Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 3, No. 12, p. 116-124, 2013
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Phylogenetic relationships between *Trigonella* species (*Bucerates* section) using ITS markers and morphological traits

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Article published on December 14, 2013

Key words: Fabaceae, molecular markers, morphological characters, *Trigonella,* Iran.

Abstract

Trigonella L. (Fabaceae) includes about 135 species worldwide and most of the species are distributed in the dry regions around Mediterranean. Phylogenetic relationships of 18 species of medicagoid and one representative each of genera *Trifolium* and *Melilotus* were estimated from DNA sequences of the internal transcribed spacer (ITS) region. Parsimony analysis of the ITS region formed a dendrogram with strong bootstrap support from two groups: *Melilotus officinalis* together with *Trigonella anguina* comprise members of subclade A. The subclade B with 100% bootstrap makes up a large clade comprising *Trigonella*, medicagoides *Trigonella* as well as *Medicago* species. Moreover, the subspecies of *T. monantha* including *T. monantha* subsp. *noeana* and *T. monantha* subsp. *geminiflora* are at a farther distance from this species. Cluster analysis of morphological characters showed two major groups that joined *Medicago* and *Trigonella* (Bucerathes) species together. Accordingly, both maximum parsimony and phenetic study joined medicagoid species more confidently with *Medicago* rather than with *Trigonella*. Based on our results, the medicagoids are better joined in *Medicago* rather than placed in a new genus and reconsideration in Iranica Flora is suggested.

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Introduction

The legume family is the third largest family of angiosperm with approximately 730 genera and over 19400 species worldwide (Marzouk and El-Bakatoushi, 2011).On the other hand, Faboideae has received the most attention, because it is the largest and most widespread of the three legume subfamilies with an estimated 470 genera and 13860 species (Wojciechowski *et al*., 2004). The genus *Trigonella* is one of the largest genera (Close to 135 species) of the tribe *Trifolieae*, subtribe *Trigonellinae* in the subfamily Faboideae. This subtribe grouped four genera *Medicago*, *Melilotus*, *Trifolium* and *Trigonella* (Bena, 2001; Dangi *et al*., 2004). Many taxonomical studies have been carried out on ascertaining the relationship and the delimitation between *Medicago* and *Trigonella*. The studies tried to solve the problem by floral, seed and pollen morphology or biochemical analyses (Small, 1986; Bena, 2001; Ahmed & Marzouk, 2002; Marzouk, 2006 and Marzouk & El-Bakatoushi, 2011). The main taxonomic problem arises from these species known as "medicagoid" *Trigonella*, whose exhibit flower and seed similarities with individuals of *Medicago*, especially these characters related to the explosive tripping pollination mechanism (Small *et al*., 1987). Some authors transfered these "medicagoid" *Trigonella* species to genus *Medicago* (Small *et al*., 1987; Small, 1987b, 1989; Boulos, 1999; Bena, 2001 and Marzouk and El-Bakatoushi, 2011). Others retained these within *Trigonella* and indicated the close similarity between them in morphological characters and flavonoids (Taeckholm, 1974; Kawashty *et al*., 1998). Also, recent studies on *Trigonella* species in Egypt showed that some species such as *T. cylindraceae* and *T. polyceratia* have some morphological characters similar to those of the genus *Medicago* (Ahmed & Marzouk, 2002; Marzouk, 2006). Iran is one of the most important centers of genetic variation of Fabaceae family specially for *Medicago* and *Trigonella* taxa. Based on Iranica flora, *Trigonella* genus consists of 58 species, distributed over different climatic regions. It has 12 section, that *Bucerates* is one of the most important section with 11 species consist of: *T. aurantiaca, T. fischeriana*, *T. tenuis*, *T. persica*, *T. astroites*, *T. crassipes*, *T. arcuata*, *T. monantha*, *T. macroglochin*, *T. orthoceras*, *T. uncinata* . The closely related between this section and *Medicago* were recognized (Baum, 1968; Small, 1987a; Jurzysta *et al*., 1988; Bena, 2001). Because these taxa have high genetic variability, it is possible to use them as a rich and valuable genetic resource for breeding farm species. Therefore, study of the reconstruction of the phylogenetic relationships among species in *Bucerates* section and *Medicago* taxa using *nr*DNA ITS sequences and morphological traits, would enhance the efficiency of "medicagoid" *Trigonella* breeding program. The aim of this study was to investigate the relationships of taxa with these two genus. To accomplish this goal, the internal transcribed spacer (ITS) region of the 18s-5.8s-28s nuclear ribosomal DNA (*nr* DNA) was sequenced. Morphological characteristics were studied and the resulting cluster compared with the molecular cluster.

