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ASSESMENT OF MOLECULAR DIVERSITY OF INTERNAL TRANSCRIBED SPACER REGION IN SOME LINES AND LANDRACE OF PERSIAN CLOVER (*TRIFOLIUM RESUPINATUM* L.)

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ABSTRACT

Clover which is an herbaceous, annual, and self-pollinated plant belongs to fabaceae family (legumes) and has become naturalized in Iran, Asia Minor and the Mediterranean eastern suburban countries. The aim of the present study is ITS molecular evaluation of the nuclear ribosomal genes of lines and landraces of Persian Clover. The sequences were aligned using ClustalW method and by MegAlign software and the dendrogram of different phylogenetic and matrix relationships between the sequences were drawn. The results showed little genetic diversity between the lines and the landrace. The conserved sequence of the analyzed gene in the Persian clover is 561 base. Totally, 740 loci (69 and 671 loci, respectively, with and without removal and addition), 9 Singletons, and 5 haplotypes were identified. The highest rate of transfer was observed in pyrimidine (%16.3). The numerical value of the ratio (dN/dS) was 0.86, and since it was less than 1, the pure selection on the studied gene happened. The lines and landraces were not separated based on their geographic locations. In general, the results indicated that the highest rate of the regional diversity belonged to the clover plants in Lorestan region. Moreover, ITS markers did not seem suitable enough for evaluating the intra- species genetic variation, but it was quite well- suited for inter-species or intergeneric evaluation.

Keywords: Persian clover; haplotype; genetic diversity; Ribosomal DNA; dN/dS

INTRODUCTION

After alfalfa, Clover, with about 100,000 hectares of cultivation area, is the most important forage plant and has a special place in the country **(Lala et al., 2018)**. They are located in three main centers of diversity: Eurasia, South Africa, and the Americas.

Iran is one of the most important centers of the clover genetic diversity at the main origin of Eurasian diversity **(Yousefi et al., 2018)**. The 60% of clover species are Eurasian- originated and 7% are endemic species of Iran, Tūrān, and Euro-Siberia **(Abbasi et al., 2012)**.

Based on the latest statistics, the total mass of forage plants kept in the National Plant Gene Bank of Iran is 5989 landrace among which clover with a mass of 1859 has got the second place, after alfalfa with a mass of 2285. In the clover collection, 416 landrace belong to the Persian clover (one- cut or multi- cut) **(Yousefi et al., 2018)**. Clover or trefoil are is a species of plants in the *leguminous pea* family in Fabaceae of the genus *Trifolium* (Latin, *tres* "three" + *Folium* "leaf"); thus, all species belong to this trifoliate genome. It consists of 238 species of plants of which 49 species are distributed medicinally in Iran **(Roma-Marzio et al., 2018)**. About a third of the clover species is annual and one of these annual species is Persian clover (*Trifolium resupinatum*) **(Hussain et al., 2017; Yousefi et al., 2018)**.

According to many classification systems, including APG III system, Fabacea species is located in Fabales with the following three sub-species **(Shahverdi, 2014; Yousefi et al., 2018)**:

1- Cercis siliquastrum subspecies (*Caesalpinioideae*), with about 166 genera and 400 species, spread all over the world: e.g. *Cesalpinia*, *Senna,Bauhinia*, and *Amherstia*

2- Caesalpinia subspecies (*Mimosoideae*), with about 60 genera and 3,200 species, mostly grow in the tropics and warm temperate Asia and America: such as *Mimosa* and *Acasia*.

3- Acacia Senegal subspecies (*Papilionoideae*), with about 430 genera and 9000 species, are scattered all over the world. Plants such as *Astragalus, Lupines*, and Persian clover (*Trifolium Resupinatum*) are also known with the same subspecies.

According to **Chapman and Oldham, (2018)** and **Yousefi et al., (2018)** clovers have different species among which the most important ones are Berseem clover (Egyptian clover) (*Trifolium alexandrium*); red clover

(*Trifolium pratense* L); white clover (*Trifolium repense* L.); Crimson clover (clove) (*Trifolium incarnatum*); Alsike clover (Lsyk) (*Trifolium hybridum*); strawberry clover (*Trifolium fragiforum*); Alpine clover (*Trifolium alpestre*); mountain white clover (*Trifolium mentanum*); Largehop clover (*Trifolium campestre*); Lesser yellow clover (*Trifolium dubium*); Harest foot or Bottlegrass clover (*Trifolium arvense*); subterranean clover (*Trifolium subterraneum*); Arrow leaf colver (*Trifolium squarosum*), Persian clover (*Trifolium Resupinatum*) (Figure 4), and wild clover (Trifolium Clusii).

