



## Molecular Phylogenetic Analysis of *Vicia* L. (Fabaceae) Taxa Growing in the Southeastern Anatolia Region of Turkey: Based on Internal Transcribed Spacer (ITS)

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### ABSTRACT

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Leguminosae or Fabaceae is the third-largest flowering plant family and is important in terms of both food production and soil fertility. Wild *Vicia* species and the genetic diversity of the Southeastern Anatolia Region provide an invaluable resource for the improvement of cultivated temperate feed and legume crops. The rapid progress of technology in recent years has now made it possible to use modern techniques in phylogenetic studies and to examine plants in a greater detail using biochemical, cytological and molecular methods to supplement purely systematic studies. The use of molecular phylogenetic analysis is the most attractive alternative strategy for a more accurate identification of the species of the *Vicia* genus. In the current study, some *Vicia* L. taxa growing naturally in the Southeastern Anatolia Region were investigated using molecular phylogenetic analysis. Internal transcribed spacers (ITS) of nuclear ribosomal DNA were sequenced in order to study the phylogenetic relationships of *Vicia* L. taxa. *Lathyrus inconspicuus* L. and *Lathyrus cassius* Boiss. were used as an outgroup. The ITS area was determined to be approximately 479-672 bp. The ITS sequences were submitted to the NCBI database and accession numbers obtained. The resulting tree clearly groups and separates the sect. *Narbonensis*, *Ervillea*, *Peregrinae*, *Lathyroides*, *Vicia* and *Cracca* species but was less able to distinguish species from sect. *Hypechusa* and *Lentopsis*. The data acquired were observed to be reliable in terms of solving the taxonomical problems of the *Vicia* L. taxa. The morphological distinctions are greatly supported by DNA sequence studies. The species-specific markers developed in this study are useful for early detection of targeted *Vicia* taxa and can act as a guide to the basic data required for the evolution of systematic breeding and conservation strategies, as well as for germplasm resources.

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## Introduction

Fabaceae (Leguminosae) is the third largest flowering plant family, comprising over 765 genera and ~19500 species (LPWG, 2017; Legume Phylogeny Working Group [LPWG]). The family is generally divided into three subfamilies, Caesalpinioideae (Paraphyletic), Mimosoideae and Papilionoideae (Monophyletic) (Davis, 1970; Heywood et al., 2007; Miller et al., 2011; Simpson, 2012).

The economically important and widespread genus *Vicia* L. comprises annual or perennial herbaceous species spread throughout the temperate regions of Europe, Asia, and North and South America. The number of species recognized in the genus varies significantly and is estimated to be between 150 and 210 (Kupicha, 1976; Hanelt and Mettin, 1989). Vavilov (1994) discussed how Turkey contains many of the diversity and gene centers of the genus in the Mediterranean and Near East. In particular,

Eastern Anatolia and some parts of southeastern Anatolia have the third-largest number of genetically differentiated examples of *Vicia* L. species in the world (Vavilov, 1950). 66 species, 22 subspecies and 29 varieties have been reported in Turkey. 11 of these taxa were endemic (Başbağ et al., 2013). After Russia, Turkey has the second most abundant growth of *Vicia* L. species (Kaplan, 2014).

It has been reported that there are a number of taxonomic problems with the *Vicia* genus, and there is as yet no monograph on this genus (Davis, 1970). As with many variable cultivated plants, there is great confusion about the taxonomy and nomenclature of *Vicia* species (Guidetta and Michelozzi, 1986). Despite the fact that the morphological characters, anatomy, and ecological and karyological (Tabur et al., 2002; Navratilova et al., 2003; Bryant and Hughes, 2011; Kahraman et al., 2013) features of *Vicia* genus have

all been studied in order to try and resolve such problems, the number of molecular studies is still not sufficient. Generally, diagnoses of the major subgeneric groupings within the subgenus *Vicia* have relied heavily on a few diagnostic characters that have been used commonly and consistently. These are: the presence of peduncle and, if present, the length of the peduncle in relation to either the flower or the subtending leaf length; the number of flowers per inflorescence; the presence of stipular nectarines; and the positioning of hairs around the style. Many more characters are used, however, to distinguish subgroups in the subgenus (Maxted, 1995). These characters are life span, plant stature, the number of flowers per inflorescence, the number of leaflets per leaf, the size of leaflets, calyx mouth shape, flower color, vexillum shape, vexillum pubescence, legume pubescence, legume protrusion, relative legume size, relative legume shape, legume suture curvature, legume hair types, seed shape, relative hilum length, relative position of the seed hilum and lens, and seed surface type. A key to *Vicia* subgenus *Vicia* sections, series, and species was presented by Maxted (1995). Hosseinzadeh et al. (2008) reported, however, that there are several specimens with intermediate morphological characters and that there are many polymorphisms in these characters that share a common habitat. This means that species diagnosis using morphological characters is a difficult task for nonspecialists and results are not always accurate (Haider et al., 2010). However, the rapid advancement of technology has provided the opportunity to use more modern methods, and taxa have begun to be evaluated using molecular techniques rather than purely systematic studies. Because none of the previous classifications completely reflects the phylogenetic relationship of flowering plants researchers have turned to DNA sequence studies and other modern studies (Felstein, 1985). Recently, the phylogenetic relationships of *Vicia* species have been studied using nucleotide sequences of different regions of DNA (Haider et al., 2012; Foladi et al., 2013; Saqer, 2014). This study was thus carried out to understand the evolutionary relationships of the taxa belonging to *Vicia* genus which have spread naturally in the Southeastern Anatolia Region and to delimit the borders of the species.

We chose to use the internal transcribed spacer (ITS; ITS1 intergenic spacer+5.8S gene+ITS2 intergenic spacer) region of nuclear DNA. ITS of 18S–26S nuclear ribosomal DNA (nrDNA) is suitable for molecular systematic studies (Aceto et al., 1999) due to its variability at the species level. The main objectives of the current study were: i) to shed further light on the systematics and evolutionary structure of *Vicia* taxa living in Southeastern Anatolia Region by using the sequence diversity of the regions of ITS; ii) to work out the phylogenetic relationships among sections and subsections including native species; iii) to understand the usefulness of the regions for the phylogeny of *Vicia* genus.

