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The Evaluations Of Taxonomic Classifications In The Genus *Trifolium* L. Based On ITS Sequences

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Abstract

The genus *Trifolium* L. belonging to family Fabaceae is represented by about 255 species in the world. Two classifications based on the sections or subgenus are commonly used by the researchers in the taxonomically evaluation of the genus *Trifolium*. However, there are still some confusing and complex situations about the taxonomy of the genus. For this reason, internal transcribed spacer (ITS) regions between rDNA genes in nrDNA were examined for 139 *Trifolium* taxa with the Maximum Parsimony (MP) method. Furthermore MP tree were evaluated based on two classification used commonly in the genus to provide contributions to taxonomic classification of the *Trifolium* species and to evaluate phylogenetic relationships of the *Trifolium* species. Study results support the status of subgenus proposed by new infrageneric classification. Also it can be stated that it is rationale to divide the section *Lotoidea* into several sections according to phylogenetic relationships of the taxa examined. Finally, this study reveals the importance of sequence information and problems in the barcoding.

Keywords: *Trifolium*, Fabaceae, Internal transcribed spacer, Maximum parsimony, *Lotoidea*

1. INTRODUCTION

The genus *Trifolium* L. (clover) belongs to the Fabaceae that are the third largest angiosperm family with approximately 20,000 species [1, 2]. *Trifolium* is one of the largest and important genus as forage crops for animals in the family Fabaceae. Besides the members of the genus have great importance for animals, the most of species belonging to the genus *Trifolium* are popular as ornamental plants [3, 4].

Trifolium taxa have cosmopolitan distribution. However, the greatest species diversity is found in the Mediterranean region and its periphery [3, 5]. The genus is represented by about 255 species which consist of herbaceous perennials or annuals commonly called clover [2, 6]. There are some confusing and complex situations about the taxonomy of the genus, especially in the

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classification of the species based on the sections or subgenus. While some researchers use the classification proposed by Zohary and Heller [3, 6, 7], some researchers use the classification proposed by Ellison *et al.* [2, 5]. In the classification of *Trifolium* taxa based on Zohary and Heller [6], the taxa are evaluated into 8 sections as *Lotoidea*, *Paramesus* (C.Presl), *Mistyllus* (C.Presl), *Vesicaria*, *Chronosemium*, *Trifolium*, *Trichocephalum* Koch, and Involucrarium Hooker. In the classification of *Trifolium* taxa based on Ellison *et al.* [5], taxa belonging to the genus *Trifolium* firstly are classified into two subgenus as *Trifolium* L. and *Chronosemium* (Ser.) Reichenb. The most of taxa are placed to subgenus *Trifolium* in this classification. Thereafter, these taxa which belong to subgenus *Trifolium* are grouped within 8 sections as *Glycyrrhizum* Bertol., *Paramesus* (C. Presl), *Lupinaster* (Fabricius) Ser., *Trifolium*, *Trichocephalum* Koch, *Vesicastrum* Ser., *Trifoliastrum* S.F. Gray, *Involucrarium* Hooker.

Especially molecular techniques based on the polymerase chain reaction (PCR) methods or DNA sequence information's are frequently used to solve taxonomic problems, to explain the genetic status and phylogenetic relationships of taxa in the genus *Trifolium* [4,5,7,8], because of morphological variations caused by ecological factors, genetic drift, gene flow, hybridization, and epigenetic mechanisms in plants. However, molecular studies show that there are still taxonomic problems and some species are not grouped together with other species belonging to the same section [8]. Furthermore it is observed in some studies that the taxa belonging to section *Chronosemium* are found together with the taxa belonging to other sections (*Vesicaria* and *Lotoidea*) [7], unlike the new infrageneric classification proposed by Ellison *et al*. [5].

In this study, 139 *Trifolium* taxa were examined based on ITS1 and ITS2 sequences informations between rRNA genes provided from NCBI (National Center of Biotechnology Information) [9]. The objective of this study is to evaluate phylogenetic relationships of the *Trifolium* species, to compare the taxonomic classifications suggested by Zohary and Heller [6] and Ellison *et al*. [5] using the results obtained from this study separately for both taxonomic classifications and finally to provide important contributions to taxonomic classification of the *Trifolium* species.

