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RESEARCH ARTICLE

Phylogenetic Analysis of Medicagoid *Trigonella* L. Species based on ITS Sequence Data

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Study Area: Tehran, Iran
Coordinates: 35°41'46"N; 51°25'23"E

Key words: Fabaceae, *Bucerates, Lunatea*

Abstract

Phylogenetic relationship between 11 species belonging to Medicagoid *Trigonella* group from Iran were studied and compared with 12 species belonging to non Medicagoid *Trigonella, Medicago, Melilotus* and *Trifolium* species (Fabaceae) using internal transcribed spacer (ITS) sequences by using Baysian inference and maximum parsimony. The results indicate that Medicagoid *Trigonella* species belong to *Bucerates* and *Lunatae* sections joined together and are monophyletic and close together rather than another *Trigonella* sections. These results supports some earlier studies. Our results confirm that use of molecular data with morphological characteristics is reliable evidence in systematic discriminations of the taxa.

Introduction:

The Fabaceae family that commonly known as Legum family is the largest family of flowering plants with about 751 genera and 19000 species that is distributed mainly in the temperate and subtropical parts of the world (Mirzaei *et al.*, 2015a). Many of the species serves as an important source of food, fodder, ornamental and raw material for industry and fixation of atmospheric nitrogen (N_2) and etc. (Mirzaei *et al.*, 2015b). Recent molecular and morphological evidences indicate the Fabaceae as a monophyletic family. The subtribe *Trigonellinae* from the tribe *Trifoliae* of family Fabaceae consists of three genera, *Trigonella, Medicago,* and *Mellilotus* that generic delimitation between *Trigonella* and Medicago has long been a matter of controversy. Members of this subtribe are united by the consistent presence of pinnately trifoliate leaves and high bootstrap support (99%) in analyses of matK (Steel *et al.*, 2003). Some of the earlier taxonomical studies for delimitation of *Trigonella* and Medicago such as floral characters, asymmetric leaves, phenolic variation, stigma morphology, pollen-ovul pattern and seed characters for discriminating between them were not successful (Bena, 2001) but Small (1987) based on some floral morphological characters transferred 23 *Trigonella* species including *Bucerates* and *Lunatea* sections to the genus Medicago and named them Medicagoid species (Gazara *et al.*, 2001). Medicagoid species are distributed in dry regions and Mediterranean climate in Asia, Europe, North and South America and North Africa (Small, 2011). Molecular markers such as the internal transcribed (ITS) of nuclear ribosomal DNA (rDNA) consists of 18s, ITS1, 5.8 S, ITS2, 28S with several hundred copies in eukaryotes is one of the most extensive sequence markers provides phylogenetic information belonging to wide systematic levels due to amplifying even from small quantities of DNA and has a high degree of variation even between closely related species.

The previous molecular studies of Medicagoid species by using analyses of the nrDNA were focused on Medicagoid species by Downie *et al.* (1998), Bena (2001), Salimpour *et al.* (2013) and Dangi *et al.* (2016). These studies proved that majority of Medicagoid species in two sections are monophyletic and are better joined to genus Medicago rather than the genus *Trigonella*.

Major aim of our study was to survey the taxonomic and phylogenetic relationships within Medicagoid species found in Iran. To reach this aim, the internal transcribed species (ITS) region of the 18s-5.8s – 28s nuclear ribosomal DNA (nrDNA) sequenced.

Materials and methods:

Taxon sampling: for molecular phylogenetic studies, leaf materials collected from various regions of Iran during May - June, 2014 and deposited species in TARI and IAUH herbaria of Iran (Table-1). The collected samples were identified at our respective research stations by using various Flora of Iran and adjacent countries. In this survey we used ITS sequence from 24 species,11 Medicagoid species and 12 non Medicagoid species belonging to

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Trigonella, *Medicago* and *Trifoilum* (Table- 1). List of taxa in our analysis with Genbank accession numbers are showed in Table- 2.