Given that the registered genotypes are so-called cultivars or varieties **(Bazdar and Sadeghi, 2018)**, the registered genotypes of Persian clover (*Trifolium Resupinatum*) which is the same crop clover is one-cut and multi- cut **(Chapman and Oldham, 2018; Yousefi et al., 2018)** with the following cultivars **(Shahverdi, 2014)**: (1) Kermanshahi 1; (2) Kermanshahi 2; (3) Haft tan; (4) P513; (5) Isfahanian triple- cut; (6) Isfahanian 7- cut; (7) Nahavandi (8) Hanedanian 7- cut; (9) Chegni Lorestan, (10) Doroud Lorestan, (11) Harati Boroujerd 1; (12) 7- cut Boroujerd; (13) Deh pir Lorestan, (14) Silakhor Boroujerd; (15) Elashtar Lorestan; (16) Kazeroon (17) Shazand; (18) Alavijan Markazi; (19) Reihan Markazi; (20) Tajra Markazi; (21) 7- cut Anaj; (22) 7- cut Qurchi Bashi Tabriz; (23) one- cut Kurdistan; (24) Surian Abade Fars; (25) Lordgan Bakhtiari, and (26) Kyambro (Persian clover cultivar in Australia). Of course, there are other cultivars which are less important, so their names will be discarded.

Genetic diversity, as the basis for the development and evolution of all creatures, is important for all those who somehow, theoretically or practically, deal with the genetic modification in organisms **(Vivodík et al., 2018; Fazeli-Nasab et al., 2013)**. Plant breeding which is both a science and an art is the most applicable science in this field and its efficient activity and continued viability depends greatly on genetic variation in the genetic species under investigation **(Sani et al., 2018; Yousef et al., 2018)**. Modifyaction of the plants was conducted with the aim of improving their quantity and quality, but it requires passing many steps among which the first and foremost ones are collecting the genetic resources of the desired plant, conserving the collection and determining the traits and the genetic diversity of the gathered samples **(McKain et al., 2018; Yousef et al., 2018)**.

ITS marker has never been used for genetic assessment of clover, whereas similar plants of the same family such as beans **(De Luca et al., 2018)**, Acacia **(Úrbez-Torres et al., 2016)**, Glycine and Flemingia **(Wu et al., 2013)**, *Brassica napus* **(Abdelmigid and El-Sayed, 2016)**, Subtribe Diocleinae **(Varela and Albornoz, 2013)**, chickpea **(Yadav et al., 2017)**, and other fungi including *Lallemantia* Kamrani, 2018 #5282}, *Myrothecium roridum* **(Jordan et al., 2018)**, *Rhizoctonia cerealis* **(Ji et al., 2017)** and *Alternaria burnsii* **(Singh et al., 2018)** were used frequently.

Presence of high levels of diversity in the first (T. *resupinatum*), the second (T. *clusii*), and the third (T. *fragiferum*) gene pools of Persian clover available in the National Plant Gene Bank of Iran, grounds for the users of this valuable germplasm to develop the superior varieties of Persian clover **(Abbasi, 2008)**. Moreover, the present study has used the clover samples gathered from different habitats of the country, especially the ones available in Borujerd Agricultural Research Center which were under modification and in the final stage of providing superior lines **(Shahverdi, 2014)**.

MATERIAL AND METHODS

Plant materials

The Persian clover lines and landraces used in this study (Table 1) are the results of a ten-year research project conducted to create superior lines in Boroujerd Agricultural Research Center **(Shahverdi, 2014)**.

