist around Sülüklü Lake at the most northwest of the province of Bolu, it is being thought that these records are incorrect as the dispersion of *C. abantensis* does not reach until the referred location.

In the Flora of Turkey, 5 taxa -*Crocus abantensis*, *C. ancyrensis*, *C. biflorus* subsp. *pulchricolor*, *C. olivieri* subsp. *olivieri* and *C. speciosus* subsp. *speciosus*- had been reported within the borders of Bolu Province. And then, *C. x paulineae* taxon – being the crossbreed of *C. ancyrensis* and *C. abantensis*- had been reported (Pasche, and Kendorff, 1999). Only *C. speciosus* subsp. *speciosus* among these taxa is the *Crocus* which blooms in autumn. Others bloom in spring (Mathew, 1984). On the other hand, one species, *C pestalozzae* Boiss., which was reported from Bolu as a

type specimen in Flora of Turkey (Mathew, 1984), could not be found in all botanical trips during this study. In the Flora of Turkey, Mathew (1984) explained that this taxon may be mixed with one of the infraspecific *C. biflorus* taxa easily. Because of these reasons it was accepted that this was an erroneous report from Bolu province. Therefore, this taxon has not been included in the study as a research material.

1.5 Some Studies Relevant to *Crocus* Taxa in Turkey and Abroad

Some floristic and vegetation studies containing the *Crocus* taxa previously carried out at the province of Bolu have been encountered. The vegetation studies of K ro lu Mountains was carried out by Akman and Keten lu in 1978 at the province of Bolu, of Bolu and Semen Mountains performed by Akman et al. in 1983, and of the surrounding of Mudurnu performed by Akman and İlarslan in the same year attracts attention. Some floristic studies are the floras of Gerede and Aktaş Forests and K ro lu Mountains by Akman and Keten lu in 1979, and of Bolu and Semen Mountains by Akman and Yurdakulol in 1981, and of Yedig ller National Park by Ekim and İlarslan in 1982. Besides these, the Flora of the Campus of Abant İzzet Baysal University were studied by Turgut and G ner in 1996, the Flora of Abant had been studied by T rker-U ar and G ner (2002), and the Flora of Yeni a a Lake had been studied by S mer in 2002. The Flora of G lc k were also studied by İkinci and G ner in 2006, the Flora of Karakiriş Mountain had been studied by Aksoy in 2009, the Flora of Kartalkaya had been studied by Sungurlu in 2011, and the Flora of S l kl  Lake and its surrounding had been studied by Kano lu (2011). In all studies

mentioned above, more or less the same taxa have been rediscovered and no new taxa was added to the current lists of *Crocus* in Bolu province.

Rudall and Mathew (1990) had performed a study where they had examined the leaf anatomy of *Crocus* species. They indicated that the anatomic characteristics of *Crocus* type may also be used in the identification of Ixioidae subfamily of Iridaceae.

In the post graduate thesis of Ersoy (1998) the morphological structures of 7 *Crocus* taxa in Thrace have been determined, and it has also been determined that four of these are at the level of species and 3 at the level of subspecies (Ersoy, 1998).

Özdemir (2000), as the result of his Ph. D. thesis, has determined the morphological, anatomical and ecologic characteristics of three subspecies (*C. speciosus* subsp. *speciosus*, *C. speciosus* subsp. *ilgazensis* Mathew, *C. speciosus* subsp. *xantholaimos* Mathew) of *Crocus speciosus*, two of which are endemic at alpine and subalpine areas of Black Sea Region, and for this purpose, he had shown through photographs and drawings the morphological structures of corm, leaf, flower, fruit and seeds of the plants.

Radjabian et al. (2001) had analyzed the buds of *C. sativus* and *C. haussknechtii* (Boiss. et Reut. ex Maw) Boiss. by thin layer chromatography (TLC) and by high performance liquid chromatography (HPLC). As the results, they showed the high pigment concentrations in the buds of *C. haussknechtii* and the carotenoid compounds of these species, similarity in between *C. sativus* L. and other *Crocus* L. species, and that it can be used in obtaining saffron.

Vurdu et al. (2002), intended to determine the growing techniques required in order to obtain the most qualified corms and stigmas from the saffron plant

(*C.sativus*). So, he has determined that the most significant factor affecting the small corm and total flower yield was the diameter of corm in other words the size of corm. They had determined that the total number of flowers increase as the size of corm increases.

Abdullaev (2002) had assessed the use of *C. sativus* L. species, saffron, as spice and as anti-carcinogenic in medicine.

Akan and Eker (2004) had examined the *C. pallasii* subsp. *turcicus* B. Mathew and *C. cancellatus* subsp. *damascenus* (Herbert) B. Mathew taxa –collected from around Şanlıurfa- in morphological and anatomic respects, and they had compared their morphological characteristics with the Flora of Turkey (Davis, 1984).

Erol et al. (2004) had performed a study on *C. wattiorum* (B.Mathew) B.F.Mathew species which was among the new taxa determined in the Flora of Turkey within the last 10 years. In this study, the external morphological characteristics and leaf anatomy of the species had been examined, and it had been tried to clarify its taxonomic state.

Morphological and anatomic characteristics of *C. danfordiae* Maw and *C.fleischeri* endemic taxa distributing at narrow areas in our country, had been examined. As the characteristics of these taxa, it had been determined that the bottom of flowers and flower tube of *C.fleischeri* was purple colored, and that the bottom part of the anthers of *C.danfordiae* was black colored. In the study performed in anatomic aspect, the root, trunk and leaf pieces of *C. danfordiae* and *C. fleischeri* have been comparatively compared and examined (Özdemir et al., 2004).

Selvi (2005), in his M.Sc. thesis, examined the taxonomic, morphological, anatomic and ecologic characteristics of *Crocus* L. species distributing in Balıkesir

Province. As a result of the study, it was determined that ten *Crocus* species (*C. chrysanthus* Herb., *C. pallasii* Goldb. subsp. *pallasii*, *C. cancellatus* Herb. subsp. *mazziaricus* (Herbert) B. Mathew, *C. pulchellus* Herb., and among the endemic species of our country *C. biflorus* Mill. subsp. *nubigena* (Herbert) B. Mathew, *C. olivieri* subsp. *istanbulensis* B. Mathew, *C. candidus* Boiss, *C. flavus* subsp. *dissectus* Baytop & B. Mathew, *C. gargaricus* Herb. *gargaricus*) was dispersed within the borders of the province of Balıkesir.

Özhatay et al. (2006) prepared a list about some additional taxa for the flora of Turkey. In this list, *Crocus biflorus* subsp. *atrospermus* Kerndorff & Pasche, *Crocus biflorus* subsp. *leucostylosus* Kerndorff & Pasche added to *Biflorus* species of *Crocus* L. type, *C. nerimaniae* Yüzbaşıoğlu and *C. xpaulineae* taxa were represented.

Antimicrobial activities of hexane, ethyl acetate and methanol extracts of *Crocus biflorus* Mill., *C. baytopiorum* B. Mathew and *C. flavus* plants had been determined by the “agar well diffusion method” by using 10 different bacteria. The findings indicated that the plant extracts were a moderate antimicrobial activity against test microorganisms used in the study, and that they can be used as alternative to synthetic antioxidants (Acar, 2006).

Twenty-nine taxa of pollen grains belonging to the genus *Crocus* L. were studied by light and electron microscopy. Three types of opening of grains, spiral-shaped, more or less wide-shaped and short-shaped, had been identified in pollen grains (Işık, and Oybak, 2006).

Şık et al. (2008) had searched the genetic variety level among *Crocus* taxa. Molecular classification had been made by using RAPD and ISSR markers in the determination of genetic variety on 56 pieces representing 19 *Crocus* taxa dispersing

at West Anatolia. *Crocus* samples had been selected from among the pieces with high genetic variety. Genetic distance (GD) values had changed in between 0.06 and 0.52. Classification of *Crocus* by cluster analysis DNA markers did not provided conformity with morphological classification. Thus, it had been specified that robust DNA indicators such as AFLP and SSR as well as DNAs of nuclear mitochondrial and chloroplast shall be reviewed again by using more taxa in respect of DNA basis classification intended for taxa.

Şık and Candan (2009) examined the ecological characteristics of *C. chrysanthus* Herb., *C. flavus* Weston subsp. *flavus*, and *C. flavus* subsp. *dissectus* T. Baytop & B. Mathew. The plants had been collected in the flowering period time along with soil samples from the areas they distributed. New distribution areas of the taxa had been determined, and it had been observed that the taxa generally grow on slightly alkaline, salt-free, clayed and fertile soils.

The chromosome numbers and morphology of *C. fleischeri*, *C. pallasii* subsp. *pallasii*, *C. cancellatus* subsp. *lycius* Mathew and *C. pulchellus* taxa growing in West Anatolia have been studied. The chromosome numbers of *C. fleischeri*, *C. pallasii* subsp. *pallasii*, *C. cancellatus* subsp. *lycius* and *C. pulchellus* were found as $2n = 20$, $2n = 14$, 16 , and 12 , respectively (Candan et al., 2009).

The relations among 15 *Crocus* taxa dispersing in Turkey had been analyzed by using 29 morphological and 4 anatomical characteristics by Coşkun et al. (2010). The most interesting result of this study was the taxonomic position of *C. pallasii*. It was included in the *Crocus* section in previous studies, but in this study, *C. pallasii* has been shown as a sister group with *C. cancellatus* group from the *Nudiscapus* section of *Reticulati* series. *C. gargaricus* from the *Reticulati* section was

also a sister group with the members of *Nudiscapus* section from among the members of *Bifloris* series (especially with *C. leichtlinii* (Dewar) Bowles). In order to reach some reliable results on the studied taxa, it had been determined that more morphological data are required along with molecular data (Coşkun et al., 2010).

The genetic variety among taxa of *C. antalyensis* B. Mathew and of some subspecies from South Anatolia had been identified by using AFLP marker system. In order to determine the polymorphism in between the samples, 22 primers had been used, and genetic variation had changed in between 0.44 – 0.69. The effect of AFLP marker system had been revealed the differences among the individuals of *C. antalyensis* samples. It had been observed that a high level of genetic variety exist among the *C. antalyensis* samples collected. It had been specified that *C. flavus* Haw. subsp. *dissectus* being the relative of subspecies of *C. antalyensis* is genetically different from those of Baytop & B. Mathew's samples. Moreover, a new subspecies of *C. antalyensis* B. Mathew had been described at the south of Turkey (Erol et al., 2011).

1.6 Aim of the Study

Six taxa such as *C. abantensis*, *C. ancyrensis*, *C. biflorus* subsp. *pulchricolor*, *C. olivieri* subsp. *olivieri* and *C. speciosus* subsp. *speciosus*, and *C. x paulineae*, being the hybridization of *C. ancyrensis* and *C. abantensis* from the Bolu province are the materials of this study. By the ITS and rDNA analyses of these taxa in the study, it has been aimed that;

1. Produce a preliminary study to determine and fix the experimental conditions for a cladistic study based on ITS of rDNA and *trnL-F* of cpDNA

analyses that will be followed for taxonomic studies on Turkish *Crocus* taxa in future.

2. Determine the taxonomic circumscriptions of taxa from the points of molecular views based on their variation spectrums.

3. Indicate that *C. x paulineae* is a hybrid between *C. ancyrensis* and *C. abantensis* from the point of molecular view, and finally,

4. Checking the existence of possible some new hybrid taxa between *C. olivieri* subsp. *olivieri* and *C. abantensis* from the molecular ways.

2. MATERIALS AND METHODS

In this study, taxonomic position of six *Crocus* taxa, *C. abantensis*, *C. ancyrensis*, *C. biflorus* subsp. *pulchricolor*, *C. olivieri* subsp. *olivieri*, *C. speciosus* subsp. *speciosus* and *C. x paulineae* hybrid, which are reported from Bolu province, were studied by using molecular analysis.

In order to carry out the procedure, gDNA was isolated from these *Crocus* taxa, then ITS region of gDNA and *trnL-F* region of cpDNA were replicated by PCR method. As a result of this molecular studies, taxa of *Crocus* genus were determined based on the inter- and intra-specific variations. ITS and *trnL-F* regions of DNA sequences were analyzed by the various statistical and numerical methods. Therefore, phylogenetic and phenetic relationships of taxa were determined.

2.1 Materials

Six *Crocus* taxa (*C. abantensis*, *C. ancyrensis*, *C. olivieri* subsp. *olivieri*, *C. biflorus* subsp. *pulchricolor*, *C. speciosus* subsp. *speciosus*, *C. x paulineae*) were collected from the province of Bolu the localities mentioned in the Flora between March-September 2013-2014. The samples were according to the diagnosis of Mathew's monograph (1982) and "Flora of Turkey" (Matthew 1984). Studied plant specimens' geographic localities, coordinates and altitudes, and photos taken during the field studies are given in Table 1, and Figure 3 respectively.

After plant materials collected from the field, fresh and clean leaves were stored in plastic bags which were filled with orange silica gel and dried for the DNA isolation. The herbarium specimens were prepared using with the standard techniques given by Davis and Heywood (1973). Multiple copies of herbarium specimens from each were conserved in the Herbarium of Abant Izzet Baysal University (the acronym of the herbarium is AIBU).

2.2 Methods

2.2.1 Application of Primers

Five individual leaf samples were used for each taxa. A total of 30 individuals were selected for DNA isolation. Genomic DNAs were isolated from silica gel-dried leaf material by using a DNA isolation kit. Genomic DNA was isolated from silica gel-dried leaf material by using a DNA isolation kit (GeneMATRIX Plant & Fungi DNA Purification Kit). The chemicals used for the study (the enzyme used in PCR reactions or other solutions) were purchased from BM and Refgen company. Primers for preparing the stock, a brief centrifugation of lyophilised sample was done to collect the sample in the bottom of the tube. Then it was dissolved with necessary amount of distilled water to make 100 pmol/ μ L primary stocks. The final concentration of each primer, 5 μ mol / μ L to be diluted. Working primer concentrations were prepared from the primer stock solution and both were kept at -20 ° C during the experiment. DNA sequences of the ITS region and *trnL*-F region primers used in this study, the melting temperatures (T_m) are given in Table 2 and Table 3, respectively.

Table 1. Localities, coordinates, altitudes and accession numbers of *Crocus* taxa used in the study.

Taxon Name (and Code)	Location	Coordinate	Altitude	Acess. No
<i>C. abantensis</i> (ABA) (ABA1)-(ABA2)-(ABA3)- (ABA4)-(ABA5)	Between Abant Lake and Mudurnu, about 1 km from lake onto Mudurnu road	40° 35.549'N 032° 16.956'E	1480 m	SÇ+MTB+EU 2013/01
<i>C. ancyrensis</i> (ANK) (ANK1)-(ANK2)-(ANK3)- (ANK4)-(ANK5)	Between Abant Lake and Mudurnu, about 1 km from lake onto Mudurnu road	40° 35.549'N 032° 16.956'E	1480 m	SÇ+MTB+EU 2013/02
<i>C. olivieri</i> subsp. <i>olivieri</i> (OLI) (OLI1)-(OLI2)-(OLI3)- (OLI4)-(OLI5)	Between Abant Lake and Mudurnu, about 1 km away from the lake onto Mudurnu road	40° 35.549'N 032° 16.956'E	1480 m	SÇ+MTB+EU 2014/01
<i>C. biflorus</i> subsp. <i>pulchricolor</i> (BIF) (BIF1)-(BIF2)-(BIF3)- (BIF4)-(BIF5)	Between Abant Lake and Mudurnu, about 1 km away from the lake onto Mudurnu road	40° 35.549'N 032° 16.956'E	1480 m	SÇ+MTB+EU 2014/02
<i>C. x paulineae</i> (PAU1) (PAU1)-(PAU2)-(PAU3)- (PAU4)-(PAU5)	Between Abant Lake and Mudurnu, about 1 km from lake onto Mudurnu road	40° 35.549'N 032° 16.956'E	1480 m	SÇ+MTB+EU 2014/03
<i>C. speciosus</i> subsp. <i>speciosus</i> (SPE) (SPE1)-(SPE2)-(SPE3)- (SPE4)-(SPE5)	Campus of the Abant Izzet Baysal University	40° 42.864'N 031° 31.166'E	850 m	SÇ+MTB+EU 2013/04



C. speciosus Bieb. subsp. *speciosus*



C. biflorus Mill. subsp. *pulchricolor*
(Herb.) Mathew



C. abantensis T. Baytop & Mathew



C. ancyrensis (Herb.) Maw



C. x paulineae Pasche & Kerndorff



C. olivieri Gay subsp. *olivieri*

Figure 3. Original pictures of the collected specimens (Photographs: M. Tekin BABAÇ, Emel USLU and Serap ÇEVİK).

Table 2. ITS primers used in the PCR

	Primer Name	Nucleotide Sequence(5'->3')	Tm values
Reverse	ITS 5A	CCTTATCATTTAGAGGAAGGAG	49.9 °C
Forward	ITS 4	TCCTCCGCTTATTGATATGC	52.1 °C

Table 3. *trnL*-F primers used in the PCR

	Primer Name	Nucleotide Sequence (5'->3')	Tm values
Reverse	<i>trnL</i> -e	GGTTCAAGTCCCTCTATCCC	54.4 °C
Forward	<i>trnL</i> -f	ATTGAACTGGTGACACGAG	52.8 °C

2.2.2DNA Isolation

Silica gel dried leaf materials were used for genomic DNA isolation by a DNA isolation kit (GeneMATRIX Plant & Fungi DNA Purification Kit). The modified kit protocol is as follows;

1. About 100 mg dried leaf material was powdered with liquid nitrogen in a mortar and pestle, and then transferred to Eppendorf tubes. The powder was centrifuged and added 400 µL of Lyse P solution.
2. For each sample 3 µl of RNase A and 10 µl Proteinase K were added.
3. Mixture was incubated in a water bath at 65 °C for 20-30 minutes. Tubes were shaken gently every 10 minutes.
4. The tubes were taken from water bath and added 130 µl Buffer AC (precipitation buffer). The mixture was incubated on ice for 5 minutes.
5. The tubes were centrifuged at 14,000 RPM for 10 minutes.
6. Supernatant was transferred to new tubes.
7. About 350 µl Sol P Buffer was added.

8. Then 250 μ L 96 % ethanol was added and mixed thoroughly by several times inverting tube.
9. The tubes were centrifuged at 12,000 RPM for 1 minutes and discarded the flow-through. Placed back the spin-column in the collection tube.
10. Wash PX Buffer, 500 μ L (wash buffer) was added then the tubes were centrifuged at 12,000 RPM for 1 minutes and discarded the flow-through.
11. Wash PX Buffer, 500 μ L (wash buffer) was added then the tubes were centrifuged at 12,000 RPM for 2 minutes and discarded the flow-through.
12. New collection tubes replaced and spin columns were placed in tubes. Then 100-200 μ L Elution Buffer was added and incubated in a water bath at 70°C for 5 minutes.
13. The tubes were taken from water bath and incubated the spin-column/collection tube assembly for 3 minutes at room temperature.
14. Centrifuged at 12000 RPM for 1 minutes to elute.
15. Extracted genomic DNAs were kept at 4 °C.

2.2.3 PCR and Electrophoresis Processes

The ITS region (ITS1, 5.8S rDNA, ITS2) was amplified using the primers ITS-4 and ITS-5A and *trnL*-F region was amplified using the primers *trnL*-e and *trnL*-f. DNA amplification was performed in a Thermal Cycler (Techne-³Prime). PCR was performed with carried out in a 25 μ L mix containing 25 ng of genomic DNA, 1X PCR buffer ((NH₄)SO₄), 2.0 mM MgCl₂, 1.0 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 1.0 μ M primer, 1.0 U of *Taq* polymerase.

The PCR cycle program for ITS was as follows: initial denaturation at 94 °C for 5 min, and then 35 cycles at 94 °C for 1 min (denaturation), 54-56.5 °C for 30

min (primer hybridization), and 72 °C for 1 min (elongation), and a final elongation at 72 °C for 10 min.

The *trnL*-F amplification reactions performed as follows: 25 ng of genomic DNA, 2.0 mM of MgCl₂, 1.0 mM of each dNTP, 1.0 μM of *trnL*_F primer, 1.0 U of *Taq* polymerase, and 1X buffer ((NH₄)SO₄) in a final volume of 25 μL. The PCR cycle program for *trnL*-F was as follows: initial denaturation at 94 °C for 5 min, then 35 cycles at 94 °C for 1 min (denaturation), 30 min at appropriate primer hybridization temperature (54-57 °C), 1 min at 72 °C (elongation), and a final elongation at 72 °C for 10 min.

In order to look at the results of genomic DNA isolation was performed by 0.8 agarose gel electrophoresis. At the beginning of electrophoresis, agarose solution is boiled until it became clear, and let it cool down at room temperature for a few minutes before pouring into gel tray. Then melted agarose is poured into a gel tray with removable comb. The agarose polymerizes formed a flexible gel when cooled. Combs are removed carefully from the gel. The gel together with tray is placed in electrophoresis chamber. Electrophoresis buffer (1X TBE) is added to cover the gel to a depth of at least 1 mm. After this process, 0.5 μg/mL ethidium bromide added to the buffer. The PCR products are placed in the wells with loading buffer and run under the influence of an electrical field through the gel at a neutral pH. DNA ladder and control samples were loaded in the first two of the wells. Inclusion of a DNA ladder (100-10,000 bp) makes it easy to determine the sizes of unknown PCR products. Blank sample is provided to negative control which was contained water instead of DNA during PCR.

About 6 μL PCR product was loaded into the wells by pipette. In order to determine the size of PCR products, 5 μL DNA size markers (1 kb DNA ladder)

was used. Products were carried out for 80 min at 100 Volts. Then, the gel images were photographed with a gel documentation system (Ultralum-Teknis) under UV light. To check the results of genomic DNA, 5 µL Genomic DNA samples were loaded into wells. Then, 5 µL DNA size markers (1kb DNA ladder) was loaded into an empty well. Then, the same procedures were repeated (Stanford et al., 2000; Şahin, 2011).

2.3 Methods of Phylogenetic Analyses and Drawing Trees

The amplified ITS and *trnL-F* regions were sent to Refgen and Makrogen company for making DNA sequencing. In order to examine the DNA sequences visually, the codoncode aligner professional computer software program was used. In this program, the DNA sequence contigs were treated visually by looking at the situation and the consensus sequence was generated. Created consensus sequences have been copied to word and was made available for sequence alignment. ClustalW program was used (free of charge on the internet) for sequence alignment. In a word copied sequences were affixed to the ClustalW program and "Execute Alignments" by command sequence alignment was performed. In order to understand the degree of kinship of studied *Crocus* taxa, aligned sequences were run with the program namely, PAUP (Phylogenetic Analysis Using Parsimony) 4.0b10 phylogenetic analysis Program (Swofford, 2002). This program commonly preferred for these kinds of studies. The analysis began with "execute" command of Nexus type data. Then, the "help" command, which opened the window of the criteria tree. In the open window, the desired criteria were selected and phylogenetic trees were obtained. To create a phylogenetic tree, character-based methods used was selected as the parsimony criterion. Also search algorithm has been selected as heuristic research

and Bootstrap. For phenetic analyses, the UPGMA and Neighbor Joining (NJ) of distance-based methods were used.

3. RESULTS

3.1 DNA Isolations

Six taxa of *Crocus* L. were used and totally 30 individuals of them were collected from the localities (Table 1). In the study *Crocus pallasii* F. Goldbach as the close outgroup and *R. bulbocodium* (L.) Seb. et Mauri, *Romulea ramiflora* Ten., as the far outgroup were used. The genomic DNAs of the plant samples were isolated by following the GeneMATRIX extraction kit protocol purchased commercially (Biolab). The agarose gel pictures of gDNAs belonging to some *Crocus* taxa are indicated in the figures 4 and 5. ITS regions of the isolated gDNAs were amplified by using the ITS4 and ITS5A primers with PCR. Some part of the ITS bands obtained as a result of PCR were given in the figures 6, 7 and 8. The *trnL-F* regions of the isolated gDNAs were amplified by using the *trnL-f* and *trnL-e* primers. Some parts of the *trnL-F* bands obtained as a result of PCR were given in the figures 9, 10 and 11.