The Fabaceae (Legumes) family, which contains important cultivars found in large parts of the world, is also very important for Turkey, which generally has an agricultural economy. Identifying the variations between species in this genus and resolving the taxonomic problems are very important tasks in terms of their economic contribution to future breeding studies.

## Material and Methods

### Plant Material

Plant material was collected from different localities in the study area from April to May, which is the vegetation periods of the plants in natural habitats, and was identified according to the diagnostic morphological characteristics described in the Flora of Turkey and the East Aegean Islands (Davis, 1970) (Table 1). The study area is located in the Southeastern Anatolia Region (the provinces of Adiyaman, Batman, Diyarbakir, Gaziantep, Mardin, Siirt, Şanlıurfa). Species were included in eight different sections; *Cracca* S.F. Gray, *Ervilia* (DC) Link, *Lentopsis* Kupicha, *Peregrinae* Kupicha, *Lathyroides* (Buchenau) Tzvel, *Hypechusa* (Alef.) Aschers & Graebner, *Vicia* L. and *Narbonensis* (Radzhi) Maxted. To construct the phylogenetic relationships of the *Vicia* genus, we analyzed sequences of ITS regions from a total of 22 specimens and 25 taxa. *Lathyrus cassius* Boiss. and *Lathyrus inconspicuus* L. were used as an outgroup. DNA sequences of the species studied were entered into the NCBI database and an accession number was obtained for each species and listed in Table 1. Plants were deposited under suitable conditions at the Herbarium of the Department of Biology (DUF), Faculty of Science and Art, Dicle University.

### Genomic DNA extraction

Using the Lefort et al. (1998) method total genomic DNA was isolated from leaf tissues that had been dried and stored at room temperature. The purity and quantity of the extracted DNA were determined using the NanoDrop 1000c UV-Vis Spectrophotometer (Thermo Scientific) at  $A_{260}/A_{280}$  nm. The isolated DNA samples were checked in 1% agarose gel electrophoresis (Figure 1).

### PCR Analysis and Agarose Gel Electrophoresis

Specific primer pairs were used to amplify the targeted region on genomic DNA. Amplification of the ITS region was carried out by using primer pairs ITS1 5' TCC GTA GGT GAA CCT GCG G (forward) / ITS4 5' TCC TCC GCT TAT TGA TAT GC 3' (reverse) (White et al., 1990). DNA amplification was performed in a 50 µL volume mixture containing genomic DNA (100 ng/µL), 10X PCR Buffer, MgCl<sub>2</sub> (25 mM), dNTP mixture (25 mM), selected primer pair (50 µM), Taq polymerase (5u/µL) and sterile water (ddH<sub>2</sub>O). PCR reaction of each region consisted of almost same amount of Buffer (5 µL), DNA (1 µL), MgCl<sub>2</sub> (1.5 µL), dNTP (1 µL), each primer (forward and reverse) (1.5 µL) and Taq polymerase (0.3 µL). PCR amplification was started with 2 min initial denaturation at 94°C, and terminated with 5 min at 72°C. Each reaction ended with a final 4°C hold step and consisted of 35 cycle numbers. Each reaction cycle consisted of a denaturation step at 94°C for 20 sec (1 min), an annealing step at 58°C for 30 sec and an elongation step at 72°C for 30 sec (1 min). Amplicons were visualized by electrophoresis on 2% agarose gel (Figure 2). After purification, the products were sequenced in both directions using an Applied 3130 Genetic Analyzer automated sequencer. All sequence chromatograms were opened using DNA dragon program. Ambiguous sites were checked manually and corrected by comparing the strands. Sequences of ITS regions were deposited in NCBI database for further studies (accession numbers were indicated in Table 1).

Table 1. The taxa of *Vicia* L. studied and their collecting sites, section, accession number and voucher name and herbarium number