2. MATERIAL AND METHODS

Internal transcribed spacer (ITS) regions between rDNA genes in nrDNA are commonly used in plant molecular systematic studies, due to these regions have high variable sites to determine the status and phylogenetic relationships of species. Furthermore, both regions (ITS1 and ITS2) appropriate for resolving taxonomic problems and understanding relationships within the genus. All sequence informations for ITS1 and ITS2 regions were provided from NCBI [9] and later these regions were analysed in point of compatibility of sequence information. All regions belonging to ITS1 and ITS2 whose sequence information is compatible were determined and used in this study (Appendix).

A total of 139 *Trifolium* taxa that is compatible were detected and examined based on sequence informations belonging to ITS1 and ITS2 regions of nrDNA in this study.

Sequences of the ITS1 and ITS2 regions which were analysed separately for the same plant samples in NCBI were taken and combined [8, 10]. Furthermore, the sequence informations of regions such as ITS1- 5,8SrRNA- ITS2 [11, 12], 18SrRNA Partial- ITS1- 5,8SrRNA- ITS2- 26SrRNA Partial [4, 13, 14, 15] and finally 18SrRNA Partial- ITS1- 5,8SrRNA- ITS2- 28SrRNA Partial [5, 16] were provided, the sequences belonging to ITS1 and ITS2 from these regions were extracted and then sequence informations of these two regions were combined for effective analysis.

Genbank codes for ITS1 and ITS2 sequences provided from NCBI are presented in Appendix. After the ITS1 and ITS2 sequence informations separately provided and combined with each other, multiple sequence alignments for 139 taxa belonging to the genus *Trifolium* were performed by using Molecular Evolutionary Genetics Analysis (MEGA X) [17]. Variable sites,

conserved sites and parsim-info sites were computed by using alignment sequences that were edited. Transitional and transversional substitutions (%) were determined in addition to transition/transversion ratios for purines-pyrimidines. Furthermore, nucleotide frequencies of the regions containing ITS1 and ITS2 for *Trifolium* taxa examined were computed by using alignment sequences. Finally, dendrogram showing the evolutionary history was inferred using the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [18]. This analysis involved 139 sequences belonging to *Trifolium* taxa and all datas stated were computed by MEGA X. Gaps analyzed in comparison of these sequences were treated as missing data. In other words, the positions containing gaps and missing data were eliminated with complete deletion option for effective analysis.

3. RESULTS AND DISCUSSIONS

The sequences which belong ITS1 and ITS2 were combined for each taxa and aligned to determine the sequence similarities and variations for each *Trifolium* taxa. Alignment length of the taxa examined was determined as 558 bp with 328 variable sites and 231 parsimony informative sites. It was observed that the rate of transitional substitutions with 58.92 % is higher than transversional substitutions. Furthermore, transition/transversion ratios are 2.01 for purines and 3.64 for pyrimidines (Table 1). The overall transition/transversion ratio is 1.44 (Table 1). Nucleotide frequencies of ITS1 and ITS2 sequences were also analysed for *Trifolium* taxa and determined as 51.83 % for A+T/U and for 48.17 G+C (Table 1).

Table 1

The informations of taxa examined based on ITS1-ITS2 sequences

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
ITS1-ITS2	139	558	328	231	58.92	41.08	2.01	3.64	1.44	51.83	48.17

Furthermore, phylogenetic tree provided from Maximum Parsimony analysis were used in the comparisons of the taxonomic classifications based on Zohary and Heller [6] (Figure 1) and Ellison *et al*. [5] (Figure 2). In this way, it was aimed to understand the taxonomy of the genus better, besides determination of problems in taxonomic classification within the genus *Trifolium*.

Trifolium taxa based on Zohary and Heller [6] are evaluated into 8 sections. All sections were represented by the taxa examined in this study. Taxa belonging to section *Chronosemium* were grouped in same clade and formed a distinct group with outmost species in phylogenetic tree. The most of the taxa belonging to the section *Trifolium* (26 taxa) were grouped in two close clade and formed the outmost groups after section *Chronosemium*. However, other taxa from the section *Trifolium* were separated from these two out groups and formed distinct clade in phylogenetic tree. While some of these taxa such as *T. alexandrinum*, *T. clypeatum*, *T. echinatum*, *T. pallidum* and *T. pannonicum* were grouped in