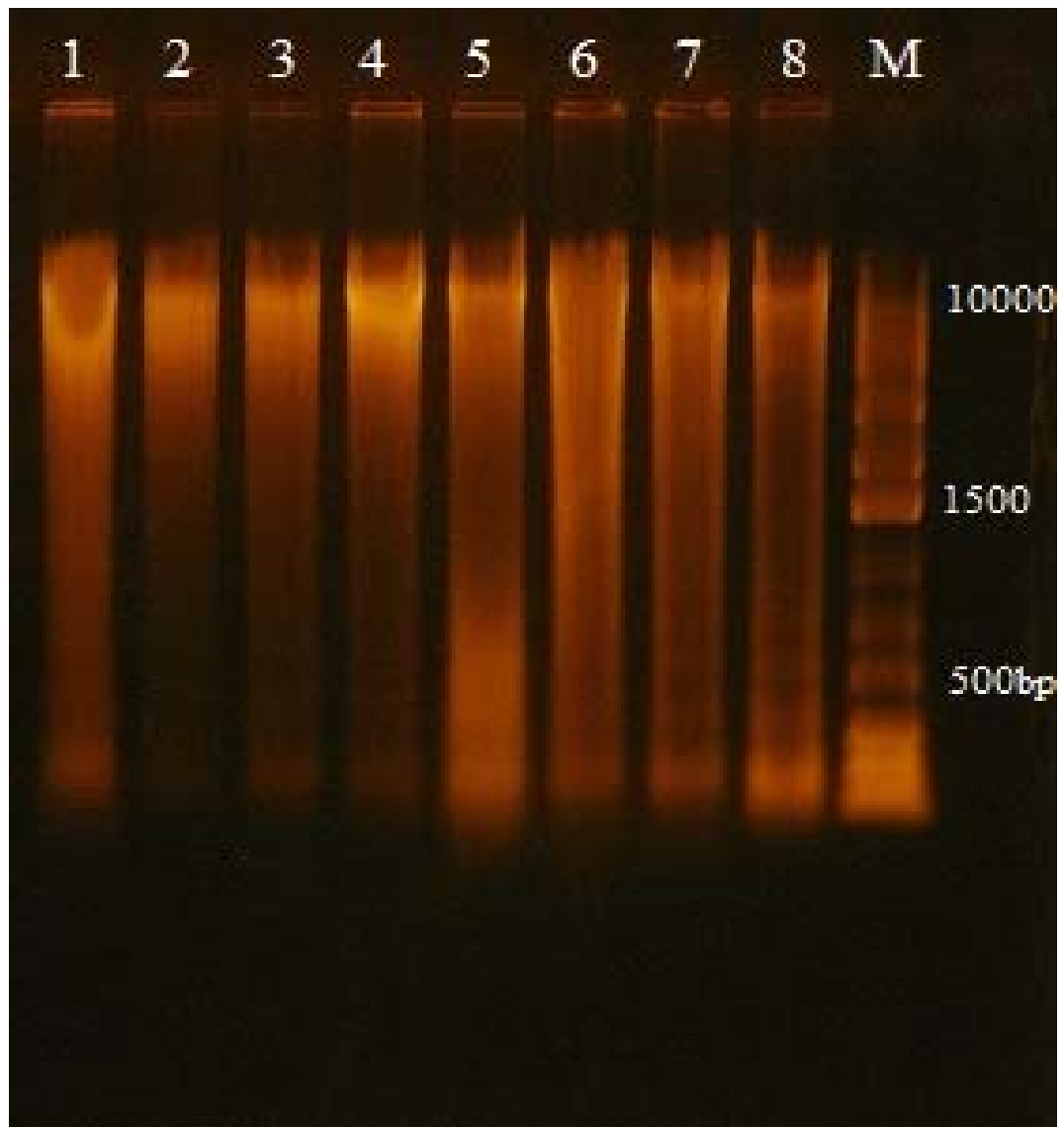


Figure 4. After isolation of genomic DNA, its quality was checked by electrophoresis (M: Marker; 1: *C. x paulineae*1, 2: *C. x paulineae*2, 3: *C. x paulineae*3, 4: *C. ancyrensis*3, 5: *C. abantensis*3, 6: *C. biflorus* subsp. *pulchricolor*3, 7: *C. olivieri* subsp. *olivieri*3, 8: *C. speciosus* subsp. *speciosus*3).

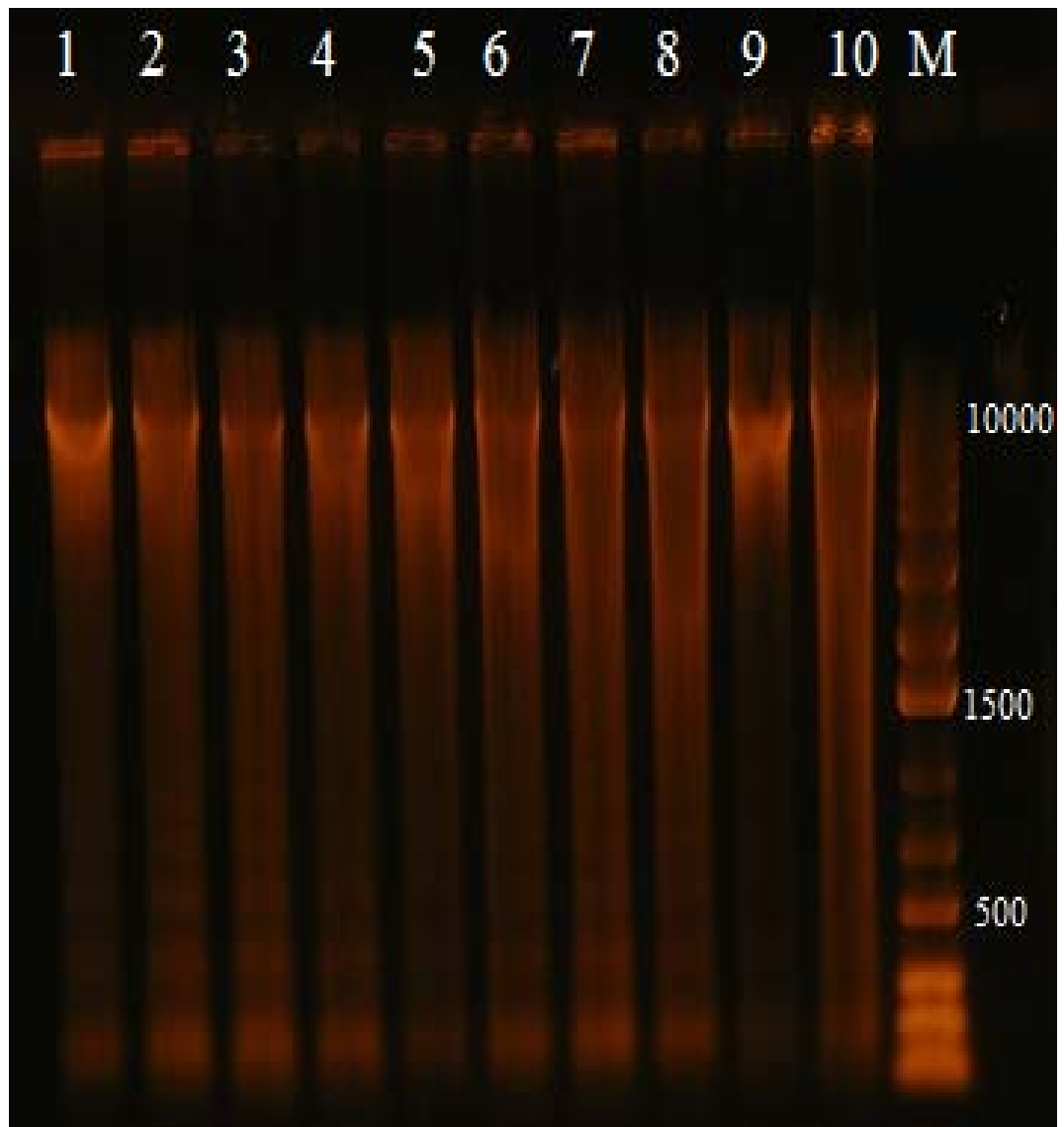


Figure 5. After isolation of genomic DNA, its quality was checked by electrophoresis (M: marker, 1: *C. ancyrensis*1, 2: *C. ancyrensis*2, 3: *C. abantensis*1, 4: *C. abantensis*2, 5: *C. biflorus* subsp. *pulchricolor*1, 6: *C. biflorus* subsp. *pulchricolor*2, 7: *C. olivieri* subsp. *olivieri*1, 8: *C. olivieri* subsp. *olivieri*2, 9: *C. speciosus* subsp. *speciosus*1, 10: *C. speciosus* subsp. *speciosus*2).

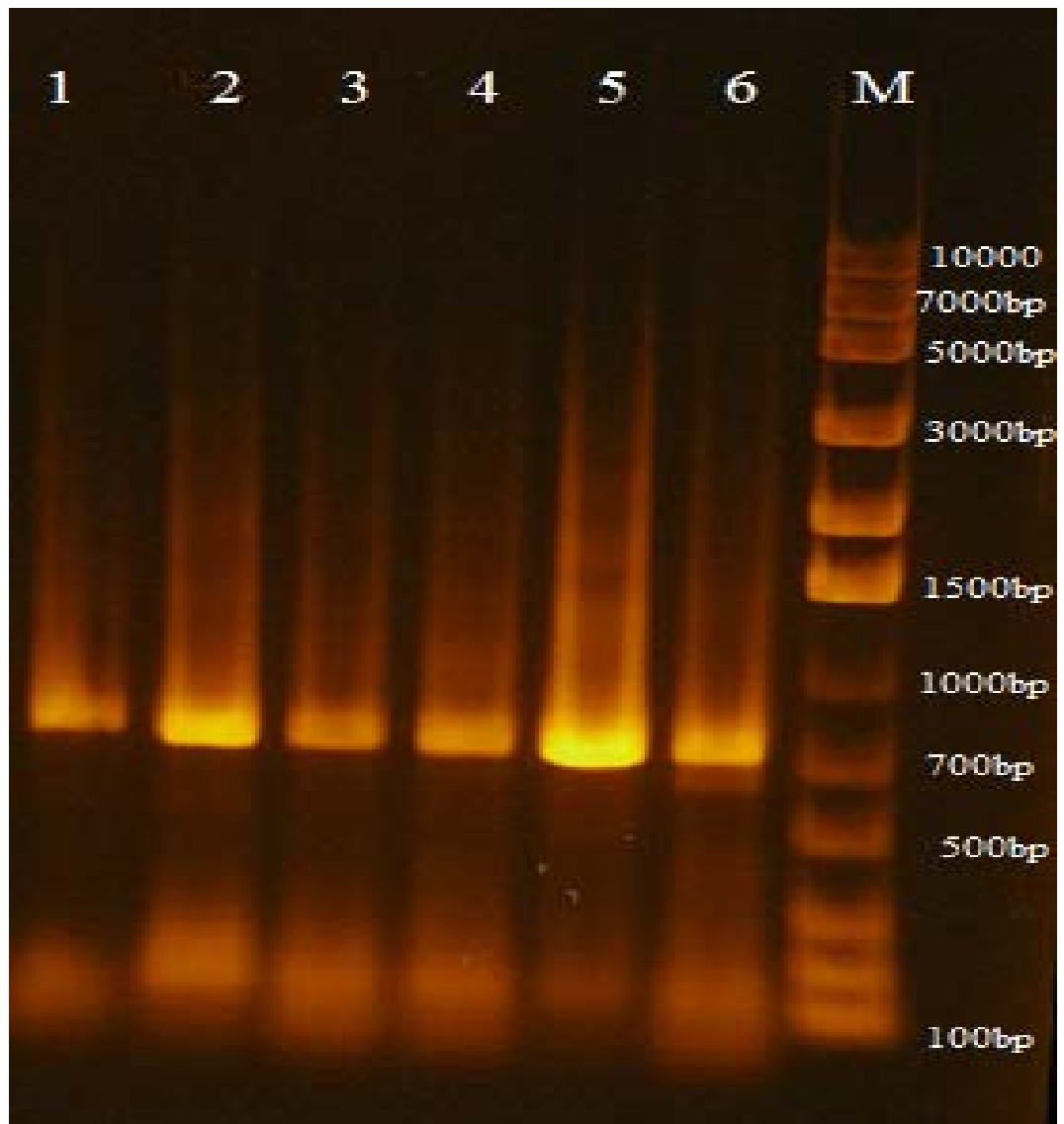


Figure 6. Some individuals of *Crocus* taxa were amplified by using the ITS4 and ITS5A primers with PCR (M: Marker, 1: *Crocus abantensis*1, 2: *C. ancyrensis*1, 3: *C. biflorus* subsp. *pulchricolor*1, 4: *C. olivieri* subsp. *olivieri*1, 5: *C. speciosus* subsp. *speciosus*1, 6: *C. x paulineae*1).

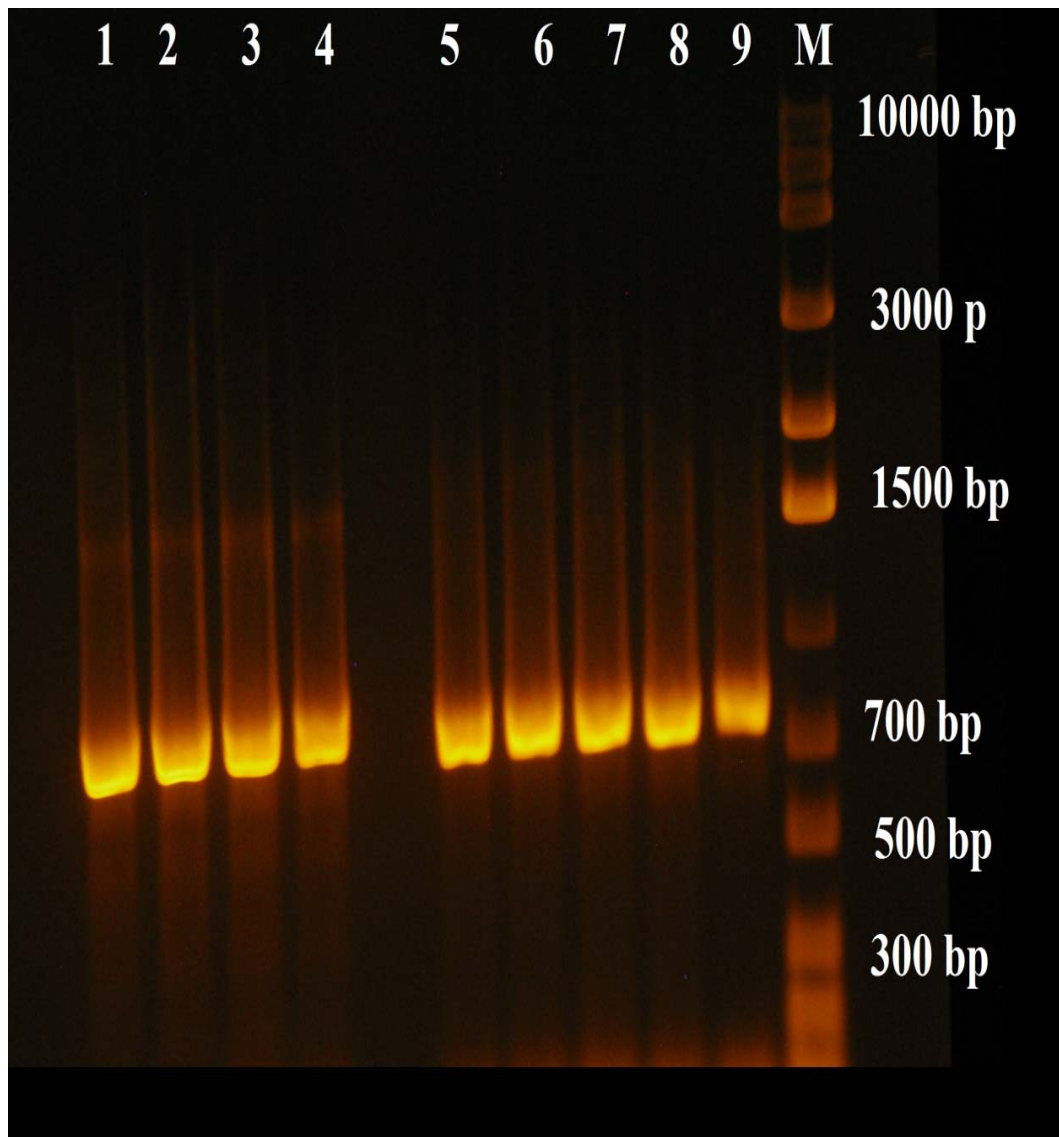


Figure 7. Some individuals of *Crocus* taxa were amplified by using the ITS4 and ITS5A primers with PCR (M: Marker, 1: *Crocus abantensis*2, 2: *Crocus abantensis*3,3: *C. ancyrensis*2, 4: *C. ancyrensis*3, 5: *C. biflorus* subsp. *pulchricolor*2, 6:*C. biflorus* subsp. *pulchricolor*3,7: *C. olivieri* subsp. *olivieri*2, 8: *C. speciosus* subsp. *speciosus*2, 9: *C. x paulineae*2).

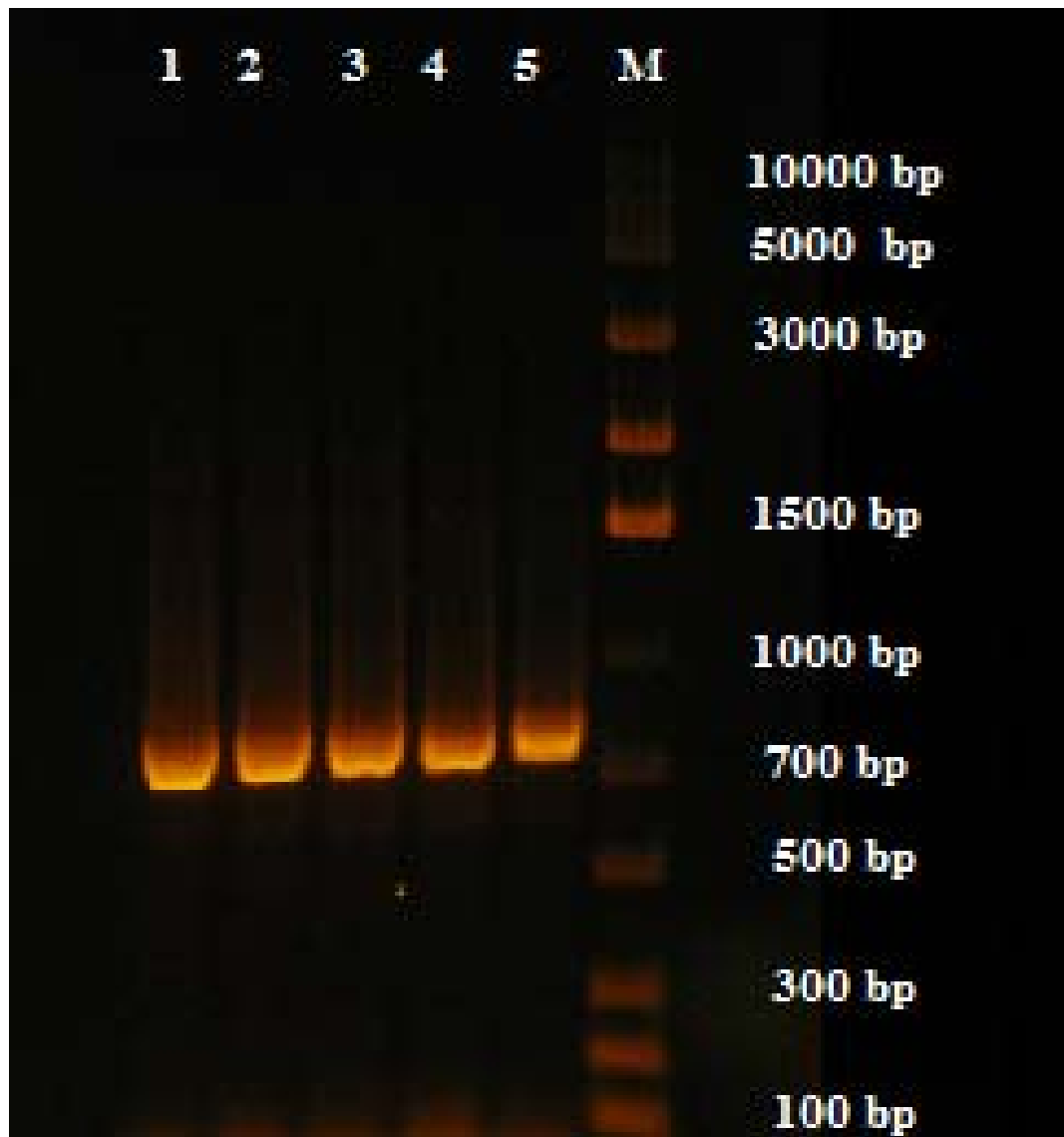


Figure 8. Some individuals of *Crocus* taxa were amplified by using the ITS4 and ITS5A primers with PCR (M: Marker, 1: *Crocus abantensis*4, 2: *C. ancyrensis*4, 3: *C. biflorus* subsp. *pulchricolor*4, 4: *C. olivieri* subsp. *olivieri*3, 5: *C. x paulineae*3).

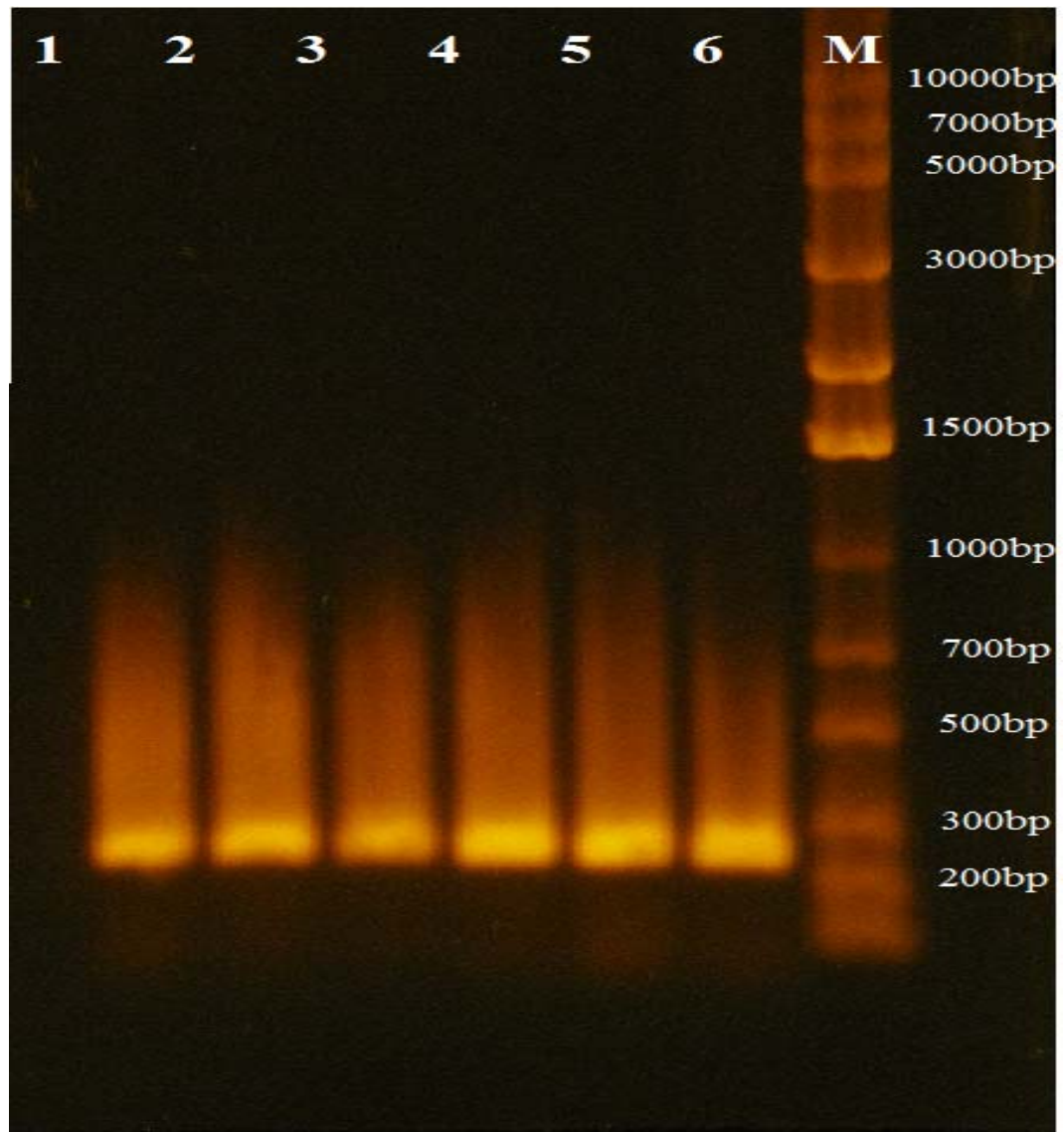


Figure 9. Some individuals of *Crocus* taxa were amplified by using the *trnL*-e and *trnL*-f primers with PCR (M: Marker, 1: *Crocus abantensis*1, 2: *C. ancyrensis*1, 3: *C. biflorus* subsp. *pulchricolor*1, 4: *C. olivieri* subsp. *olivieri*1, 5: *C. speciosus* subsp. *speciosus*1, 6: *C. x paulineae*1).