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA extraction method was performed based on SDS **(Dellaporta et al., 1983)**. DNA quality and quantity were determined respectively by using agarose gel (1%) and a UV–VIS spectrophotometer (LUV-300, Labnika, USA) and for amplification of the ITS1, ITS2 and 5.8 S fragments, the AB101primer with a forward sequence of 5'-ACGAATTCATGGTCCGGTGAAGTGTTCG-3' and the AB102 primer with a backward sequence of 5'-TAGAATTCCCCGGTTCGCTCGCCGTTAC-3' (as reported by **Robinson et al., (2001)**) were used. The PCR mixture provided by **Li et al., (2010)** methods and contained 20 ngDNA, 5 µL mastermix (Amplicon, Denmark), 0.7 µL of each primer (10 pmol) and water to a final volume of 15 µL. PCR amplification was carried out on Eppendorf Thermal Cycler (22331 Humburg) with the thermal profile: dena-turation step at 95°C for 5 min, then followed by 35 cycles of 55 s at94°C, the optimized annealing temperature (60°C) for 50 s and72°C for 55 s, finishing extension at 72°C for 10 min. To ensure successful amplification of 2% agarose gel, staining was performed using the Gel Red Staining.

Sequencing and data analysis

The final PCR products were sent to Macrogen Co. in South Korea for sequencing. To make corrections in the sequences, Sequencing Analysis Software ver 5.1 software and Chromas 2.3 software were used. Moreover, to assess individual sequences with the sequences in the NCBI database, BLAST online software was carried out. The sequences were aligned and clustered and their similarity and genetic distance were defined using ClustalW method and by DNASTAR software package and MEGA6 software.

Table 1 The name of Lines and Landrace of Persian Clover were used.

Sample Code	Line/ Landrace	Sample Name	Province	Accession Number	Geography Area
1	Line	Kazeroon	Fars	KY021172	N, 51° 39′ 29.99″ E, 29° 37′ 5.99″
2	Line	Kordestan	Kordestan	KY021173	N, 46° 59′ 45.6″ E, 35° 18′ 40.68″
3	Line	Eghlid	Fars	KY021168	N, 52° 41′ 9.27″ E, 30° 54′ 21.79″
4	Line	Abadeh	Fars	KY021161	N, 52° 40′ 12″ E, 31° 10′ 48″
5	Line	Aligodarz	Lorestan	KY021163	N, 49° 40′ 12″ E, 33° 22′ 12″
6	Line	Ghoorchi Bashi	Markazi	KY021170	N, 49° 52′ 22.08″ E, 33° 39′ 10.08″
7	Line	Aleshtar	Lorestan	KY021162	N, 48° 15′ 0″ E, 33° 52′ 12″
8	Line	Dorood	Lorestan	KY021167	N, 48° 42′ 0″ E, 33° 24′ 0″
9	Line	Dochin Borujerd	Lorestan	KY021166	N, 48° 30′ 0″ E, 33° 48′ 0″
10	Line	Shazand	Markazi	KY021175	N, 49° 25′ 12″ E, 33° 55′ 48″
11	Line	Harati Borujerd	Lorestan	KY021171	N, 48° 50′ 34″ E, 33° 55′ 46″
12	Line	Dehkalan	Kordestan	KY021165	N, 47° 24′ 57.6″ E, 35° 16′ 30″
13	Line	Lordegan	Charmahal-Bakhteiari	KY021164	N, 50° 49′ 41.88″ E, 31° 30′ 34.92″
14	Line	Hafti Chin Borujerd	Lorestan	KY021169	N, 48° 35′ 23″ E, 33° 54′ 59″
15	Landrace	Native Borujerd	Lorestan	KY021174	N, 48° 30′ 0″ E, 33° 48′ 0″

DnaSP5 software was also used to perform and calculate additional analyses including dN and ds. Nucleotide substitution was also calculated based on the Tamura-Nei model pattern **(Tamura and Nei, 1993; Tamura et al., 2011)** as transition and Transversion.

RESULTS

Sequencing of ITS1-5.8s rRNA-ITS2 regions

After sequencing, the homology rate of the examined sequences with the sequences of the reference species available in the NCBI was measured and their overall similarity was in the range of 98 – 99%. Following the sequence alignment, a 695 bp fragment was obtained in which 561 was preserved. It should be noted that all the sequences were registered in the NCBI and their access numbers are mentioned in Table 1.

Using the sequences obtained with the help of DnaSP software, a total of 740 positions (671 positions without removal and addition (10 polymorphic loci, 661 monomorphic loci) and 69 loci with removal and addition), 9 Singletons (29 33 37 59 622 623 624 628 698) were identified. The proportion of 5 haplotype (haplotype diversity index 77/0) were identified.

Nucleotide substitution was calculated as transmission and cross in which the maximum and the minimum transfer rates belonged respectively to pyrimidine (16.03%) and purine (9.76%) (Table 2). Moreover, the ratio of various nucleotides to the entire nucleotides was calculated (Table 3), and the average ratios of 26.8% (thymine), 22.5 % (cytosine), 23.7% (adenine), and 26.9% (guanine) was obtained in the population.

