# Phylogenetic study of tribe *Vicieae* based on Internal Transcribed Spacer (ITS)

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**ABSTRACT**

*Vicieae is one of the most important tribe in Faboideae that consists of Vicia, Lathyrus, Lens and Pisum genera. Molecular reassessment of relationships within Vicieae using electrophoretic and immunochemical techniques suggested the classification of Cicer under Vicieae rather than a seperate tribe Cicerideae. To resolve the relationships among some Iranian taxa of this tribe, 19 species of this tribe were analyzed using nuclear ribosomal internal transcribed spacer (ITS). Trifolium pratense was used as outgroup. Based on the Maximum Likelihood(ML), Cicer species formed a seperate group and within the Cicer clade, C. spiroceras, C. kermanense and C. oxyodon constitute the unresolved group and showed polytomy. Results presented here provide strong support for a monophyletic Vicieae. Morphological features are supported these data.*

**Keywords:** Fabaceae, Phylogeny, ITS, Iran

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## INTRODUCTION

*Vicieae* was first delineated as a "section" including *Aphaca* ,*Cicer* , *Clymenum*, *Ervum*, *Lathyrus*, *Lens*, *Nissolia* , *Orobus* ,*Pisum* and *Vicia*. Later on, it was given tribe rank [1]. In 1865, Bentham recognized six genera in the tribe *Vicieae*: *Cicer*, *Vicia*, *Lens*, *Lathyrus*, *Pisum* and *Abrus*. The morphological , anatomical and karyological data advocated that *Abrus* should be excluded from the tribe *Vicieae* and placed in its own tribe *Abreae* [23,16,2,6,5]. More recent treatments of the *Vicieae*, recognise the following genera; *Lathyrus*, *Lens* ,*Pisum*, *Vavilova* and *Vicia* [13,7]. Following anatomical , morphological ,Pollen grain morphology, karyological , isoflavonoid and isoezymatic data, it was suggested that *Cicer* must be assigned to a seperate tribe ,The Cicerideae [12,4]. This was recently supported by molecular phylogenies based on *matk* sequences [26]. However, immunological and electrophoretic data of the total seed proteins of the members of the tribe *Viciea*e including *Cicer* displayed high similarity, supporting that *Cicer* should be included in *Vicieae* [19]. Molecular reassessment of relationships within *Vicieae* using electrophoretic and immunochemical thecniques suggested the classification of *Cicer* under *Vicieae* rather than a seperate tribe Cicerideae [20].In Iran There are five genera of this tribe including *Vicia*, *Cicer*, *Lens*, *Pisum* and *Lathyrus* [18].Curently, there is no consensus on the taxonomy of the Iranian taxa of this tribe. Therefore, this study aimed to use Internal transcribed spacer (ITS) to examine the relationships of the genus *Cicer* with the tribe *Vicieae*. Also, resolve the phylogenetic affinities among some species in this tribe.

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## MATERIALS AND METODS

**Taxa  Samples**
The plant material was collected from  the natural habitat (Table 1). Taxa of *Vicieae* including eight species of *Vicia*, eight species of *Lathyrus*, one species of *Cicer* and one species from *Lens* and *Pisum* were used in this analysis. *Trifolium  pratense* was used as outgroup.

**Table1- The species of *Vicieae* tribe studied and their collecting sites**

| Species | Locality and voucher specimen no. |
|---|---|
| *Cicer arietinum*  L. | ....................... |
| *Cicer chorassanicum*      Boiss . | ....................... |
| *Cicer insicum* Willd. | ....................... |
| *Cicer oxyodon*  Boiss. | Qazvin:Alamut,1500m,Mazooji- Salimpour,13538 |
| *Cicer spiroceras*  Juab. | ....................... |
| *Cicer  kermanense* Bornm. | ....................... |
| *Cicer  tragacanthoides*    Juab . | ....................... |
| *Vicia  cracca* L. | Tehran:Fasham,Roodbare Qasran,  2020m , Qasemi, 284. |
| *Vicia ciceroideae* Boiss. | Tehran:Dizin, 3000m, Mazooji, 13517. |
| *Vicia canesense*  Labill. | Alborz: Karaj,Shahrestanak,3000m Karafarin,176. |
| *Vicia ervillia* L. | Tehran: moution  sohanak,2010m, Kazemi, 13503. |
| *Vicia  leucophaea* L. | ....................... |
| *Vicia monantha* Retz. | Mazandaran:Chalus,1700m,Karafarin, 150. |
| *Vicia sojakii* Chr kova | Alborz:Shahrestanak,2500m,Karafarin,199. |
| *Vicia variabilis* Freyn | Qazvin:Alamut,1500m, Mazooji-Salimpour,70. |
| *Lens orientalis* Boiss. | Mazandaran:  Kiasar, vavsar , 2100m, Fooladi,13518. |
| *Lathyrus aphaca* L. | Tehran: Varamin,1600m ,Salimpour –Karafarin, 13536 |
| *Lathyrus  cicera* L. | Qazvin: Alamut,1600m,Mazooji,13528. |
| *Lathyrus inconspicuus* L. | Tehran: Firoozkooh,2500m,Mazooji,13543. |
| *Lathyrus  pseudocicera* Pamp. | Tehran:Galandook,1780m, Salimpour,13530. |
| *Lathyrus rotundifolius* Willd. | Mazandaran:Siah bisheh, 2410m,Salimpour- Karafarin,13521. |
| *Lathyrus roseus* Stev | Mazandaran:  Chapdarreh,2500m, Salimpour-Mazooji,13540. |
| *Lathyrus  sativus*  L. | Qazvin: Niag village ,2100m,Mazooji,13543. |
| *Pisum sativum*  L. | Mazandaran:Tonekabon, 2100m,Taremi,13512. |