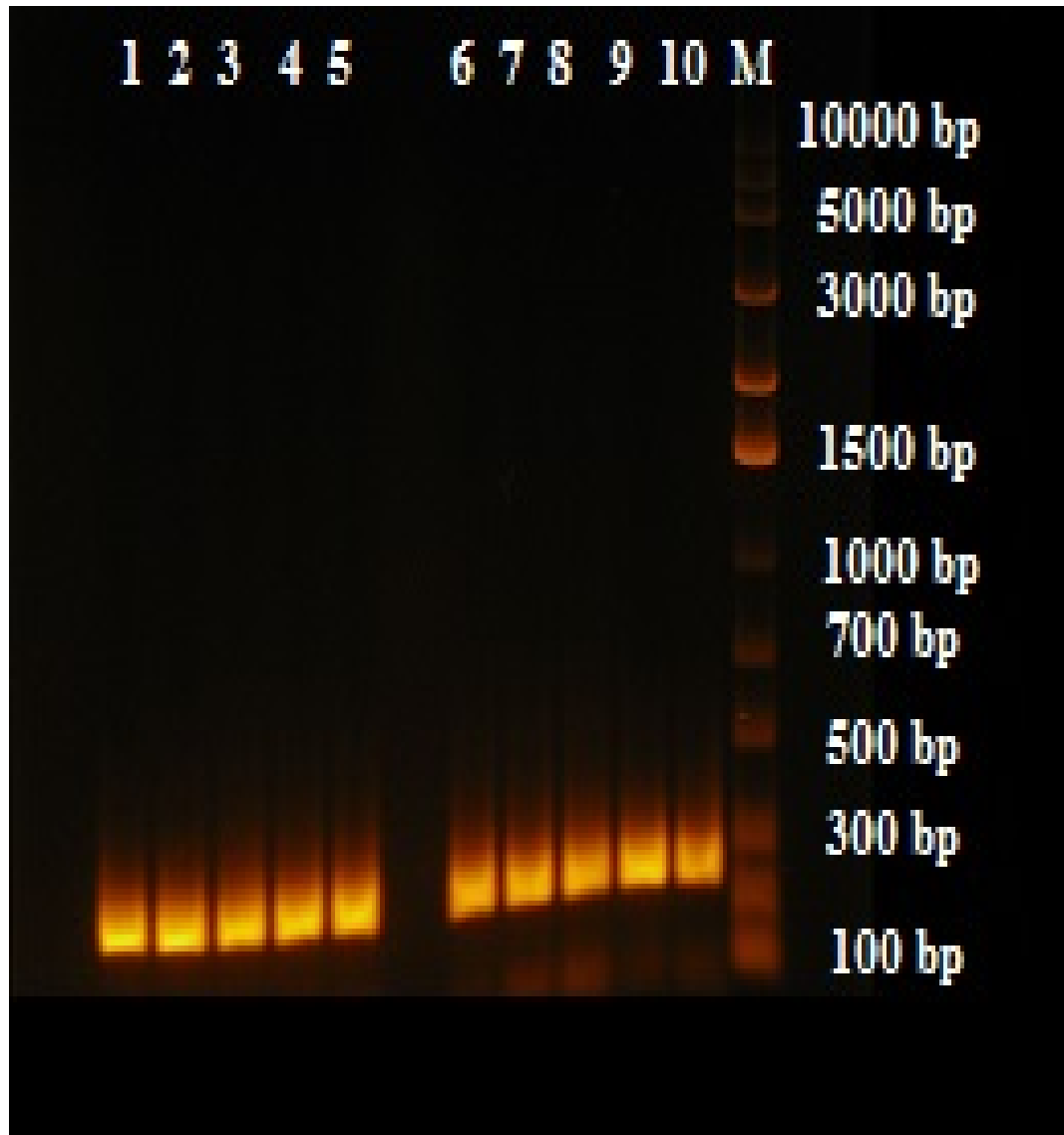


Figure 10. Some individuals of *Crocus* taxa were amplified by using the *trnL*-e and *trnL*-f primers with PCR (M: Marker, 1: *Crocus abantensis*2, 2: *C. ancyrensis*2, 3: *C. biflorus* subsp. *pulchricolor*2, 4: *C. olivieri* subsp. *olivieri*2, 5: *C. speciosus* subsp. *speciosus*2, 6: *C. x paulineae*2, 7: *Crocus abantensis*3, 8: *C. ancyrensis*3, 9: *C. biflorus* subsp. *pulchricolor*3, 10: *C. olivieri* subsp. *olivieri*3).

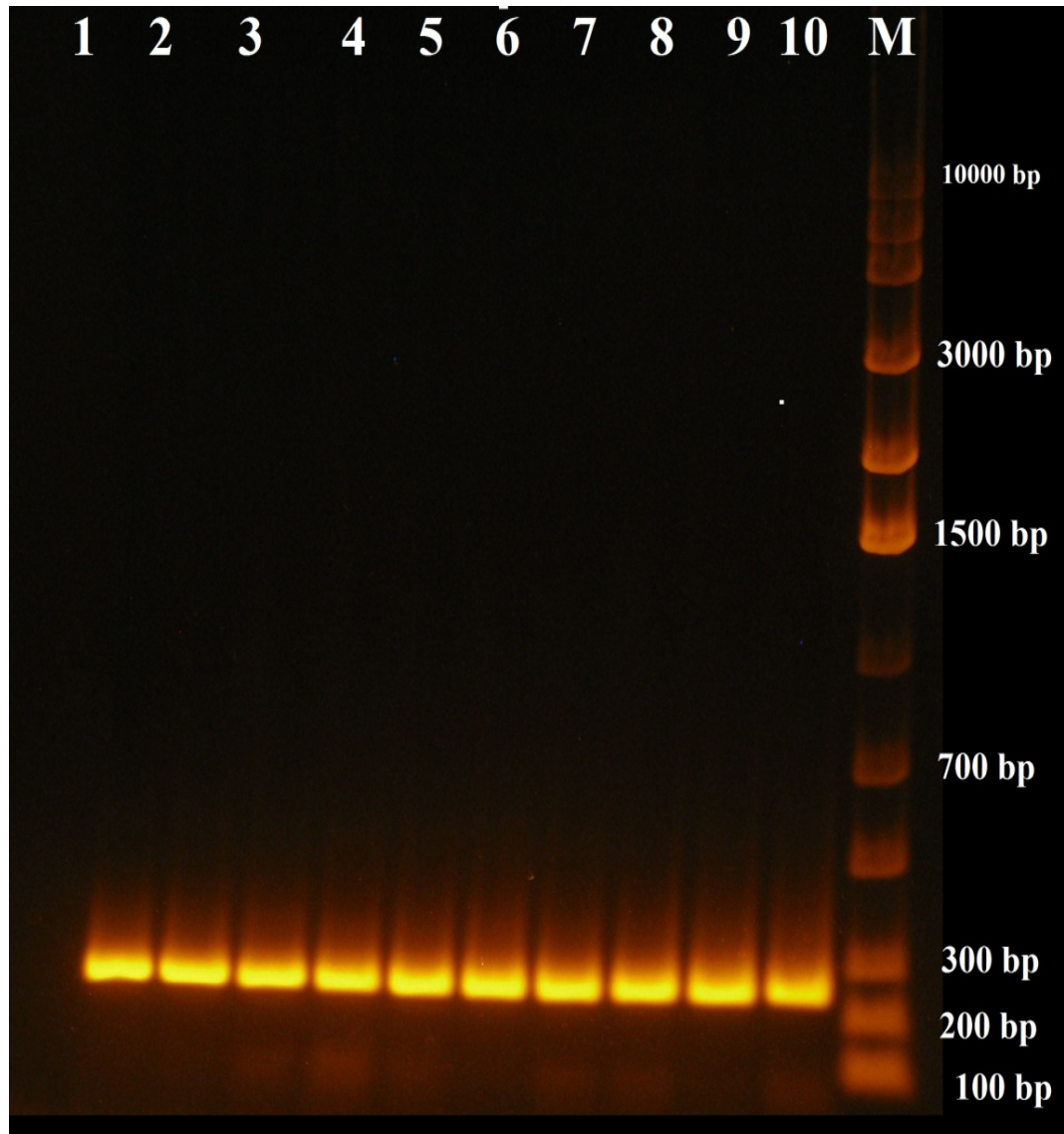


Figure 11. Some individuals of *Crocus* taxa were amplified by using the *trnL*-e and *trnL*-f primers with PCR (M: Marker, 1: *Crocus abantensis*4, 2: *C. ancyrensis*4, 3: *C. biflorus* subsp. *pulchricolor*4, 4: *C. olivieri* subsp. *olivieri*4, 5: *C. speciosus* subsp. *speciosus*3, 6: *C. speciosus* subsp. *speciosus*4, 7: *C. x paulinae*3, 8: *C. x paulinae*4, 9: *Crocus abantensis*5, 10: *C. ancyrensis*5).

3.2 DNA Sequencing Reactions

After the ITS and *trnL*-F regions were amplified with PCR reactions, DNA sequences of them were made by Makrogen and Refgen Companies for the

purification and sequencing reactions (Cycle sequencing). The results of the sequences were received from the companies in the *ABI prism* formats.

3.3 Processing of the DNA Sequences

Codoncode Aligner program (Anonymous, 2014) was used to process the DNA sequences that came in files with *ABI prism* format. Contig was created in forms of the sequences of forward and reverse for each type. Some of the bases misread by the device which sequencing reactions were carried out were corrected manually with the help of Codoncode Aligner and BioEdit programs (Hall, 1997-2013) by taking the strength and clearness of the signals in the chromatogram and contig sequences were obtained (Figure 12).

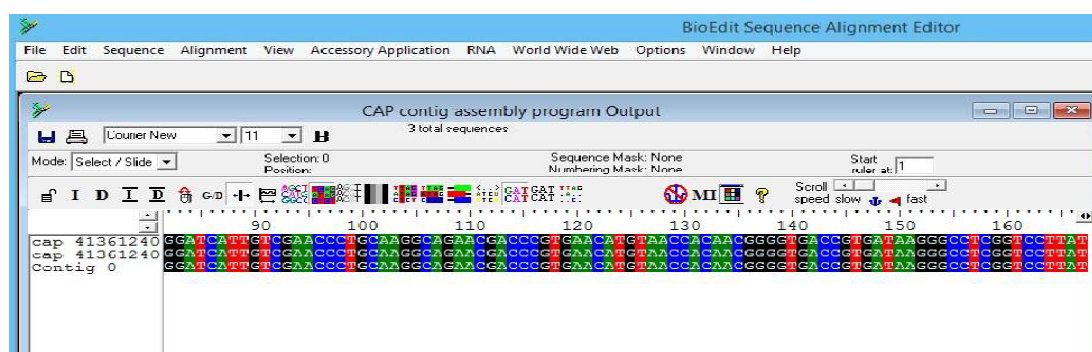


Figure 12. Series of images from BioEdit program

3.4 Alignments of the DNA Sequences

ClustalW (Thompson et al., 1994) program was used for the alignment of the sequences. Since this program operates in Fasta format, the DNA sequences obtained were converted into Fasta format with the help of Microsoft Office Word program and prepared to be ready for alignment. DNA base sequences were aligned with the

relevant commands in ClustalW program and the base differences between the types were identified (Figure 13).

```

ABA1_ITSS      --GTAACAAGGTTTCOGTAGGTGAA-CCTGCGGAAGGATCATTGTGAGA 47
ABA5_ITSS      -CGTAACAAGGTTTCOGTAGGTGAA-CCTGCGGAAGGATCATTGTGAGA 48
ABA2_ITSS      -CGTAACAAATTTTCOGTAGGTGAA-CCTGCGGAAGGATCATTGTGAGA 48
ABA3_ITSS      ---GAACAAGGTTTCOGTAGGTGAA-CCTGCGGAAGGATCATTGTGAGA 46
PAU2_ITSS      --GAAGCACGGTAGGAATAGGTGAA-CCTGCGGAAGGATCATTGTGAGA 47
PAU5_ITSS      -----
PAU4_ITSS      -----
ANK2_ITSS      -TGTAACAAGGTTTCOGTAGGTGAA-CCTGCGGAAGGATCATTGTGAGA 48
ANK5_ITSS      ---AACAAAGGTTTCOGTAGGTGAA-CCTGCGGAAGGATCATTGTGAGA 45
ANK1_ITSS      -CGTAACAAGGTTTCOGTAGGTGAA-CCTGCGGAAGGATCATTGTGAGA 48
PAU1_ITSS      -----GGTGA---CTGCGGAAGGATCATTGTGAGA 28
ANK4_ITSS      -CGTAACAAGGTTTCOGTAGGTGAA-CCTGCGGAAGGATCATTGTGAGA 48
ANK3_ITSS      TCGTAACAAGGTTTCOGTAGGTGAA-CCTGCGGAAGGATCATTGTGAGA 49
PAU3_ITSS      TCGTAACAAGGTTTCOGTAGGTGAA-CCTGCGGAAGGATCATTGTGAGA 49
SPE1_ITSS      -----

```

Figure 13. Alignment of the DNA sequences

3.5 Phylogenetic Analyses

PAUP 4.0b10 (Swofford, 2002) program was used for phylogenetic analyses. This program operates in Nexus format. The sequences aligned in ClustalW program were prepared to be ready for phylogenetic analyses in PAUP 4.0b10 program by converting into the Nexus formats.

MAC computer was used in the study and phylogenetic analyses were carried out in the MAC (Apple Macintosh) version of PAUP. Phylogenetic trees were easily created by selecting the criteria and the analyses required to be carried out from the windows occurring in the upper part of the screen in MAC computer after PAUP opened.

Parsimony which is one of the character based methods was selected from the criteria and Heuristic research was carried out and finally the trees created were recorded. The mutual consensus trees from the formed trees were created by

following the necessary ways from the window and recorded. Again the bootstrap analysis was carried out in Parsimony criterion and the trees were recorded.

Then UPGMA (Unweighted Pair Group Method Using Arithmetic Average) (Sneath and Sokal, 1973) and NJ (Neighbor Joining) (Abbott et al., 1985) analyses were carried out by using the distance based method.

3.5.1 Phylogenetic Analyses Based on the ITS Sequences

Heuristic research has been made by using the maximum parsimony from the character based methods. The maximum tree number to be formed and scanned in this method has been selected as 10,000. The algorithm used to evaluate the branches was selected as TBR (Tree-Bisection-Reconnection). In this analysis, all character types were adjusted to be in equal weight and unordered.

As a result of the analysis, 10,000 trees have been obtained and totally 3 of them were presented such as 1st, 500th and 1000th in this thesis. The Figures of 14, 15, and 16 formed according to the result of parsimony criteria are indicated the 1st, 500th, and 1000th trees, respectively.

Totally 844 bases of the characters were examined in the parsimony analysis and 645 of them were the constant characters. Their variable values, and informative values were found as 75, and 124, respectively. Results of Heuristic searches formed totally 73,805,110 different tree topologies and the score of the best parsimonious trees (digit number) was found to be 272.

Romulea ramiflora and *R. bulbocodium* species which were selected as the distant out group obtained at the lower part of the trees (Figures 14, 15, and 16) and *Crocus* taxa were separated obviously as monophyletic group based on the parsimony

analysis. The *Crocus pallasii* species was also selected as the close out group, showed a very close relation with the taxa forming our internal group. The taxa of the *Crocus* formed 5 different but monophyletic clades where *C. olivieri* subsp. *olivieri* and *C. pallasii* taxa included in the first clade. Some of the *C. abantensis* individuals and *C. x paulineae* hybrids included in the second clade together, and the third clade contained the remained *C. anycrensis* and the hybrid *C. x paulineae* individuals (Figures 14-16). The remained two clades comprised *C. speciosus* subsp. *speciosus* and *C. biflorus* subsp. *pulchricolor* taxa. It was an interesting result that 3 *C. x paulineae* and 3 *C. abantensis* taxa, which are the one parent, were grouped under the same clade, though the other 2 *C. x paulineae* taxa grouped with all the *C. anycrensis* individuals, the other parent, together in the same clade. It is clear evidence that *C. x paulineae* is the hybrid of *C. abantensis* and *C. anycrensis*.

Heuristic search completed

Total number of rearrangements tried = 73805110

Score of best tree(s) found = 272

Number of trees retained = 10000

Time used = 00:01:00

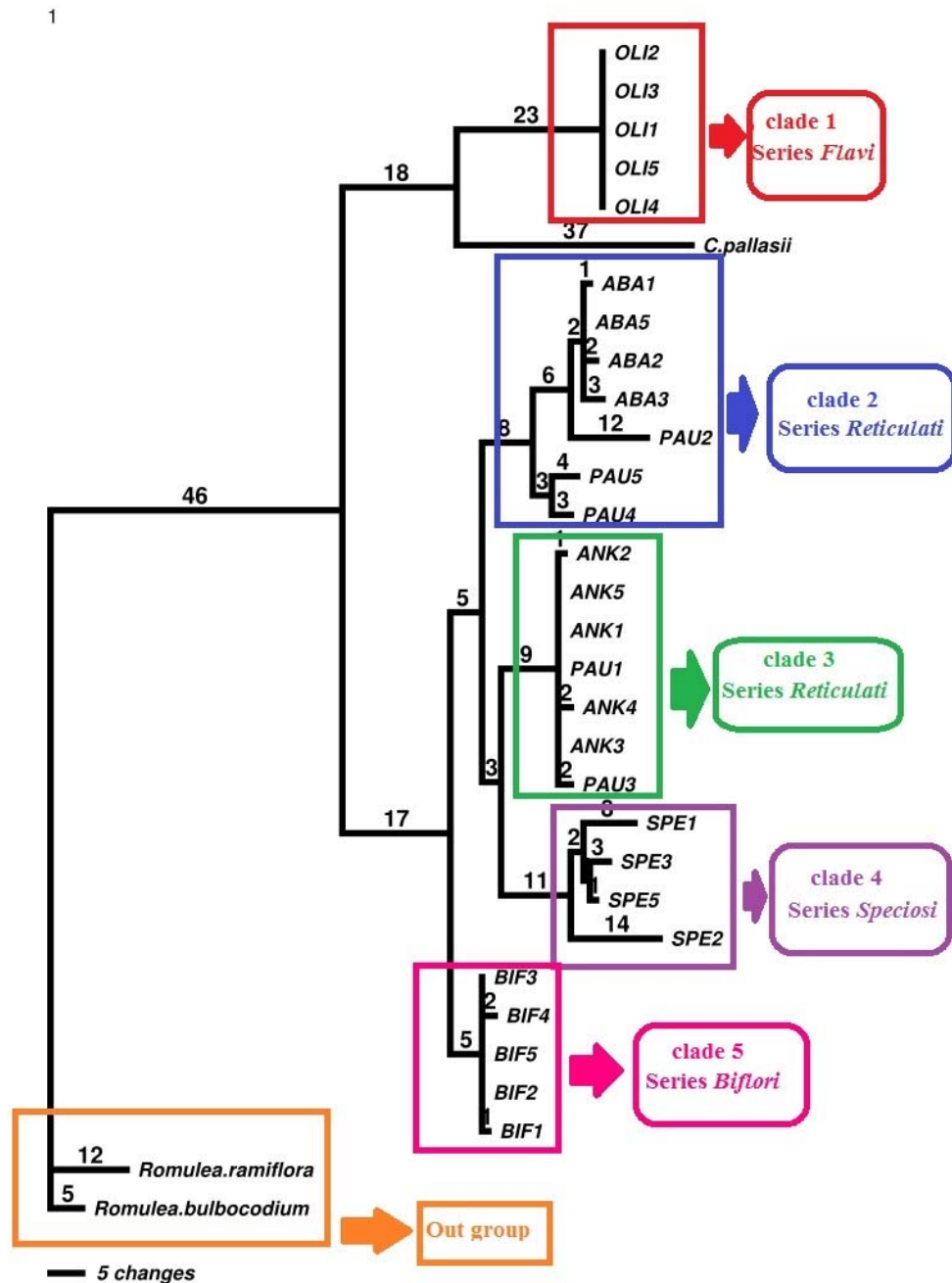


Figure 14. The tree number 1 obtained by using the Parsimony Criterion

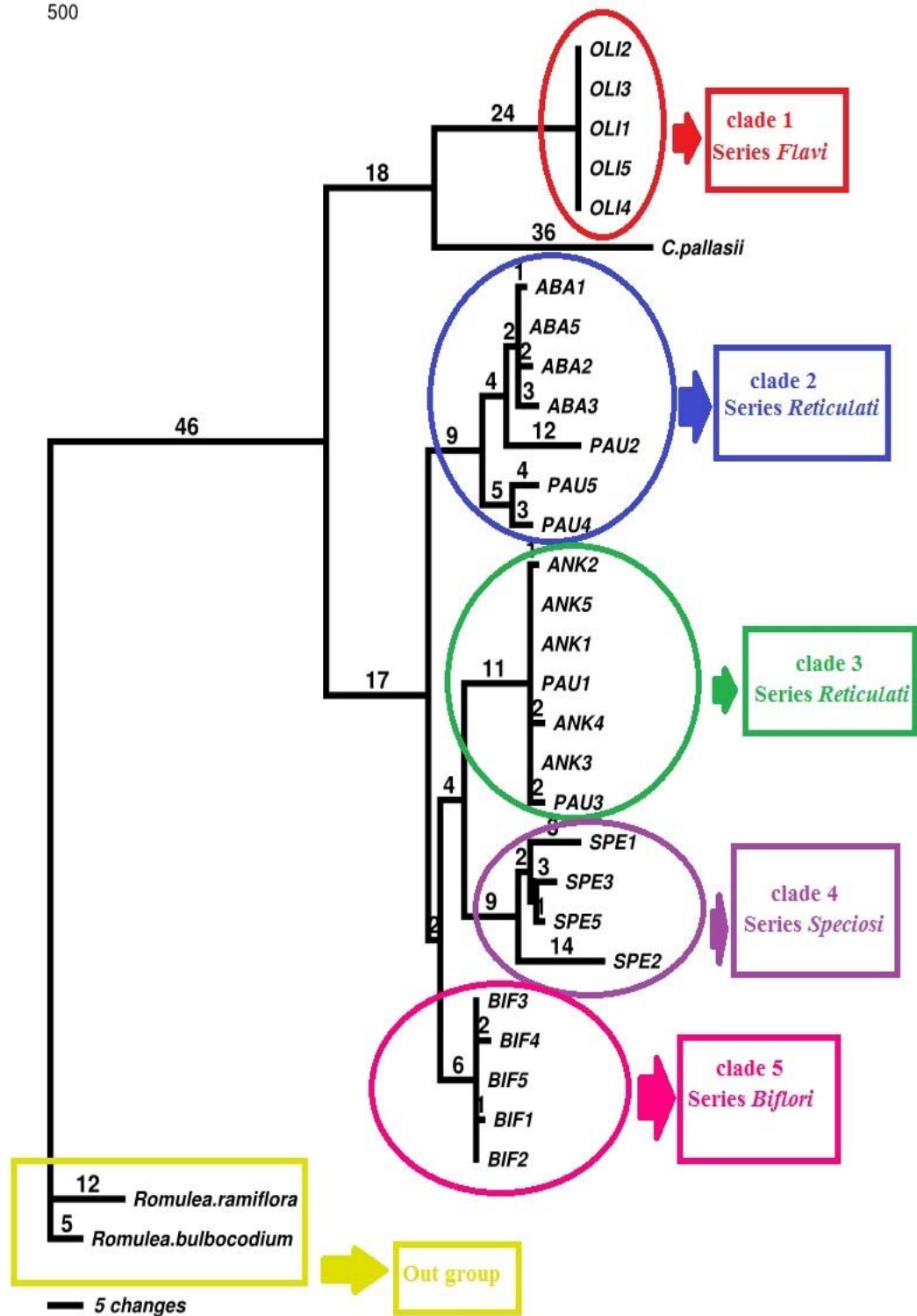


Figure 15. The tree number 500 obtained by using the Parsimony Criterion

1000

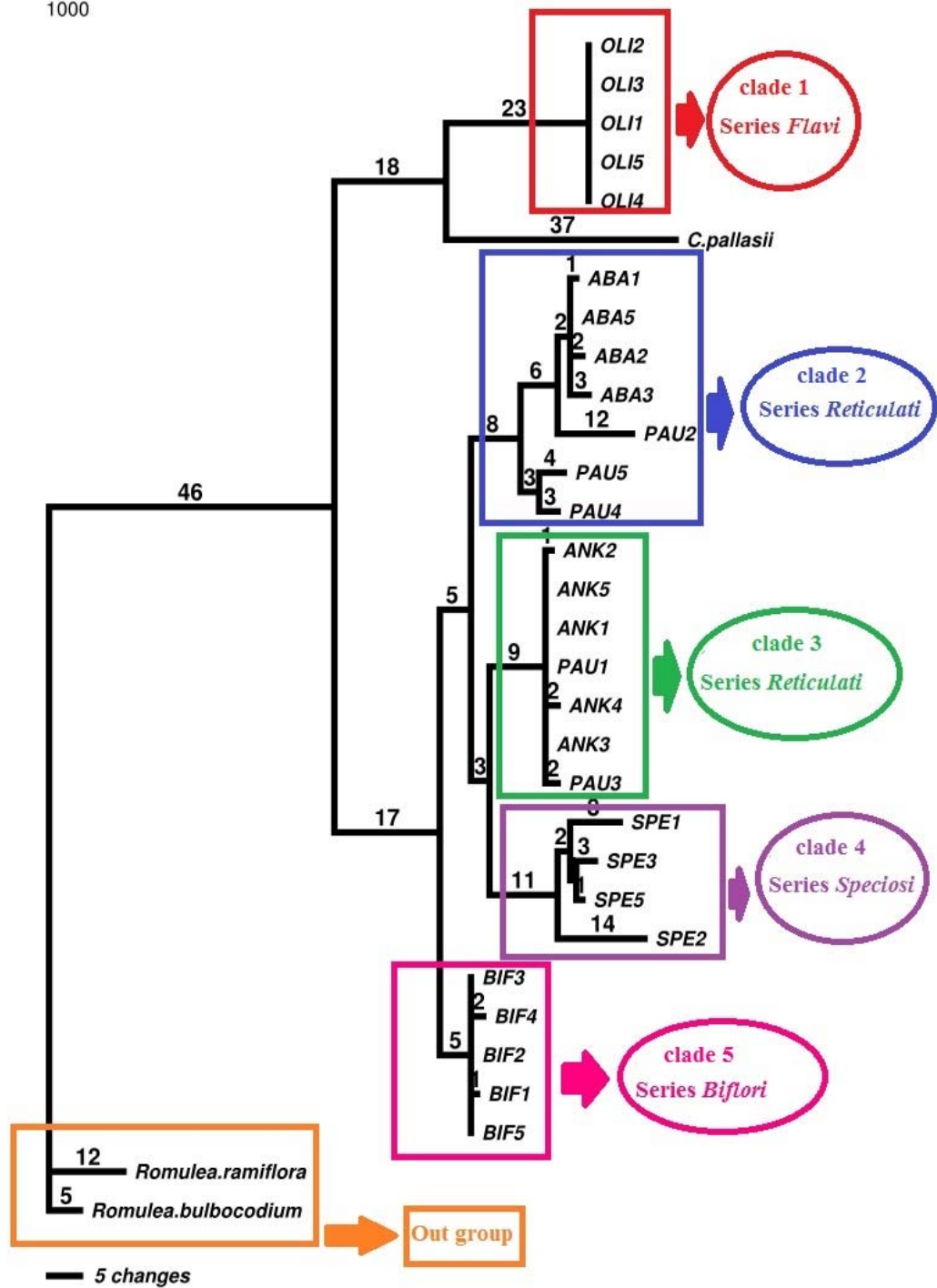


Figure 16. The tree number 1000 obtained by using the Parsimony Criterion

Apart from these analyses, the bootstrap analysis in the maximum parsimony method was also carried out. This method is used to determine the most reliable branches in terms of statistics by using the parsimony criteria of the tree branches obtained. It indicates at what rate of each branch was supported in percentages. If the supporting value is below 50%, the program will downfall so, this branch as it will not be reliable by qualifying it too weak, and the non-cascaded branching formed as a result of collapsing branch is called polytomy.