In comparison with the Nucleotide variations that have no impact on the resulting amino acid (dS), the results of the Nucleotide variations that alter the amino acids (dN) can be a more useful and highly efficient method for detection process of natural selection during genetic evolution. If the ratio is greater than one, the selection is positive, if less than one, it is a pure selection, and if equal to one, it will show a neutral selection during the evolution of these genes (Dasmeh et al., 2014). In this study, the numerical value of this ratio (dN/dS) was 0.86 which was less than one indicating that the pure selection has occurred on the desired gene without any key changes.

Comparing Genetic Data between Paired Regions

Genetic distance matrix between the studied samples indicated that the genetic distance among the samples was from 0 to 0.15 (Table 4). It was found the lines and the landraces in clover were not clearly separated, but Aligoodarz line was in completely separated group (Figure 1). Moreover, the highest rate of the regional diversity was observed in the clovers of Lorestan region (Table 5), so it can be concluded that Lorestan could be regarded as the origin of clover.

Of the samples collected from different areas in the same clusters of genetic similarity or physical interchange seed can due to between different regions is the interpretation based on physical currency is more justified than genetic similarity **(Ninou et al., 2017)**.

Since ITS was unable to separate the lines of a species from each other, the sequences of some plants in other sub-families of *Fabaceae* available at NCBI were used for further assessments of the ITS ability in assessing the genetic diversity of different crops. They were then compared with the sequences of the present study. It was also found that each sub-species of the family was in a separate group (Figure 2); therefore, it can be concluded that ITS is a useful tool for genetic assessments, both inter-species and intergeneric.

Due to the fact that Iran has two types of clover dominant Persian clover (*Trifolium Resupinatum / persian clover*) and Wild clover (*Trifolium Clusii*) and it is likely that Persian clover has originated from the wild species, categorizing the Iranian clovers beside the wild clover (*Trifolium Clusii*) (Figure 2) proves the accuracy of this testing, so it can be concluded that there is a lot of genetic similarity between the Persian clover and the wild clover.

In the subsequent studies, the accuracy of the evolutionary process of the emergence of Persian clover from the wild clover will be discussed.

Table 2 The Percentage of displacement nucleotide sequences of ribosomal genes Persian clover on the pattern of Tamura-Nei model (**Tamura and Nei, 1993**).

	A	T	C	G
A	-	*6.83*	*5.65*	**11.43**
T	*5.65*	-	**13.26**	*6.62*
C	*5.65*	**16.03**	-	*6.62*
G	**9.76**	*6.83*	*5.65*	-

Note: Black and italicized numbers represent transition and transversion substitutions respectively.

Table 3 The base pair ratio of ITS sequences in all lines and landrace of Persian Clover.

Genotypes	T(U)	C	A	G
1	27.1	22.6	23.3	27.1
2	27.0	22.6	23.4	27.1
3	26.7	22.4	23.8	27.1
4	26.8	22.3	23.8	27.1
5	27.5	23.0	22.3	27.2
6	26.8	22.3	23.7	27.2
7	26.8	22.4	23.7	27.1
8	26.7	22.4	24.0	27.0
9	26.8	22.3	24.1	26.8
10	26.7	22.6	24.1	26.7
11	26.8	22.4	24.1	26.7
12	26.7	22.6	24.1	26.7
13	26.7	22.6	24.1	26.7
14	27.0	22.6	23.4	27.1
15	26.7	22.6	24.1	26.7

Table 4 Distance Matrix of ITS region sequence variation among genotypes of Persian Clover.

Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14
2	0.000													
3	0.001	0.001												
4	0.001	0.001	0.000											
5	0.015	0.015	0.014	0.014										
6	0.001	0.001	0.001	0.001	0.015									
7	0.001	0.001	0.001	0.001	0.015	0.000								
8	0.001	0.001	0.001	0.001	0.015	0.001	0.001							
9	0.001	0.001	0.001	0.001	0.015	0.000	0.000	0.001						
10	0.001	0.001	0.000	0.000	0.014	0.001	0.001	0.001	0.001					
11	0.001	0.001	0.001	0.001	0.015	0.000	0.000	0.001	0.000	0.001				
12	0.001	0.001	0.000	0.000	0.014	0.001	0.001	0.001	0.001	0.000	0.001			
13	0.001	0.001	0.000	0.000	0.014	0.001	0.001	0.001	0.001	0.000	0.001	0.000		
14	0.000	0.000	0.001	0.001	0.015	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	
15	0.001	0.001	0.000	0.000	0.014	0.001	0.001	0.001	0.001	0.000	0.001	0.000	0.000	0.001

Table 5 Distance Matrix of ITS region sequence variation among genotypes of Persian Clover based on provinces that were collected.