No	Taxon names	Section	Location	Accession number	Voucher name and herbarium no
1	<i>V.narbonensis</i> L.var. <i>narbonensis</i>	<i>Narbonensis</i>	Batman, Batman University, central campus, 570 m	MN733368	A.Kaplan DUF 90
2	<i>V. hybrida</i> L.	<i>Hypechusa</i>	Batman, Batman University, central campus, 570 m	MN733398	A.Kaplan DUF 88
3	<i>V. villosa</i> Roth subsp. <i>varia</i> (Host) Corb.	<i>Cracca</i>	Adıyaman, Bozova to Adıyaman, 18.5 km, 589m	MN733439	A.Kaplan DUF 48
4	<i>V. mollis</i> Boiss. & Hausskn.	<i>Hypechusa</i>	Adıyaman, Hilvan to Bozova, Külünçe village, 5 km.600m	MN733450	A.Kaplan DUF 40
5	<i>V. galeata</i> Boiss.	<i>Hypechusa</i>	Adıyaman, Hilvan to Bozova, Külünçe village, 5 km, 600m	MN736208	A.Kaplan DUF 44
6	<i>V. sativa</i> L. subsp. <i>sativa</i>	<i>Vicia</i>	Diyarbakır, Dicle University campus, Bakbako stream around, 630-640m	MN736336	A.Kaplan DUF 82
7	<i>V. sativa</i> L. subsp. <i>nigra</i> L.(Ehrh.) var. <i>nigra</i> L.(Ehrh.) <i>V. michauxii</i>	<i>Vicia</i>	Diyarbakır, Çermik to Çüngüş, 6-7 km,703m	MN738731	A.Kaplan&A.S.Ertekin DUF 35
8	Sprengel.var. <i>stenophylla</i> Boiss.	<i>Peregrinae</i>	Şanlıurfa, Şanlıurfa to Suruç, 18km,666m.	MN736412	A.Kaplan DUF 08
9	<i>V. monantha</i> Retz subsp. <i>monantha</i> Retz.	<i>Cracca</i>	Şanlıurfa, Akçakale,382m.	MN741043	A.Kaplan DUF 09
10	<i>V. anatolica</i> Turril.	<i>Hypechusa</i>	Mardin, Exit of Mardin 2 km,1095m.	MN736415	A.Kaplan DUF 12
11	<i>V. peregrina</i> L.	<i>Peregrinae</i>	Siirt, Çatılı village, 1475m	MN736414	A.Kaplan DUF 73
12	<i>V. noeana</i> Reuter ex Boiss var. <i>noeana</i> Reuter ex Boiss.	<i>Hypechusa</i>	Diyarbakır, Ergani to Çermik, 13km, 870-875m	MN741159	A.Kaplan&A.S.Ertekin DUF 16
13	<i>V. sericocarpa</i> Fenzl. var. <i>sericocarpa</i> Fenzl.	<i>Hypechusa</i>	Diyarbakır, Ergani to Çermik 13km,870-875m	MN741118	A.Kaplan&A.S.Ertekin DUF 18
14	<i>V. lathyroides</i> L.	<i>Lathyroides</i>	Diyarbakır, Çermik to Çüngüş, 11.5- 12km, 905m	MN736416	A.Kaplan&A.S.Ertekin DUF 39
15	<i>V. ervilia</i> (L.) Willd.	<i>Ervilia</i>	Diyarbakır, Ergani to Çermik, 16- 17km, 880m	MN736417	A.Kaplan&A.S.Ertekin DUF 21
16	<i>V. galilaea</i> Plitm.Et. Zoh.	<i>Narbonensis</i>	Diyarbakır, Çermik to Siverek, 1km, 732m	MN736418	A.Kaplan&A.S.Ertekin DUF 32
17	<i>V. cracca</i> L. subsp. <i>Stenophylla</i> Vel.	<i>Cracca</i>	Adıyaman, Ferryboat to Siverek, 4km,588m	MN741044	A.Kaplan DUF 56
18	<i>V. palaestina</i> Boiss.	<i>Cracca</i>	Diyarbakır, Çermik to Çüngüş 11.5- 12km, 905m	MN736419	A.Kaplan&A.S.Ertekin DUF 38
19	<i>V. lutea</i> var. <i>hirta</i> (Balbis) Lois.	<i>Hypechusa</i>	Diyarbakır, Devegeçidi picnic area, 732m.	MN736420	A.Kaplan&A.S.Ertekin DUF 59
20	<i>V. esdraelonensis</i> Warb. & Eig	<i>Hypechusa</i>	Diyarbakır, the way of Bilgekişla, 3.5 km, 740m	MN736422	A.Kaplan&A.S.Ertekin DUF 60
21	<i>V. villosa</i> Roth subsp. <i>Eriocarpa</i> (Hausskn) P.Ball.	<i>Cracca</i>	Adıyaman, Hilvan to Bozova, Külünçe village, 5km, 600m	MN736423	A.Kaplan DUF 41
22	<i>V. sativa</i> L. subsp. <i>Amphicarpa</i> (Dorth.) Aschers. & Graebn.	<i>Vicia</i>	Diyarbakır, Dicle University campus, 630-640m	MN736424	A.Kaplan DUF 80
23	<i>V. assyriaca</i> Boiss.	<i>Hypechusa</i>	Şanlıurfa, Siverek Avurtepe village, Otlı avenue, 1080m	MN736425	A.S.Ertekin DUF 79
24	<i>V. montbretii</i> Fisch. & C.A.Mey.	<i>Lentopsis</i>	Şanlıurfa, Siverek Avurtepe village, Otlı avenue, 1080m	MN736426	A.S.Ertekin DUF 78
25	<i>V. caesarea</i> Boiss.& Bal.	<i>Lentopsis</i>	Siirt, Şirvan to Madenköy, 4-5km, 1020m	MN736432	A.S.Ertekin DUF 184
26	<i>Lathyrus cassius</i> Boiss.	<i>Lathyrus</i>	Diyarbakır, the way of Bilgekişla, 3.5 km, 740m	MN736435	A.S.Ertekin DUF 4241
27	<i>Lathyrus inconspicuus</i> L.	<i>Linearicarpus</i>	Gaziantep, Islahiye Bahçe to Gaziantep, 22km	MN738502	A.S.Ertekin DUF 5249