The bootstrap tree formed in Figure 17 was obtained by selecting the maximum tree number as 1000 and repeating 100 times (replicates). Since the taxon number is excessive 1000 repetition number was selected as 100. When the repetition number and/or maximum tree number are increased, the capacity of the computer was insufficient since it lasted too long.

The mutual decision (consensus) of 10,000 trees was formed that were created as a result of heuristic research in maximum parsimony criterion. From these the Strict consensus tree is seen in Figure 18, Majority Rule tree as seen in Figure 19 and Adams consensus tree is as seen in Figure 20.

The bootstrap percentages were processed onto the obtained Maximum Parsimony tree as seen in Figure 17. Here, it is seen that 6 taxa created 5 groups basically and these groups were supported as 100%, 99%, 98% and 67% in bootstrap analysis. While it is not possible to solve the real phylogeny very well, differentiating on the surface of the species namely being different from one another is clearly observed.

Again the taxa of the distant out group formed a monophyletic clade within themselves and this branch received 100% bootstrap value. *C. pallasii*, selected as close out group, was found to be closed to *C. olivieri* subsp. *olivieri* taxa and this monophyletic clade supported as 67% bootstrap value.

In these trees which are the parsimony tree, strict consensus tree, majority rule tree, and adams consensus trees (Figures 16–20), five monophyletic groups were obtained with higher supporting values, such as *C. olivieri* subsp. *olivieri* together with the close out group taxon -*C. pallasii* (called as Olivieri group), *C. abantensis* and *C. x paulineae* individuals (called as Abantensis + Paulineae group), polytomy group of *C. anycrensis* individuals and some *C. x paulineae* individuals (called as Ancyrensis + Paulineae group), *C. speciosus* subsp. *speciosus* individuals (as Speciosus group) and another polytomy group of *C. biflorus* subsp. *pulchricolor* individuals (called as Biflorus group). All these monophyletic groups formed by five different polytomies.

Heuristic search completed

Total number of rearrangements tried = 7371646

Score of best tree(s) found = 272

Number of trees retained = 1000

Time used = 6.12 sec

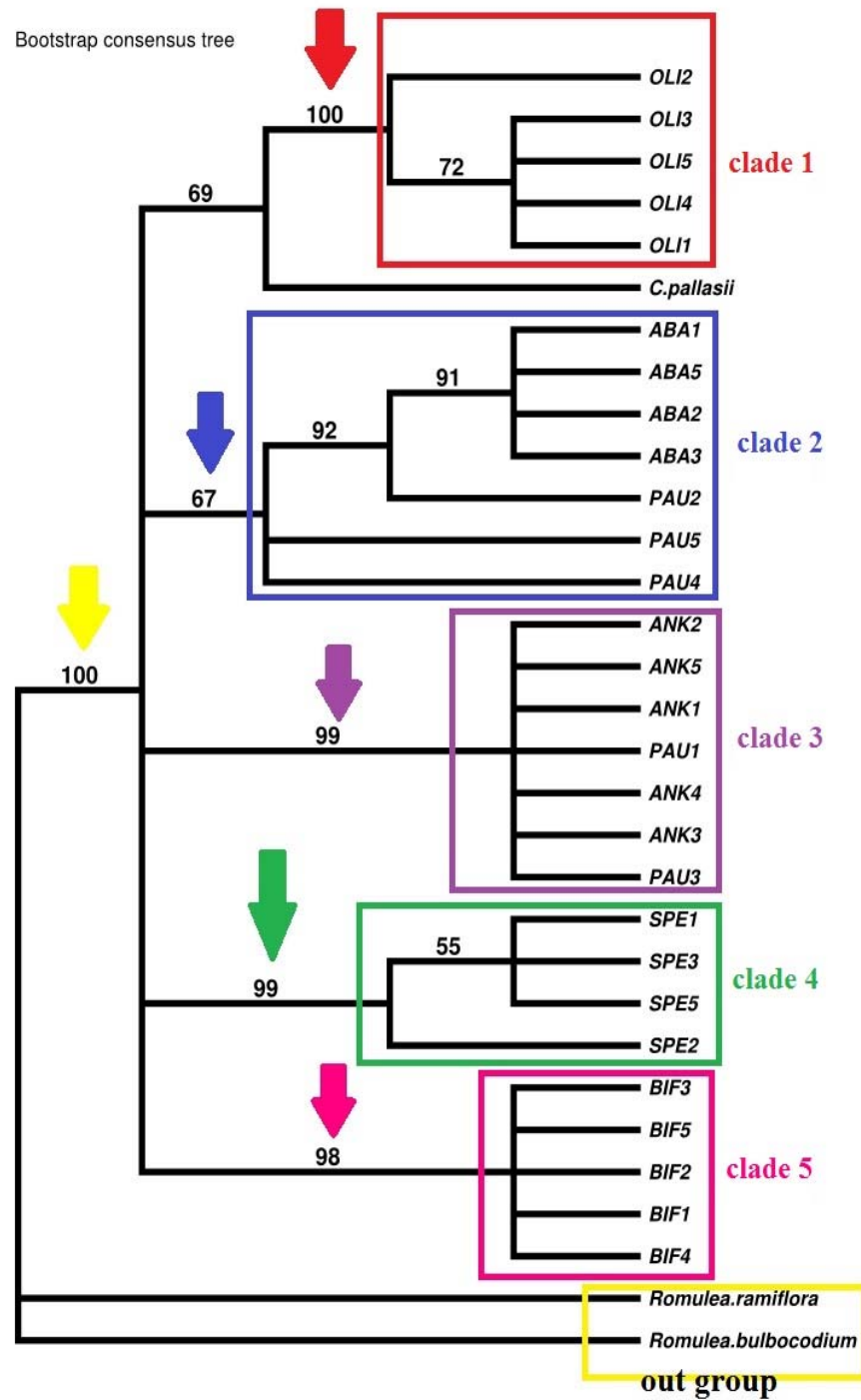


Figure 17. Parsimony tree formed after a Bootstrap analysis

Strict consensus tree

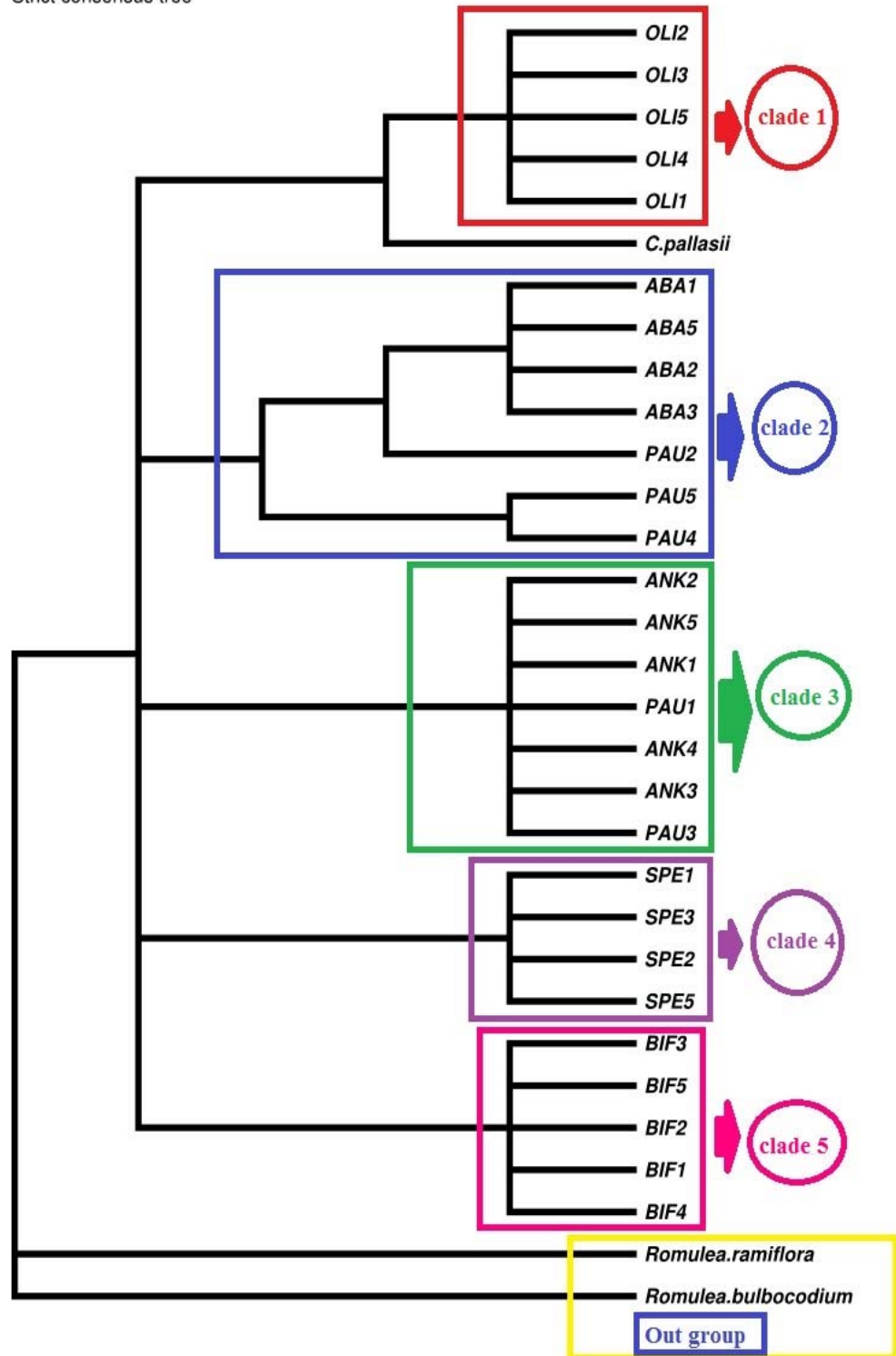


Figure 18. Strict Consensus of 10000 trees obtained with Parsimony

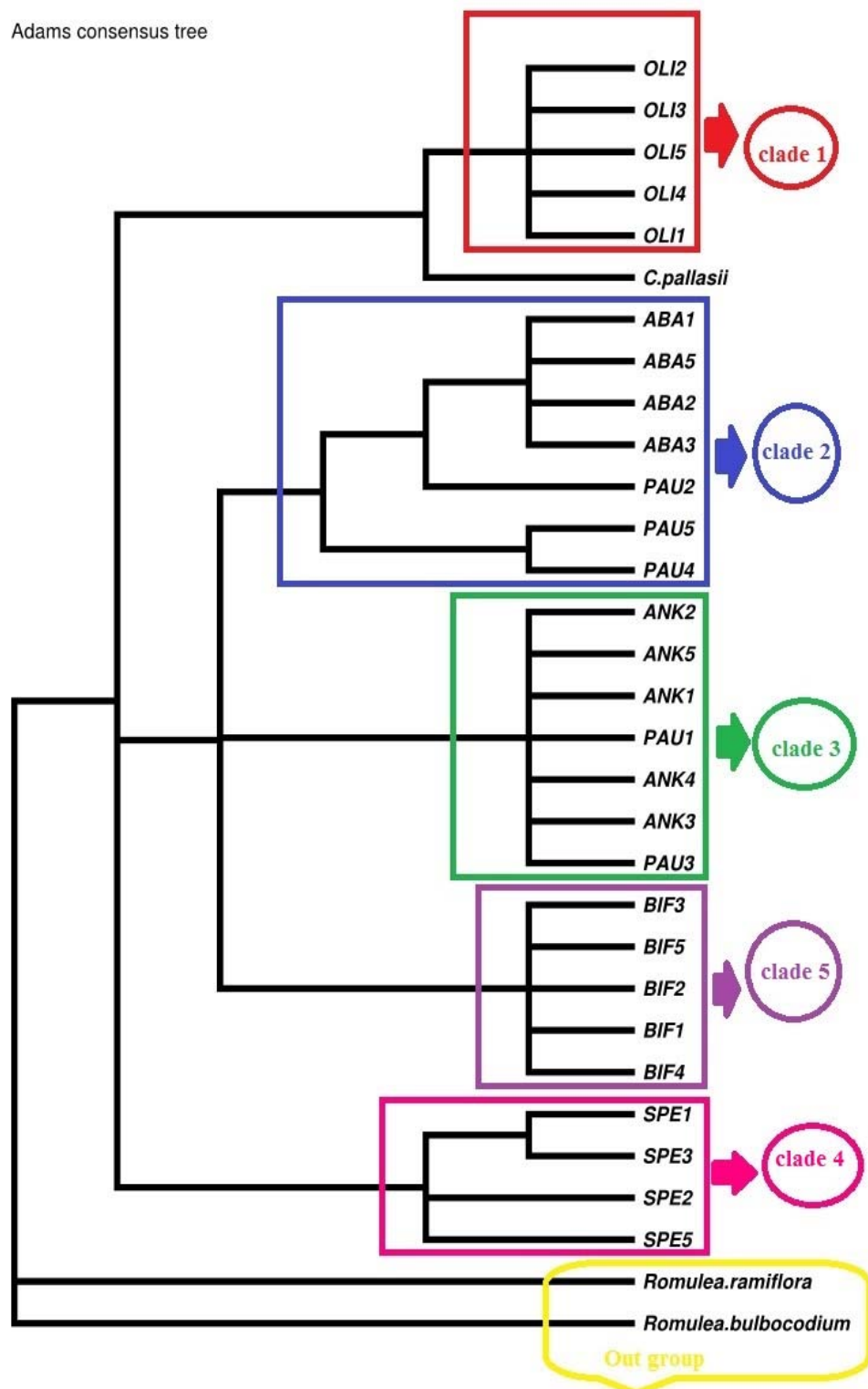


Figure 20. Adams Consensus of 10000 trees obtained with Parsimony

In addition to the Maximum Parsimony method, UPGMA and NJ analyses were also carried out under the HKY85 model which is the distance based methods. The genetic distance of the taxa were calculated with HKY85 model algorithm and the values were provided in appendices part as table (Appendix V). Phylogenetic trees formed as the results of HKY85, UPGMA, and NJ analyses are given in Figures 21, 22, and 23, respectively.

Distance methods are based on phylogenetic tree formation for all the taxa according to the distance scores in matrix subsequently by forming a data matrix with evolutionary distances obtained as a result of aligning the sequences (Felsenstein, 1987). The number of the changes emerging along the branches of the tree show the distance between the sequences (Başbüyük et al., 2000). The preferred trees are the ones that reduced that distance between the taxa to the minimum (Mount, 2001). The algorithms used in this method are divided into two branches as set or optimal situation (optimum) based. Set of these algorithms form the phylogenetic tree by calculating based on a distance matrix starting with the most similar sequenced pairs. These algorithms include the UPGMA and NJ method by using the arithmetical average.

In Figure 22, it was observed that the lengths of the branches are different from each other in the phylogenetic tree formed as a result of NJ analysis. Branch lengths being different in phylogram indicated what species come out before or after. The long branches in the phylogenetic tree indicates the closest ancestor to today, the short branches indicate the oldest (primitive) ancestor (Felsenstein, 1987).

In Figure 23, the branch lengths in UPGMA analysis are equally calculated. This method is the one used in the early evolutionary analyses and it has revealed the measuring of the distances with Neighbor Joining method since the conditions of going through mutation at the same rate of the sibling taxa in today and it is widely used (Başibüyük et al., 2000).

Almost all trees have yielded the same result by ITS sequence analyses either with character based or the distance based methods. We clearly see that 6 taxa divide into five monophyletic groups and in all analyses the *C. x paulineae* individuals was also the hybrid of the *C. abantensis* and *C. anycrensis* taxa. Because all these taxa have taken place in the same group.

Heuristic search completed

Total number of rearrangements tried = 8486

Score of best tree(s) found = 0.01113

Number of trees retained = 1

Time used = 0.11 sec

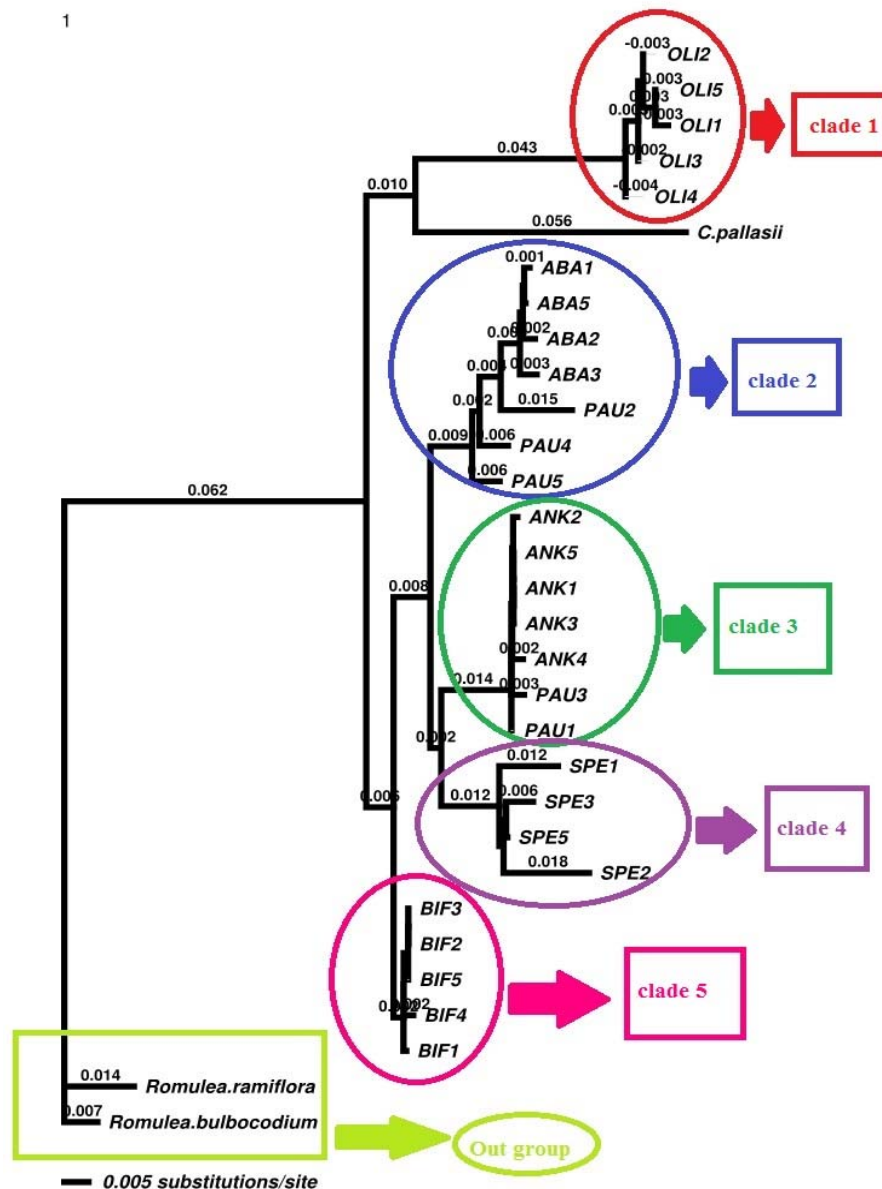


Figure 21. The tree formed as a result of Parsimony analyses using Branch-and-Bound search algorithm

NJ tree

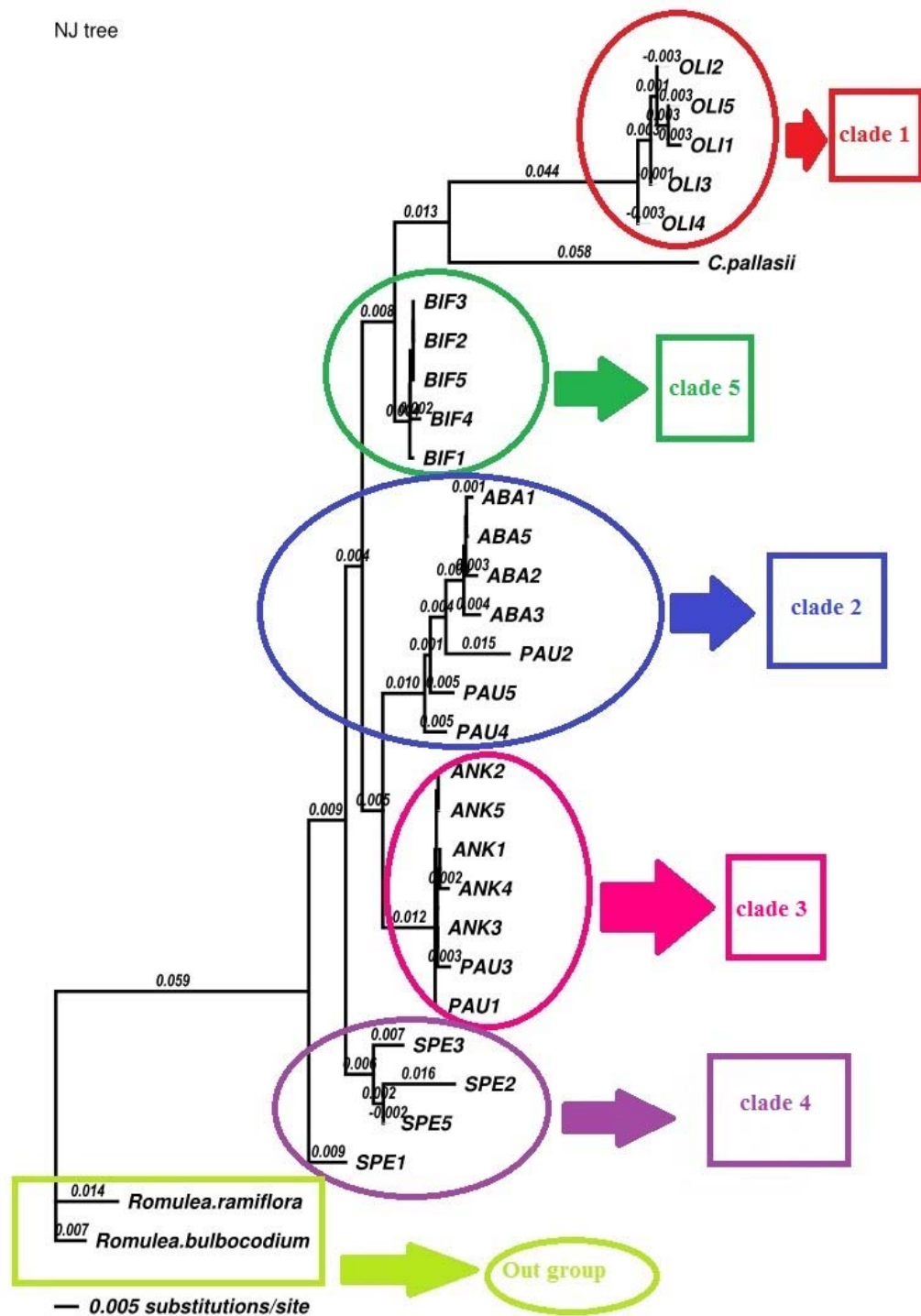


Figure 22. ITS nrDNA Neighbor-Joining (NJ) genetic distance tree

3.5.2 Phylogenetic Analyses Based on the *trnL-F* Sequences

Heuristic research had been made by using Maximum Parsimony criterion from among character based methods. Here, the number of trees to be composed and scanned had been selected as 10,000. The algorithm, used in order to evaluate the branches to compose in the scan to be performed, had been selected as TBR (Tree Bisection Reconnection). All character types had been adjusted as to be unordered and equal weights. As the result of the analysis made, 10,000 trees had been obtained, and 3 of these –as being trees with number 1, 500 and 1000 composed as the result of Parsimony criterion- are shown in Figures 24, 25, and 26, respectively.