DNA extraction PCR and ITS sequencing: DNA extraction was obtained from leaves, dried in silica gel by using the Nucleo Spin Plant Kit (Macherey-Ngel GmbH & Co. KG, Duren, Germany) following the manufacturer`s protocol. Concentration and quality of extracted DNAs were checked by 1% agarose gel electrophoresis. We amplified the ITS region (ITS1-5.8S- ITS2) of the nuclear ribosomal DNA by using primer combinations AB101 and AB102 primers: a forward primer AB 101 annealing, 5`-ACG AAT TCA TCG TCC GGT GAA GTG TTC G-3` and a reverse primer AB 102 annealing, 5`-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3` (Kang *et al.*, 2003).

The PCR amplification were performed in 25µL, reaction volumes containing 1µL template DNA 10.5 µL dd H_2O .10 ×buffer + $Mgcl_2$ + Taqpolymeras +Tween 20 + dNTP = 12.5 µL and 0.5 µL each of the both primers. Thermal cycles were performed with 5 min denaturation step at 95°C, followed by 35 cycles at 95°C for 1 min, 51.5°C for 1 min and 72°C for 1.5 min, followed by 7 min final extension at 72°C for completion of primer extention. The PCR products were resolved by electrophoresis in 1% agarose gel and then visualized under UV light.

Phylogenetic analysis: forward and reverse sequences were compared, edited and initially aligned using Sequencher 4 software (Gene Codes Corporation, Ann Arbor, MI USA). All ITS sequences were assembled and aligned using Mac Clade 4 (Maddison & Maddison, 2010).

Maximum Parsimony analyses (MP): these were used by applying PAUP ver.4.0 (Swofford, 2002) using following criteria: 100 heuristic search replicates, the random stepwise addition of taxa and tree-bisection-reconnection (TBR) branch swapping. These parsimonious trees were used to calculate the consensus tree. Bootstrap analyses (BS) were applied to determine the clade support. BS for clades was calculated using PAUP with 100 replicates of heuristic searches, and the randomly stepwise addition of taxa. Clades with a bootstrap value of 70% or more considered as robustly supported nodes.

Baysian analysis: the BA analyses of the ITS datasets were performed using Mrbayes ver.3 (Huelsenbeck & Ronquist, 2001). In order to find an appropriate model of DNA substitution.

Bayesian inference: Bayesian inference of phylogenetic tree was analyzed by some parameters from Modeltest and included in the analysis. The option was set up using 5,000,000 generations of Markov Chain Monte Carlo (MCMC) searches and a sample frequency of 100. Saturation was readed after a burn-in of 1000 generations. The clade support was assessed using Baysian posterior probabilities employing MrBayes version 3.0 (Huelsenbeck & Ronguist 2001).

Table-1: List of species investigated and voucher specimens information
(IAUH:Islamic Azad university Herbarium. TARI:The research of forests and Rangeland)

Species	Locality	voucher specimen no.
A: Trigonella **1-Sec.Bucerates**		
T. arcuata C.A.Mey	Iran. Prov: West Azarbayjan, 1470m	Kasebi, 14502 (IAUH)
T. aurantiaca Boiss.	Iran. Prov: Khozestan, 160 m	H. Foroughi, 3277(TARI)
T.carassipes Boiss.	Iran. Prov: Kurdestan	Maroof & Mansori & Sh-Naseri, 5770 (TARI)
T. fischeriana Ser	Iran. Prov: Charmahal and Bakhtiari 2400 m	Mozaffarian, 54602 (TARI)
T. macroglochin Durieu.	Iran. Prov: .Fars , 1900 m	Dini Arazm, 16865 (TARI)
T. monatha sub sp. *monantha*	Iran. Prov: West Azarbayjan, 1700 m	Khandani, 14514 (IAUH)
T. orthoceras Kar & Kir.(EA)	Iran. Prov: bastazarbaijan, 1500 m	Kasebi, 14519 (IAUH)
T. orthoceras Kar & Kir.(WA)	Iran. Prov: West Azarbayjan, 1700m m	Khandani, 14518 (IAUH)
T. persica Boiss.	Iran. Prov: Charmahal and Bakhtiari, 1750m	Mozaffariam, 54563 (TARI)
T. tenuis Fisch.	Iran. Prov: Ardebil, 1800 m	Ranjbar & Hajmoradi, 19601 (TARI)
T. uncinata Banks &Soland.	Iran. Prov: Lorestan, 1350m	Hamzeh, 71867 (TARI)
2- Sec. Bieberteinianae		
T. coerulescens (M.Bieb.) Halacsy.	Iran. Prov: Tehran, Chitgar	8017 (IAUH)
3- Sec. Callicerates		
T.calliceras Fisch.	Iran , Prov.EastAzarbaijan, 1350m	Assadi & Maassoumi, 20091 (TARI)
4 - sec. Cylindrica		
T.spruneriana Boiss.	Iran. Prov: Teharn, Lavizan	8010 (IAUH)
5 Sec. Elliptica		
T. elliptica Boiss.	Iran. Prov: Charmahal and Bakhtiari , 2400 m	Mozaffarian, 54578 (TARI)
6 Sec. Falcatulae		
T.uncataBoiss&Noe.	Iran. Prov: Khuzestan	7991 (IAUH)
7. Sec.Lunatae		
T. brachycarpa Fisch.	Iran. Prov: West Azarbayjan, 400m	Hamzeh&Asri 79167 (TARI)

