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Phylogenetic Relationships of The *Trifolium* L. Species Based on cpDNA Sequences

Aykut YILMAZ[1]✉, Yudum YELTEKİN[2]
[1,2]Department of Molecular Biology and Genetics, Faculty of Science and Arts, Uşak University, Uşak 64200, Türkiye
[1]https://orcid.org/0000-0002-0327-8388, [2]https://orcid.org/0000-0003-3891-8717
✉: aykut.yilmaz@usak.edu.tr

ABSTRACT

DNA barcoding is an important molecular approach in the determination of species diversity, evaluation of phylogenetic relationships and identification of taxonomically problematic species because of insufficient diagnostic characters. It has great importance to determine the barcoding regions that will give the best result in the evaluation of phylogenetic relationships in species like *Trifolium* that spread over wide geographical regions in the world and show high variation accordingly. For this aim, matK (maturase K) and rbcL (ribulose bisphosphate carboxylase gene) regions belonging to cpDNA(chloroplast DNA) was used to determine the barcoding ability and evaluate the taxonomy of the genus *Trifolium*. 63 taxa from matK region and 47 taxa from rbcL region were determined and used in this study. It was observed that transitional substitutions for matK and rbcL regions are higher than transversional substitutions with the rate of 51.52 % and 70.69 %, respectively. It can be stated that both of barcoding regions are valuable to reveal the phylogenetic relationships, in addition to their grouping ability the species as taxonomically. However, especially the using of matK sequence informations that have high variable sites (158) and grouping ability clearly for all taxa is strongly recommended.

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ÖZET

DNA barkodlama; tür çeşitliliğinin tayininde, filogenetik ilişkilerin değerlendirilmesinde ve yetersiz ayırt edici karakterlerden dolayı taksonomik açıdan problemli türlerin kimliklendirilmesinde önemli bir moleküler yaklaşımdır. Geniş coğrafik bölgelere yayılan ve buna bağlı yüksek varyasyon gösteren *Trifolium* gibi türlerde, filogenetik ilişkilerin değerlendirilmesinde en iyi sonucu verecek barkodlama bölgelerini tayin etmek büyük bir öneme sahiptir. Bu amaçla, cpDNA'ya (chloroplast DNA) ait matK (maturase K) ve rbcL (ribulose bisphosphate carboxylase gen) bölgeleri *Trifolium* cinsinin taksonomisini değerlendirmek ve barkodlama yeteneğini tayin etmek için kullanıldı. Bu çalışmada matK bölgesinden 63 taxa ve rbcL bölgesinden 47 taxa belirlendi ve kullanıldı. Transisyonel değişimlerin, matK ve rbcL bölgeleri için transversiyonel değişimlerden sırasıyla % 51.52 ve % 70.69 ile daha yüksek olduğu gözlemlendi. Heriki barkodlama bölgesinin taksonomik olarak türleri gruplama yeteneklerinin yanısıra, filogenetik ilişkileri ortaya çıkarmak adına değerli olduğu ifade edilebilir. Bununla birlikte, özellikle bütün taksonlar için açık bir şekilde gruplama yeteneğine ve yüksek oranda değişken bölgelere sahip (158) matK sekans bilgilerinin kullanımı tavsiye edilir.

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INTRODUCTION

The genus *Trifolium* belonging to the family Fabaceae is represented by about 255 species which have cosmopolitan distribution (Gillet & Taylor, 2001; Choi et al., 2020; Kalinkina et al., 2020). However, the greatest species diversity within the genus *Trifolium* is

observed in the Mediterranean region and its periphery (Ellison et al., 2006; Uslu & Babaç, 2019).

The most of *Trifolium* species are used as ornamental plants, medical plants and foods for animals (Zohary & Heller, 1984; Eroğlu et al., 2013; Uslu & Babaç, 2019).