**DNA extraction**
DNA extraction, PCR amplification and sequencing the leaf material used for the extraction of genomic DNA were dried and stored at room temperature.The extraction method used was a stightly modifies version of  that of Tsumura et al.[25].The nuclear ribosomal region encompassing  the  ITS-1,5.8S rRNA and  ITS2 spacers was amplified using the primers 18S and 28S [14].Each 25 μL of PCR reagent contained  1 μL of the 5′ and the 3′ primer, 1μL of dNTP,0.5 μL Taq DNA polymerase, and 2.5 μL 10 X PCR Buffer.

DMSO was added to a final 10% in the ITS amplifications to increase the specificity of the PCR fragments and the intensity of the sequence peak profiles. All amplifications were carried out using a thermocycler.The PCR cycles involved an initial denaturing step at 94 °C for 3′,35 cycles at 94 °C for 45″ and 56 °C for 1′and at 72°C for 2′. An additional extention was performed at 72 °C for 5′ and then coded to 4 °C. The purification and sequencing of the PCR products were performed in South Korea.

**Sequence aligment and phylogenetic analyses**
The sequences were edited and aligment with Sequencher ver 4.1.4 and Mesquite ver. 2.73 the phylogenetic analyses Maximum Parsimony (MP) and Maximum Likelihood (ML) were conducted using PAUP *4.0b[24] and Bayesian Inference (BI) using MrBayes version 3.ob4(Huelsenbeak and Ronquist, 2007). Heuristic parsimony were performed using equally weighted characters, tree-bisection-reconnection (TBR) branch. Swapping , random addition of sequence (1000 replicates),and with no limit to the number of trees saved. The substitution models for ML and Bayesin analyses were obtained using Modeltest ver.3.4 [17] with both Hierarchial Likelihood Ratio Tests(hLRTS) and Akaike Information  Criterion (AIC) methods. The remaining trees were saved and imported into PUAP* for the construction  of a majority rule consensus trees. The posterior probability for each clade was obtained to evaluate the branch support in the resulting trees.

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## RESULTS

The aligned ITS data set consists of 310 nucleotide characters and of these,128 characters were informative. Based on the maximum like lihood (ML) analysis using the SYM+G model, data matrix with equal weighted characters, resulted 182 trees whit a length of 458 steps, having a Consistency Index CI=%827 and a Retention Index RI=%646(Fig.1).
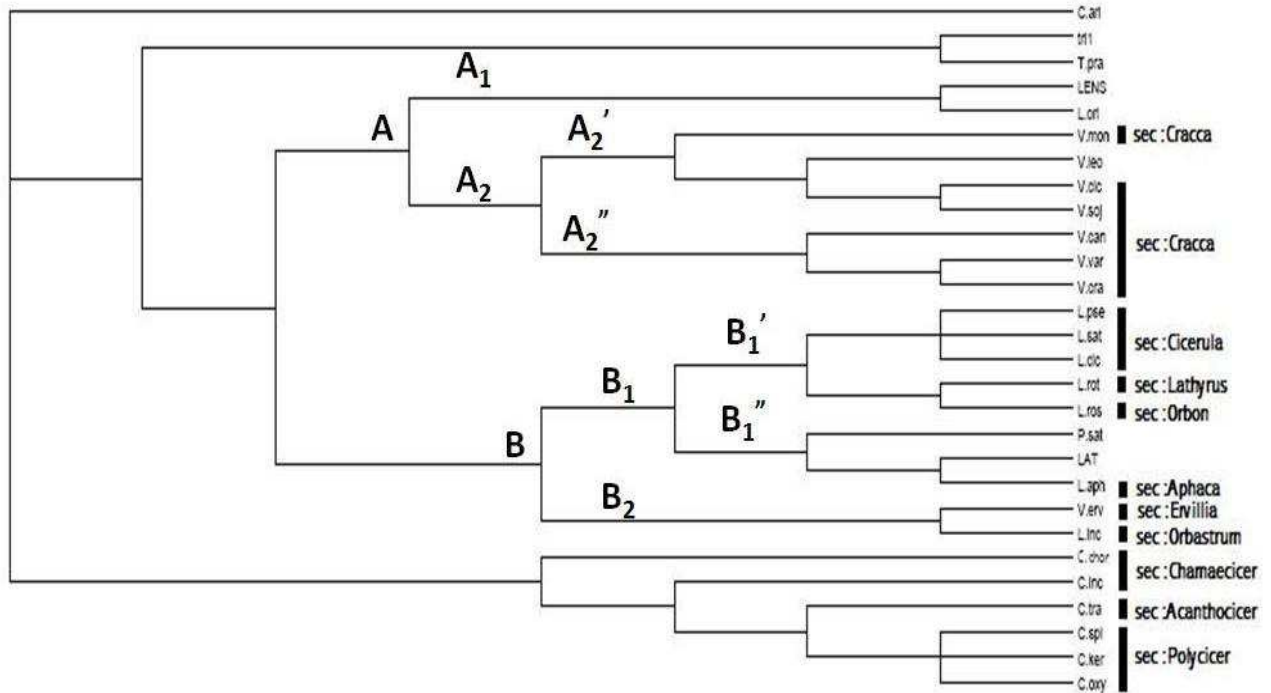