Material and methods

Taxon sampling

To conduct molecular phylogenetic studies during 2012, leaf materials were sampled from herbarium specimens deposited in the herbarium Islamic Azad University, North Tehran Branch (IAUN). In this study, 20 species including 10 species and three supspecies of *Bucerates* sect., 6 species of *Medicago* were selected. Also, *Trifolium scabrum* and *Melilotus officinalis* were chosen as out-groups. Details of these species, including accession identities geographical origins are given in Table 1.

DNA extraction, PCR and sequencing

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 slightly modifies version of that of Tsumura *et al*. (Tsumura *et al*., 1995). The nuclear ribosomal region encompassing

the ITS-1,5.8S rRNA and ITS2 spacers was amplified using the primers 18S and 28S (Muir and Schlottere, 1999). 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 alignment and data analysis

The *nr*DNA ITS Sequences were aligned by Sequencher Ver. 4.1.4 and Mesquite Ver. 2.73. The phylogenetic analyses Maximum Parsimony (MP) were conducted using PAUP *4.0b (Swofford, 2001) and MEGA 5.10 software (Tamura *et al*., 2011). 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.

Morphological analysis

A total of 60 quantitative/qualitative traits related to vegetative and reproductive organs were studied in 20 species of the *Trigonella* genus (12 species), *Medicago* genus (6 species), *Melilotus* genus (1 species) and *Trifolium* genus (1 species) (Table 2). For statistical analysis, the qualitative traits were initially encoded according to the multi-state method, and the related means were considered for quantitative characters, which were standardized. Phenetic analysis was carried out using MVSP Ver. 3.2 (Kovach, 1985-2002) and the UPGMA method. phenograms of these species were prepared by analyzing morphological character variation in all species in each section.

Results

The length of *nr*DNA ITS ranges from 621 base pairs (bp) in *T. crassipes* to 705 bp in *T. astroites*. Maximum Parsimony (MP) analysis of the data matrix with equally weight resolted in a phylogenetic tree with the length of 211, Consistency Index (CI)= 843, Retention index (RI)=0.77 and Rescaled Consistency (RC)=0.65. The phylogenetic tree includes 12 *Trigonella* species as inner group, 6 *Medicago* species as sister group and two species of the *Trifolium* and *Melilotus* as an out – group (See Fig. 1). In this tree, *T. scabrum* was separated as a single clade which is identified as an out-group for the other studied species. As Fig. 1 shows, the cladogram is divided into two subclad, namely, A and B, where *Melilotus officinalis* together with *Trigonella anguina* comprise members of subclade A. The subclade B with 100% bootstrap makes up a large clade comprising *Trigonella*, medicagoides *Trigonella* as well as *Medicago* species. Moreover, the subspecies of *T. monantha* including *T. monantha* subsp. *noeana* and *T. monantha* subsp. *geminiflora* are at a farther distance from this species. Also, *T. coerulescens* belonging to the *Biebersteinianae* section which is accepted as *Trigonella* species in the International Plant Name Index (IPNI), shows polytomy with *M. orthoceras* and *M. crassipes*. This result confirms the status of this taxon in *Trigonella* genus. *Trigonella filipes* belong to *cylindricae* section with a similar status to *T. coerulescens*.

In morphological analysis, 12 species of this genus, 6 species of *Medicago*, 2 species of *Trifolium* and *Melilotus* genera were analyzed based on 60 morphological traits. Figure 2 presents a phenogram with UPGMA method. Morphological analysis showed two main clades. *M. polymorpha* forms a separate group at a farther distance from other species. The second main clade is divided into two groups where in group A, *Melilotus officinalis* and *Trifolium scabrum* are placed in subclade A_1 adjacent to three species of *Trigonella*. In group B, *Medicago* species are placed at a separate clade and

all species which are related to *Trigonella*-medicagoid based on morphological features, form a separate group.