The taxonomy of the genus, classification and systematic position of some species within the genus are still problematic and confusing. One of the most important reasons is cosmopolitan distribution of the species and variations caused by wide geographical factors. Two classifications proposed by Zohary and Heller (1984) and Ellison et al. (2006) are commonly used in the taxonomy of the genus. The species of the genus *Trifolium* are evaluated into two subgenus as *Trifolium* L. and *Chronosemium* (Ser.) in the classification proposed by Ellison et al. (2006), whereas they are classified into eight sections without subgenus in the classification proposed by Zohary and Heller (1984): *Lotoidea*, *Trifolium*, *Chronosemium*, T*richocephalum* Koch, *Involucrarium* Hooker, *Vesicaria, Paramesus* (C.Presl) and *Mistyllus* (C.Presl). Both of classifications are still commonly used by many researchers in the evaluation of taxonomy and phylogenetic relationships within the genus (Uslu et al., 2013; Uslu & Babaç, 2019; Choi et al., 2020; Kalinkina et al., 2020). For this aim, studies based on morphologic, cytogenetic and especially different molecular techniques like PCR methods and DNA sequences belonging to nuclear and plastid genom are frequently performed to provide considerable informations about taxonomy of the genus *Trifolium* (Watson et al., 2000; Ellison et al., 2006; Kalinkina et al., 2020).

Cytogenetic studies show that basic chromosome number in the genus *Trifolium* is x=8 for the majority of the species examined (Goldblatt, 1981; Ellison et al., 2006; Kıran et al., 2010), although anaploidy (x= 5, 6 and 7) and polyploidy (4x, 6x and 12x) are detected in some species (Ellison et al., 2006; Vizintin et al., 2006; Kıran et al., 2010; Uslu, 2012).

In cases where morphological datas are insufficient due to differences in ecological conditions, geographical factors and variations in morphological characters in plants caused by these, molecular systematic studies based on DNA sequence informations are commonly performed to determine the status and phylogenetic relationships of some problematic taxa. It is expected that the DNA sequence information belonging to the region prefered should contain enough nucleotide changes to identify the species, in addition to enough nucleotide similarities to reveal the relationship between species and to collect the species in the correct systematic categories. Therefore, especially cpDNA regions which contain gene and spacer sequences between genes are used in plant systematic studies. In this study, two regions belonging to cpDNA (matK and rbcL) frequently proposed were examined to provide contribution to taxonomy of the genus and to determine the species identification ability of the regions examined. All sequences from past to present for matK and rbcL regions belonging to *Trifolium* taxa were provided from NCBI (National Center of Biotechnology Information). In addition to understand the taxonomy of the genus *Trifolium*, it is aimed in this study to reveal the importance of regions selected in DNA barcoding because the same DNA regions examined for barcoding in different plant groups may show separation ability in different taxonomic categories.

MATERIALS and METHODS

All DNA sequence informations belonging to matK and rbcL genes from cpDNA were collected from NCBI and analysed according to compatibility of their sequence information. In other words, sequence informations which are compatible with each other were determined and used in this study. Totally 63 *Trifolium* taxa from matK region (Burgess et al., 2011; Schaefer et al., 2011; Bruni et al., 2012; Elliott & Davies, 2014; Kajtoch et al., 2015; Kuzmina et al., 2017; Thornhill et al., 2017) and 47 *Trifolium* taxa from rbcL region (Burgess et al., 2011; Schaefer et al., 2011; Bruni et al., 2012; de Vere et al., 2012; Kajtoch et al., 2015; Manton, 2016; Kuzmina et al., 2017) were examined based on sequence informations (Appendix) and analysed their species identification and separation abilities the taxa in the genus *Trifolium*.

Exclusion of problematic taxa and selection of taxa according to the results of phylogenetic tree is a situation observed commonly. For this reason, some species which have sequence informations in high level in NCBI were represented by a few taxa to show compatibility of the taxa in phylogenetic tree. Furthermore, attention was paid in the selection of these taxa to the fact that they were analysed by different researchers.

After the determination of sequences for both regions examined, these sequences belonging to *Trifolium* taxa for matK and rbcL were aligned and performed by using Molecular Evolutionary Genetics Analysis (MEGA X) (Kumar et al., 2018).