Province	Genetic distance
Fars	0.000994657
Kordestan	0.001491986
Lorestan	0.004567513
Markazi	0.001494834

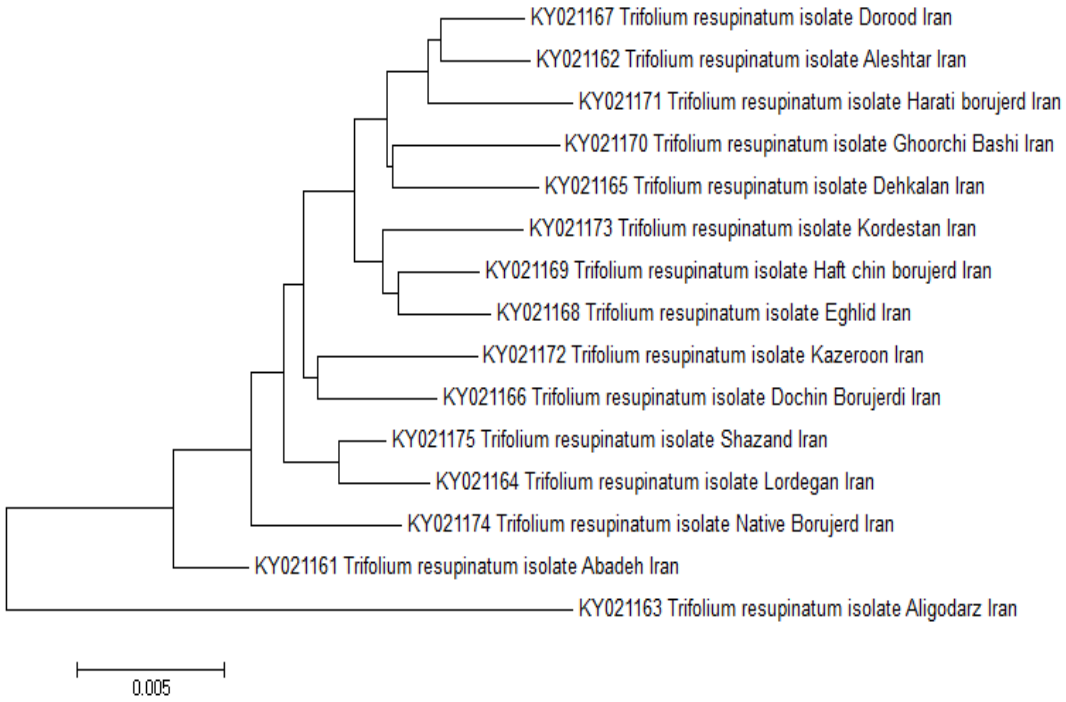

Figure 1 Cluster analysis of the genotypes of Persian Clover based on ITS sequences using ClustalW and NJ methods using MEGA6 software.

