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Phylogenetic Relationship Investigation of Some Medicinal Plants Using Nuclear ITS Barcodes

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ABSTRACT

DNA barcoding is a straightforward strategy that uses short orthologous genetic sequences and standard genomes to specify species. This technique has the capability of molecular identification, detection of living species and discovery of unfamiliar species, preservation of genetic resources, identification of genetic diversity and phylogenetic characterization, and testing of differentiated existing plant species, as well as assuring the safety and efficacy of pharmaceuticals. In this experiment, the allelic diversity of 7 classes of medicinal plants viz. fenugreek, local fenugreek, waybread, cumin, flax, fixweed and sesame, from Ilam and Khuzestan provinces; west and south of Iran, respectively, was carried out employing this technique and with the aid of primers designed and established on ITS nuclear barcodes (ITS1 and ITS2 genes). The results indicated that there was a great difference between the fragments and the duplicated sequences of ITS1 and ITS2 barcodes in different samples. Moreover, the nucleotide searching of the sequences showed that there was a very high similarity (more than 90%) between the acquired sequences and their equivalents in the NCBI database. The nucleotide sequence of the ITS1 gene of fenugreek showed the highest similarity (78.2%) with native fenugreek. Regarding the ITS1 gene, more amount of G~C content than A~T was observed and, in the waybread plants the amount of C base was higher than G, and for native fenugreek, the amount of A~T content was more than G~C. In the case of ITS2 position, in all examined samples (except the fixweed plant, which had higher A~T), the values of G~C content were higher than A~T. The output of the cluster analysis with the UPGMA algorithm showed the precise grouping and separation of species and the high potential of these sequences using the barcode system in the phylogenetic evaluation of medicinal plant species.

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1. Introduction

In recent years, there has been a great desire to investigate the physiological and pharmacological effects of herbal extracts, and the use of herbal medicines in the world, especially in Iran. Factors such as fewer side effects, diversity of effective compounds in plants, lower economic costs, development of industries related to the cultivation of medicinal plants, prevention of foreign exchange from the country, creation of useful work, and especially the suggestion of the use of medicinal plants by the World Health Organization (WHO), are the reasons for the global approach to herbal medicine (Asadbegy *et al*., 2011). Therefore, considering the special value and importance that medicinal plants have in providing health and community health, their recognition in the fields of natural resources of each region is one of the fundamental steps in the field of sustainable development of medicinal plants and can provide researchers basic important information. Of course, some medicinal plants, including fenugreek and cumin, have edible and consumption aspects as well (Cazzola and Cestaro, 2014; Fritz *et al*., 1993; Nakaziba *et al*., 2021; Nikravesh and Jalali, 2003).

Genetic identification and registration of different plant cultivars are considered one of the important pillars of protection and correct utilization of genetic resources, which will be a difficult task in most plants using morphological characteristics in the early stages (Asadi *et al*., 2015). Bayer and Starr (1998) pointed out

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that the great diversity of plants in the Asteraceae family makes it difficult to identify them at the species level, and to solve this problem, a simple and accurate method to determine the identity of species is necessary ensuring the food and medicinal safety of such plants that are traded internationally. In this regard, the technique of DNA barcoding (a very short standard DNA sequence of a well-known gene or intergenic region, which can be used to determine the species of any animal, plant or fungus has a high potential in the detection and identification of species and is considered a new and attractive tool in taxonomic research. It also has been widely used in the identification of plant species since 2003 (Hebert *et al.*, 2003). In other words, in DNA barcoding, information located in a common region of a gene or an intra or intergenic region is used in all species, and after determining their sequence, it tries to form a global network to exchange information between researchers and a successful method in identifying and documenting the biological diversity of organisms is considered to be proposed by Hebert et al. (2003) (Asadi *et al.*, 2015). Because of the amplification of the target sequence, this method can distinguish very close species from each other.

The primary goal of DNA barcoding is to identify a specific sequence for each species and to form a barcode database of living organisms so that all researchers in various specialized fields can access this database (Khahramanzadeh, 2011). Many of the proposed barcodes in plants are based on a single region of chloroplast or the content of different chloroplast or nuclear regions, which have been tested by many researchers in identifying and verifying the plant diversity of ITS (Ahmadi *et al.*, 2022; Fazeli-Nasab *et al.*, 2020; Jomeh-Ghasem-Abadi *et al.*, 2019; Pahlavan *et al.*, 2021), trnH-psbA, matK, and rbcL barcodes (Chen *et al.*, 2010).

In a report, 105 *Fallopia multiflora* plant samples from different regions of China were studied using three ITS, rbcL, matK, and one trnH-psbA intergenic location (Sun *et al.*, 2013). Soleimani et al. (2011) also used the DNA barcoding method to identify the Iranian species of meadow rue (*Thalictrum*) genus using nuclear selective DNA barcode (nrDNA ITS) and two chloroplast DNA barcodes (trnH-psbA, nrDNA ITS) and the results showed that the nrDNA IT nuclear sequences had more differentiate potential than the two chloroplast DNA barcodes in order to identify *Thalictrum* species. The trnL-F region is one of the important regions in the chloroplast genome that has been used in many studies to investigate the phylogenetic relationships between plant species. According to the reports, many studies reported the relationship between cyanobacteria, algae, and plants, as a result of evaluating the evolutionary history of introns, the relationships of angiosperms, as well as the molecular evolution of the trnL-F region of plants (Quandt *et al.*, 2004). In 2009, the barcoding consortium of living organisms proposed the use of chloroplastic genes rbcl and matK as the standard plant barcode, which has good sequence quality and high levels of species separation for plants (Galimberti *et al.*, 2013; Mahadani and Ghosh, 2013). Also, the researchers' findings suggest trnH-psbA intergenic location barcode and ITS nuclear gene location barcode as complementary s to general barcodes in plants (Hollingsworth *et al.*, 2011). Therefore, investing in the DNA barcoding technique can provide researchers with a powerful tool for identifying and separating plant samples. However, other genetic markers such as SSR (Yousefi *et al.*, 2017) and ISSR (Ahmadi *et al.*, 2017; Khoramifard *et al.*, 2017; Mohammadi *et al.*, 2014) can be employed in such works.