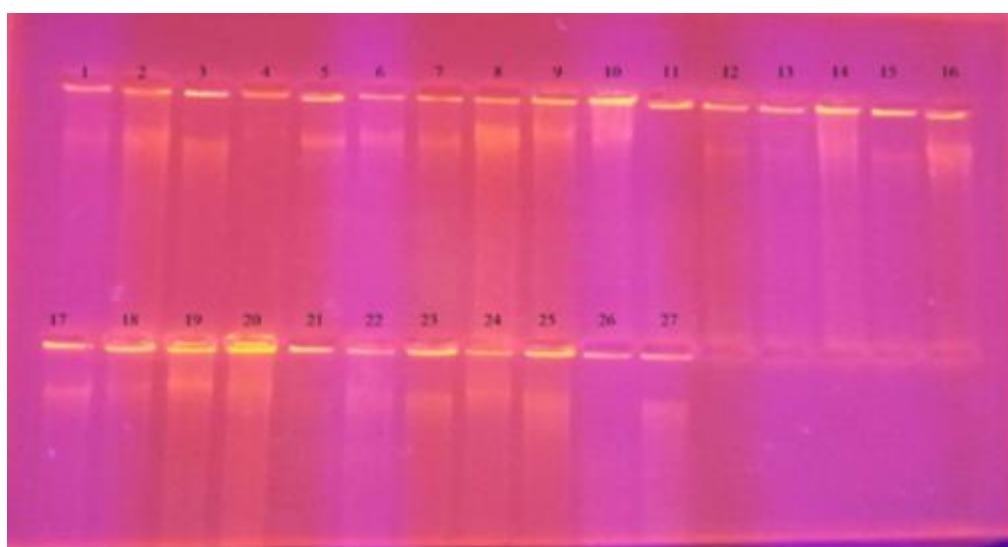


Figure 1. 1% gel image of *Vicia* taxa genomic DNA samples

1) *V. narbonensis* var. *narbonensis* 2) *V. hybrida* 3) *V. villosa* subsp. *varia* 4) *V. mollis* 5) *V. galeata* 6) *V. sativa* subsp. *sativa* 7) *V. sativa* L. subsp. *nigra* var. *nigra* 8) *V. michauxii* var. *stenophylla* 9) *V. monantha* subsp. *monantha* 10) *V. anatolica* 11) *V. peregrina* 12) *V. noeana* var. *noeana* 13) *V. sericocarpa* var. *sericocarpa* 14) *V. lathyroides* 15) *V. ervilia* 16) *V. galilaea* 17) *V. cracca* subsp. *stenophylla* 18) *V. palaestina* 19) *V. lutea* var. *hirta* 20) *V. esdraelonensis* 21) *V. villosa* subsp. *eriocarpa* 22) *V. sativa* subsp. *amphicarpa* 23) *V. assyriaca* 24) *V. montbretii* 25) *V. caesarea* 26) *Lathyrus cassius* 27) *Lathyrus inconspicuus*

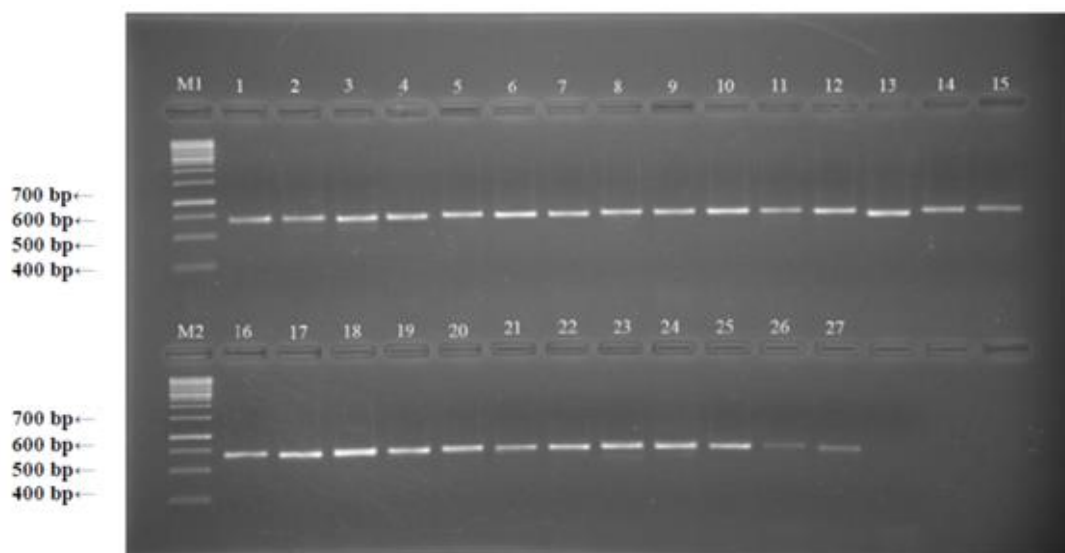


Figure 2. 2% agarose gel image of PCR products of *Vicia* taxa belonging to ITS region

1) *V. narbonensis* var. *narbonensis* 2) *V. hybrida* 3) *V. villosa* subsp. *varia* 4) *V. mollis* 5) *V. galeata* 6) *V. sativa* subsp. *sativa* 7) *V. sativa* L. subsp. *nigra* var. *nigra* 8) *V. michauxii* var. *stenophylla* 9) *V. monantha* subsp. *monantha* 10) *V. anatolica* 11) *V. peregrina* 12) *V. noeana* var. *noeana* 13) *V. sericocarpa* var. *sericocarpa* 14) *V. lathyroides* 15) *V. ervilia* 16) *V. galilaea* 17) *V. cracca* subsp. *stenophylla* 18) *V. palaestina* 19) *V. lutea* var. *hirta* 20) *V. esdraelonensis* 21) *V. villosa* subsp. *eriocarpa* 22) *V. sativa* subsp. *amphicarpa* 23) *V. assyriaca* 24) *V. montbretii* 25) *V. caesarea* 26) *Lathyrus cassius* 27) *Lathyrus inconspicuus*

### Sequence Alignment and Phylogenetic Analysis

Total nucleotide length (base pair, bp) (Table 2), distance matrix among *Vicia* species were determined using Molecular Evolutionary Genetics Analysis software (MEGA 5.1; [Beta 2] (Table 3). DNA sequences were aligned using the Clustal W program. The sequence data was analyzed using the UPGMA method (Figure 3) based on the Kimura model based on the Kimura model (Kimura 1980) and bootstrap analysis with 1000 replications (Felsenstein, 1985). According to the Kress et al. (2005) bootstrap support values, > 85% is defined as very strong, 70-85% as strong, 50- 70% as weak and <50% as very weak. A bootstrap support of 70% or greater is often associated with the identification of the correct phylogeny. If the bootstrap support for a certain branch is below 50%

it is concluded that the relationship between the species is not fully clarified and the branching model is not determined. As a result, this branch will be determined in the tree as a fork (polytomy = uncertainty point) rather than a single node (Freeman and Herron, 1999).

### Results and Discussion

Molecular methods have been used as an important tool in recent years in classifying plants and understanding their evolution (Soltis et al., 1992). Classification studies of angiosperms at the level of genus and species have focused on using ITS sequence analysis studies. For this reason, ITS sequences have been a valuable method in new phylogenetic organizations of angiosperms.