Base substitutions among the taxa examined were determined for matK and rbcL sequences, separately. Variable sites and parsim-info sites were defined for two regions examined in addition to alignment lengths (Table 1). Sequence substitution probabilities among *Trifolium* taxa examined for both of matK and rbcL regions were determined. Afterwards, transitional and transversional base substitutions range were computed in addition to transition/transversion ratios for purines-pyrimidines. Finally nucleotide frequencies for the regions belonging to matK and rbcL of *Trifolium* taxa were determined (Table 1).

Table 1. The comparisons of all studied DNA regions
Çizelge 1. Tüm çalışılan DNA bölgelerinin karşılaştırmaları

DNA regions	Taxon (number)	Alignment lenght (bp)	Variable site	Parsim-info site	Transitional substitutions (%)	Transversional substitutions (%)	Transition/Transversion rate Purines (k_1)	Pyrimidines (k_2)	Overall (R)	Nucleotide freq. (%) A+T/U	G+C
matK	63	514	158	86	51.52	48.48	0.40	3.56	0.94	70.47	29.53
rbcL	47	332	65	31	70.69	29.31	2.77	6.76	2.33	58.62	41.38
Total	110										

Maximum Parsimony method were performed to show phylogenetic relationships of *Trifolium* taxa, to provide contribution to understanding of *Trifolium* taxonomy, to find out grouping abilities with species in different branches in phylogenetic tree for matK and rbcL regions besides the species identification abilities (Figure 1, 2).

This analysis involved 63 *Trifolium* taxa from matK region and 47 *Trifolium* taxa from rbcL region. Gaps and missing data analyzed in sequences of taxa examined were eliminated for effective analysis.

RESULTS and DISCUSSION

All sequence informations for *Trifolium* taxa examined in this study were provided from NCBI. Firstly all sequences belonging to *Trifolium* taxa from past to present were collected for two regions (matK and rbcL) from cpDNA. Afterwards, sequences which is compatible with each other according to nucleotide lengths were determined and preferred for analysis. Finally, 63 taxa from matK region and 47 taxa from rbcL region were determined and used for further analysis.

While 158 variable sequences and 86 parsimony informative sites in the comparison of the taxa examined were determined for matK region (Table 1), it was observed 65 variable sequences and 31 parsimony informative sites for rbcL region (Table 1). After the elimination of the sequences containing gaps and missing data, alignment length of the *Trifolium* taxa for matK and rbcL regions were determined as 514 bp and 332 bp, respectively.

The rates of transitional and transversional substitutions were shown in Table 1 for matK region. The highest base substitutions were observed between Cytosine and Thymine bases with the rate of 47.03 %. In other words, it can be said that the most of the sequence variations belonging to matK region for *Trifolium* taxa examined were caused by transitional substitutions with the rate of 51.52 %. Transition/transversion ratios were computed as 0.40 for purines, 3.56 for pyrimidines and 0.94 for overall (Table 1). Nucleotide frequencies for matK region of *Trifolium* taxa were determined as 31.67% (A), 38.80% (T/U), 15.61% (C), and 13.92% (G). As a result, it can be stated that the most of the sequence informations for matK region in *Trifolium* taxa consist of A and T bases (Table 1).

The probability of substitutions from one base to another were shown in Table 1 for rbcL region. The highest base substitutions were observed between Cytosine and Thymine bases with the rate of 50.95 %. The rate of transitional substitution in Table 1 were computed as 70.69 %. In other words, it can be said that the rate of transitional substitution is higher than transversional substitutions with the rate of 70.69 %. Transition/transversion ratios for purines, pyrimidines and overall were determined as 2.77, 6.76 and 2.33, respectively (Table 1). Finally, nucleotide frequencies of rbcL sequences for *Trifolium* taxa were also determined as 26.63 % for Adenine, 31.99 % for Thymine/Uracil, 19.44 % for Cytosine and 21.94 % for Guanine.

Maximum Parsimony (MP) method was performed using by sequence informations of matK and rbcL regions belonging to cpDNA to provide contribution to taxonomy of the genus and to determine the species identification ability of the regions examined (Figure 1, 2).