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Species	Locality	voucher specimen no.
8. Sec. Reflexae		
T. monspelica L.	Iran. Prov: Golestan, 300 m	Assadi & Maassoumi, 55572 (TARI)
9- Sec. Verae		
T. turkemena M. Popov.	Iran Prov: Esfehan, 2600m.	Babakhanlo & Amin, 16913 (TARI)
10-Sec. Capitatae		
T. caerulea (L.) ser	Iran. Prov: Ardebil	Barazandeh (TARI)
B: Medicago		
M.radiata (L.) Boiss.	Iran. Prov: Kohgiloyeh	Mehregan & Yeganeh, 13978 (IAUH)
C: Melilotus		
M. officinalis(L.) pall.	Iran. Prov: Shandasht	Mozaffarian, 4122 (TARI)
D:Trifolium		
T. pratense L.	Iran. Prov: West Azarbaijan	Ghasemi & Salimpour, 8006(IAUH)

Table 2- List of taxa with GenBank number used in our analysis

Code	Species	ITS Gene Bank accession number
1	*Medicago astroites*	KJ530710.1
2	*Medicago aurantiaca*	KJ530711.1
3	*Medicago brachycarpa*	GQ246121.1
4	*Medicago crassipes*	KJ530713.1
5	*Medicago lunata*	JX274123.1
6	*Medicago orthoceras*	KJ530716.1
7	*Medicago pamphylica*	JX274199.1
8	*Medicago persica*	KJ530717.1
9	*Medicago plicata*	JX274119.1
10	*Medicago radiata*	KF938696.1
11	*Melilotus albus*	DQ006009.1
12	*Melilotus altissimus*	KP987767.1
13	*Melilotus elegans*	KP987765.1
14	*Melilotus tauricus*	KP987764.1
15	*Trifolium buckwestiorum*	AF053148.1
16	*Trifolium ochroleucum*	DQ312107.1
17	*Trifolium palmeri*	DQ312061.1
18	*Trifolium rueppellianum*	DQ307478.1
19	*Trigonella coerulsence*	KJ530712.1
20	*Trigonella orthoceras*	KJ530716.1
21	*Trigonella crassipes*	KJ530713.1
22	*Trigonella anguina*	JX274190.1
23	*Trigonella arabica*	Jx274192.1
24	*Trigonella medicaginoides* (T. arcuata)	Ay256395.1
25	*Trigonella balansa*	JX274195.1- JX274133.1
26	*Trigonella caerulea*	JX274203.1
27	*Trigonella calliceras*	JX274204.1
28	*Trigonella coelesyriaca*	JX274198.1
29	*Trigonella coerulescens*	JX274206.1-.1KJ530712.1
30	*Trigonella cretica*	JX274209.1
31	*Trigonella cylindracea*	JX274210.1
32	*Trigonella filipes*	JX274214.1
33	*Trigonella foeum-graecum*	HM176644.1
34	*Trigonella geminiflora*	KJ530715.1
35	*Trigonella gladiata*	HE602408.1
36	*Trigonella grandiflora*	JX274109.1
37	*Trigonella maritima*	JX274113.1
38	*Trigonella mesopotamica*	JX274115.1
39	*Trigonella schlumbergeri*	JX274121.1
40	*Trigonella spicata*	JX274124.1
41	*Trigonella stellata*	JX274127.1
42	*Trigonella strangulata*	JX274128.1
43	*Trigonella suavissima*	JX274131.1

Results:

The data set of the ITS region included 411 characters, out of which 334 characters are constant, 47 variable characters are parsimony uninformative and 118 characters were parsimony informative. Strict consensus phylogeny trees with 361 length was included consistency index (CI) = 0.623 , retention index (RI) = O.881.