Table 2. ITS region sizes in *Vicia* L. and *Lathyrus* L. (outgroup) taxa

Taxon names	T(U)	C	A	G	Total
<i>Vicia narbonensis</i> var. <i>narbonensis</i>	26.9	23.9	22.3	26.9	624.0
<i>Vicia hybrida</i>	29.2	22.8	22.4	25.6	504.0
<i>Vicia villosa</i> subsp. <i>varia</i>	26.7	24.0	22.2	27.0	603.0
<i>Vicia mollis</i>	29.9	21.9	22.1	26.1	479.0
<i>Vicia galeata</i>	27.4	23.2	23.0	26.3	638.0
<i>Vicia sativa</i> subsp. <i>sativa</i>	27.7	23.7	22.1	26.5	620.0
<i>Vicia sativa</i> subsp. <i>nigra</i> var. <i>nigra</i>	27.5	23.8	22.3	26.4	622.0
<i>Vicia michauxii</i> var. <i>stenophylla</i>	27.7	23.6	22.0	26.7	632.0
<i>Vicia monantha</i> subsp. <i>monantha</i>	27.0	24.1	22.2	26.7	626.0
<i>Vicia anatolica</i>	28.5	22.2	22.8	26.5	635.0
<i>Vicia peregrina</i>	27.5	23.7	22.0	26.7	632.0
<i>Vicia noeana</i> var. <i>noeana</i>	27.9	23.1	22.2	26.8	631.0
<i>Vicia sericocarpa</i> var. <i>sericocarpa</i>	27.8	23.0	22.6	26.6	625.0
<i>Vicia lathyroides</i>	27.1	23.7	21.8	27.3	641.0
<i>Vicia ervilia</i>	28.5	22.2	22.3	27.0	641.0
<i>Vicia galilaea</i>	26.6	24.3	22.0	27.1	672.0
<i>Vicia cracca</i> subsp. <i>stenophylla</i>	26.9	23.8	22.4	26.9	621.0
<i>Vicia palaestina</i>	27.3	23.8	22.2	26.7	630.0
<i>Vicia lutea</i> var. <i>hirta</i>	27.3	24.0	21.7	27.0	641.0
<i>Vicia esdraelonensis</i>	27.5	23.5	22.5	26.4	639.0
<i>Vicia villosa</i> subsp. <i>eriocarpa</i>	26.7	24.1	22.4	26.8	615.0
<i>Vicia sativa</i> subsp. <i>amphicarpa</i>	27.6	24.0	21.6	26.7	633.0
<i>Vicia assyriaca</i>	28.1	23.0	22.6	26.3	634.0
<i>Vicia montbretii</i>	28.1	23.9	21.1	26.9	587.0
<i>Vicia caesarea</i>	28.1	23.5	21.0	27.4	620.0
<i>Lathyrus cassius</i>	26.9	24.3	22.0	26.9	674.0
<i>Lathyrus inconspicuus</i>	27.8	23.0	22.4	26.8	634.0
Avg.	27.6	23.5	22.2	26.7	620.5

Table 3. Distance matrix among *Vicia* L. and *Lathyrus* L. (outgroup) taxa

	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>Vicia narbonensis</i> var. <i>narbonensis</i>													
<i>Vicia hybrida</i>	0.025												
<i>Vicia villosa</i> subsp. <i>varia</i>	0.032	0.032											
<i>Vicia mollis</i>	0.030	0.018	0.032										
<i>Vicia galeata</i>	0.032	0.035	0.034	0.030									
<i>Vicia sativa</i> subsp. <i>sativa</i>	0.035	0.032	0.037	0.028	0.035								
<i>Vicia sativa</i> subsp. <i>nigra</i> var. <i>nigra</i>	0.030	0.028	0.032	0.023	0.030	0.005							
<i>Vicia michauxii</i> var. <i>stenophylla</i>	0.023	0.016	0.025	0.016	0.021	0.025	0.021						
<i>Vicia monantha</i> subsp. <i>monantha</i>	0.028	0.027	0.018	0.028	0.032	0.028	0.023	0.021					
<i>Vicia anatolica</i>	0.037	0.035	0.035	0.021	0.032	0.039	0.035	0.023	0.035				
<i>Vicia peregrina</i>	0.025	0.018	0.027	0.018	0.023	0.028	0.023	0.002	0.023	0.025			
<i>Vicia noeana</i> var. <i>noeana</i>	0.030	0.028	0.030	0.023	0.016	0.028	0.023	0.016	0.025	0.030	0.018		
<i>Vicia sericocarpa</i> var. <i>sericocarpa</i>	0.035	0.028	0.037	0.023	0.039	0.037	0.032	0.025	0.032	0.030	0.028	0.032	
<i>Vicia lathyroides</i>	0.025	0.023	0.027	0.023	0.030	0.023	0.018	0.016	0.018	0.030	0.018	0.023	0.028
<i>Vicia ervilia</i>	0.039	0.037	0.039	0.030	0.042	0.039	0.035	0.028	0.039	0.042	0.030	0.035	0.044
<i>Vicia galilaea</i>	0.007	0.018	0.025	0.023	0.025	0.028	0.023	0.016	0.021	0.030	0.018	0.023	0.028
<i>Vicia cracca</i> subsp. <i>stenophylla</i>	0.030	0.030	0.014	0.025	0.030	0.035	0.030	0.018	0.016	0.032	0.021	0.023	0.035
<i>Vicia palaestina</i>	0.030	0.030	0.025	0.025	0.030	0.035	0.030	0.018	0.021	0.032	0.021	0.023	0.035
<i>Vicia lutea</i> var. <i>hirta</i>	0.025	0.023	0.025	0.023	0.025	0.028	0.023	0.016	0.021	0.030	0.018	0.018	0.028
<i>Vicia esdraelonensis</i>	0.030	0.028	0.030	0.023	0.016	0.028	0.023	0.016	0.025	0.030	0.018	0.005	0.032
<i>Vicia villosa</i> subsp. <i>eriocarpa</i>	0.032	0.032	0.005	0.032	0.034	0.037	0.032	0.025	0.018	0.039	0.027	0.030	0.037
<i>Vicia sativa</i> subsp. <i>amphicarpa</i>	0.032	0.030	0.039	0.030	0.032	0.011	0.007	0.028	0.030	0.0042	0.030	0.025	0.040
<i>Vicia assyriaca</i>	0.030	0.028	0.030	0.023	0.016	0.028	0.023	0.016	0.025	0.030	0.018	0.005	0.032
<i>Vicia montbretii</i>	0.027	0.027	0.018	0.027	0.025	0.032	0.028	0.016	0.018	0.030	0.018	0.025	0.032
<i>Vicia caesarea</i>	0.032	0.032	0.027	0.027	0.037	0.032	0.027	0.025	0.023	0.039	0.027	0.030	0.037
<i>Lathyrus cassius</i>	0.047	0.044	0.049	0.044	0.049	0.044	0.039	0.032	0.044	0.051	0.035	0.044	0.039
<i>Lathyrus inconspicuus</i>	0.054	0.046	0.056	0.046	0.054	0.051	0.046	0.039	0.051	0.054	0.042	0.046	0.049
	14	15	16	17	18	19	20	21	22	23	24	25	26
<i>Vicia ervilia</i>	0.035												
<i>Vicia galilaea</i>	0.018	0.035											
<i>Vicia cracca</i> subsp. <i>stenophylla</i>	0.025	0.037	0.023										
<i>Vicia palaestina</i>	0.025	0.037	0.023	0.018									
<i>Vicia lutea</i> var. <i>hirta</i>	0.018	0.035	0.018	0.023	0.023								
<i>Vicia esdraelonensis</i>	0.023	0.035	0.023	0.023	0.023	0.018							
<i>Vicia villosa</i> subsp. <i>eriocarpa</i>	0.027	0.039	0.025	0.014	0.025	0.025	0.030						
<i>Vicia sativa</i> subsp. <i>amphicarpa</i>	0.025	0.042	0.025	0.037	0.037	0.030	0.025	0.039					
<i>Vicia assyriaca</i>	0.023	0.035	0.023	0.023	0.023	0.018	0.005	0.027	0.025				
<i>Vicia montbretii</i>	0.023	0.039	0.021	0.016	0.021	0.021	0.025	0.018	0.035	0.025			
<i>Vicia caesarea</i>	0.025	0.035	0.025	0.025	0.025	0.025	0.030	0.027	0.035	0.030	0.023		
<i>Lathyrus cassius</i>	0.039	0.056	0.039	0.044	0.046	0.035	0.044	0.049	0.047	0.044	0.039	0.044	
<i>Lathyrus inconspicuus</i>	0.046	0.059	0.046	0.049	0.049	0.046	0.046	0.056	0.054	0.046	0.051	0.051	0.037