Table 1. Locality and voucher specimen number of studied species**.**

Species	Locality and voucher specimen no.
Trigonella anguina Del.	Khuzestan: 32 Km from Ahvaz towards Ramhormoz, 16m, Safi al-Din, 16711.
Trigonella astroites Fischer & C. Meyer	Qazvin: Abyek,1300m, Safi al-Din, 16713.
Trigonella aurantiaca Boiss.	Khuzestan: Haf Tappeh, 160m, Safi al-Din, 16707.
Trigonella coerulescens M. B.	Qazvin: Abyek,1300m, Safi al-Din, 16703.
Trigonella crassipes Boiss.	Lurestan: 45 Km NW Khorramabad, 1700m, Safi al-Din, 16702.
Trigonella filipes Boiss.	Lurestan: 75 Km from Khorramabad towards Dow Rud, 1550m, Safi al-Din, 16701.
Trigonella monspeliaca L.	Qazvin: Abyek,1300m, Safi al-Din, 16705.
Trigonella monantha C. Meyer	Qazvin: Abyek,1300m, Safi al-Din, 16714.
Trigonella monantha C. Meyer subsp. *Noeana* (Boiss.)	Qazvin: Karaj,1400m, Safi al-Din, 16716.
Trigonella monantha C. Meyer subsp. *Geminiflora* (BUNGE) RECH.	Lurestan: Dow Rud, 1600m, Safi al-Din, 16718.
Trigonella orthoceras Kar. & Kir.	Lurestan: 45 Km NW Khorramabad, 1700m, Safi al-Din, 16702
Trigonella persica Boiss.	Lurestan: 75 Km from Khorramabad towards Dow Rud, 1700m, Safi al-Din,16718.
Medicago radiate L.	Tehran: Sade Latyan, 1780m, Safi al-Din, 16725.
Medicago sativa L.	Tehran: Sorkheh Hesar national park, 1500m, Zanjani, 16726.
Medicago laciniata L.	Khuzestan: Dezful, Shahyoon village, 500m, Safi al-Din, 16727.
Medicago lupulina L.	Tehran: Abbas Abad, 1350m, Kuchaki Panah, 16728.
Medicago polymorpha L.	Lurestan: Bisheh, 1500m, Safi al-Din, 16729.
Medicago truncatula Gaetn.	.....
Trifolium scabrum L.	Khuzestan: Andimeshk, 300m, Safi al-Din, 16731.
Melilotus officinalis L.	Isfahan : Golpayegan, 2079m, Feizy, 16730.

Table 2. Morphological characteristics of studied species.

Row	Plant characteristics	Score
1	Longevity	0= perennial; 1= annual
2	Stem habit	0= ascending; 1= prostrate
Stipule		
3	Shape of stipule	0= ovate; 1= lanceolate; 2=sagittate; 3=oblong
4	Marginal shape	0= entire; 1=dentate; 2= laciniate
5	Length of stipule	0 ≤ 4; 1= 4-6; 1>6
Leaf		
6	Leaflet form	0= obovate-cuneate; 1= oblanceolate; 2= oblong
7	Length of leaflet (mm)	0= 5; 1=5-8; 2=5-15; 3>15
8	Size of leaflet width (mm)	0= 5; 1=5-8; 2>8
9	Length to wide ratio (mm)	0≤1; 1>1
10	Marginal shape	0= dentate; 1= serrate
11	State of tip of the leaflet	0= rounded; 1= emarginate
12	Number of hairs/mm², adaxial surface	0=1-5; 1=1-10
13	Number of hairs/mm², abaxial surface	0=1-10; 1>10
14	Length of petiole (cm)	0< 5; 1=5-10; 2=10-15; 3>15
15	Length of petiole of terminal leaflet (mm)	0≤3; 1>3
Inflorescence		
16	Type of inflorescence	0= solitary or paired; 1= raceme; 2= umbel
17	Peduncle	0=without Peduncle or very short; 1= with Peduncle
18	Presence of hair	0= glabrous; 1= hairy or ciliate
19	Length of Peduncle (cm)	0<10; 1≥10
20	Length of Pedunculate	0<1; 1>1
21	Orientation of Pedunculate	0=upward; 1=down
Calyx		
22	State of teeth of calyx	0=equal-subequal; 1=unequal
23	Teeth figure of calyx	0= lanceolate; 1=subulate; 2= triangular
24	Length of calyx (mm)	0≤3; 1>3
25	Cover of calyx	0= glabrous; 1 = sparsely pubescent