**Fig 1- Maximum Likelihood analyses of ITS sequences in some Iranian Vicieae species**

Based on the Maximum Likelihood(ML), *Trifolium pratense* as a outgroup, form a seperate clade and *Cicer* species form a group that are sister to other *Vicieae* species. Within the *Cicer* clade, *C.spiroceras*, *C.kermanense* and *C. oxyodon* constitute the unresolved group and show polytomy. The ingroup consists of two main clades that labeled as a A and B. Clade A comprises two clades including $A_1$ and $A_2$ . in clade $A_1$ , *Lens orientalis* form a separate group. In clade $A_2$, the species of *Cracca* section of *Vicia*, recognize in two subclades $A'_1$ and $A''_2$ , respectively. Clade B consisting of the species of *Lathyrus* genus, in two clade. In clade $B'_1$ the species of *Cicerula* section form a polytomy. *L. rotundifolius* and *L. roseus* are the sister taxa to them. *Pisum sativum* formed a seperate clade in subclade $B''_1$ .In $B_2$ clade, *V.ervillia* and *L. inconspicuus* are grouped together.

## DISCUSSION

In the ML tree obtained in present study, *T. pratense* is presumed to be a outgroup of the *Vicieae*. This conclusion indicates that *Vicieae* is an ingroup of *Trifolieae* and not part of an outgroup to *Trifolieae* [3]. Using the data of Figure 1 , clade A was composed of three clade : in clade $A_1$, *Lens* species formed a clade near to *Vicia* species. Steele and Wojciechowski in their molecular phylogenetic analysis examined two *Lens* species with *Vicia*. In their work, these two taxa formed a clade with three species of the subgenus *Vicia* and *V. american* in their analysis, with bootstrap supports form 100 replicates [22]. So our results show that *Lens* is close genus to *Vicia*. Based on our results, *Cicer* species divided into seperate clade. Cladistic analysis of phylogenetic relationships among tribes *Cicereae*, *Trifolieae* and *Vicieae* showed that *Cicereae* and *Vicieae* are monophyletic group and *Trifolieae* is its sister group [3]. Morphological data support this result using the differences in shape of style, leaflets, inflorescense and legume. Also the sculpture of seed is different in *Cice*r [9]. The molecular data based on sequences of the plastid gene *matk*, strongly support that *Vicieae* as currently delimited consisting of *Vicia*, *Lathyrus*, *Pisum* and *Lens*,with the exclusion of *Cicer* [22]. So our study well- supported these results. On the other hand, *Vicia* species is used in

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this study, is classified into section *Cracca* of subgenus *Cracca* [12] . Most species of this section have Le–tpye styles. In clade A$_2'$, *V. sojakii*, as an endemic species in Iran, classified near to *V. cicerideae* . These two species have similarities in morphological character such as the shape of leaflet and stipulate, color of corolla and the shape of calyx. The present study, support the close relationship between these two taxa. In flora of Iran, *V. canesense* is placed in section *Varigata*. In our study, this taxon is placed in *Cracca* section and near to other species such as *V. monanta*, *V. canesense* and *V. cracca*. This results different from Nemati et al [15]. In Figure1, *Lathyrus* taxa formed a seperate clade near to *Pisum sativum*. Morphological and molecular data supported that *Pisum* is sister to a monophyletic *Lathyrus* [22]. In clade B, *L. aphaca* formed separate group. Morphological characters such as leafy stipulate and sessil leaf, support this result. In Flora of Iran, *L. sativus* and *L. cicera*, *L. psuedocicerae* , *L. roseus* and *L. rotundifolius* are in three different sections (Figure 1). But the molecular data based on sequences of the ITS with high bootstrap, support that these species are placed in section *Lathyrus* [10]. Our results provide this results. In conclusion, It seems that further investigation of the poorly resolved nodes within the *Vicieae* will provide important insights into the interrelationships of each of the species and consequently the genera of this tribe.

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