same clade, other taxa (*T. hirtum*, *T. scabrum*, *T. cherleri* and *T. scutatum*) showed unexpected positions in phylogenetic tree. The *Lotoidea* with 76 taxa is the section represented by the highest species number in this study. These taxa were grouped in many clades according to MP tree. It can be stated that relationships of species within the section *Lotoidea* are very complex and confusing. While some species from section *Lotoidea* formed the wide clades which consist of many species, some clades contained a few species from section *Lotoidea*. Section *Paramesus* represented by *Trifolium strictum* was embedded among *Lotoidea* clusters. This situation show similarity with the result of Watson *et al*. [8] and supports the suggestion of Zohary and Heller [6] that section *Paramesus* needs to be included within section *Lotoidea* [8].

The species belonging to sections *Mistyllus* and *Vesicaria* were grouped together in two separate clades. It is reported by Badr [19] that these sections have the same chromosome number with 2n = 16. The result is consistent in this aspect. Section *Trichocephalum* was represented by two species in this study (*T. subterraneum* and *T. eriosphaerum*). These two species were grouped together in same clade with a few species from section *Lotoidea*. Section *Involucrarium* was represented by *T. depauperatum* and *T. buckwestiorum*. These species were grouped together and embedded within wide clade which consist of many species belonging to section *Lotoidea*.

In addition to classification based on Zohary and Heller [6], *Trifolium* taxa in this study was evaluated based on classification of Ellison *et al*. [5]. The 15 taxa belonging to subgenus *Chronosemium* formed a distinct group with outmost species in MP tree and clearly separated from other taxa examined. This result show similarity with Ellison *et al*. [5]. It can be said that the most of taxa from section *Trifolium* (26 from 37 taxa) were grouped together into two close clade. Furthermore, these taxa consist of outmost species within subgenus *Trifolium*. In other words, *Trifolium* is a section showing the most differences in comparison to others. However, other taxa from the section *Trifolium* were grouped separately from these two clades in unexpected positions in phylogenetic tree. These discrepancies can be caused by the situations such as labelling errors in accessions or misidentifications of the taxa. Consequently, it can be stated that there are similarity with the results of Ellison *et al*. [5] in point of the problematic taxa within the section *Trifolium*. The section *Vesicastrum* was represented by 27 taxa in this study. The taxa from section *Vesicastrum* were grouped together and formed three clades. Four taxa: *T. semipilosum*, *T. resupinatum*, *T. vesiculosum* and *T. clusii* showed differences from other taxa within the section and grouped with the taxa belonging to other sections. The section *Involucrarium* was represented by 27 taxa and all taxa were grouped in same clade. It is observed that the section *Involucrarium* is close to section *Vesicastrum*, unlike Ellison *et al*. [5] classification. The section *Trifoliastrum* was represented by 20 taxa and all taxa were closely grouped with each other. These taxa were previously classified within the section *Lotoidea* based on Zohary and Heller [6] and formed the problematic species belonging to the *Lotoidea* section in phylogenetic tree. As a result, it can be stated that it is rationale to divide the largest section *Lotoidea* into several sections like Ellison *et al*. [5]. The section *Lupinaster* were represented by 9 taxa in this study. Wide geographical distribution and variations observed due to theirs distribution is the basic reason of issues related to the *Lupinaster* [4].