		2 = notably pubescent
26	Teeth to tube ratio	0<1; 1=equal; 2>1; 3= twice
Corolla		
27	Color of flower	0= violet-blue; 1=yellow; 2=white
28	Tripping mechanism	0= absence; 1= presence
29	Corolla rather than calyx	0=equal; 1 = a little more, 2 = 1/5 times, 3 = 2 to 3 times
Standard		
30	Figure of standard	0= widely elliptic; 1=oblong; 2= obovate; 3= lanceolate
31	State of tip	0= rounded; 1= obtuse 2= emarginated or cleft
32	Presence of claw	0=without claw; 1= with claw
33	Length of standard (mm)	0≤3; 1>3
34	Lamina rather than claw	0= equal; 1= 1-1.5 times ; 2=2-3 times
35	Standard rather than wing	0= equal; 1= a little more ; 2=1.5-2 times
36	Number of major vein clusters on basal standard	0=3; 1 >3
Wing		
37	Length of wing	0≤2.5; 1>2.5
38	Figure of wing	0= narrowly oblong or oblong-linear; 1=ovate-rhomboid or ovate-triangular
39	Lamina rather than claw	0 =shorter; 1= equal-subequal; 2= longer
40	Size of auricle	0=small; 1= large
41	Presence of thumb	0=without thumb; 1= with thumb
42	Size of thumb	0=small; 1= large
Keel		
43	keel rather than wing	0 =shorter; 1= equal-subequal; 2= longer
44	Length of keel	0≤3; 1=3-4; 2>4
45	State of keel and wing to each other	0=released; 1= connected
46	Apex of androecium	0=Straight; 1 = conical
Pod		
47	Curvature	0=straight; 1=falcate; 2= semicircular
48	Orientation	0=erect; 1=reflexed
49	Orientation of tipe	0=upward; 1=down
50	Orientation of areoles	0=smooth; 1= oblique or undefined; 2= longitudinal
51	Thickness of marginal suture	0= fine; 1= thick
52	Stellate-spreading pod	0= absence; 1= presence
53	Spine or hook	0= absence; 1= presence
54	Presence of rostrume	0=without rostrume; 1= with rostrume
55	Corolla	0= absence; 1= presence
Seed		
56	Cotyledons pulvinate	0= absence; 1= presence
57	Length (mm)	0≤2.5; 1>2.5
58	Sculpture of surface	0=smooth; 1= rugose
59	Color of seed	0 =yellow, 1 yellow- green, 2 = yellow- brown to brown
60	Shape	0=oblong; 1= cylindrical; 2= ovate; 3= reniform

Discussion

The phylogenetic tree resulted from MP analysis, showed that *Trifolium scabrum* in a separate group rather than as an out-group. This is in agreement with the result reported by Bena *et al.* (1998) and steel *et al.* (2003). In morphological analysis also this species is placed in a separate group beside *Melilotus* taxon. *Melilotus officinalis* is also placed as an out-group but at a closer distance from *Trifolium*. Our results are in agreement with previous studies (Bena *et al.*, 1998; Steel and Wojciechowski, 2003; Wojciechowski *et al.*, 2004). Palynological studies (Small *et al.*, 1981; Ferguson and Skvarla, 1981; Taia, 2004); morphological studies (Steel *et al.*, 1997); seed protein studies (Gazara *et al.*, 2001) and molecular studies through *ETS* (Bena, 2001) also confirm the result of the present study. The close relation between *Trigonella* and *Medicago* genera is shown in both MP and UPGMA analyses. Based on ITS, ETS and matK gene analyses, morphological studies and seed protein assay, these two genera are considered as sister-groups (Small and Jomphe, 1989; Bena *et al.*, 1998; Gazara *et al.*, 2001; Wojciechowski, 2000; Steel and Wojciechowski, 2003). A number of studies on the evolutional relation of *Bucerates* section (*T. monantha*, *T. geminiflora*, *T. noeana*, *T. astroites*, *T. crassipes*, *T. aurantiaca, T. persica* and *T. orthoceras*), suggest