In the parsimony analysis made, 268 characters in total have been examined, and among these 253 characters have been found as constant, and 10 characters found as inconstant. The numbers of characters comprising parsimony info were found as 5. As the result of heuristic search, 53,052,295 different tree topologies were composed in total, and the score (digits) of the best parsimony trees were also found as 15.

When look at Figures 24, 25, and 26, they could not resolve the real phylogeny and could just be able to indicate the recently occurring changes. The reasons of this is both the shortness of *trnL-F* region of cpDNA, and its status of being conservative, thus it has provided some shallow information about the real phylogeny.

When it was considered these three trees composed by the parsimony criterion, it was observed that the *Romulea ramiflora* and *C. pallasii*, which were selected as out group taxa, materially differs from our internal groups. It can be said that *C. biflorus* subsp. *pulchricolor*, *C. olivieri* subsp. *olivieri* and *C. speciosus* subsp. *speciosus* taxa within the internal groups, differed from each other by making monophyletic clades at least within themselves.

When we consider the Figures 24 and 25, majority of *C. abantensis* and *C. ancyrensis* taxa along with the crossbreed taxon among them composes a single clade, and it had been observed that the *C. xpaulineae* is the crossbreed of these two taxa. But one from each of *C. abantensis* and *C. ancyrensis* (ANK5 and ABA4) had left this clade, and had been located at different parts of the tree. One individual of *C. speciosus* subsp. *speciosus* (SPE4) indicating monophyletic had been located at a different part as leaving its individuals. When the tree provided in Figure 26 is examined, most of the *C. abantensis* and *C. ancyrensis* individuals have been grouped in two different clades along with the crossbreed in between themselves. And the other three taxa, *C. speciosus* subsp. *speciosus*, *C. olivieri* subsp. *olivieri*, and *C. biflorus* subsp. *pulchricolor* have been separated as monophyletic clades within their own individuals as in Figures 24 and 25, and an individual of *C. speciosus* subsp. *speciosus* species (SPE4) has again left the clade it has formed a different group consisting of one individual.

Heuristic search completed

Total number of rearrangements tried = 53052295

Score of best tree(s) found = 15

Number of trees retained = 10000

Time used = 00:06:12

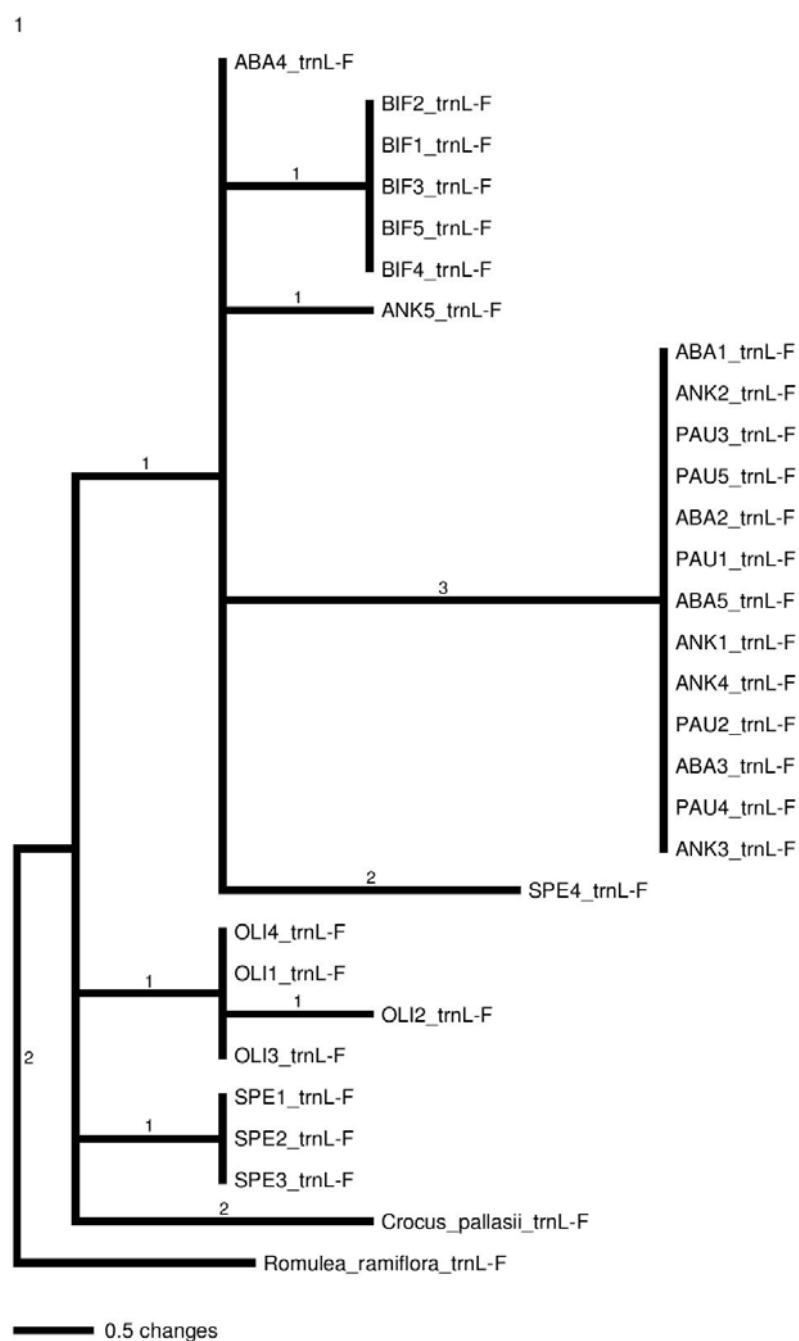


Figure 24. The tree number 1 obtained by using the Parsimony Criterion

500

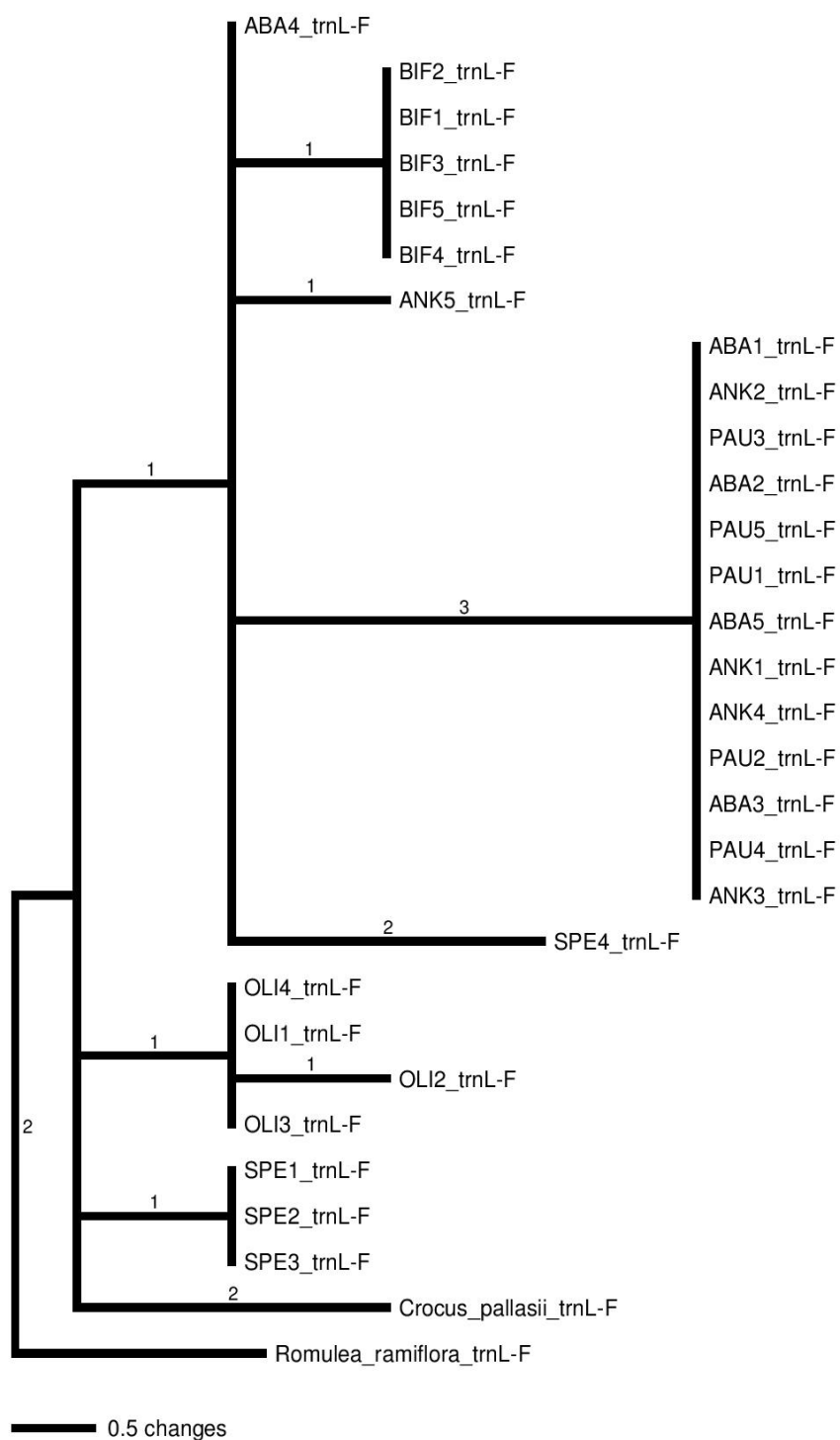


Figure 25. The tree number 500 obtained by using the Parsimony Criterion

1000

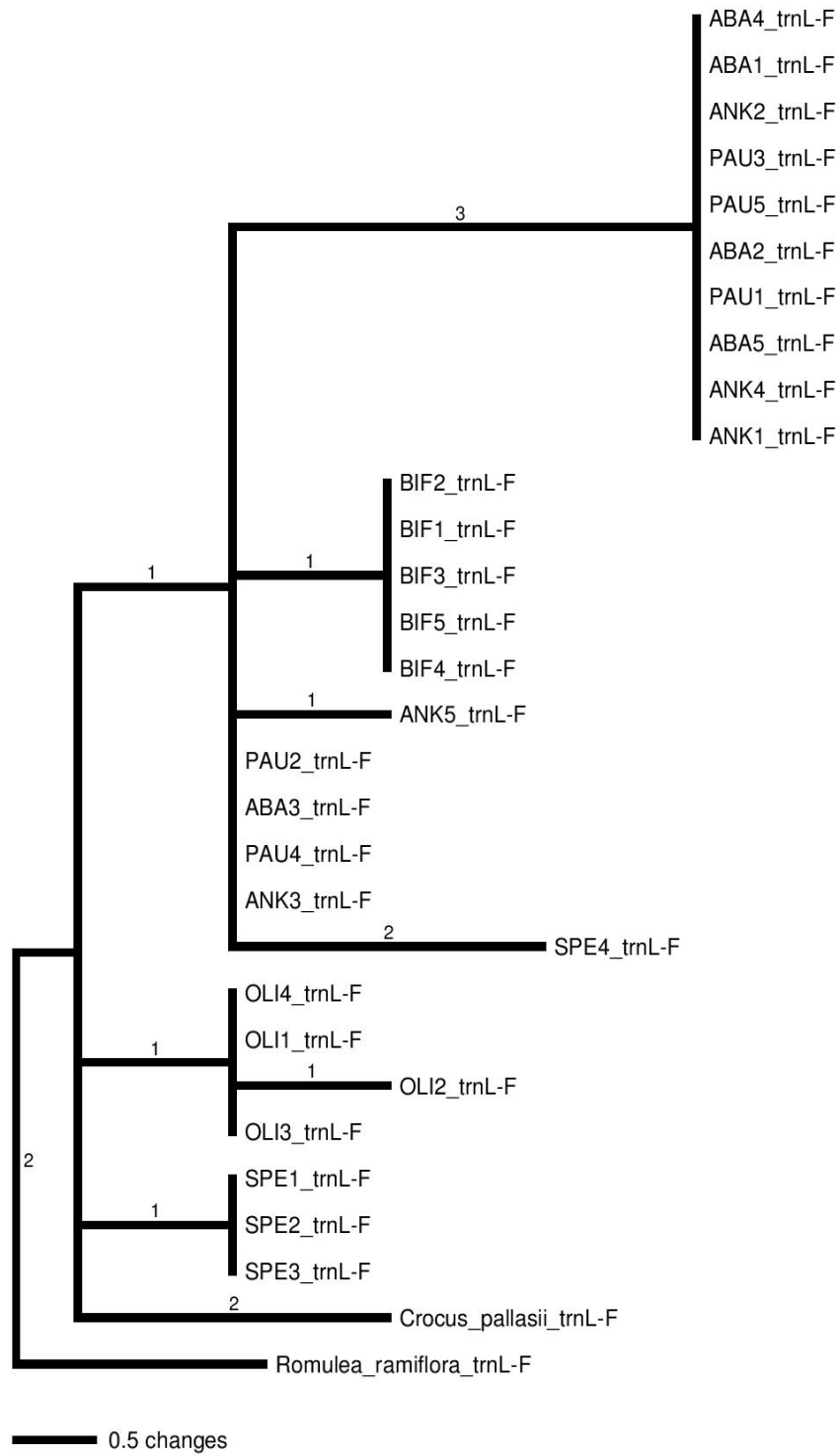


Figure 26. The tree number 1000 obtained by using the Parsimony Criterion

Besides these analyses, bootstrap analysis had been made by the maximum parsimony method. Here, the maximum number of trees to be composed and scanned had been selected as 100. The algorithm used to evaluate the branches to be composed in the scan to be performed had been selected as TBR (Tree Bisection Reconnection). All character types had been adjusted as unordered and as to be of equal weights. In the bootstrap analysis made, again 268 characters in total had been examined, and among these 253 characters had been found as constant, and 10 characters had been found as inconstant. And the number of characters comprising parsimony info had been found as 5. As the result of Heuristic search, 5,228,891 different tree topologies had composed in total and the score (digits) of the best parsimony trees had been found as 15. The composed bootstrap tree is given in Figure 27, and in the Strict Consensustree composed with the Heuristic search method, the maximum number of trees has been selected as 1000 and the numbers of iterations have been selected as 1000. The composed Strict Consensus tree is given in Figure 28.

When the bootstrap analysis (Figure 27) is examined, it could not completely resolve the interrelation of species. Thus, even there exist separations and groupings on the basis of species, the supporting values of these had either been in between 58% and 68%, or they have not provided the bootstrap values. *C. biflorus* subsp. *pulchricolor* has composed a monophyletic clade along with its own individuals, and it has supported with 58% bootstrap value.

C. speciosus subsp. *speciosus* taxon have composed the other 4th clade among themselves except one of its individuals (SPE4), and it has gained a bootstrap value of 65%. *C. olivieri* subsp. *olivieri* has also composed the monophyletic 3rd clade covering its own individuals with a bootstrap value at a rate of 68%.

The 2nd clade consisting of *C. abantensis* and *C. ancyrensis* individuals and the complex composed along with the *C. x paulineae* crossbreed composed by them has been covered a bootstrap value of under 50%. Thus, a reliable comment cannot be made for this group as they compose a polytomy. But it can be said that they indicate polytomy.

UPGMA and NJ analyses performed on the basis of distance had been provided in Figures 29 and 30. The comparative statistical analyses of the topologies of the closest adjacent trees specify the separation priority of each unit of taxa specified on the tree as per each other. In these two analyses, some branches differentiated from each other. The NJ tree, as in the analyses made with parsimony criterion, could not resolve very well the detailed phylogeny because five polymorphic characters had been found, and this could not completely resolve the interrelations of species. But it can be specified that taxa differentiate from each other on the basis of species. It has been observed that *C. pallasii* selected as close out group as per UPGMA tree and *Romulea ramiflora* species selected as far out group have differentiated from our internal group taxa. As the *trnL-F* region of cpDNA indicates maternal heritage, it has again been revealed by the UPGMA and NJ analyses that *C. x paulineae* species is a crossbreed in between *C. abantensis* and *C. ancyrensis* as well.

Bootstrap consensus tree



Figure 27. *trnL-F* cpDNA Bootstrap Consensus of Parsimony tree

Heuristic search completed

Total number of rearrangements tried = 5228891

Score of best tree(s) found = 15

Number of trees retained = 1000

Time used = 31.25 sec

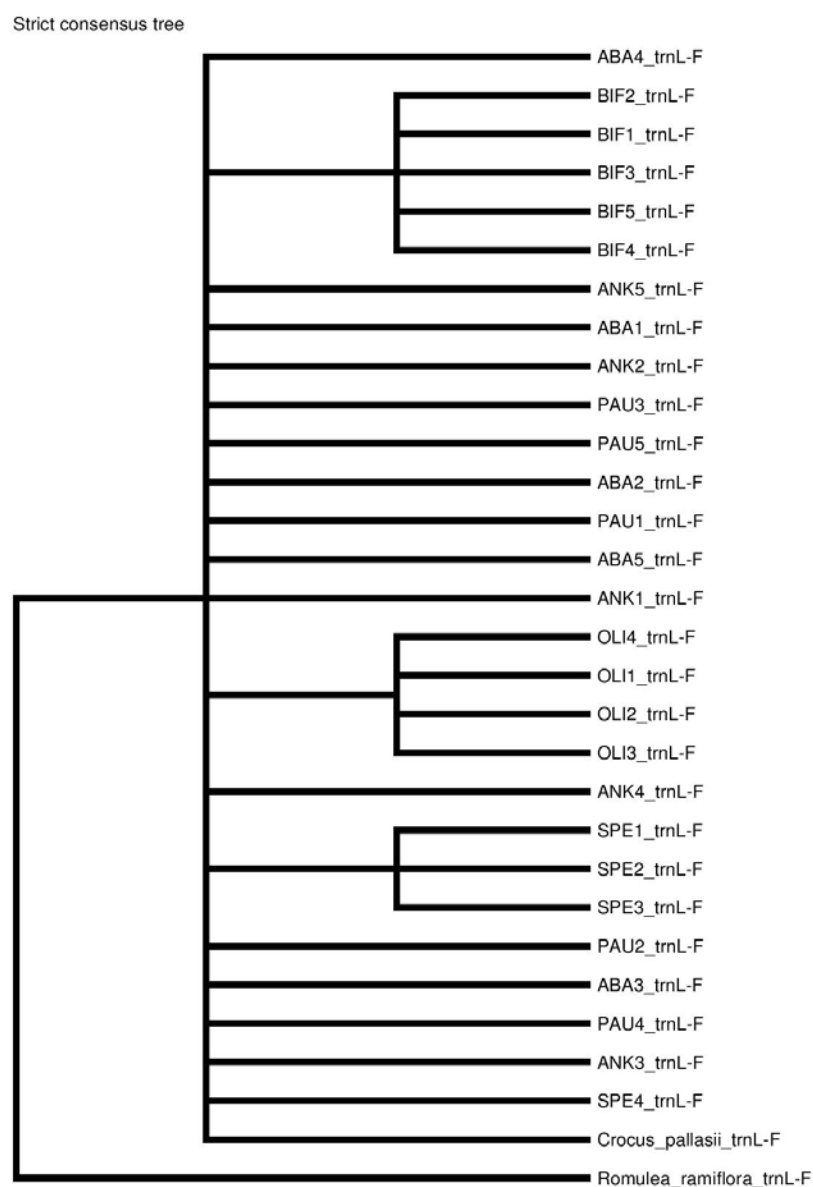


Figure 28. Strict Consensus of 1000 *trnL-F* cpDNA trees obtained with analysis

Phylogenetic tree showing the relationships between various *trnL-F* gene sequences. The scale bar represents 0.0005 substitutions/site. The tree is rooted at the bottom left with *Romulea ramiflora_trnL-F*. The species names are listed on the right side of the tree.

Species names (from top to bottom):

- ABA4_trnL-F*
- Crocus_pallasii_trnL-F*
- ANK1_trnL-F*
- BIF2_trnL-F*
- BIF1_trnL-F*
- BIF3_trnL-F*
- BIF5_trnL-F*
- BIF4_trnL-F*
- ANK5_trnL-F*
- PAU4_trnL-F*
- PAU2_trnL-F*
- OLI4_trnL-F*
- OLI3_trnL-F*
- OLI1_trnL-F*
- OLI2_trnL-F*
- SPE1_trnL-F*
- SPE3_trnL-F*
- SPE2_trnL-F*
- ABA3_trnL-F*
- ANK3_trnL-F*
- PAU1_trnL-F*
- ANK2_trnL-F*
- PAU5_trnL-F*
- ABA5_trnL-F*
- PAU3_trnL-F*
- ABA1_trnL-F*
- ABA2_trnL-F*
- ANK4_trnL-F*
- SPE4_trnL-F*
- Romulea_ramiflora_trnL-F*

Bootstrap values (from top to bottom):

- 0.002
- 0.002
- 0.002
- 0.005
- 0.005
- 0.002
- 0.001
- 0.002
- 0.001
- 0.004
- 0.002
- 0.002
- 0.002
- 0.005
- 0.002
- 0.008
- 0.009

Scale bar: 0.0005 substitutions/site

71

Phylogenetic tree showing relationships between various trnL-F sequences. The tree is rooted at the bottom left and branches upwards. Bootstrap values are indicated at several nodes: 0.007, 0.006, 0.002, 0.003, and 0.002. The sequences listed on the right include ABA4_trnL-F, ABA1_trnL-F, ANK2_trnL-F, PAU3_trnL-F, PAU5_trnL-F, ABA2_trnL-F, PAU1_trnL-F, ABA5_trnL-F, ANK1_trnL-F, ANK4_trnL-F, PAU2_trnL-F, ABA3_trnL-F, PAU4_trnL-F, ANK3_trnL-F, ANK5_trnL-F, SPE1_trnL-F, SPE2_trnL-F, SPE3_trnL-F, BIF2_trnL-F, BIF1_trnL-F, BIF3_trnL-F, BIF5_trnL-F, BIF4_trnL-F, OLI4_trnL-F, OLI1_trnL-F, OLI3_trnL-F, OLI2_trnL-F, SPE4_trnL-F, *Crocus_pallasii*_trnL-F, and *Romulea_ramiflora*_trnL-F.

— 0.0005 substitutions/site

72

4. DISCUSSION AND CONCLUSION

Today it is known that molecular studies contribute significantly to the solution of the problems of the plants of their systematic and evolutionary histories (Baldwin, and Markos, 1998; Froslev et al., 2005; Fior et al., 2006). The phenetic and the phylogenetic classifications carried out based on the morphological characters of the plant species may differ to a certain degree (they can be incongruent) and may not be sufficient sometimes (Abbott et al., 1985). It is because, the classifications carried out may show some differences according to the age of the plants, their physiological status and the environmental factors (Havey, 1991). The developed molecular genetic markers are successfully used to correct these negations that come up in respect of morphological properties and to reveal the genetic diversity in plants and to determine the phylogenetic relationships properly (Yang, and Quiros, 1993). In recent years, ITS and *trnL-F* gene regions are widely used in the studies of molecular plant systematics (Baldwin, and Markos, 1998).