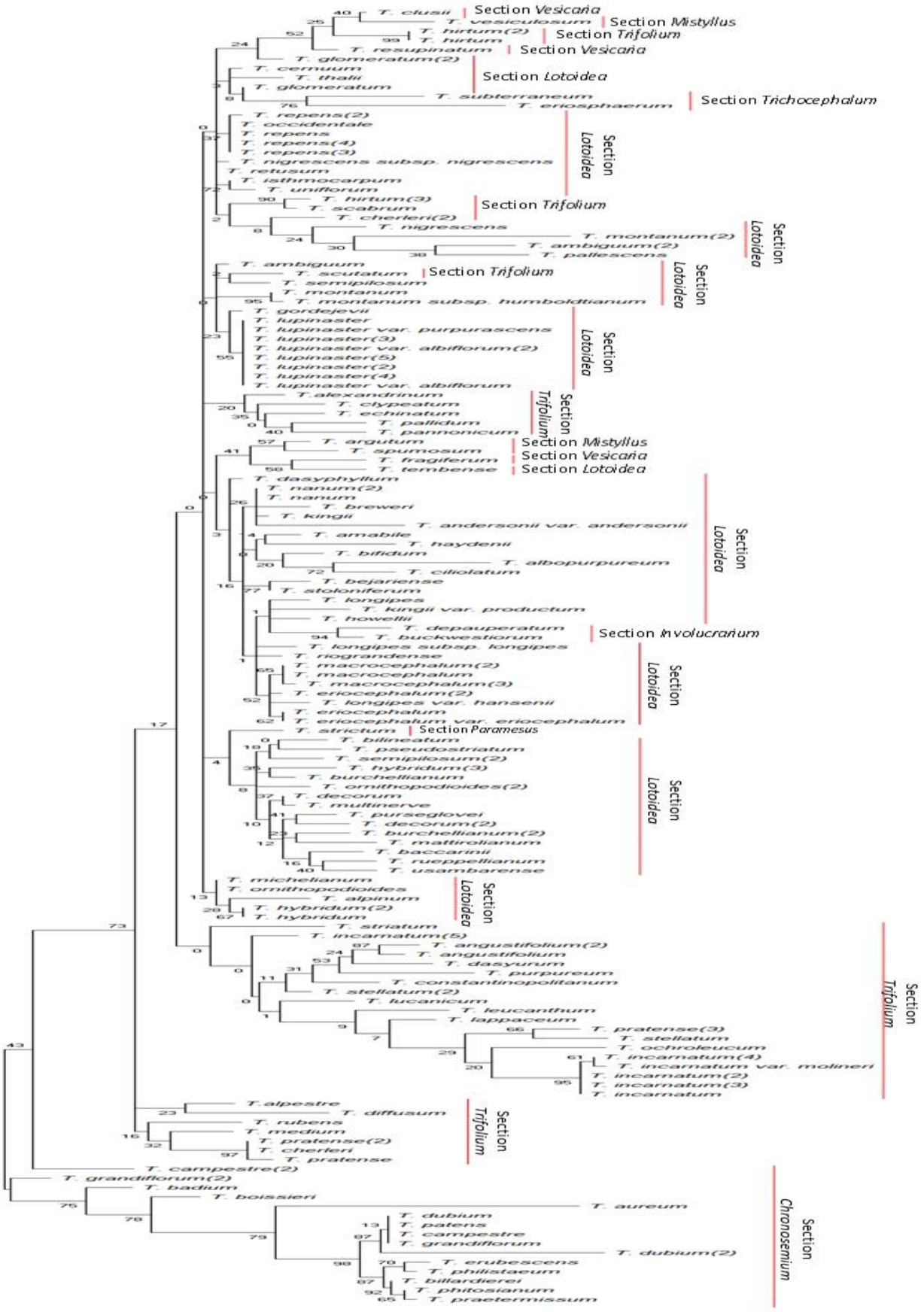

Figure 1 Phylogenetic relationships based on Zohary and Heller (1984)