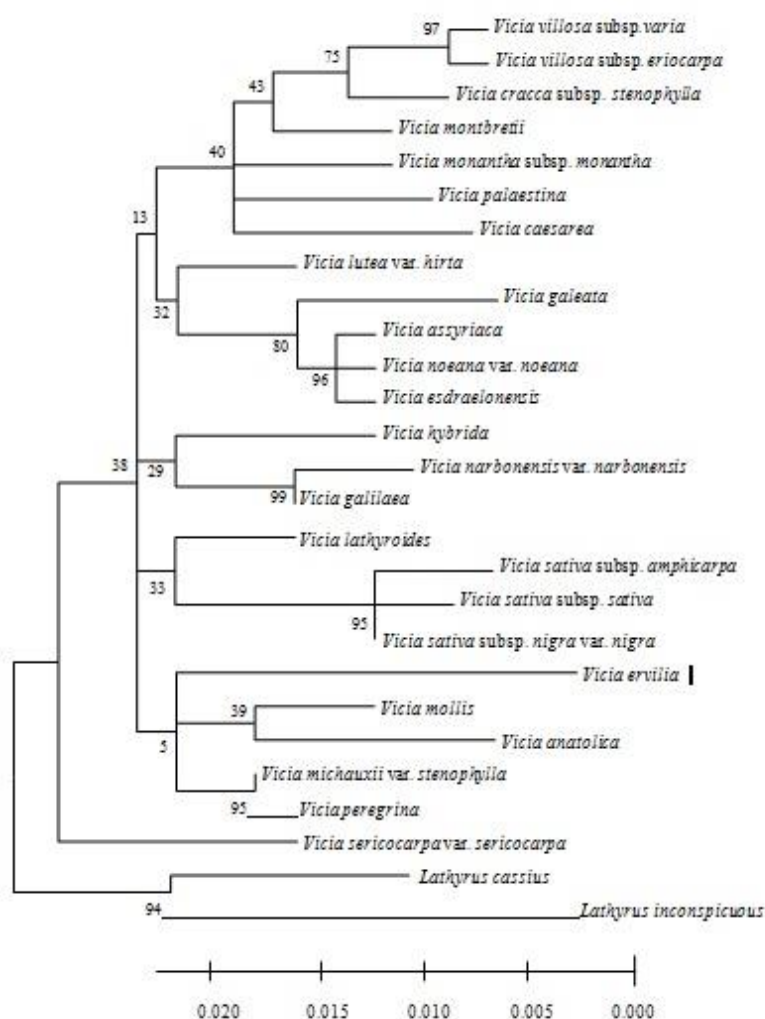


Figure 3. Phylogenetic tree of *Vicia* taxa based on (UPGMA) analysis of the ITS region. *Lathyrus cassius* and *Lathyrus inconspicuus* were used as a outgroup. Bootstrap analysis of UPGMA was based on 1000 replicates. Percentage of each branch shows the support rate of that branch (Bootstrap value)

The data obtained as a result of the analysis of ITS base sequences make important contributions to the solution of problems existing in taxonomic categories. Generally, by looking at the ITS variations of taxa, the attempt can be made to identify relatives of the related taxa (Baldwin et al., 1995).

As the first parameter that constitutes the first step of the study, PCR was performed on gDNA samples of targeted species, the presence of bands was visualized using 1% agarose gel electrophoresis (Figure 1). Amplicons (ITS region) were then visualized by electrophoresis on 2% agarose gel (Figure 2).