In this study, ITS region of nrDNA and *trnL-F* region of cpDNA were analyzed for the purpose of revealing the relationships of *Crocus* taxa distributed in the province of Bolu. There is no such research carried out before according to the literature of *Crocus* taxa in Bolu. In this study, firstly, the gDNAs were isolated from the *Crocus* taxa then ITS of nrDNA and *trnL-F* of cpDNA were amplified with PCR methods.

Parsimony is a method which is most frequently used for the evaluation of the data in similar studies and creating the cladograms (Freeman, and Herron, 1999). This method is based on the each of different criteria or scores to evaluate or select all possible trees. Maximum parsimony is a useful tool to determine the most prudent tree that reflects the relationships of the taxa in the most realistic way (Klug et al., 2000).

Other than this analysis, the genetic distance based on the differences and distances between the base pairs were calculated of the sequences of ITS and *trnL-F* in the taxa. The determined distances were used to form the distance matrix between the taxa. According to the algorithms, it was determined that the most suitable one by comparing many tree topologies with UPGMA and NJ were carried out by using the similar sequence pairs (Felsenstein, 1987; Başibüyük et al., 2000; Mount, 2001).

As for the trees obtained with Bootstrap analysis, it is aimed to find the most reliable branches statistically by using the criteria of parsimony. It was analyzed that what percentage rate was supported to the tree branches obtained from the study. Bootstrap values changes between 0-100%. According to the bootstrap support criteria, the scales are defined as $\geq 85\%$ strong, 70-85% fair, 50-70% weak, and $< 50\%$ very weak by Kress et al. (2005).

According to phylogenetic analyses based on the ITS sequences, almost all trees have yielded the same result by ITS sequences analyses either with character based or the distance based methods. In the parsimony trees (Figure 14-16), it is clearly seen that six taxa were divided into five different monophyletic groups or clades. Firstly, *C. olivieri* subsp. *olivieri* individuals belonging to the series of *Flavi* were separated from other monophyletic groups which creates the clade 1 and this

clade has received 100% support values in the bootstrap analysis (Figure 17). Individuals of *C. abantensis* and the hybrid *C. x paulineae* belonging to the series of *Reticulati* and separated from other monophyletic groups, that creates the clade 2 and this group has received 67% support values in the bootstrap analysis (Figure 17). *C. anycrensis* and hybrid *C. x paulineae* individuals in *Reticulatis* series were separated from other monophyletic groups as well. It creates the clade 3 and has received 99% support values in the bootstrap analysis (Figure 17). The individuals of *Speciosi* series, *C. speciosus* subsp. *speciosus* formed the monophyletic clade 4 and it has received 99% support values in the bootstrap analysis (Figure 17). Finally, the last monophyletic group which is the 5th one, *C. biflorus* subsp. *pulchricolor* individuals of *Biflori* series, has received 98% support values in the bootstrap analysis (Figure 17).

According to obtained trees by the distance based methods, NJ (Figure 22) and UPGMA (Figure 23), the all studied taxa were grouped into five monophyletic clades. In the all trees, the the individuals of *C. x paulineae* hybrid were distributed within both clades 2 and 3 which contained the *C. abantensis* and *C. anycrensis* individual, respectively. These results supported that *C. x paulineae* was the hybrids between *C. abantensis* and *C. anycrensis* species as published in the morphological studies of Pasche and Kerndorff (1999).

Considered by the morphological features; the close out group taxon *C. pallasii* subsp. *pallasii* belonged to the Section *Crocus*, the taxa with a basal prophyll. Inside of this section, it is in the series *Crocus* with autumn-flowering, anthers yellow, style distinctly three-branched diagnostic characteristics. This taxon grouped together with the *C. olivieri* subsp. *olivieri* individuals (Clade1, Figure 14 – 16) but it is in the Section *Nudiscapus* taxa without a basal prophyll. On the other hand, *C.*

olivieri subsp. *olivieri* individuals also belongs to the series *Flavi* with tunics of the corms membranous, split into parallel fibers, numerous leaves, style much divide. It might be accepted that these two taxa were the most primitive ones within the analyzed taxa. In the *Abantensis* + *Paulineae* group (Clade 2, Figure 14 – 16), taxa of *C. abantensis* and the hybrid *C. x paulineae* and in the *Anycrensis* + *Paulineae* group (Clade 3, Figure 14 – 16), *C. anycrensis* and hybrid *C. x paulineae* taxa were reclassified in the series *Reticulati* of the Section *Nudiscapus* (Mathew, 1982, 1983 and 1984). The diagnostic characteristics of the series with corm tunic, mostly covered by the reticulate forms of fibers, flowering season in winter and in spring, containing with three-forked or sometimes much divided styles. It is reasonable that the parents of the taxa, *C. x paulineae* with some large purple spots or patches on yellow flower colors was a hybrid form between the former taxon with lilac flower color (*C. abantensis*) and the latter taxon with yellow flower color (*C. anycrensis*) (Figure 3). This hybrid form has been reported by Kerndorff and Pasche (1999) as the first time. The members of the other two groups belonged to the different series of the Section *Nudiscapus* (Mathew, 1982). *C. speciosus* subsp. *speciosus* individuals (Clade 4, Figure 14 – 16), in series *Speciosi*, holding the diagnostic characters with corm tunics with rings at the bases, leathery or membranous structures, foliage after the autumn-flowers, style with many parts divided, pale blue flower color (Figure 3). Finally, the last group, the taxa of biflorus group (Clade 5, Figure 14 – 16), *C. biflorus* subsp. *pulchricolor*, belongs to the series *Biflori*. The series of the taxa also contain the diagnostics characters such as tunics of corms with rings at the base containing entire or sometimes tooth-like projections, leathery structures, blooming in spring or late winter, with three-forked style, dark bluish lilac flower color (Mathew, 1982), (Figure 3).

All these trees showed that there were no unclear taxonomic groups based on ITS sequences analyses. So the results supported the traditional taxonomic classifications made by Mathew (1982, 1983, and 1984). At that point, the circumscriptions of the taxa may be obtained by the ITS analyses in this study.

According to phylogenetic analyses based on the *trnL-F* sequences; the parsimony analyses (Figur 24, 25, and 26) could not resolve the real phylogenies of the taxa, thus, they could not be able to indicate the orders of the deviations branches of the tree. When the bootstrap analysis (Figure 27) is examined, it could not completely resolve relationships of the taxa. But only the three monophyletic groups which are *C. speciosus* subsp. *speciosus*, *C. biflorus* subsp. *pulchricolor*, and *C. olivieri* subsp. *olivieri* have received between 58% and 68% supporting values in the bootstrap analysis. Because of this reasons, some polytomies masked the phylogenetical separation orders of the taxa. In addition to these analyses, NJ and UPGMA methods on the basis of distance also gave the same results with those of character-based methods (Figures 29, and 30). It has again been revealed by the UPGMA and NJ analyses that *C. x paulineae* species was a crossbred between *C. abantensis* and *C. ancyrensis* as well.

It can be observed that the character- and the distance-based methods of ITS analyses gave better results than those of *trnL-F* analyses. When it was considered, in the ITS analyses, the taxa have been separated from each other and have formed monophyletic clades covering their own individuals. It is clearly observed that *C. x paulineae* took place along with their parents of *C. abantensis* and *C. ancyrensis*. Some of the hybrids provided monophyletic groups with *C. abantensis*, and others also formed another monophyletic group with *C. ancyrensis*. Consequently, we can see that the 6 taxa being studied have been separated into the 5

monophyletic clades. Although the results of *trnL*-F analyses could not give efficient phylogenetic relationships of the taxa, it showed some similar results with those of ITS analyses.

If the results obtained from these analyses have been compared by the results of Harpke et al. (2013), and Harpke et al. (2014), it could be observed the similarities in somewhat where the taxa belonging to the studying area were grouped under their normal series (Harpke et al., 2013). In their studies, phylogenetic tree of *Crocus* was prepared using a Bayesian analysis of combined sequences of the ITS and *trnL*-F regions. As results of their studies, *Crocus* genus was divided in two Sections of *Crocus* and *Nudiscapus* supporting to Mathew's classification (1982, 1983, and 1984) and the most of the series supported the Mathew's studies. On the other hand, in another study (Harpke et. al., 2014), series *Reticulati* taxa formed different polytomy clades where *C. anycrensis* and *C. abantensis* clades have fallen slightly away from each other in the phylogenetic tree obtained by combined sequences of the nuclear rDNA ETS and ITS regions.

These results have also shown some similarities to the phylogenetic trees formed from the results of UPGMA analyses in this study. The taxonomic circumscription of infrageneric and infraspecific taxa gave a good results overlapped with those of boundaries of taxa obtained from the studies of Mathew (1982, 1983, and 1984), Harpke et al. (2013) and Harpke et al. (2014) very well. On the other hand, when the results obtained from this study were compared the results of other studies (Mathew, 1982, 1983, and 1984; Kerndorff, 1993; Pasche, 1993; Kerndorff and Pasche, 2003; Kerndorff and Pasche, 2004a and 2004b; Kerndorff and Pasche, 2006; Petersen et al., 2008; Kerndorff and Pasche, 2011; Kerndorff et al., 2011; Kerndorff et al., 2012; Harpke et al., 2013; Harpke et al., 2014), it can be observed

that the taxonomic relationships between the taxa have shown a high congruencies with the relationships.

As the result of this study may be concluded as follows:

According to the results obtained from this preliminary study indicated that in a phylogenetic study of infrageneric *Crocus* taxa in Turkey the ITS and *trn*-L regions, consisting of the *trn*L gene with its intron and intergenic spacer between the *trn*L and *trn*F genes, because of containing more base pairs than those of *trn*L-F intergenic spacer, may be safely used in future following the experimental protocols adopted in this study.

The taxonomic boundaries of the *Crocus* taxa from Bolu province were determined in a high accuracy from the points of molecular views in spite of their morphologic variabilities.

The results have strictly supported hybrid status of *C. x paulineae* taxon between the parental taxa of *C. abantensis* and *C. ancyrensis* species from the point of molecular view, as well.

Existence of another hybrid taxon was not being possible between *C. olivieri* subsp. *olivieri* and *C. abantensis* taxa in the studying area.

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APPENDIX

APPENDIX I- ITS Sequences

>ABA1 ITS5

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GTTATATATTTGCTAACCAAAACAACCTTCGTTTCGTCCCGCCGTCCCTCGCGGCGCGGCGTGGCGATACGGAGGA
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>ABA2 ITS5

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>ABA3 ITS5

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>ABA5 ITS5

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>ANK1 ITS5

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>ANK2 ITS5

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>Crocus_pallasii ITS5

TCGAGACCCGAACGAACGGACGATCGCGAACGTGTTACACCCTACTTACTTACTTACGAC
TCCGTCCCGTCCCGTCGCTCCTCGCGGCGTGGCGAGACGGAGGAAACGAAACCCCGGGCGC
AGTGGGCGCAAGGAACACTTCTTGAAGCGCCGTCGCGGCCCTTCCACCTCCCTTTCT
GTCACACGATAGGGAGGGAGGGAGGAGGAGGAACGCGGGCGTGGATATACATATCGTATT
CTGTACGACTCTCGGCAACGGATATCTAGGCTCTCGCATCGATGAAGAACGTAGCGAAAT
GCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGC
GCCCCAGGCCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCCCC
ACAGCCGTGCGGATGCGGAGATTGGCCCAACCGTGCTCCGTGCGCGCGGGTGAAGTGTG
GGCCGTGTCGGGCCCTGGCGCGGCGAATGGTGGACGAATACATCGTTGTTTGTGCCTACT
CTCCGTGTCCTTGCCCTCAACAATGCGACGTGTCGTGTCGCGACCCCTACCAAGGACTC
TTCCGGTTTCTGAATAAGAAGAAGGAACCGTCCTCGAAACG

>Romulea_ramiflora ITS5

TCGAGACCCGAACGAACGGACGATCGCGAACGTGTTATATCACCACATCTACATCGCCTT
CGCCTCGTCTTGCCCGTATCCCACCGTGGTGCGGCACGGCGAGGCGGAGGGAACGAAACC
CCGGCGCGGTGGGGCGCCAAGGAACACTTTTTGGAAGCGCCGTCGCGGCCTCCGTCCTTCC
TCGGGGAGGGGCGGGGGAACGCGGGCGTGGATATAGATATCGTATTTGTACGACTCTCG
GCAACGGATATCTAGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGT
GAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAGGCCATCC
GGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCCCCACACACGCACACGC
ACTCGTGTGCATGCGTGC GGATGCGGAGATTGCCCCGCCGTGCCTCGTGCGGGCGGGTC
GAAGTGGGGCCGTCGTCGGGCCGGGCGCGGCGAATGGTGGACGAACGGACCGTTTGTGC
CTGCTCTTCGCGTCCCGGACCCAAATAACGCGACGTGCCGTCGTCGGACCCCTCACCAAG
AATACGAGATCTCTCGTGTCTCGGAACG

>Romulea_bulbocodium ITS5

TCGAGACCCGAACGAACGGACGATCGCGAACGTGTTATATCACCACATCTCTACATCACC
TTCGCTCGTCTGCCCCTATCCCACCGCGGTGCGGCACGGCGAGGCGGAGGGAACGAAA
CCCCGGCGCGGTGGGGCGCCAAGGAACACTTTTTGGAAGCGCCGTCGCGGCCTCCGTCCTT
CCCTTCCTTAGGGTAGGGAGGGGCGGGGGAACGCGGGCGTGGATATAGATATCGTATTTT
GTACGACTCTCGGCAACGGATATCTAGGCTCTCGCATCGATGAAGAACGTAGCGAAATGC
GATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGC
CCGAGGCCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCCCCAC
ACACGCACACACACACAAAAGTGTGTGTGCATGCGTGC GGATGCGGAGATTGCCCCACC
GTGCCTCGTGCGCGCGGGTCGAAGTGGGGCCGTCGTCGGGCCGGGCGCGGCGAATGGT
GGACGAACATAACGTTTGTGCCTACTCTTCGCGTCCCGGACCCAAATAATGCGACGTGCC
GTGTCGGACCCCTACCAAGAATACGAGATCTCTCGTGTCTCGGAACG

APPENDIX II- Aligment of ITS sequences

CLUSTAL 2.1 multiple sequence alignment

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OLI2      TCGTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 49
OLI3      -----TTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 38
OLI5      --GTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 47
OLI4      TCGTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 49
OLI1      -CGTAACARGRTTTCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 48
C.pallasii -----TCGAGA 6
ABA1      --GTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 47
ABA5      -CGTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 48
ABA2      -CGTAACAAAATTTCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 48
ABA3      ---GAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 46
PAU2      --GAAGCACGGTAGGAATAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 47
PAU5      -----
PAU4      -----
ANK2      -TGTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 48
ANK5      ----AACAAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 45
ANK1      -CGTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 48
PAU1      -----GGTGA--CTGCGGAAGGATCATTGTCGAGA 28
ANK4      -CGTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 48
ANK3      TCGTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 49
PAU3      TCGTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 49
SPE1      -----
SPE3      ----AACAAAGGTTTCCGTAGGTGAT-CCTGCGGAAGGATCATTGTCGAGA 45
SPE2      -----ACAAGATRTCTTAGGTGAA-CTTGCGGAAGGATCATTGTCGAGA 43
SPE5      -----GTGAA-CCTGCGGAAGGATCATTGTCGAGA 29
BIF3      --GTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 47
BIF5      --GTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 47
BIF2      ---TAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 46
BIF1      -----ACAAGGTTTCCGTAGGTGAAACCTGCGGAAGGATCATTGTCGAGA 45
BIF4      -CGTAACAAGGTTTCCGTAGGTGAA-CCTGCGGAAGGATCATTGTCGAGA 48
Romulea.ramiflora -----TCGAGA 6
Romulea.bulbocodium -----TCGAGA 6

OLI2      CCCGAACGAACGGACGATCGCGAACGCGTTACA---TACTTGCATACCAA 96
OLI3      CCCGAACGAACGGACGATCGCGAACGCGTTACA---TACTTGCATACCAA 85
OLI5      CCCGAACGAACGGACGATCGCGAACGCGTTACA---TACTTGCATACCAA 94
OLI4      CCCGAACGAACGGACGATCGCGAACGCGTTACA---TACTTGCATACCAA 96
OLI1      CCCGAACGAACGGACGATCGCGAACGCGTTACA---TACTTGCATACCAA 95
C.pallasii CCCGAACGAACGGACGATCGCGAACGTTTACACCCTACTTACTTACTTA 56
ABA1      CCCGAACGAACGGACGATCGCGAACGTTGTTATA---TATTTGCTAACCAA 94
ABA5      CCCGAACGAACGGACGATCGCGAACGTTGTTATA---TATTTGCTAACCAA 95
ABA2      CCCGAACGAACGGACGATCGCGAACGTTGTTATA---TATTTGCTAACCAA 95
ABA3      CCCGAACGAACGGACGATCGCGAACGTTGTTATA---TATTTGCTAACCAA 93
PAU2      CCCGAACGAACGGACGATCGCGAACGTTGTTATA---TATTTGCTAACCAA 94
PAU5      -----
PAU4      -----
ANK2      CCCGAACGAACGGACGATCGCGAACGTTGTTATA---TATTTGCTAACCAA 95
ANK5      CCCGAACGAACGGACGATCGCGAACGTTGTTATA---TATTTGCTAACCAA 92
ANK1      CCCGAACGAACGGACGATCGCGAACGTTGTTATA---TATTTGCTAACCAA 95
PAU1      CC-GAACGAACGGACGATCGCGAACGTTGTTATA---TATTTGCTAACCAA 74
ANK4      CCCGAACGAACGGACGATCGCGAACGTTGTTATA---TATTTGCTAACCAA 95
ANK3      CCCGAACGAACGGACGATCGCGAACGTTGTTATA---TATTTGCTAACCAA 96
PAU3      CCCGAACGAACGGACGATCGCGAACGTTGTTATA---TATTTGCTAACCAA 96
SPE1      -----
SPE3      CCCGAACGAACGGACGATCGTGAACGTTGTTATA---TATTTGCTAACCAA 92
SPE2      CYCGAACGAACGGACGATCGRGAAACGTTGTTATA---TATTTGCTAACCAA 90
SPE5      CCCGAACGAACGGACGATCGTGAACGTTGTTATA---TATTTGCTAACCAA 76
BIF3      CCCGAACGAACGGACGATCGCGAACGTTGTTATA---T--TTGCCAACCAA 92

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BIF5	CCCGAACGAACGGACGATCGCGAACGTGTTATA---T--TTGCCAACCAA 92
BIF2	CCCGAACGAACGGACGATCGCGAACGTGTTATA---T--TTGCCAACCAA 91
BIF1	CCCGAACGAACGGACGATCGCGAACGTGTTATA---T--TTGCCAACCAA 90
BIF4	CCCGAACGAACGGACGATCGCGAACGTGTTATA---T--TTGCCAACCAA 93
Romulea.ramiflora	CCCGAACGAACGGACGATCGCGAACGTGTTATA---TCACCACATCT--A 51
Romulea.bulbocodium	CCCGAACGAACGGACGATCGCGAACGTGTTATA---TCACCACATCTCTA 53
OL12	AA-CAACTCCGGTTCGTCCCGTCG----CGCCTCGCGGCGCGGCGTGCG 141
OL13	AA-CAACTCCGGTTCGTCCCGTCG----CGCCTCGCGGCGCGGCGTGCG 130
OL15	AA-CAACTCCGGTTCGTCCCGTCG----CGCCTCGCGGCGCGGCGTGCG 139
OL14	AA-CAACTCCGGTTCGTCCCGTCG----CGCCTCGCGGCGCGGCGTGCG 141
OL11	AA-CAACTCCGGTTCGTCCCGTCG----CGCCTCGCGGCGCGGCGTGCG 140
C.pallasii	---CGACTCCGTCCCGTCCCGTCG----CTCCTCGCGGCG----TGCG 94
ABA1	AA-CAACTTCGTTTCGTCCCGCCG----TCCCTCGCGGCGCGGCGTGCG 139
ABA5	AA-CAACTTCGTTTCGTCCCGCCG----TCCCTCGCGGCGCGGCGTGCG 140
ABA2	AA-CAACTTCGTTTCGTCCCGCCG----TCCCTCGCGGCGCGGCGTGCG 140
ABA3	AA-CAACTTCGTTTCGTCCCGCCG----TCCCTCGCGGCGCGGCGTGCG 138
PAU2	AA-CAACTTCGTTTCGTCCCGCCG----TCCCTCGCGGCGCGGCGTGCG 139
PAU5	-----ACTTCGTTTCGTCCCGCCG----TCCCTCGCGGCGCGGCGTGCG 41
PAU4	-----TCGTCCCGCCG----TCCCTCGCGGCGCGGCGTGCG 33
ANK2	AA-CAACTTCGACTCGTCCCGTCG----CCCCTCGTGCGCGACGTGCG 140
ANK5	AA-CAACTTCGACTCGTCCCGTCG----CCCCTCGTGCGCGACGTGCG 137
ANK1	AA-CAACTTCGACTCGTCCCGTCG----CCCCTCGTGCGCGACGTGCG 140
PAU1	AA-CAACTTCGACTCGTCCCGTCG----CCCCTCGTGCGCGACGTGCG 119
ANK4	AA-CAACTTCGACTCGTCCCGTCG----CCCCTCGTGCGCGACGTGCG 140
ANK3	AA-CAACTTCGACTCGTCCCGTCG----CCCCTCGTGCGCGACGTGCG 141
PAU3	AA-CAACTTCGACTCGTCCCGTCG----CCCCTCGTGCGCGACGTGCG 141
SPE1	-----
SPE3	AA-CAACTTCGTTTCGTCCCGCCG----CCCCTCGCGGGGCGACGTGCG 137
SPE2	AA-CAACTTCGTTTCGTCCCGCCG----CCCCTCGCGGGGCGACGTGCG 135
SPE5	AA-CAACTTCGTTTCGTCCCGCCG----CCCCTCGCGGGGCGACGTGCG 121
BIF3	AA-CGACTTCGGTTCGTCCCGCCG----CCCCTCGCGGGGCGGCGTGCG 137
BIF5	AA-CGACTTCGGTTCGTCCCGCCG----CCCCTCGCGGGGCGGCGTGCG 137
BIF2	AA-CGACTTCGGTTCGTCCCGCCG----CCCCTCGCGGGGCGGCGTGCG 136
BIF1	AA-CGACTTCGGTTCGTCCCGCCG----CCCCTCGCGGGGCGGCGTGCG 135
BIF4	AA-CGACTTCGGTTCGTCCCGCCG----CCCCTCGCGGGGCGGCGTGCG 138
Romulea.ramiflora	CATCGCCTTCGCCTCGTCTTGCCCGTATCCACCGTGGTGCGGCACGGCG 101
Romulea.bulbocodium	CATCACCTTCGCCTCGTCTTGCCCGTATCCACCGCGGTGCGGCACGGCG 103
OL12	AGACGGAGGAAACGAAACCCCGGCGCGATGGGCGCCAAGGAACACTTTTT 191
OL13	AGACGGAGGAAACGAAACCCCGGCGCGATGGGCGCCAAGGAACACTTTTT 180
OL15	AGACGGAGGAAACGAAACCCCGGCGCGATGGGCGCCAAGGAACACTTTTT 189
OL14	AGACGGAGGAAACGAAACCCCGGCGCGATGGGCGCCAAGGAACACTTTTT 191
OL11	AGACGGAGGAAACGAAACCCCGGCGCGATGGGCGCCAAGGAACACTTTTT 190
C.pallasii	AGACGGAGGAAACGAAACCCCGGCGCAGTGGGCGCCAAGGAACACTTCTT 144
ABA1	ATACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 189
ABA5	ATACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 190
ABA2	ATACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 190
ABA3	ATACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 188
PAU2	ATACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 189
PAU5	ATSCGGAGGAAACGAAACCCCGGCGCGGTGSRCGCCAMGGAACACTTTTT 91
PAU4	ATACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 83
ANK2	AGCCGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 190
ANK5	AGCCGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 187
ANK1	AGCCGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 190
PAU1	AGCCGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 169
ANK4	AGCCGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 190
ANK3	AGCCGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 191
PAU3	AGCCGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGMACACTTYTT 191
SPE1	-----GGCGCGGTGGGCGCCAAGGAACACTTTTT 29
SPE3	AGACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 187
SPE2	AGACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 185
SPE5	AGACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 171
BIF3	AGACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 187
BIF5	AGACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 187
BIF2	AGACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 186
BIF1	AGACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 185
BIF4	AGACGGAGGAAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 188
Romulea.ramiflora	AGGCGGAGGGAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 151
Romulea.bulbocodium	AGGCGGAGGGAACGAAACCCCGGCGCGGTGGGCGCCAAGGAACACTTTTT 153