Using the data of Fig.- 1. *Trifoiulm buckwestiorum, T.palmeri* and *T.ochroleucum* taken as an outgroup form a separate clade. The ingroup consist of two main clades that labeled as A and B. Clade A includes all of Medicagoid and closest non-Medicagoid species with posterior probability (pp) of pp=1 and bootstrap 100 makes up three subcladesa, bandc. Subclade a separately comprises all Medicagoid species sections *Bucerates* (expect *Trigonella coerulsence, Trigonella geminiflora* is a synonymous of *Trigonella monantha*). The support occurred in subclade a (pp) = 0.7 and bootstrap 100.

Subclade a that comprises subclades d and e and subclade d comprise subclade f and g with pp= 0.55 and subclade f comprises subclades h and I with pp= 0.96 and subclade h makes up subclade J and k with pp= 1 and subclade k comprises subclades land n inlcudes all of Medicagoid species in Bucerates section with pp= 0.72. *Medicago astroites*is outgroup for subcladel.

Subclade b comprises Medicaid species (*Medicago radiata* and *Medicago plicata*) with post prior pp=0/88 and subclade c includes two members of Lunatae section *Medicago rostrata* and *Trigonella brachycarpa* with pp=1 and bootstrap= 100%. Clade B with pp=1 makes up subcladesa andsubclade b and comprises all non-Medicagoid species. Subclade a makes up three subclades c, d and e with pp=0/77 and subclade b comprise subclade k and l with pp=0/5 that all of the their members are non-

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Medicagoid. Subclade c forms separately monophyletic group and includes Melilotus species as sister group for clade A with pp=1. Other subclades were placed in clade B are non-Medicagoid *Trigonella* species with various sections in genus *Trigonella* (Figure- 1).