The base ratios obtained from approximately 620 bp of ITS genes analyzed in the series are given in Table 2. When Table 2 is examined, the sizes of the ITS regions can be seen to vary from 479 bp (*Vicia mollis*) to 672 bp in *Vicia galilaea*. In order of magnitude this appears to be: A: 22.2%, G: 26.7%, C: 23.5%, T (U):27.6%. DNA sequences related to the samples have a G - C ratio (50.2%) and an A - T ratio (49.8%) and the values are close to each other. The distance between the samples obtained using the MEGA program and the Kimura 2-Parameter base change model is given in Table 3. When Table 3 is examined, the smallest genetic distance value (0.005) was observed between 21-3, 7-6 and 20-12, while the largest genetic distance value

(0.044) was observed between samples 15 and 13. These values are genetically significant in the genus and this shows that there are no differences.

The phylogenetic results from ITS region sequences were found to be generally useful for understanding the evolutionary relationships of species and sections of *Vicia*. In the unweighted pair group method with arithmetic mean (UPGMA) tree obtained in the present study (Figure 3), it was observed that the *Lathyrus* species used as an outgroup were the first to be separated and this branch was supported with a bootstrap value of 94%. The ingroup consists of two main clades. The first clade is divided into five subclades, consisting of the first subclade: *V. villosa* subsp. *varia*, *V. villosa* subsp. *eriocarpa*, *V. cracca* subsp. *stenophylla*, *V. monantha* subsp. *monantha*, *V. montbretii*, *V. caesarea*, and *V. palaestina*; the second subclade: *V. lutea* var. *hirta*, *V. galeata*, *V. assyriaca*, *V. esdraelonensis*, and *V. noeana* var. *noeana*; the third subclade: *V. hybrida*, *V. narbonensis* var. *narbonensis*, and *V. galilaea*; the fourth subclade: *V. sativa* subsp. *sativa*, *V. sativa* subsp. *nigra* var. *nigra*, *V. sativa* subsp. *amphicarpa*, and *V. lathyroides* and the fifth subclade, *V. mollis*, *V. michauxii* var. *stenophylla*, *V. anatolica*, *V. peregrina*, and *V. ervilia*. The second clade is *V. sericocarpa* var. *sericocarpa*.

Using the data in Figure 3, the first subclade was found to consist of two sections of species. The first was sect. *Cracca* (*V. villosa* subsp. *varia*, *V. villosa* subsp. *eriocarpa*, *V. cracca* subsp. *stenophylla*, *V. monantha* subsp. *monantha*, and *V. palaestina*), and the second was sect. *Lentopsis* (*V. montbretii*, *V. caesarea*). The species belonging to sect. *Cracca* were in the same group. We can see that the reliability of the branch containing *V. villosa* subsp. *eriocarpa* and *V. villosa* subsp. *varia* is 97%. *V. cracca* subsp. *stenophylla* was linked to this branch with a 75% reliable bootstrap value. Molecular data based on sequences of the ITS with high bootstrap values supports these species being placed in the same group. Other species belonging to the section are linked with weak bootstrap values. The species belonging to sect. *Lentopsis* are scattered within the group. Since the bootstrap values are as low as 43% and 40%, there can be no clear interpretation about the relationship between these taxa. We can say that polytomy has been found since the reliability values are below 50%. Therefore, the parser has not provided any information. Schaefer et al. (2012), in their study of sect. *Lentopsis*, examined the species in the same branch of the tree (maximum likelihood). The morphological data obtained in this study revealed that the variable characteristics of the peduncle length, the number of flowers per peduncle, flower sizes, the number of leaflet pairs, the leaflet shape, the fruit shape and the aspect ratio were the most common among these species. Leht (2009), examined the *V. palaestina*, *V. villosa*, and *V. cracca* taxa, which are among the *Cracca* section taxa, in the phylogenetic tree that he obtained in his study, and made a new cladistic analysis of 91 morphological data. In parallel with our work, Binzaf (2012), Foladi et al. (2003), Schaefer et al. (2012) all examined sect. *Cracca* taxa within the same group in their studies on the molecular phylogeny of *Vicia* species.

The second subclade included *V. lutea* var. *hirta*, *V. galeata*, *V. assyriaca*, *V. esdraelonensis*, and *V. noeana* var. *noeana*. These species belong to the section *Hypechusa*. In this subclade, *V. esdraelonensis*, *V. noeana* var. *noeana* and *V. assyriaca* were supported with a bootstrap value of 96% reliability, while *V. assyriaca* connection to this group was supported with a bootstrap value of 80% reliability. However, *V. lutea* var. *hirta* connection to this group was supported with a bootstrap value of 32% reliability and it was not informative. Molecular data based on sequences of the ITS with high bootstrap values support placing these species in this section. Maxted and Douglas (1997) found that in sect. *Hypechusa* the peduncle length can be divided into two series according to the shape and size of the corolla, the hairiness of the flag and the strength of the wings at the basal. These series are *Hypechusa* and *Hyrceanicae*. *V. assyriaca*, *V. galeata*, *V. esdraelonensis*, *V. noeana* var. *noeana* are included in the *Hyrceanicae* series. This information was confirmed in our tree through the supporting branches with high bootstrap values. Castiglione et al. (2007), in their cytological and molecular characterization (ITS region located in nrDNA) studies of *V. esdraelonensis*, found that the species belongs strictly in the section of *Hypechusa*, and that it is closely related to *V. noeana*. This finding coincides with those in our study. Schaefer et al. (2012), in their molecular phylogeny

studies, determined that taxa belonging to the section, such as *V. hyrcanica*, *V. noeana*, *V. galeata*, *V. lutea*, *V. esdraelonensis*, were included in the same group. They reported that the *Hypechusa* section was not monophyletic. Caputo et al. (2006) also stated that the *Hypechusa* section is a heterogeneous section, not a monophyletic section, but noted that some species in the section are monophyletic. The phylogenetic tree we obtained supports this assertion.