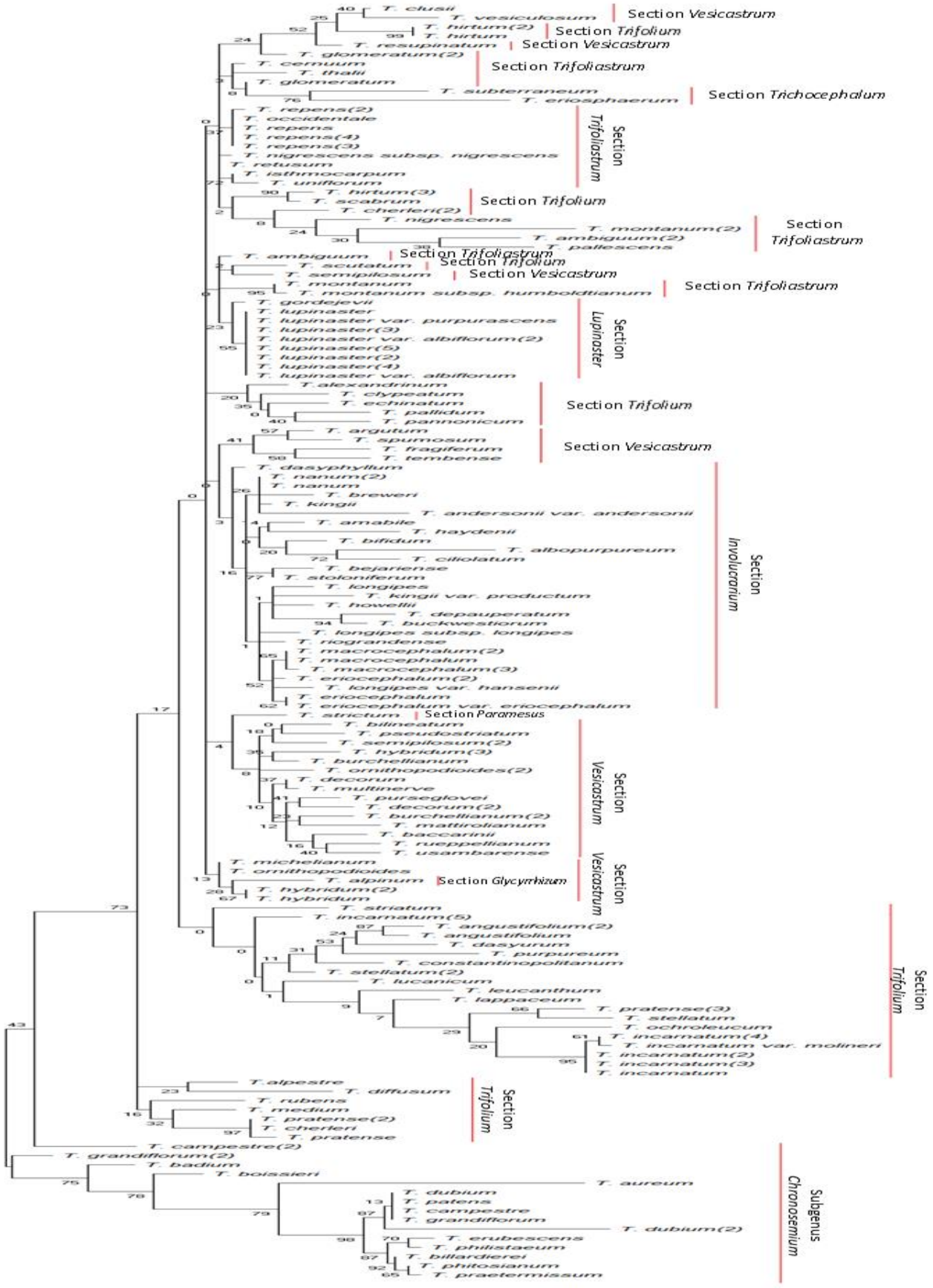

Figure 2 Phylogenetic relationships based on Ellison *et al.* (2006)

The taxa from section *Lupinaster* were grouped together in same clade and the relationships among taxa were observed to be quite similar. Section *Trichocephalum* was represented by two species in this study (*T. subterraneum* and *T. eriosphaerum*). These two species were grouped together in same clade with a few species from section *Trifoliastrum.* Section *Glycyrrhizum* was represented by *T. alpinum* and it was embedded within the clade which consist of *Vesicastrum* species. Section *Paramesus* was represented by *T. strictum*. This taxon merged from outermost to the clade which consist of *Vesicastrum* species.

4. CONCLUSIONS

When sequence information of ITS1 and ITS2 regions added by many researchers to NCBI were examined, it was observed that sequence lengths for *Trifolium* species are quite compatible, although the sequence determinations were made at different times by many researchers. However, ITS sequences of Ellison *et al*. [5] did not match with other researches. Therefore, sequence informations of only 15 taxa were used in this study with other sequence informations.

As a result of this study, firstly it can be said that the taxa belonging to *Chronosemium* formed outmost clade in MP tree and showed the highest differences in the comparison of other taxa. In other words, study result supports the classification in the status of subgenus proposed by Ellison *et al*. [5]. Second highest variations were observed in the section *Trifolium*. While the most of taxa from the section *Trifolium* were grouped in two clades with outmost species, a group exhibited irregular distribution in MP tree. In the study of Vizintin *et al*. [20] based on genetic characterization of selected *Trifolium* species, it is stated that there is quite a high variation within the section *Trifolium*. Similar results are stated by Uslu and Babaç [3] in their studies based on pollen and seed characteristics. The section *Lotoidea* based on Zohary and Heller [6] was represented by highest taxa in this study. It was observed that the taxa from the section *Lotoidea* were grouped together but in many distinct clades (see Figure 1). Consequently, it can be stated that it is rationale to divide the largest section *Lotoidea* into several sections like Ellison *et al*. [5]. However, when the relationships between the sections are examined in terms of phylogenetic, it is observed some differences with the results of the Ellison *et al*. [5].