Phylogenetic trees provided from matK and rbcL were used to evaluate the genus *Trifolium* based on the taxonomic classification suggested by Ellison et al. (2006).

The Evaluation of Phylogenetic Relationships Among *Trifolium* Species Based on matK Region:

New infrageneric classification proposed by Ellison et al. (2006) separated the genus *Trifolium* into two subgenus as *Chronosemium* and *Trifolium* on the contrary of classification proposed by Zohary and Heller (1984) which separate the species as only sectional without subgenus.

Subgenus *Chronosemium* were represented by 9 taxa in this study. These taxa evaluated within the subgenus *Chronosemium* formed the outmost groups in phylogenetic tree. In other words, the highest variation in MP tree was observed in the species belonging to *Chronosemium*. This supports the classification based on Ellison et al. (2006).

Subgenus *Trifolium* is represented by 8 sections: *Trifolium*, *Vesicastrum*, *Involucrarium, Trifoliastrum*, *Lupinaster*, *Trichocephalum*, *Glycyrrhizum* and

Paramesus. In this study, taxa examined were grouped within the 6 sections as *Paramesus*, *Vesicastrum*, *Trifolium*, *Trichocephalum*, *Trifoliastrum* and *Involucrarium*.

Section *Paramesus* was represented by one species (*T. strictum*) in MP tree. *T. strictum* formed the most distinct group after *Chronosemium* in dendrogram and showed affinity to the clade which consist of *Vesicastrum* species.