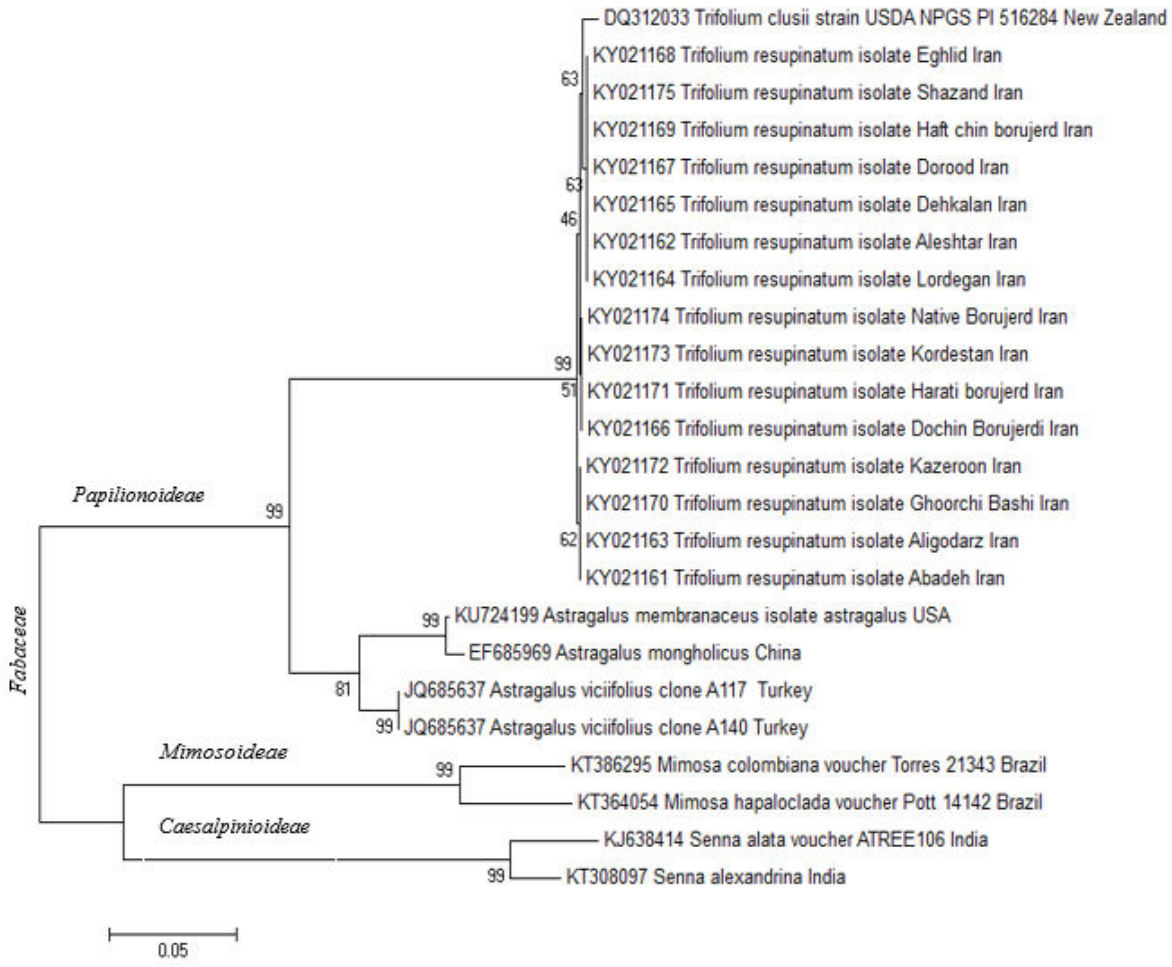

Figure 2 Cluster analysis of the genotypes of Persian Clover in this research with some other sub-family's clover ITS sequences were related from NCBI using ClustalW and NJ methods using MEGA6 software.

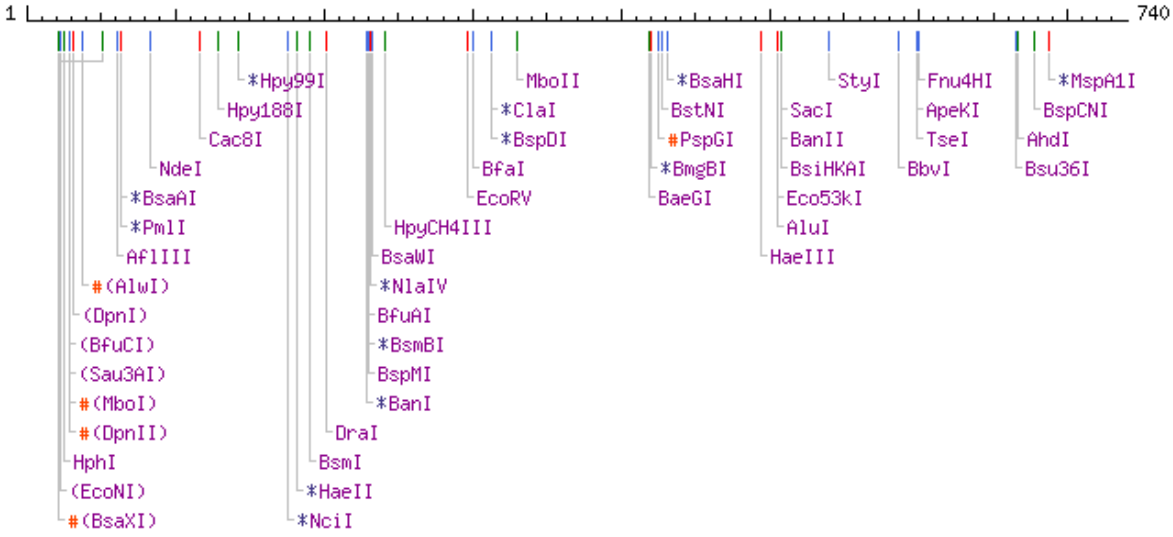

Figure 3 The restriction enzymes cut position on ITS gene sequence.