The third subclade included *V. hybrida* (sect. *Hypechusa*), *V. narbonensis* var. *narbonensis* and *V. galilaea* (sect. *Narbonensis*). In our tree, the branch with *V. narbonensis* var. *narbonensis* and *V. galilaea* species was supported with a 99% bootstrap value. Molecular data based on sequences of the ITS with high bootstrap values support placing these species in this section. *V. hybrida* was connected to this branch with a bootstrap value of 29%. Since this value was below 50%, the branch collapsed and did not provide any exact information. In many studies (Enneking and Maxted, 1995; Fennel et al., 1998; Potokina, 1999; Schaefer et al., 2012), species belonging to sect. *Narbonensis* have been studied within the same branch. These results are in line with our study. Schaefer et al. (2012) reported that the section was monophyletic. Our study supports the finding that the section is monophyletic.

The fourth subclade included *V. sativa* subsp. *sativa*, *V. sativa* subsp. *nigra* var. *nigra*, *V. sativa* subsp. *amphicarpa* (sect. *Vicia*), and *V. lathyroides* (sect. *Lathyroides*). The branch with *V. sativa* subsp. *sativa*, *V. sativa* subsp. *nigra* var. *nigra*, *V. sativa* subsp. *amphicarpa* species was supported with a bootstrap value of 95% and this value indicates that a meaningful result has been achieved. Molecular data based on sequences of the ITS with high bootstrap support placing these species in this section. *V. lathyroides* was connected to this branch with a weak bootstrap value of 33%. Since the bootstrap value of this branch was below 50%, no clear information was provided and this branch collapsed. Van de Wouf et al. (2001) found that *V. sativa* subsp. *amphicarpa* and *V. sativa* subsp. *nigra* var. *nigra* are located close to each other, while *V. sativa* subsp. *sativa* is located farther away in the UPGMA phylogenetic tree they obtained. Potokina (1999) reported that the *V. sativa* complex coexists in the dendrograms they obtained in their study. Leht (2009), Potokina (1999) and Schaefer et al. (2012) reported that this section is not monophyletic but polyphyletic. However, Potokina et al. (1999) examined *V. lathyroides* species as a separate branch in their study and found 53% support for the branch where this species was located. They also found it close to the *Vicia* section in all the trees they created. Jaaska (2008) and Leht (2009) reported that they found the species to be close to the *Vicia* section. Although the bootstrap value of the *V. lathyroides* species was low, a close connection has been established with sect. *Vicia* in the studies mentioned above. Schaefer et al. (2012) determined that the section was not monophyletic. The results we obtained support those of the other recent studies.

The fifth subclade included *V. mollis*, *V. anatolica* (sect. *Hypechusa*) *V. michauxii* var. *stenophylla*, *V. peregrina* (sect. *Peregrinae*), and *V. ervilia* (sect. *Ervilia*). The branch with the highest bootstrap value in the group was the branch with *V. michauxii* var. *stenophylla* and *V.*

*peregrina*, which was supported with a bootstrap value of 95%. Molecular data based on sequences of the ITS with high bootstrap, support placing these species in this section. Many studies (Fennel et al., 1998; Potokina et al., 1999; Leht, 2009; Emre, 2011; Schaefer et al., 2012) found that these species belonged to the same branch and examined them in the *Peregrinae* section. Schaefer et al. (2012) found that the section was monophyletic. This result is in line with our study. Since the branch containing the *V. mollis* and *V. anatolica* species was only supported by a bootstrap value of 39%, the branch collapsed and did not provide any discriminatory information. *V. ervilia* is located on a single, separate branch (monotypic clade) of the tree. Although it appears to be different from the other distributed species, there is no bootstrap support. Jaaska (2005) positioned the *V. ervilia* taxon in a separate branch on the tree in the dendrogram obtained in his study. He found that the group with which a taxon connection was formed was a group external to the other groups. Leht (2009) examined *V. ervilia* as a single-branch monotypic clade. According to Schaefer et al. (2012) dendrograms obtained from the ITS region, *V. ervilia* belonged in sect. *Ervilia* as a monotypic clade. According to Binzat (2012), genus *Vicia* members have compound leaves. Most of them have a leaf ending with tendrils. Only two of them have no tendrils. Instead of having tendrils, they have a mucro at the leaf's rachis ending (*V. truncatula* and *V. ervilia*). This property of the leaves is useful in infrageneric grouping. In terms of the monotypic nature of *V. ervilia*, we think that the information obtained in the ITS region may represent this morphological difference.

The second clade, *V. sericocarpa* var. *sericocarpa*, acted as the outer group of the second clade and has no bootstrap value. In the current study, this taxon was located separately. This is different from the findings of Schaefer et al. (2012), Leht (2009), and Binzat (2012). It has been generally studied within the *Hypechusa* section. However, El-Shanshoury and Soliman (1996) reported that *V. sericocarpa* was separate from the *Hypechusa* section and was located on the same branch as *V. palaestina*. According to the phylogenetic tree we obtained with the ITS region, this species has not yet been successfully parsed. Finally, the main branches formed in the phylogenetic tree obtained in the current study almost completely support the divisions into sections made according to the morphology of the genus.

In the field of taxonomy, the validity of hypotheses created with only morphological data may remain limited. We can say that the ITS region we used in our study is largely successful. The high genetic variation of the ITS region may be due to rapid concerted evolution and crossing over.

Consequently, a leguminous plant which *Vicia* L. a good source of nutrients that for animals, 14 to close the farming of its kind in Turkey, used as edible (*V. faba*), are used as green manure (The amount of nitrogen detected by the vet per decare through its nodosity at the roots is 10-12 kg.) as well as the same features a good time for the nectar of wild bees and honey bees (nectar) any research due to be held in *Vicia* sources will contribute to increasing the economic importance of this plant in Turkey. However, the fact that *Vicia* L. taxa, especially in some parts of Eastern Anatolia and Southeast Anatolia, is one of the third genetic

differentiation regions in the world adds another importance to our study. It is important for the whole world to carefully conserve the gene resources of *Vicia* L. species, which are almost as old as human history, and many species are cultivated, belonging to the legume family. We hope that the economic integration studies, which are carried out at the end of the inventory and documentation studies in basic sciences all over the world, will now be carried out effectively in our country and that this information will serve this purpose.

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