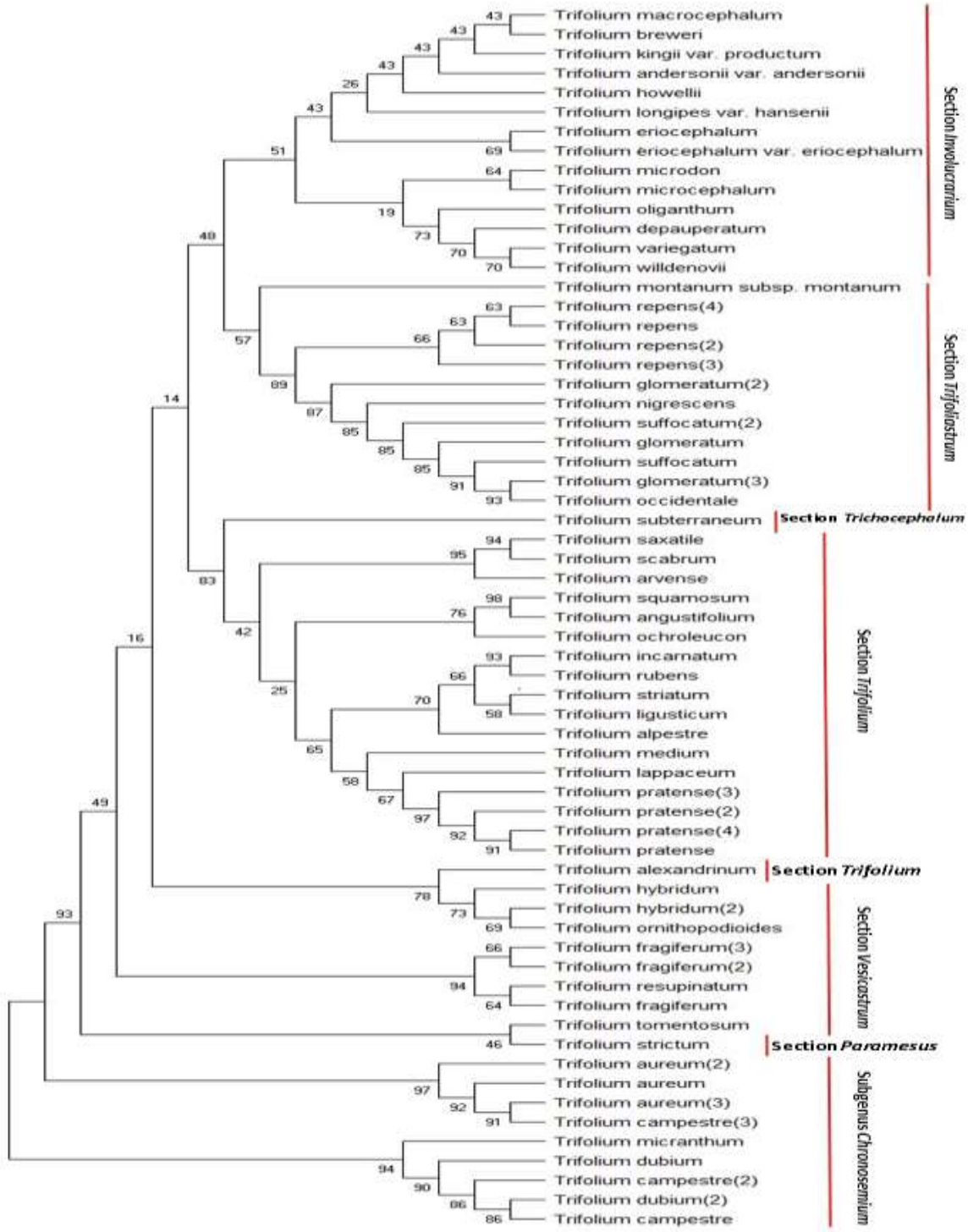

Figure 1. MP tree provided from matK sequence of *Trifolium* taxa
Şekil 1. Trifolium taksonlarının matK sekansından elde edilen MP ağacı