Table 6 Introduce the affect restriction enzymes on ITS gene in this research.

Enzyme Name	Cut Number	Cut Site	Fragment Length
EcoNI	1	5'..CCTNN▼NNNAGG…3' 3'..GGANNN▲NNTCC…5'	24 Nucleotide and 716 Nucleotide
MspA1I	1	5'..CMG▼CKG…3' 3'..GKC▲GMC…5'	51 Nucleotide and 689 Nucleotide
HphI	1	5'..GGTGA(N)^{8}▼…3' 3'..CCACT(N)7▲…5'	26 Nucleotide and 714 Nucleotide

Figure 4 Persian clover *Trifolium resupinatum* L., Photography by: Dr. Mohammad shahverdi.

In Silico Analysis of Clover Samples

Consensus Sequence of all samples, analyzed by using BioEdit Sequence Alignment Editor 7 software **(Hall, 1999)**, was entered in Nebcutter (http://tools.neb.com/NEBcutter2/) to identify the specific restriction enzymes that are able to recognize haplotypes (Figure 3). The results showed that different human enzymes are capable of cutting in the amplified area, so three types of restrictions enzymes (EcoNI, MspA1I and HphI) can cut and distinguish the amplified area in different samples (Table 6).

DISCUSSION

The results of the present study showed that the clove lines and landrace were not separated based on their geographic loci, yet the highest intra-region diversity belonged to the clovers in Lorestan region. The results showed that ITS marker was not suitable for intraspecific evaluation but it was useful for inter-species and intergeneric assessments. Moreover, the Persian clover (*Trifolium Resupinatum / Persian clover)* and wild clover (*Trifolium Clusii*) were put together in a separate group and given that Iran's dominant species of clover are *Trifolium Resupinatum / persian clover* and *Trifolium Clusii*, it is likely that the crop clover has emerged from wild clover.

Given that most genes responsible for resistance to diseases, pests, environmental stresses and genes responsible for product quality are usually found in the centers of diversity, so plant breeders having accurate information about the genetic diversity of each plant can engage more effectively using the genetic resources, so they can directly collect the required genetic resources. In addition, such information has led the plant breeders and those involved in conservation of these plant resources to the issue of biodiversity issues to use them. Moreover, according to Vavilov's theory, the origin of these plants belongs to the centers with the greatest diversity (Lalramnghaki). Therefore, in collecting clover germplasm, it is highly recommended to pay more attention to these geographic regions of Iran (especially Lorestan).

Several ecological factors have led to the accumulation of genetic differences between both populations. Different geographical locations vary in terms of some ecological characteristics, including latitude and longitude, temperature, and humidity. These factors, so- called eco-geographic, cause genetic variation between the two populations. According to the results, using more populations in the geographical areas is necessary to confirm the existing pattern.

Due to new combinations of genes, in the cross-pollinated species, the high genetic flow and the environmental pressure governing the region have established a series of specific genes, but the genetic distance within the populations is so sparse. Instead, the diversity of the population is relatively low **(Rauf et al., 2010)**. However, in the self-pollinated species, because of the different alleles within a population, there are more changes within populations **(Mirzaei and Mirzaghaderi, 2017; Petrova et al., 2017)**. In general, in both self-pollinated and cross-pollinated plants, the variation within the population was mostly greater than the variation between the populations, but this variation in self-pollinated plants was greater than in the cross-pollinated species.

Some key factors used to explain the greater intra-population diversity in the plants are self-pollination, annual, increased by seed, number of studied allelic sites studied, allelic and genotypic loci of the population, type of crosses, and population size **(Thormann et al., 2017)**. Analysis of the genetic diversity in populations faces difficulties due to the interference of several factors, including integration, cross kinship, migration and differences between the individuals of the populations **(Ndjiondjop et al., 2018)**.