It was determined that some species represented by a few taxa were not grouped together in same clade or some species from same section formed distinct clade in far distance. These discrepancies can be caused by the situations such as labelling errors in accessions, missing data caused by sequencing or misidentifications of the taxa.

Appendix

AF154381.1, AF154605.1, AF154400.1, AF154624.1, AF154379.1, AF154603.1, AF154353.1, AF154578.1, AF154388.1, AF154612.1, AF154365.1, AF154589.1, AF154370.1, AF154594.1, AF154405.1, AF154629.1, AF154373.1, AF154597.1, AF154406.1, AF154630.1, AF154372.1, AF154596.1, AF154403.1, AF154627.1, AF154357.1, AF154582.1, AF154362.1, AF154586.1, AF154383.1, AF154607.1, AF154386.1, AF154610.1, AF154387.1, AF154611.1, AF154401.1, AF154625.1, AF154382.1, AF154606.1, AF154366.1, AF154590.1, AF154360.1, AF154584.1, AF154404.1, AF154628.1, AF154359.1, AF154932.1, AF154376.1, AF154600.1, AF154392.1, AF154616.1, AF154395.1, AF154619.1, AF154394.1, AF154618.1, U56017.1, U56018.1, AF154390.1, AF154614.1, AF154399.1, AF154623.1, AF154352.1, AF154577.1, U50859.1, U50860.1, AF154356.1, AF154581.1, AF154397.1, AF154621.1, AF154377.1, AF154601.1, AF154354.1, AF154579.1, AF154385.1, AF154609.1, AF154384.1, AF154608.1, AF154396.1, AF154620.1, AF154375.1, AF154599.1, AF154389.1, AF154613.1, AF154371.1, AF154595.1, AF154361.1, AF154585.1, AF154398.1, AF154622.1, AF154369.1, AF154593.1, AF154358.1, AF154583.1, AF154380.1, AF154604.1, AF154374.1, AF154598.1, AF154364.1, AF154588.1, AF154393.1, AF154617.1, AF154391.1, AF154615.1, AF154378.1, AF154602.1, AF154367.1, AF154591.1, AF154368.1, AF154592.1, AF154363.1, AF154587.1, AF004305.1, JX506163.1, AF004304.1, AF004302.1, AF004303.1, AF004301.1, AF004300.1, AF053143.1, AF053144.1, AF053145.1, MF963884.1, EF667004.1, AF053147.2, AF053156.1, MF963868.1, AF053148.1, AF053149.1, AF053150.1, AF053151.1, AF053152.1, KX254378.1, AF053153.1, AF053154.1, MF964020.1, MF963886.1, AF053155.1, KX254380.1, AF053157.1, AF053158.1, MF963885.1, AF053159.1, EU348779.1, AF053160.1, AF053161.1, KX254379.1, MF963869.1, AF053162.1, MF963887.1, KX254386.1, KX254385.1, KX254384.1, KX254375.1, AF053163.1, KX254376.1, KX254373.1, KX254383.1, KX254372.1, MF964097.1, MF963870.1, AF053164.1, AF053165.1, AF053146.1, AF053166.1, KX254374.1,

AF053167.1, AF053168.1, AF053169.1, AF053171.1, EU348780.1, AF053172.1, KY968960.1, AF053173.1, AF053174.1, AF053175.1, AF053176.1, AF053177.1, AF053178.1, AF053179.1, DQ312005.1, DQ312015.1, DQ312025.1, DQ312041.1, DQ312047.1, DQ312050.1, DQ312051.1, DQ312062.1, DQ312098.1, DQ312116.1, DQ312121.1, DQ312122.1, DQ312137.1, KC572140.1, DQ311962.1, DQ312165.1

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The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the authors.

Authors' Contribution

The authors contributed equally to the study. A.Y: Data collection, literature research, writing the article, Y.Y: data collection, literature research.

The Declaration of Ethics Committee Approval

This study does not require ethics committee permission or any special permission.

The Declaration of Research and Publication Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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