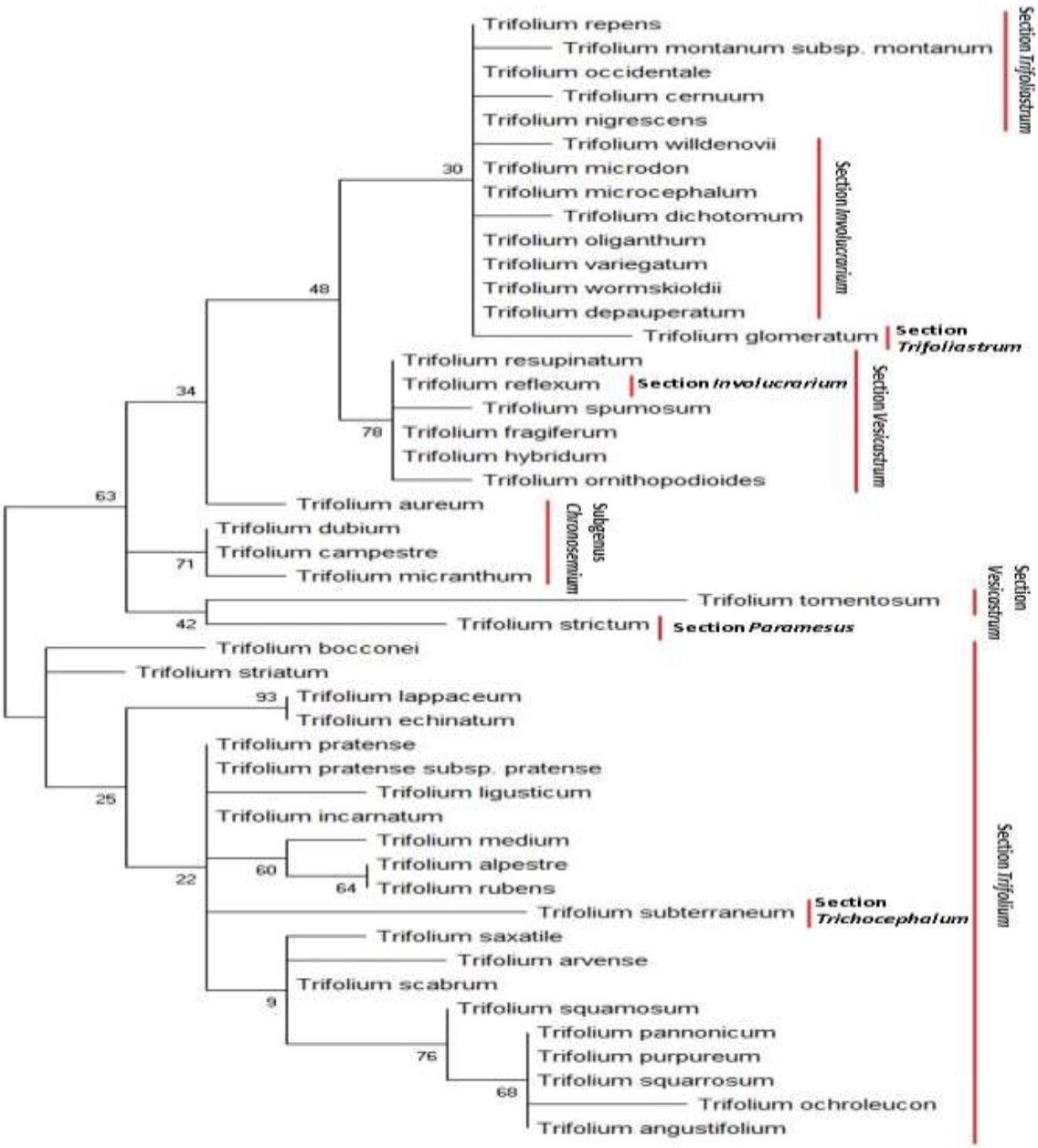

Figure 2. MP tree provided from rbcL sequence of *Trifolium* taxa
Şekil 2. Trifolium taksonlarının rbcL *sekansından elde edilen MP ağacı*

The section *Vesicastrum* was represented by 8 taxa in this study. These taxa belonging to the section *Vesicastrum* were grouped together although they formed different clades between section *Paramesus* and *Trifolium*. Section *Paramesus* and *Vesicastrum* formed the outmost groups in dendrogram with subgenus *Chronosemium*. Other sections (*Trifolium*, *Trichocephalum*, *Trifoliastrum* and *Involucrarium*) show more similarities with each other although each section was grouped in distinct clades.

Section *Trifolium* was represented by 18 taxa. It can be said that all taxa were grouped together in dendrogram although taxa belonging to the section *Trifolium* formed few clades.

Section *Trichocephalum* was represented by only one species (*T. subterraneum*) in this study. *T. subterraneum* merged from outermost to the clade which consist of *Trifolium* taxa. Furthermore, this section was separated from other taxa and formed

different clade although it was represented by only *T. subterraneum*.

Section *Trifoliastrum* and *Involucrarium* were represented by 12 and 14 taxa, respectively. These two sections formed distinct two groups and all taxa examined were grouped as sectional.

Moreover, section *Trifoliastrum* and *Involucrarium* showed similarity as phylogenetically in dendrogram (Figure 1).

As a result, it was observed that all sections examined were separated from each other and formed distinct clades. Finally, it can be stated that sequence information for matK region is significant to understand of taxonomy of the genus *Trifolium* and have enough information to group the species as sectional.

The Evaluation of Phylogenetic Relationships Among *Trifolium* Species Based on RbcL Region:

Subgenus *Chronosemium* was represented by 4 species in this study and species belonging to *Chronosemium* were grouped together in separate clade from other taxa. However, species evaluated within the subgenus *Chronosemium* did not formed outmost clade in dendrogram, unlike the matK.

Section *Trifolium* was represented by 20 taxa. All taxa examined were grouped together in distinct clade. MP tree provided from rbcL region consists of two main groups and one of these main groups is section *Trifolium*. In other words, species which show the most differences among the all taxa examined belong to section *Trifolium*.

Section *Trichocephalum* was represented by only one species (*T. subterraneum*) for rbcL region and *T. subterraneum* was embedded among *Trifolium* species (Figure 2).

Second main group in dendrogram consist of four sections (*Trifoliastrum*, *Involucrarium*, *Vesicastrum* and *Paramesus*) except subgenus *Chronosemium*.

Species belonging to section *Trifoliastrum* and *Involucrarium* were grouped together in same clade.

Species evaluated within the section *Vesicastrum* formed distinct clade from other taxa and showed affinity with the clade which consist of the species of section *Trifoliastrum* and *Involucrarium*.