In some studies, it has been indicated that ITS marker was suitable for both inter-species and intergeneric assessments so that in a research a different species of 6 genera belonging to the family *Subtribe Diocleinae* were evaluated using the 5.8S ribosomal genome, distance matrix, and Furthest Neighbor method and finally, it was concluded that the genomes so- called *Calopogonium* and *Pachyrhizus* which belonged to this family till the beginning of this study were clearly separated and diagnosed Meanwhile, the phytochemical and morphological results were obtained after this separation and the accuracy of the ITS result was also confirmed and it was concluded that ITS in section of 5.8S could be a useful tool for evaluating various plants in the genus level **(Varela et al., 2004)**.

In another study, four species of Glycine and two species of *Flemingia* were evaluated using ITS1 and ITS2. Fragments with a length of 595 to 622 were replicated and the cluster analyses of two species were placed in separate clusters. It was also concluded that ITS can be useful for intraspecific evaluation (Wu et al., 2013). Using ITS sequencing data in the nuclear ribosomal DNA as well as plastid DNA sequencing (*psbA-trnH intergenic spacer* regions) and morphological markers (the number of edges and seam width of fruit), the phylogenetic relationships between 39 species of *Bunium* were studied and the results showed that the genome *Bunium* has more than one subtype. It is composed of two main sub-branches. The results of molecular, morphological and carbologic studies in this study have shown similar patterns of phylogenetic relations of the species and have also confirmed each other **(Degtjareva et al., 2009)**.

In another study, 5 lanraces of *Dracocephalum* with 2 landraces of *D. moldavica* and *Basil* plants (as excluded plants) were studied using ITS regions (Internal Transcribed Spacers) and then the results showed that ITS marker was appropriated to intergeneric evaluation **(Haidari et al., 2014)**.

In other studies, it was also indicated that ITS marker is not suitable for intra species evaluation so that in a research, 44 population of Egyptian bean along with 5 other species were assessed using ITS. ITS marker showed an unexpectedly kinship between *Angustifolia* and *Lutei*. Likewise, based on the geographic areas, the results of ITS made a distinction between the clovers in the East and West regions of the US, but the other groups were various and even in the group of the South East of America, there were perennial beans. In general, the results indicated that except for some cases, intergeneric phylogenetic is not resolved by ITS **(Ainouche and Bayer, 1999)**.

Meanwhile, in another study, 51 species of Acacia of the family Phylodina (*leguminous* family) and a different kind of *Lysiloma divaricate* were assessed by using ITS. Cluster analysis showed that the two groups were in two main species: the first species were quite unified in terms of the morphologic features, whereas the second group were different **(Brown et al., 2010; Murphy et al., 2003)**.

In another study, six different breeds of species *Glycine tomentella* were evaluated using ITS, yet no significant difference was observed between ITS regions of these diverse species (Rauscher et al., 2004). Potential variability in the ITS region, ribosomal DNA of the isolates of *Fusarium solani* of potato and its relation to pathogenesis and its geographical origin in the provinces of Khorasan and Northern Razavi were investigated and the results showed that PCR-ribotyping technique showed no relationship in separate groups between the geographical areas and pathogenesis of the isolates **(Baghaee Ravari et al., 2007)**.

CONCLUSION

According to the results of the ITS marker in lack of separation of the lines and the landraces in Persian clover based on the geographical location and other key traits, it can be concluded that the ITS marker is not so suitable for analyzing the intraspecific genetic diversity, but on the basis of a comparison between the lines and the landraces used in this study as well as other plants of different genera of the *Fabaceae* family (provided on NCBI site), it was determined that ITS can be regarded as a useful tool for genetic assessments, both inter-species and intergeneric. In addition, in this study, the numerical value ratio (dN/dS) was 0.86, indicating that the pure selection has occurred on the studied gene, yet has made no key changes. On the other hand, of 740 loci, 671 loci were without removal and addition and only 69 loci had removal and addition. As a result, the fact that the ratio of dN/dS is less than 1 and there are few loci for removal and addition indicates that there are small variations between different lines; thus, it can be the possible reason for the inability of ITS in separating the lines and the landraces.

Since the source or origin of the plants belong to the centers with the highest diversity and regarding the point that Lorestan lines are the most diverse lines, it is necessary to reconsider this region while collecting clover germplasm used for exploitation of eugenics.

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Availability of data and material

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