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OL12	AGAAGCGCCGTCGCGGGCCTCCTCCCGCTCCTCCTTCCCCCCCATCTATCG	241
OL13	AGAAGCGCCGTCGCGGGCCTCCTCCCGCTCCTCCTTCCCCCCCATCTATCG	230
OL15	AGAAGCGCCGTCGCGGGCCTCCTCCCGCTCCTCCTTCCCCCCCATCTATCG	239
OL14	AGAAGCGCCGTCGCGGGCCTCCTCCCGCTCCTCCTTCCCCCCCATCTATCG	241
OL11	AGAAGCGCCGTCGCGGGCCTCCTCCCGCTCCTCCTTCCCCCCCATCTATCG	240
C.pallasii	GGAAGCGCCGTCGCGGGCCTCTCCA-----CCTCCCTTTCTGTCA	184
ABA1	AGAAGCGTCATCGCGGCCTCCTCCT-----CCTCGTCC-CCTACCT	229
ABA5	AGAAGCGTCATCGCGGCCTCCTCCT-----CCTCGTCC-CCTACCT	230
ABA2	AGAAGCGTCATCGCGGCCTCCTCCT-----CCTCGTCC-CCTACCT	230
ABA3	AGAAGCGTCATCGCGGCCTCCTCCT-----CCTCGTCC-CCTACCT	228
PAU2	AGAAGCGTCATCGCGGCCTCCTCCT-----CCTCGTCTACCTCCCT	230
PAU5	AGAAGTGTATCGYGGYCTCCTTCT-----CCTCAGYTACCTMCCT	132
PAU4	AGAAGCGTCATCGCGGCCTCCTCCT-----CCTCTCTACTTACCT	124
ANK2	AGAAGCGCCGTCGCGGCCTCCTCCTT-----CCTCCCTACCTACCT	232
ANK5	AGAAGCGCCGTCGCGGCCTCCTCCT-----CCTCCCTACCTACCT	228
ANK1	AGAAGCGCCGTCGCGGCCTCCTCCT-----CCTCCCTACCTACCT	231
PAU1	AGAAGCGCCGTCGCGGCCTCCTCCT-----CCTCCCTACCTACCT	210
ANK4	AGAAGCGCCGTCGCGGCCTCCTCCTY-----CCTCCCTACCTACCT	232
ANK3	AGAAGCGCCGTCGCGGCCTCCTCCT-----CCTCCCTACCTACCT	232
PAU3	AGAAGCGCCGTCGCGGCCTCCTCCT-----CCAACCCTACCTACCT	232
SPE1	AGAAGCGCCGTCGCGGCCTCCTTCT-----CCTCCYCTACCT----	66
SPE3	AGAAGCGCCGTCGCGGCCTCCTTCT-----CCTCCCTACCT----	224
SPE2	AGAAGCGCCGTCGCGGCCTCCTTCT-----CCTCCCTACCT----	222
SPE5	AGAAGCGCCGTCGCGGCCTCCTTCT-----CCTCCCTACCT----	208
BIF3	AGAAGCGCCGTCGCGGCCTCCTCC-----	211
BIF5	AGAAGCGCCGTCGCGGCCTCCTCC-----	211
BIF2	AGAAGCGCCGTCGCGGCCTCCTCC-----	210
BIF1	AGAAGCGYCGTCGCGGCCTCCTCC-----	209
BIF4	AGAAGCGCCGTCGCGGCCTCCTCC-----	212
Romulea.ramiflora	GGAAGCGCCGTCGCGGCCTCCGTC-----CTTCCTC-----	182
Romulea.bulbocodium	GGAAGCGCCGTCGCGGCCTCCGTC-----CTTCCTTCTC-----	188

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OL12	TAGGTAGGTAGGGAGGGAGGAAGGA----GGGAGGTGGAACGCGGGCGTG	287
OL13	TAGGTAGGTAGGGAGGGAGGAAGGA----GGGAGGTGGAACGCGGGCGTG	276
OL15	TAGGTAGGTAGGGAGGGAGGAAGGA----GGGAGGTGGAACGCGGGCGTG	285
OL14	TAGGTAGGTAGGGAGGGAGGAAGGA----GGGAGGTGGAACGCGGGCGTG	287
OL11	TAGGTAGGTAGGGAGGGAGGAAGGA----GGGAGGTGGAACGCGGGCGTG	286
C.pallasii	CAC---GATAGGGAGGGAGGGAGGA----GGA----GGAACGCGGGCGTG	223
ABA1	---TAGGGAGGGAGGGAGGAAGGA----GGG----GGAACGCGGGCGTG	267
ABA5	---TAGGGAGGGAGGGAGGAAGGA----GGG----GGAACGCGGGCGTG	268
ABA2	---TAGGGAGGGAGGGAGGAAGGA----GGG----GGAACGCGGGCGTG	268
ABA3	---TAGGGAGGGAGGGAGGAAGGA----GGG----GGAACGCGGGCGTG	266
PAU2	---TAGGGAGGGAGGGGGGGAGGAAGGAGGG----GGAACGCGGGCGTG	272
PAU5	---TAGGGAGGGAGGGAGGAAGGA-GGAGGG----GGAACGCGGGCGTG	173
PAU4	CCTGKAGGGAGGRAGGGAGGAAGGA----GGG----GGAACGCGGGCGTG	166
ANK2	CATGGAGGGAGGAAGGGAGGAAGGA----GGG----GGAACGCGGGCGTG	274
ANK5	CATGGAGGGAGGAAGGGAGGAAGGA----GGG----GGAACGCGGGCGTG	270
ANK1	CATGGAGGGAGGAAGGGAGGAAGGA----GGG----GGAACGCGGGCGTG	273
PAU1	CATGGAGGGAGGRAGGGAGGAAGGA----GGG----GGAACGCGGGCGTG	252
ANK4	CATGGAGGGAGGAAGGGAGGAAGGA----GGG----GGAACGCGGGCGTG	274
ANK3	CATGGAGGGAGGAAGGGAGGAAGGA----GGG----GGAACGCGGGCGTG	274
PAU3	CATGGAGGGAGGAAGGGAGGAAGGA----GGG----GGAACGCGGGCGTG	274
SPE1	---TAGGGTG---GGGATGACGRA----GGG----GGAACGCGGGCGTG	101
SPE3	---TAGGGTG---GGGATGACGGA----GGG----GGAACGCGGGCGTG	259
SPE2	---TAGGGTG---GGGATGAAGGA----GGG----GGAACGCGGGCGTG	257
SPE5	---TAGGGTG---GGGATGACGGA----GGG----GGAACGCGGGCGTG	243
BIF3	-----GGG----GGAACGCGGGCGTG	228
BIF5	-----GGG----GGAACGCGGGCGTG	228
BIF2	-----GGG----GGAACGCGGGCGTG	227
BIF1	-----GGG----GGAACGCGGGCGTG	226
BIF4	-----GGG----GGAACGCGGGCGTG	229
Romulea.ramiflora	-----G---GGGAGG---GGC----GGG----GGAACGCGGGCGTG	209
Romulea.bulbocodium	---TAGGGTA---GGGAGG---GGC----GGG----GGAACGCGGGCGTG	221

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OL12	GACATAGATATCGTATTTTGTA-CGACTCTCGGCAACGGATATCTAGGCT	336
OL13	GACATAGATATCGTATTTTGTA-CGACTCTCGGCAACGGATATCTAGGCT	325
OL15	GACATAGATATCGTATTTTGTA-CGACTCTCGGCAACGGATATCTAGGCT	334
OL14	GACATAGATATCGTATTTTGTA-CGACTCTCGGCAACGGATATCTAGGCT	336
OL11	GACATAGATATCGTATTTTGTA-CGACTCTCGGCAACGGATATCTAGGCT	335
C.pallasii	GATATACATATCGTATTTTGTA-CGACTCTCGGCAACGGATATCTAGGCT	272
ABA1	GATATAGATATCGTATTTTGTA-CGACTCTCGGCAACGGATATCTAGGCT	316
ABA5	GATATAGATATCGTATTTTGTA-CGACTCTCGGCAACGGATATCTAGGCT	317
ABA2	GATATAGATATCGTATTTTGTA-CGACTCTCGGCAACGGATATCTAGGCT	317
ABA3	GATATAGATATCGTATTTTGTA-CGACTCTCGGCAACGGATATCTAGGCT	315

PAU2	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	321
PAU5	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	222
PAU4	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	215
ANK2	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	323
ANK5	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	319
ANK1	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	322
PAU1	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	301
ANK4	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	323
ANK3	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	323
PAU3	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	323
SPE1	GATATAGATATCGTATTTTGTGTAACKACTMTCGGCAACGGATATCTAGGCT	151
SPE3	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	308
SPE2	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	306
SPE5	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	292
BIF3	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	277
BIF5	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	277
BIF2	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	276
BIF1	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	275
BIF4	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	278
Romulea.ramiflora	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	258
Romulea.bulbocodium	GATATAGATATCGTATTTTGTGTA-CGACTCTCGGCAACGGATATCTAGGCT	270

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OLI2	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	386
OLI3	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	375
OLI5	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	384
OLI4	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	386
OLI1	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	385
C.pallasii	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	322
ABA1	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	366
ABA5	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	367
ABA2	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	367
ABA3	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	365
PAU2	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	371
PAU5	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	272
PAU4	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	265
ANK2	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	373
ANK5	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	369
ANK1	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	372
PAU1	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	351
ANK4	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	373
ANK3	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	373
PAU3	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	373
SPE1	CTCGCATCGATGAAGAACATAACGAAATGCGATACTTGGTGTGRATTGCA	201
SPE3	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	358
SPE2	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	356
SPE5	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	342
BIF3	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	327
BIF5	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	327
BIF2	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	326
BIF1	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	325
BIF4	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	328
Romulea.ramiflora	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	308
Romulea.bulbocodium	CTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA	320

OLI2	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	432
OLI3	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	421
OLI5	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	430
OLI4	GAATCCCGTGAACCATCGGCCAAGTCTTTGMWSGYMAGTTGCGCMCGAGG	436
OLI1	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	431
C.pallasii	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	368
ABA1	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	412
ABA5	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	413
ABA2	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	413
ABA3	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	411
PAU2	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	417
PAU5	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	318
PAU4	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	311
ANK2	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	419
ANK5	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	415
ANK1	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	418
PAU1	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	397
ANK4	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	419
ANK3	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	419
PAU3	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCCGAGG	419

SPE1	GAATCCCGTGAACCATCA----AGTCTTTGAACGSAAGTTGCGSCCGAGG 247
SPE3	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCGAGG 404
SPE2	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCGAGG 402
SPE5	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCGAGG 388
BIF3	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCGAGG 373
BIF5	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCGAGG 373
BIF2	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCGAGG 372
BIF1	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCGAGG 371
BIF4	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCGAGG 374
Romulea.ramiflora	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCGAGG 354
Romulea.bulbocodium	GAATCCCGTGAACCATCG----AGTCTTTGAACGCAAGTTGCGCCCGAGG 366

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OL12	CCATCCGGCCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 482
OL13	CCATCCGGCCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 471
OL15	CCATCCGGCCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 480
OL14	CCATCCGGCCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 486
OL11	CCATCCGGCCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 481
C.pallasii	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 418
ABA1	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 462
ABA5	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 463
ABA2	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 463
ABA3	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 461
PAU2	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 467
PAU5	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 368
PAU4	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 361
ANK2	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 469
ANK5	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 465
ANK1	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 468
PAU1	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 447
ANK4	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 469
ANK3	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 469
PAU3	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 469
SPE1	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 297
SPE3	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 454
SPE2	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 452
SPE5	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 438
BIF3	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 423
BIF5	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 423
BIF2	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 422
BIF1	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 421
BIF4	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 424
Romulea.ramiflora	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 404
Romulea.bulbocodium	CCATCCGGTCGAGGGCACGCCTGCCTGGGCGTCACGCCTCCCGTCGCTCC 416

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OL12	CCACAGCCG-----TGAGGATGCG 501
OL13	CCACAGCCG-----TGAGGATGCG 490
OL15	CCACAGCCG-----TGAGGATGCG 499
OL14	CCACAGCCG-----TGAGGATGCG 505
OL11	CCACAGCCG-----TGAGGATGCG 500
C.pallasii	CCACAGCCG-----TGCGGATGCG 437
ABA1	CCACACCC-----TGTGCGGATGCG 483
ABA5	CCACACCC-----TGTGCGGATGCG 484
ABA2	CCACACCC-----TGTGCGGATGCG 484
ABA3	CCACACCC-----TGTGCGGATGCG 482
PAU2	CCACACCC-----TGTGCGGATGCG 488
PAU5	CCACACCC-----TGTGCGGATGCG 389
PAU4	CCACACCC-----TGTGCGGATGCG 382
ANK2	CCACACCC-----TGTGCGGATGCG 490
ANK5	CCACACCC-----TGTGCGGATGCG 486
ANK1	CCACACCC-----TGTGCGGATGCG 489
PAU1	CCACACCC-----TGTGCGGATGCG 468
ANK4	CCACACCC-----TGTGCGGATGCG 490
ANK3	CCACACCC-----TGTGCGGATGCG 490
PAU3	CCACACCC-----TGTGCGGATGCG 490
SPE1	CCACACCC-----TGTGCTGATGCG 319
SPE3	CCACACCC-----TGTGCGGATGCG 475
SPE2	CCACACCC-----TGTGCGGATGCG 473
SPE5	CCACACCC-----TGTGCGGATGCG 459
BIF3	CCACACCC-----TGTGCGGATGCG 444
BIF5	CCACACCC-----TGTGCGGATGCG 444
BIF2	CCACACCC-----TGTGCGGATGCG 443
BIF1	CCACACCC-----TGTGCGGATGCG 442
BIF4	CCACACCC-----TGTGCGGATGCG 445
Romulea.ramiflora	CCACACACGCACACGCACTC-----GTGTGCATGCGTGCGGATGCG 445

Romulea.bulbocodium CCACACACGCACACACACACAAAGTGTGTGTGCATGCGTGCGGATGCG 466
 ***** ** *****

OL12 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 548
 OL13 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 537
 OL15 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 546
 OL14 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 552
 OL11 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 547
 C.pallasii GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 484
 ABA1 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 530
 ABA5 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 531
 ABA2 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 531
 ABA3 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 529
 PAU2 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 535
 PAU5 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGCGKCC 439
 PAU4 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 429
 ANK2 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 537
 ANK5 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 533
 ANK1 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 536
 PAU1 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 515
 ANK4 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 537
 ANK3 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 537
 PAU3 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 537
 SPE1 GAGATTGGMCMCCGTCCTCGTGCAGCGGCGGGTCAAGTGCGGGC---C 366
 SPE3 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 522
 SPE2 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 520
 SPE5 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 506
 BIF3 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 491
 BIF5 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 491
 BIF2 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 490
 BIF1 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 489
 BIF4 GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 492
 Romulea.ramiflora GAGATTGGCCCCCGCGTCCCTCGTGCAGCGGCGGGTCAAGTGCGGGC---C 492
 Romulea.bulbocodium GAGATTGGCCCCACCGTGCTTCGTGCGCGGCGGGTCAAGTGCGGGC---C 513
 ***** ** ***** ***** ***** ***** ** *

OL12 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 598
 OL13 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 587
 OL15 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 596
 OL14 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 602
 OL11 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 597
 C.pallasii GTCGTCGGGCGCTGGCGCGGCGAATGGTGGACGAATACATCGTTGTTTGTG 534
 ABA1 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 580
 ABA5 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 581
 ABA2 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 581
 ABA3 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 579
 PAU2 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 585
 PAU5 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 489
 PAU4 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 479
 ANK2 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 587
 ANK5 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 583
 ANK1 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 586
 PAU1 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 565
 ANK4 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 587
 ANK3 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 587
 PAU3 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 587
 SPE1 GTCGTCGGGCGGGCGCGGCGAATGGKGGACGAATACTTCGTTGTTTGTG 416
 SPE3 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 572
 SPE2 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 570
 SPE5 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 556
 BIF3 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 541
 BIF5 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 541
 BIF2 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 540
 BIF1 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 539
 BIF4 GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAATACTTCGTTGTTTGTG 542
 Romulea.ramiflora GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAACGGACC---GTTTGTG 539
 Romulea.bulbocodium GTCGTCGGGCGGGCGCGGCGAATGGTGGACGAACATACC---GTTTGTG 560
 ***** ***** ***** * *****

OL12 CCT-ACTCTCCGTGCCCCGGCCCTCAACAATGCGACGTGCCGTGCGTGG 647
 OL13 CCT-ACTCTCCGTGCCCCGGCCCTCAACAATGCGACGTGCCGTGCGTGG 636
 OL15 CCT-ACTCTCCGTGCCCCGGCCCTCAACAATGCGACGTGCCGTGCGTGG 645
 OL14 CCT-ACTCTCCGTGCCCCGGCCCTCAACAATGCGACGTGCCGTGCGTGG 651
 OL11 CCT-ACTCTCCGTGCCCCGGCCCTCAACAATGCGACGTGCCGTGCGTGG 646
 C.pallasii CCT-ACTCTCCGTGTCCTTGCCCTCAACAATGCGACGTGCTGTCGCG 583
 ABA1 CCT-ACTCTCCGTGCCCCGGCCCTCAGCAATGCGACGTGCCGTGCGTGG 629

ABA5 CCT-ACTCTCCGTGCCCCGCCCCCTCAGCAATGCGACGTGCCGTCGTCGGA 630
 ABA2 CCT-ACTCTCCGTGCCCCGCCCCCTCAGCAATGCGACGTGCCGTCGTCGGA 630
 ABA3 CCT-ACTCTCCGTGCCCCGCCCCCTCAGCAATGCGACGTGCCGTCGTCGGA 628
 PAU2 CCT-ACTCTCCGTGCCCCGCCCCCTCAGCAATGCGACGTGCCGTCGTCGGA 634
 PAU5 CCT-ACTCTCCGTGCCCCGCCCCCTCARCAATGCGACGTGCCGTCGTCGGA 538
 PAU4 CCT-ACTCTCCGTGCCCCGCCCCCTCAGCAATGCGACGTGCCGTCGTCGGA 528
 ANK2 CCT-ACTCTCCGTGCCCCGCCCCCTCAACAATGCGACGTGCCGTCGTCGGA 636
 ANK5 CCT-ACTCTCCGTGCCCCGCCCCCTCAACAATGCGACGTGCCGTCGTCGGA 632
 ANK1 CCT-ACTCTCCGTGCCCCGCCCCCTCAACAATGCGACGTGCCGTCGTCGGA 635
 PAU1 CCT-ACTCTCCGTGCCCCGCCCCCTCAACAATGCGACGTGCCGTCGTCGGA 614
 ANK4 CCT-ACTCTCCGTGCCCCGCCCCCTCAACAATGCGACGTGCCGTCGTCGGA 636
 ANK3 CCT-ACTCTCCGTGCCCCGCCCCCTCAACAATGCGACGTGCCGTCGTCGGA 636
 PAU3 CCT-ACTCTCCGTGCCCCGCCCCCTCAACAATGCGACGTGCCGTCGTCGGA 636
 SPE1 CCTACTCTCCGTGCCCCGCCCCCTCAACAATGCGACGTGCCGTCGTCGGA 466
 SPE3 CCT-ACTCTCCGTGCCCCGCCCCCTCAACAATGCGACGTGCCGTCGTCGGA 621
 SPE2 CCT-ACTCTCTGTGCCCGCMCTCGACAATGCGACGTGCCGTCGTCGGA 619
 SPE5 CCT-ACTCTCCGTGCCCCGCCCCCTCAACAATGCGACGTGCCGTCGTCGGA 605
 BIF3 CCT-ACTCTCCGTGCCCCGCCCC-CAACAACGCGACGTGCCGTCGTCGGA 589
 BIF5 CCT-ACTCTCCGTGCCCCGCCCC-CAACAACGCGACGTGCCGTCGTCGGA 589
 BIF2 CCT-ACTCTCCGTGCCCCGCCCC-CAACAACGCGACGTGCCGTCGTCGGA 588
 BIF1 CCT-ACTCTCCGTGCCCCGCCCC-CAACAACGCGACGTGCCGTCGTCGGA 587
 BIF4 CCT-ACTCTCCGTGCCCCGCCCC-CAACAACGCGACGTGCCGTCGTCGGA 590
 Romulea.ramiflora CCT-GCTCTTCGCGTCCCGGACCCAAATAACGCGACGTGCCGTCGTCGGA 588
 Romulea.bulbocodium CCT-ACTCTTCGCGTCCCGGACCCAAATAATGCGACGTGCCGTCGTCGGA 609
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OLI2 CCCCTCACCAAGGAAGGACTATTTCTTGCTTGCTTTTGTGCTTGCTT 697
 OLI3 CCCCTCACCAAGGAAGGACTATTTCTTGCTTGCTTTTGTGCTTGCTT 686
 OLI5 CCCCTCACCAAGGAAGGACTATTTCTTGCTTGCTTTTGTGCTTGCTT 695
 OLI4 CCCCTCACCAAGGAAGGACTATTTCTTGCTTGCTTTTGTGCTTGCTT 701
 OLI1 CCCCTCACCAAGGAAGGACTATTTCTTGCTTGCTTTTGTGCTTGCTT 696
 C.pallasii CCCCTCACCAAGGA---CTCTTC---GGTTTCTG----- 612
 ABA1 CCCCTCACCATGGAAGGATTCCGTCTC---GCTTTC----- 662
 ABA5 CCCCTCACCATGGAAGGATTCCGTCTC---GCTTTC----- 663
 ABA2 CCCCTCACCATGGAAGGATTCCGTCTC---GCTTTC----- 663
 ABA3 CCCCTCACCATGGAAGGATTCCGTCTC---GCTTTC----- 661
 PAU2 CCCCTCACCATGGAAGGATTCCGTCTC---GCTTTC----- 667
 PAU5 CCCCTCACCARGGAAGGAGTTCTGTCTC---GCTCTC----- 571
 PAU4 CCCCTCACCAAGGAAGGAGTYCGTCTC---GCTCTC----- 561
 ANK2 CCCCTCACCAAGGAAGGAGTTCGTCTC---GCTCTC----- 669
 ANK5 CCCCTCACCAAGGAAGGAGTTCGTCTC---GCTCTC----- 665
 ANK1 CCCCTCACCAAGGAAGGAGTTCGTCTC---GCTCTC----- 668
 PAU1 CCCCTCACCAAGGAAGGAGTTCGTCTC---GCTCTC----- 647
 ANK4 CCCCTCACCAAGGAAGGAGTTCGTCTC---GCTCTC----- 669
 ANK3 CCCCTCACCAAGGAAGGAGTTCGTCTC---GCTCTC----- 669
 PAU3 CCCCTCACCAAGGAAGGAGTTCGTCTC---GCTCTC----- 669
 SPE1 CCCCTCACCAAGGAAGGATTCCGTCTA---GCTCTC----- 499
 SPE3 CCCCTCACCAAGGAAGGATTCCGTCTA---GCTCTC----- 654
 SPE2 CCCCTAACCAAGGAAGGATTCCGTCTA---GCTCTC----- 652
 SPE5 CCCCTCACCAAGGAAGGATTCCGTCTC---GCTCTC----- 638
 BIF3 CCCCTCACCAAGGAATGATTCCGTCTC---GCTTTC----- 622
 BIF5 CCCCTCACCAAGGAATGATTCCGTCTC---GCTTTC----- 622
 BIF2 CCCCTCACCAAGGAATGATTCCGTCTC---GCTTTC----- 621
 BIF1 CCCCTCACCAAGGAATGATTCCGTCTC---GCTTTC----- 620
 BIF4 CCCCTCACCAAGGAATGATTCCGTCTC---GCTTTC----- 623
 Romulea.ramiflora CCCCTCACCAAGAA---TACGAGAT---CTCTC----- 615
 Romulea.bulbocodium CCCCTCACCAAGAA---TACGAGAT---CTCTC----- 636