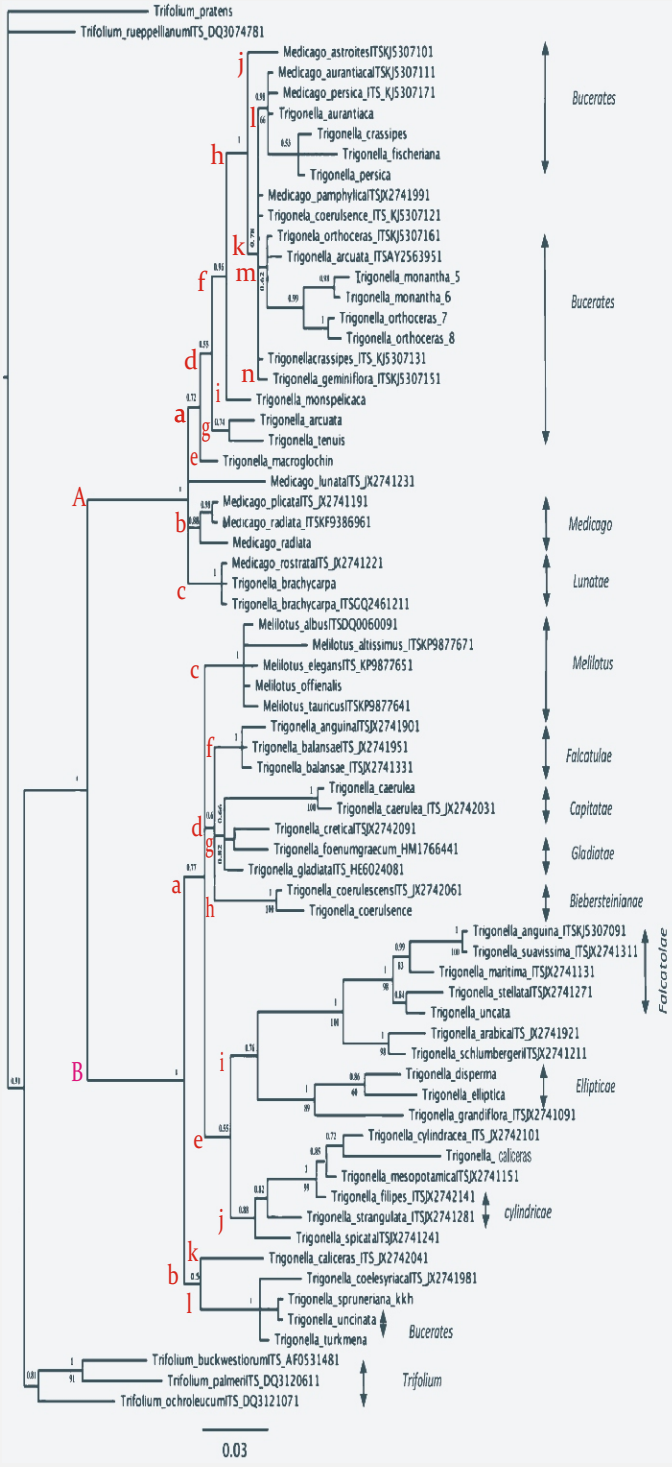

Figure -1: Bayesian consensus phylogram for combined data (Numbers above branches are Bayesian posterior probabilities. Numbers below branches are bootstrap values

Discussion:

ITS molecular results proved that *Bucerates* and *Lunatae* sections are monophyletic taxa with pp=1 and bootstrap 100%, they are also separated from non-Medicagoid species and they are close to *Medicago radiata* rather than non-Medicagoid *Trigonella* species . These results are in agreement with ITS sequencing results reported by Downie *et al.* (1998) that showed some members of section *Bucerates* and *Lunatae* are monophyletic and placed them close to *Medicago radiata* and also as can be concluded from the phylogenetic ITS and ETS study by Bena (2001), 23 Medicagoid species belong to *Bucerates* and *Lunatae* sections formed strongly supported group.

Further, our results also supports Salimpour *et al.* (2013) that represented a subclade comprises some Bucerates section members formed a group based on ITS region sequencing with 100% bootstrap.

The results of the present work are consistent with Dangi *et al.* (2016) showing some members of Medicagoid species with well-support 99% from other sections *Trigonella* and the genera of *Trifolium* and *Melilotus* based on ITS and ITS +*trn*L sequencing.

In the same studies based on other molecular markers such as loss of plastid rpo4 intron by Downie *et al.* (1998) and plastid *trn*L-*mat*K and nuclear GA30X1 gene sequences by Steel *et al.* (2010) and both nuclear genes CNGC5 and B-copsequence by Mauriea-Butler *et al.* (2008) obtained the same results of phylogenetic relationships Bucerates and Lunatae section members.

Our work strengthens Sirjaev (1923-1934) and Small (1986) and Small (2011) and Flora Iranica treatment based on morphological charachters that placed some Medicagoid *Trigonella* species into two sections *Bucerates* and *Lunatae* sections such as clade A that comprises two sections *Bucerates* and *Lunatae*. But in this study *Trigonella monspeliaca* from Reflexae section is placed next to *Bucerates* section. In addition, *Trigonella uncinate* is placed close to another non-Medicagoid sections such as *Verae* and *Cylindricae* rather than *Bucerates* section contrast of FloraIranica treatment placing it to *Bucerates* section. Therefore both of these results are not in agreement of Flora Iranica treatments (Huber-Morath 1970; Rechinger, 1984).

In this study *Melilotus* species formed a seperate group with pp=1 and and considered as a sister group for Medicagoid *Trigonella* species which is consistent with molecular ITS and ETS results by Bena *et al.* (2000) and ITS result by Dangi *et al.* (2016) and *mat*K plastid gene sequences results by Steele *et al.* (2003). Also in our work *Trifolium* species are next to Medicagoid species that is in agreement with Downie *et al.* (2008) based on ITS and Dangi *et al.* (2016) base on ITS and TIS +*trn*L intron results.

According to Dangi *et al.* (2016) complex analyses from *nr*DNA and plastid *trn*L in tron Trifolium and *Trigonella* +

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Melilotus are sister groups that is in agreement with Ellison *et al.*(2006) treatment placing *Trifolium* into *Trifolieae* tribe that this study supports them. As a conclusion our results indicate that molecular data agree with morphological data and will be most effective to complete in systematic discriminations of the taxa.

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