that a close relation between this section and *Medicago* genus based on morphological features as well as the results obtained from molecular data (Small, 1987b, 1989; Bena *et al*., 1998; Downi *et al*., 1998; Maureira-Butler *et al*., 2008; Steel *et al*., 2010; Small, 2011; Ranjbar & Hajmoradi, 2011 and Marzouk, 2011). *T. monspeliaca* belongs to *Reflexae* section and all analyses with 100% statistical, support classify it with other species of medicagoides. According to East and Russian flora (Boissier, 1872; Komarove, 1945), Sirjeav (1928-1934), Small (1987b) and as the results of molecular studies (Bena, 2001; Steel *et al*., 2010; Small, 2011) and morphological characters such as Presence of "thumb and pocket," respectively, on wing and keel petals; Number of major vein clusters on standard petal; Androecia apex shape and Tripping mechanism, This species is categorized under *Bucerates* section in medicagoid group. Steel *et al*. (2010) using matK/trnK markers, also reported similar results to our study. Regarding chemical composition and based on matK/trnK genome, Simon (1969) and Saleh *et al*. (1982) suggested a closer relationship between *M. radiata* and *Trigonella* genus. It should be noted that this species was introduced as *Trigonella radiata* (L.) by Boissier (1872). Also, in Russia flora, this species was introduced as *Trigonella radiate*, section *Pectinatae* (Komarove, 1945).

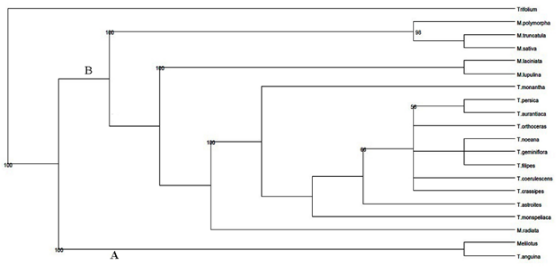

Fig. 1. Dendrogram generated from a phylogenetic analysis of DNA sequence data from internal transcribed spacers of the *nr*DNA of 12 *Trigonella* species, 6 species of *Medicag* as sister-group and two species of *Trifolium* and *Melilotus* as an out-group. Letters above the branches indicate clades. Bootstrap values are indicated above and below the branches.

However, morphological features such as fruit with ciliate-spines on the sutures, supporet the results of this present study and classify this species under *Medicago* genus (Scofield 1908; Heyn, 1959; Small and Jomphe, 1989; Small, 2011). Based on MP analyses and morphological data, *T. monantha* ssp. *noeana* and *T. monantha* ssp. *geminiflora*, are separated from *T. monantha* .This result does not agree with Turkey and Iranica Flora which assume these taxa as subspecies (Huber-Morath, 1970; Rechinger, 1984).

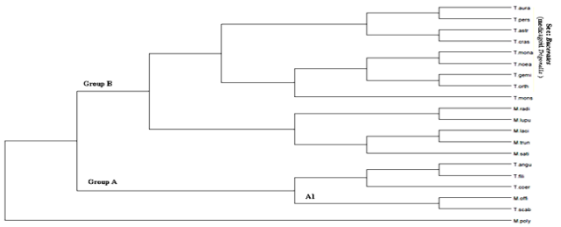

Fig. 2. Phenogram based on analyzing morphological data of 20 species (12 species of *Trigonella*, 6 species of *Medicago*, 2 species of *Trifolium* and *Melilotus* genera in Iran representing two groups (A and B).

Moreover, morphological characters such as length of stipule, shape of marginal serrations of leaflet, length of corolla, figure and length of standard petal, standard to keel and wing petales ratio and orientation and thickness of areoles on the surface of fruits, on *T. geminiflora* confirm separation of *T. monantha* from its subspecies. Since Rechinger (1984) determined the distribution of *T. monantha* and its subspecies, Small and Fawzy (1992) based on the geographic longitudes and latitudes as well as pod sculpture, Areole dimension ratio, located these subspecies in four regions, namely west (subsp. *monantha*); Center and west (subsp. *noeana*), south of Iran, Pakistan and central Asia (subsp. *geminiflora*) and India, Afghanestan (subsp. *incisa*) and eventually defined two distribution regions in east and west for these taxa. Therefore, dividing these subspecies into independent species confirm the results of the present study. Accordingly, reconsideration in Iranica Flora is suggested. This study shows that nucleotide sequence data from the

internal transcribed spacer (ITS) can be applied to investigate *Trigonella* genetics, as indicated by other relevant research (e. g. Safaei Chaei Kar *et al*., 2012). These data have high potential to reveal genotypic diversity and in the longer term, to provide molecular markers that could be linked to phenotypic properties. Because of different climate condition, Iran is considered an one of center in genetic diversity of *Medicago* and *Trigonella* genera. Identification of these species facilitates selection of suitable and compatible genes. Breeders and biotechnology experts could transfer these genes to agronomic species and thereby develop drought tolerant varieties. Evolution of phylogenetic relationships and traits can be a useful tool for determining the possibility of success in intergenomic crosses.

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