T. strictum (section *Paramesus*) with *T. tomentosum* (section *Vesicastrum*) formed outmost clade in second main group and showed the most differences phylogenetically among the species examined.

Firstly, MP tree obtained from the sequence informations belonging to the matK region of *Trifolium* taxa shows that all taxa examined were clearly grouped as sectional and formed distinct clusters (Figure 1).

Subgenus *Chronosemium* consists of taxa that exhibit the most differences in comparison to other sections. This situation is compatible with the study of Ellison et al. (2006).

Phylogenetic tree provided from nrDNA ITS sequences and trnL intron of cpDNA used in the study of Ellison et al. (2006) show the major clades of subgenus *Trifolium* and their relationships. The most of the relationships between sections for matK region in this study is compatible with Ellison et al. (2006). Here, the clades of section *Trifoliastrum* and *Involucrarium* show similarity. Furthermore, the section *Trifolium* and *Trichocephalum* exhibit similarity in dendrogram (Figure 1).

The *Paramesus* which consists of only one species (*T. strictum*) is the section showing the highest variation among the sections of subgenus *Trifolium*.

All results based on matK sequence informations show compatibility with the new infrageneric classification. In this aspect, it can be said that the using of matK as a barcoding region is strongly recommended for further studies in the genus *Trifolium*. Results provided from the groupings of taxa examined support the phylogenetic classification suggested by Ellison *et al.* (2006), unlike the classification of Zohary and Heller (1984). Similarly, phylogenetic relationships of the taxa in this study show differences with Watson et al. (2000).

The MP tree provided from rbcL sequence informations of *Trifolium* taxa formed two main groups (Figure 2). While one of these groups consist of section *Trifolium* and section *Trichocephalum* represented by *T. subterraneum*, second main group contains other sections (*Trifoliastrum*, *Involucrarium*, *Vesicastrum* and *Paramesus*) and subgenus *Chronosemium*. In other words, we found quite a high variation in the section *Trifolium* in comparison to others. The relationships between section *Trifolium* and *Trichocephalum* represented in first main group show similarity with the study of Ellison et al. (2006). Furthermore, it can be said that species examined were generally separated as sectional in MP tree provided from rbcL region. However, this sectional separation and phylogenetic relationships of taxa is not clear like information obtained from matK.

CONCLUSION

Although the use of barcoding regions is very important in the evaluation of phylogenetic relationships between species besides the solution of taxonomic problems, the DNA region preferred for analysis is one of the most important points in successful of the study. Moreover, it is necessary to know that this situation can show variability in different plant groups. In other words, a DNA region which show good separation in some plant groups can

be inadequate for other plant groups. As a result of this study, it can be stated that taxa from the genus *Trifolium* are well grouped for both of barcoding regions in aspect of their common features. However, especially the using of matK sequence informations is strongly recommended for their ability to reveal the phylogenetic relationships, in addition to well grouping of the species as sectional. Furthermore, results provided from this study support the phylogenetic classification suggested by Ellison et al. (2006) in aspect of the evaluation of the genus *Trifolium* into two subgenus as *Chronosemium* and *Trifolium*, relationships between sections and finally phylogenetic relationships of taxa.

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Author's Contributions

The contribution of the authors is equal.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

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APPENDIX

matK:

KX677782, KX677632, KX677625, KX677553, KX677538, KX677534, KX677517, KX677456, KX677428, KX677425, KX677330, KX677305, KX677243, KX677240, MF543558, MG221073, MG220863, MG220733, HQ593473, KP210441, KP210439, KJ593139, KJ593138, KJ204551, KJ204549, KP402304, JX518122, HQ619811, MK926203, MK926192, MK520770, KY607384, KY607383, MF963693, MF963637, MF963516, MF963515, MF963514, MF963513, MF963503, MF963502, FJ395427, KJ746140, HM851164, HM851162, HM851161, HM851160, HM851159, HM851156, HM851155, HM851153, HM851152, HM851150, HM851148, HM851147, HE970749, HE967505, HE967503, JN895800, JN895605, JN894444, JN894443, JN894249

rbcL:

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