OLI2 GCTCGCTCGCGACAAGG-AAGGAAGCAAGGAAGGAAGTGAGAAGGAGCCG 746
 OLI3 GCTCGCTCGCGACAAGG-AAGGAAGCAAGGAAGGAAGTGAGAAGGAGCCG 736
 OLI5 GCTCGCTCGCGACAAGG-AAGGAAGCAAGGAAGGAAGTGAGAAGGAGCCG 744
 OLI4 GCTCGCTCGCGACAAGG-AAGGAAGCAAGGAAGGAAGTGAGAAGG----- 745
 OLI1 GCTCGCTCGCGACAAGG-AAGGAAGCAAGGAAGGAAGTGAGAAGGAGCCG 745
 C.pallasii -----AATAAG-----AAGAAGGAACCGTCTCGAAACG----- 641
 ABA1 -----GACAAGG-AAGGGAGAAGGATCCGTCTCTCGGAACGCGACC 701
 ABA5 -----GACAAGG-AAGGGAGAAGGATCCGTCTCTCGGAACGCGACC 702
 ABA2 -----GACAAGG-AAGGGAGAAGGATCCGTCTCTCGGAACGCGACC 702
 ABA3 -----GACAAGG-AAGGGAGAAGGATCCGTCTCTCGGAACGCGACC 700
 PAU2 -----GACAAGG-AAGGGAGAAGGATCCGTCTCTCGGAACGCGACC 706
 PAU5 -----GACAAGA-RAGGGAGAAGGATCCGT----- 595
 PAU4 -----GACAAGA-RAGGGAGAAGGATCCGTCTCTCGGAACGCGACC 600
 ANK2 -----GATAAGA-GAGGGAGAAGGATCCGTCTCTCGGAACGCGACC 708
 ANK5 -----GATAAGA-GAGGGAGAAGGATCCGTCTCTCGGAACGCGACC 704
 ANK1 -----GATAAGA-GAGGGAGAAGGATCCGTCTCTCGGAACGCGACC 707
 PAU1 -----GATAAGA-GAGGGAGAAGGATCCGTCTCTCGGAACGCGACC 686

ANK4	-----GATAAGA-GAGGGAGAAGGATCCGTCCTCGGAACGCGACC 708
ANK3	-----GATAAGA-GAGGGAGAAGGATCCGTCCTCGGAACGCGACC 708
PAU3	-----GATAAGA-GAGGGAGAAGGATCCGTCCTCGGAACGCGACC 708
SPE1	-----GACGAAA-GAGGGAGAAGGAGCCGTCCTCGGAACGCGACC 538
SPE3	-----GACCCGA-GAGGGAGAAGGAGCCGTCCTCGGAACGCGACC 693
SPE2	-----GACAAGA-GAGGGAGAAGGAGCCGTCCTCGGAACGCGACC 691
SPE5	-----GACAAGA-GAGGGAGAAGGAGCCGTCCTCGGAACGCGACC 677
BIF3	-----GACAAGG-AAGGGAGAAGGACACGTCCTCGGAACGCGACC 661
BIF5	-----GACAAGG-AAGGGAGAAGGACACGTCCTCGGAACGCGACC 661
BIF2	-----GACAAGG-AAGGGAGAAGGACACGTCCTCGGAACGCGACC 660
BIF1	-----GACAAGG-AAGGGAGAAGGACACGTCCTCGGAACGCGACC 659
BIF4	-----GACAAGG-AAGGGAGAAGGACACGTCCTCGGAACGCGACC 662
Romulea.ramiflora	-----GTGTCCTCGGAACG---- 629
Romulea.bulbocodium	-----GTGTCCTCGGAACG---- 650
	*
OLI2	TCC----- 749
OLI3	T----- 737
OLI5	TCCTC----- 749
OLI4	-----
OLI1	TCCTCGGAATGCGA----- 759
C.pallasii	-----
ABA1	CC-AGGTC-AGGC-GGGGCTACCCGCTAAGTTTAAGCA----- 736
ABA5	CC-AGGTC-AGGC-GGGGCTACCCGCTGAG----- 729
ABA2	CC-AGGTC-AGGC-GGGGCTACCCGCTGA----- 728
ABA3	CC-AGGTC-AGGC-GGGGCTACCCGCTGA----- 726
PAU2	CC-AGGTC-AGGC-GGGGCTACCCGCT----- 729
PAU5	-----
PAU4	-----
ANK2	CC-AGGTC-AGGC-GGGGCTACCCGCTGA----- 734
ANK5	CCCAGGTC-AGGC-GGGGCTACCC----- 726
ANK1	CC-AGGTC-AGGSCGGGGCTACCCGYTGA----- 734
PAU1	CC-AGGTC-AGGY----- 697
ANK4	CC-AGGTC-AAGGCGGGGCTACCCGCTG----- 734
ANK3	CC-AGGTC-AGGC-GGGGCTACCMGCTG----- 733
PAU3	CC-AGKTC-AGGC-KGSGCTACCCGCTGA----- 734
SPE1	CCGAGGTC-ASGC-GGG-CTACC-GCTGAGTT----- 566
SPE3	CC-AGGTC-AGGC-GGGGCTACCCGCTGCG----- 720
SPE2	CC-AG-TC-AGGT-GAGGCTAC----- 709
SPE5	CC-AGGTC-AGGC-GGGG----- 692
BIF3	CC-AGGTCCAGGC-GGGGCTACCCGCTGAGTTTAAGCATAT--- 700
BIF5	CC-AGGTC-AGGC-GGGGCTACCCGCTGAGTTTAAGCATATCAA 702
BIF2	CCCAGGTC-AGGC-GGGGCTACCCGCTGAGTTTAAGCATAT--- 699
BIF1	CC-AGGTC-AGGC-GGGGCTACCCGCTGAGTTAAAGCATAT--- 697
BIF4	GC--RGTC-AGGC-SGGGCTWYCCGCTGAGCT-AAGCATATC-- 699
Romulea.ramiflora	-----
Romulea.bulbocodium	-----

APPENDIX III- *trnL*-F Sequences

>ABA1_*trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGGG
GACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTCTATTAGGATGATGCG
CGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATCCTCGTGTCCAG

>ABA2_*trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGGG
GACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTCTATTAGGATGATGCG
GCGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATCCTCGTGTCC

>ABA3_*trnL*-F

AAAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGG
GGACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTCTATTAGGATGATGCG
GCGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAA

>ABA4_*trnL*-F

TTCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGGGGACTATGCCAAAT
TTTTAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTCTATTAGGATGATGCGCGGGAAATAGTCG
GGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATCCTCGTGT

>ABA5_*trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGGG
GACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTCTATTAGGATGATGCG
CGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATCCTCGT

>ANK1_*trnL*-F

GTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGGGGA
CTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTCTATTAGGATGATGCGCG
GGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATCCT

>ANK2_*trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGGG
GACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTCTATTAGGATGATGCG
CGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATCCTCGTGT

>ANK3_*trnL*-F

TAAAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCG
GGGACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTCTATTAGGATGAT
GCGCGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATCCT

>ANK4_*trnL*-F

AAAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGG
GGACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTCTATTAGGATGATGCG
GCGGGAAATAGTCGGGATAGCTCAGTKGGTAGAGCAGAGGACTGAAAATCCTCGTGTC

>ANK5_*trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGGG
GACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTCTATTAGGATGATGCG
CGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATCCTCGTGTCACCACTT

>OLI1_*trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTAGGG
GACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTCTATTAGGATGATGCG
CGGGAAATAGTCGGGATAGCTCAGTTGGGCTAGAGCAGAGGACTG

>OLI2_*trnL*-F

AGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTTAGGGG
ACTATGCCAAATTTTAAATACTTTGGCAAGTCTCTTTAATTCCATAGATTCAAGTACTTCTATTAGGATGATGC
GCGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAAA

>OLI3_*trnL*-F

AAAAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTTAG
GGGACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTTCTATTAGGATGATG
CGCGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAAATCC

>OLI4_*trnL*-F

AGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGGAAGATCTAAAACTAAGAAATTTAGGG
GACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTTCTATTAGGATGATGCG
CGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAAATCCTCGT

>BIF1_*trnL*-F

TAAAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGGAAGATCTAAAACTAAGAAATTT
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GCGCGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAAATCCTCGT

>BIF2_*trnL*-F

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ACTATGCCAAATTTTAAATACTTTGGCAGTATCTTTAATTCCATAGATTCAAGTACTTCTATTAGGATGATGCGC
GGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAAATCCTCGTGTCACCAGTT

>BIF3_*trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTTGCGG
GACTATGCCAAATTTTAAATACTTTGGCAGTATCTTTAATTCCATAGATTCAAGTACTTCTATTAGGATGATGCG
CGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAA

>BIF4_*trnL*-F

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GGACTATGCCAAATTTTAAATACTTTGGCAGTATCTTTAATTCCATAGATTCAAGTACTTCTATTAGGATGATGCG
CGCGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAAATCCTCGTG

>BIF5_*trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTTGCGG
GACTATGCCAAATTTTAAATACTTTGGCAGTATCTTTAATTCCATAGATTCAAGTACTTCTATTAGGATGATGCG
CGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAA

>PAU1_*trnL*-F

AGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTTGCGGG
ACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTTCTATTAGGATGATGCGC
GGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAAAT

>PAU2_*trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTTGCGG
GACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTTCTATTAGGATGATGCG
GCGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAA

>PAU3_*trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTTGCGG
GACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTTAATTCCATAGATTCAAGTACTTCTATTAGGATGATGCG
CGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAAATCCTCGTGT

>PAU4_ *trnL*-F

AAAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGGG
GGACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTAAATTTCCATAGATTCAAGTACTCTATTAGGATGATGCG
GCGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGA

>PAU5_ *trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGGG
GACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTAAATTTCCATAGATTCAAGTACTCTATTAGGATGATGCG
CGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAAATCCTCGTG

>SPE1_ *trnL*-F

GTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGGGGA
CTATGCCAAATTTTAAATACTTTGGCAGTCTCTTAAATTTCCATAGATTCAAGTACTCTATTAGGATGATGCGCG
GAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAA

>SPE2_ *trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGGG
GGACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTAAATTTCCATAGATTCAAGTACTCTATTAGGATGATGCG
GCGGGAAATAGTCGGGATAGCTCAGTKGGTAGAGCAGAGGACTGAA

>SPE3_ *trnL*-F

AAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTCGGG
GACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTAAATTTCCATAGATTCAAGTACTCTATTAGGATGATGCG
GCGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAA

>SPE4_ *trnL*-F

TAAAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATTC
GGGACTATGCCAAATTTTAAATACTTTGGCAGTCTCTTAAATTTCCATAGATTCAAGTACTCTATTAGGATG
ATGCGCGGGAAATAGTCGGGATAGCTCAGTTGGTAGAGCAGAGACCTGAAAA

>Crocus_ *pallasii* *trnL*-F

GAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAGGTCCATTTTACTTCCTAACGATTCTATTATTCTAT
TCTTTTTGAATGAAATGAAGATCTAAAACTAAGAAATTCGGGGACTATGCCAAATTTTAAATACTTTGGCAGTC
TCTTAAATTC

>Romulea_ *ramiflora* *trnL*-F

ATAAAAAGTCCATTTTACTTCCTAACGATTCTATTATTCTATTCTTTTTGAATGAAGATCTAAAACTAAGAAATT
TCGGGGACTATGCTAAATTTTAAATACTTTGGCAGTCTCTTAAATTTCCATAGATCCAAGTACTCTATTAGGATG
ATGCGCGGGAAATAGTCGGG

APPENDIX IV- Alignment of *trnL*-F sequences

CLUSTAL 2.1 multiple sequence alignment

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ABA4_trnL-F -----
B1F2_trnL-F -----AGTCCATTTTA 11
ANK5_trnL-F -----AAGTCCATTTTA 12
ABA1_trnL-F -----AAGTCCATTTTA 12
ANK2_trnL-F -----AAGTCCATTTTA 12
PAU3_trnL-F -----AAGTCCATTTTA 12
PAU5_trnL-F -----AAGTCCATTTTA 12
ABA2_trnL-F -----AAGTCCATTTTA 12
PAU1_trnL-F -----AGTCCATTTTA 11
ABA5_trnL-F -----AAGTCCATTTTA 12
ANK1_trnL-F -----GTCCATTTTA 10
OL14_trnL-F -----AGTCCATTTTA 11
ANK4_trnL-F -----AAAGTCCATTTTA 13
SPE1_trnL-F -----GTCCATTTTA 10
B1F1_trnL-F -----TAAAGTCCATTTTA 14
B1F3_trnL-F -----AAGTCCATTTTA 12
B1F5_trnL-F -----AAGTCCATTTTA 12
B1F4_trnL-F -----AAAGTCCATTTTA 13
SPE2_trnL-F -----AAGTCCATTTTA 12
SPE3_trnL-F -----AAGTCCATTTTA 12
PAU2_trnL-F -----AAGTCCATTTTA 12
OL11_trnL-F -----AAGTCCATTTTA 12
OL12_trnL-F -----AGTCCATTTTA 11
OL13_trnL-F -----AAAAGTCCATTTTA 14
ABA3_trnL-F -----AAAGTCCATTTTA 13
PAU4_trnL-F -----AAAGTCCATTTTA 13
ANK3_trnL-F -----TAAAGTCCATTTTA 14
SPE4_trnL-F -----TAAAGTCCATTTTA 14
Romulea_ramiflora_trnL-F -----ATAAAAAGTCCATTTTA 17
Crocus_pallasii_trnL-F GAAATCGTGAGGGTCAAGTCCCTCTATCCCAATAAAAGGTCCATTTTA 50

ABA4_trnL-F -TTCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 43
B1F2_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 55
ANK5_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
ABA1_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
ANK2_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
PAU3_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
PAU5_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
ABA2_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
PAU1_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 55
ABA5_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
ANK1_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 54
OL14_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATGG----AAGATC 56
ANK4_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 57
SPE1_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 54
B1F1_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATGG----AAGATC 59
B1F3_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
B1F5_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
B1F4_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 57
SPE2_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
SPE3_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
PAU2_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
OL11_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 56
OL12_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 55
OL13_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 58
ABA3_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 57
PAU4_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 57
ANK3_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 59
SPE4_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 58
Romulea_ramiflora_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATG----AAGATC 61
Crocus_pallasii_trnL-F CTTCCCTAACGATTTCATTATTCTATTCTTTTGAATGAAATGAAGATC
99

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ABA4_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 90
 B1F2_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 102
 ANK5_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 103
 ABA1_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 103
 ANK2_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 103
 PAU3_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 103
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 ABA5_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 103
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 OL14_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 103
 ANK4_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 104
 SPE1_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 101
 B1F1_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 106
 B1F3_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 103
 B1F5_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 103
 B1F4_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 104
 SPE2_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 104
 SPE3_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 103
 PAU2_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 103
 OL11_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 103
 OL12_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 102
 OL13_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 105
 ABA3_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 104
 PAU4_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 104
 ANK3_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 107
 SPE4_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 107
 Romulea_ramiflora_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCTAAATTTTAACTTT-G 108
 Crocus_pallasii_trnL-F TAAAACTAAGAAA-TTTCGGGG-ACTATGCCAAATTTTAACTTT-G 146

ABA4_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 137
 B1F2_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 149
 ANK5_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 150
 ABA1_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 150
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 ABA5_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 150
 ANK1_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 148
 OL14_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 150
 ANK4_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 151
 SPE1_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 148
 B1F1_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 153
 B1F3_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 150
 B1F5_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 150
 B1F4_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 152
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 SPE3_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 151
 PAU2_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 151
 OL11_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 150
 OL12_trnL-F GCAAGTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 151
 OL13_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 152
 ABA3_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 151
 PAU4_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 151
 ANK3_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 154
 SPE4_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 155
 Romulea_ramiflora_trnL-F GCA-GTCTCTTTAATTT-CCATAGATTCAAGTACT-CTATTAGGATGATG 155
 Crocus_pallasii_trnL-F GCA-GTCTCTTTAATTT-C----- 163

ABA4_trnL-F CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 185
 B1F2_trnL-F CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 197
 ANK5_trnL-F CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 198
 ABA1_trnL-F CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 198
 ANK2_trnL-F CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 198
 PAU3_trnL-F CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 198
 PAU5_trnL-F CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 198
 ABA2_trnL-F CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 199
 PAU1_trnL-F CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 197
 ABA5_trnL-F CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 198

ANK1_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 196
OLi4_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 198
ANK4_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTKGG--TAGAGCAGAGGACTGAAA 199
SPE1_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 196
BIF1_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 201
BIF3_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 198
BIF5_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 198
BIF4_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 200
SPE2_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTKGG--TAGAGCAGAGGACTGAA- 198
SPE3_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 199
PAU2_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 199
OLi1_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGGGCTAGAGCAGAGGACTGA-- 198
OLi2_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 199
OLi3_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 200
ABA3_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 199
PAU4_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGA-- 197
ANK3_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGGACTGAAA 202
SPE4_trnL-F	CGCGGGAAATAGTCGGGATAGCTCAGTTGG--TAGAGCAGAGACTGAAA 203
Romulea_ramiflora_trnL-F	CGCGGGAAATAGTCGGG----- 172
Crocus_pallasii_trnL-F	-----
ABA4_trnL-F	ATCCTCGTGT----- 195
BIF2_trnL-F	ATCCTCGTGTCACCAGTT 215
ANK5_trnL-F	TTCCTCGTGTCACCAGTT 216
ABA1_trnL-F	ATCCTCGTGTCACCAG--- 213
ANK2_trnL-F	ATCCTCGTGT----- 208
PAU3_trnL-F	ATCCTCGTGT----- 208
PAU5_trnL-F	ATCCTCGTG----- 207
ABA2_trnL-F	ATCCTCGTGTC----- 211
PAU1_trnL-F	AT----- 199
ABA5_trnL-F	ATCCTCGT----- 206
ANK1_trnL-F	ATCCT----- 201
OLi4_trnL-F	ATCCTCGT----- 206
ANK4_trnL-F	ATCCTCGTGTC----- 210
SPE1_trnL-F	A----- 197
BIF1_trnL-F	ATCCTCGT----- 209
BIF3_trnL-F	A----- 199
BIF5_trnL-F	-----
BIF4_trnL-F	ATCCTCGTG----- 209
SPE2_trnL-F	-----
SPE3_trnL-F	-----
PAU2_trnL-F	-----
OLi1_trnL-F	-----
OLi2_trnL-F	AA----- 201
OLi3_trnL-F	ATCC----- 204
ABA3_trnL-F	-----
PAU4_trnL-F	-----
ANK3_trnL-F	ATCCT----- 207
SPE4_trnL-F	A----- 204
Romulea_ramiflora_trnL-F	-----
Crocus_pallasii_trnL-F	-----

APPENDIX V - HKY85 distance matrix

HKY85 distance matrix

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 OLI2	-															
2 OLI3	0.00000	-														
3 OLI5	0.00000	0.00000	-													
4 OLI4	0.00000	0.00000	0.00000	-												
5 OLI1	0.00000	0.00000	0.00000	0.00000	-											
6 C.pallasii	0.10129	0.10133	0.10129	0.10175	0.10133	-										
7 ABA1	0.08304	0.08411	0.08614	0.07779	0.09331	0.10445	-									
8 ABA5	0.08279	0.08402	0.08606	0.07755	0.09304	0.10432	0.00139	-								
9 ABA2	0.08605	0.08409	0.08930	0.08080	0.09470	0.10431	0.00414	0.00276	-							
10 ABA3	0.08478	0.08395	0.08787	0.07951	0.09185	0.10425	0.00554	0.00415	0.00691	-						
11 PAU2	0.10055	0.09695	0.10363	0.09539	0.11073	0.10140	0.01974	0.01964	0.02251	0.02253	-					
12 PAU5	0.07839	0.07806	0.07839	0.07881	0.07829	0.10358	0.01978	0.01986	0.01984	0.01985	0.01966	-				
13 PAU4	0.09340	0.09296	0.09340	0.08912	0.09338	0.10259	0.01739	0.01731	0.01734	0.01730	0.02223	0.01227	-			
14 ANK2	0.08889	0.08848	0.09051	0.08369	0.09900	0.09265	0.03692	0.03677	0.03972	0.03974	0.05012	0.02807	0.02588	-		
15 ANK5	0.08614	0.08699	0.08918	0.08106	0.09628	0.09242	0.03584	0.03572	0.03868	0.03859	0.04737	0.02812	0.02589	0.00000	-	
16 ANK1	0.08582	0.08696	0.08902	0.08063	0.09580	0.09249	0.03695	0.03533	0.03827	0.03831	0.05013	0.02808	0.02587	0.00138	0.00000	-
17 PAU1	0.08731	0.08738	0.09045	0.08198	0.09753	0.09078	0.03585	0.03572	0.03577	0.03859	0.03696	0.02607	0.02583	0.00000	0.00000	0.00000
18 ANK4	0.08716	0.08831	0.09037	0.08196	0.09871	0.09250	0.03984	0.03823	0.04120	0.03979	0.05300	0.02810	0.02588	0.00411	0.00275	0.00136
19 ANK3	0.08564	0.08706	0.08910	0.08046	0.09601	0.09258	0.03696	0.03534	0.03830	0.03977	0.05008	0.02808	0.02587	0.00137	0.00000	0.00000
20 PAU3	0.08759	0.08909	0.09103	0.08245	0.09795	0.09108	0.03991	0.03829	0.04124	0.04276	0.05317	0.03212	0.02806	0.00412	0.00275	0.00273
21 SPE1	0.10344	0.09986	0.10745	0.09483	0.11688	0.10906	0.05320	0.05121	0.05127	0.05511	0.05364	0.04410	0.04700	0.03708	0.03927	0.03707
22 SPE3	0.08687	0.08587	0.09000	0.08168	0.09734	0.10478	0.04615	0.04447	0.04751	0.04746	0.05517	0.04014	0.04028	0.03426	0.03303	0.03426
23 SPE2	0.10068	0.09632	0.10380	0.09550	0.11116	0.10699	0.05891	0.05888	0.05891	0.06037	0.06188	0.04248	0.04279	0.04813	0.04819	0.04809
24 SPE5	0.08440	0.08433	0.08760	0.07907	0.09494	0.10110	0.03894	0.03878	0.03886	0.04179	0.03858	0.03460	0.03484	0.02819	0.02814	0.02815
25 BIF3	0.05964	0.06039	0.06288	0.05416	0.07184	0.07810	0.02634	0.02507	0.02814	0.02970	0.03754	0.03276	0.02948	0.02992	0.03012	0.02987
26 BIF5	0.05957	0.06031	0.06281	0.05408	0.07041	0.07798	0.02633	0.02506	0.02812	0.02968	0.03754	0.03269	0.02948	0.02990	0.03010	0.02985
27 BIF2	0.05958	0.06042	0.06281	0.05422	0.07041	0.07811	0.02636	0.02508	0.02815	0.02969	0.03758	0.03276	0.02948	0.02994	0.03008	0.02989
28 BIF1	0.05988	0.06037	0.06315	0.05437	0.07078	0.07805	0.02655	0.02378	0.02687	0.02676	0.03471	0.03096	0.02772	0.03006	0.03015	0.03001
29 BIF4	0.05967	0.06054	0.06113	0.05410	0.06863	0.07815	0.02948	0.02661	0.02972	0.03130	0.03919	0.03282	0.02948	0.03293	0.03173	0.03141
30 Romulea.ramiflora	0.13328	0.13277	0.13328	0.13421	0.13311	0.13276	0.12344	0.12287	0.12330	0.12283	0.12437	0.10313	0.10349	0.10355	0.10338	0.10333
31 Romulea.bulbocodium	0.12763	0.12729	0.12763	0.12849	0.12748	0.12692	0.11313	0.11267	0.11299	0.11262	0.11213	0.09308	0.09371	0.09788	0.09770	0.09767

HKY85 distance matrix (continued)

	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
17 PAU1	-														
18 ANK4	0.00281	-													
19 ANK3	0.00000	0.00273	-												
20 PAU3	0.00289	0.00549	0.00275	-											
21 SPE1	0.03803	0.04034	0.03716	0.04145	-										
22 SPE3	0.03296	0.03575	0.03287	0.03729	0.01802	-									
23 SPE2	0.03604	0.04968	0.04814	0.05139	0.02865	0.02736	-								
24 SPE5	0.02824	0.03115	0.02816	0.03134	0.01680	0.00583	0.01479	-							
25 BIF3	0.03152	0.03290	0.02990	0.02991	0.03543	0.02836	0.04428	0.02174	-						
26 BIF5	0.03150	0.03287	0.02988	0.02989	0.03542	0.02842	0.04426	0.02174	0.00000	-					
27 BIF2	0.03153	0.03293	0.02992	0.02993	0.03731	0.02838	0.04430	0.02175	0.00000	0.00000	-				
28 BIF1	0.03152	0.03306	0.03005	0.03006	0.03548	0.02853	0.04432	0.02176	0.00145	0.00145	0.00145	-			
29 BIF4	0.03317	0.03447	0.03143	0.03149	0.03971	0.02997	0.04611	0.02334	0.00289	0.00287	0.00289	0.00288	-		
30 Romulea.ramiflora	0.10327	0.10339	0.10342	0.10631	0.08297	0.10898	0.11785	0.10861	0.08970	0.08975	0.08977	0.08976	0.08961	-	
31 Romulea.bulbocodium	0.09767	0.09772	0.09776	0.10055	0.07298	0.09543	0.10382	0.09512	0.08565	0.08565	0.08571	0.08567	0.08558	0.02122	-