This research aimed as to investigate the ability of nuclear barcodes to distinguish between species using the DNA barcode method in some medicinal plant samples from the south and west of Iran.

2. Materials and methods

The seeds of the investigated medicinal samples were prepared from different regions of Ilam and Khuzestan provinces and then planted in 2016, May, in the research greenhouse of the Faculty of Agriculture of Ilam University (Table 1). Genomic DNA extraction from fresh and young leaves was performed according to the method of Doyle and Doyle (1987) in the Biotechnology Laboratory of the Faculty of Agriculture of the University. Polymerase chain reaction in the final volume of 25 microliters included 3 microliters of genomic DNA, 1.8 microliters of 20 mM magnesium chloride, 1.8 microliters of 1 mM dNTPs, 1 microliter of forward and reverse primers with a concentration of 100 pmol (Table 2), 0.4 microliters of Taq DNA polymerase enzyme (5 units), 2 microliters of reaction buffer with 10x concentration

and finally with 14 microliters of double distilled water.

PCR amplification was performed in the Eppendorf thermal cycler (https://www.eppendorf.com) for 2 minutes and 30 seconds of initial denaturing at 94°C. After that, 35 cycles including 30 seconds of denaturing at 94°C, 1 minute at 60°C (recommended temperature to anneal the primers, 1 minute at 72°C for extension and amplification, and the final extension stage was done for 5 minutes at 72°C. The resulting products were analyzed using horizontal electrophoresis on a 1.2% agarose gel (Fig. 1). Polymerase chain reaction and gel staining were done in 1X TAE buffer, and ethidium bromide solution, respectively.

After PCR and amplification of the ITS gene, the PCR product was purified and sequenced. For sequencing, samples were first purified and then sequenced by the South Korean Bioneer Company (https://eng.bioneer.com). The results indicated the high quality of the sequence of the amplified fragment, the PCR product, and the purity of the examined samples. Following sequencing and determining the sequence of ITS1 and ITS2 genes of the investigated species, the sequences were compared with of NCBI database, and BioEdit, MegAlign, CLC Sequence Viewer 6 and MEGA 5.2 software was used for statistical analyses. Next, the MEGA 6 program was used to form a phylogenetic tree using bootstrap maximum likelihood analysis (1000 times) (Tamura *et al.*, 2013).

Table 1. Medicinal plants used in this study

Eng. name	Scientific name	Sampling location
Fenugreek	*Trigonella foenum-graecum* L.	Khuzestan/Ilam
Local fenugreek	*Trigonella foenum-graecum* L.	Ilam
Waybread	*Plantago major* L.	Ilam
Cumin	*Cuminum cyminum* L.	Khuzestan/Ilam
Flax	*Linum usitatissium* L.	Ilam
Fixweed	*Descurainia Sophia* L.	Khuzestan/Ilam
Sesame	*Sesamum indicum* L.	Khuzestan

Table 2. Sequences of primers used for multiplication of intra or intergenic locations (Asadi *et al.*, 2015; Robinson *et al.*, 2001)

Nucleotide sequence	Primer sequence
ITS1(F)	5'ACGAATTCATGGTCCGGTGAAGTGTTCG3'
ITS1(R)	5'GTTGCCGAGAGTCGT 3'
ITS2(F)	5'TAGAATTCCCCGGTTCGCTCGCCGTTAC3'
ITS2(R)	5'GCCTGGGCGTCACGC3'

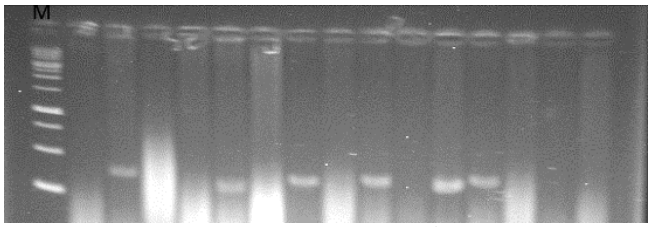

Figure 1. Agarose gel (1.2%) electrophoresis of PCR products using a specific primer on 1.2% gel. M: size marker.

3. Results and discussion

3.1. ITS1 gene

The success rate of ITS1 gene replication in the examined samples was 41.2. In addition, the length of the amplified fragment in the studied samples was very different, and for fenugreek, native fenugreek, flax, waybread, and fixweed, were 636, 383, 387, 335, and 411 nucleotides, respectively. Then, the acquired sequences were aligned by the CLUSTALW method and using BioEdit software. The alignment results showed a very high correlation between the length of the amplified fragment in the examined samples and the scan of the PCR product. Alignment of ITS1 sequences showed that there was a high similarity of nucleotides from the beginning to nucleotide 130 in the studied plants, but after that, a clear difference was observed in terms of nucleotide order (Fig. 2-4). Moreover, searching the sequences obtained in the NCBI database showed that for the ITS1 and ITS2 genes in the studied plants, the highest homology was obtained in fenugreek and native fenugreek compared to the sequences available in NCBI. Phylogenetic analysis of the samples using the MegAlign program showed that the nucleotide sequence of the ITS1 gene of fenugreek showed the highest similarity (78.2%) with native fenugreek. Fenugreek and cumin showed the lowest nucleotide similarity (51.5).

The observed high nucleotide diversity and low similarity are due to the diversity in the studied samples' genus, species, and family. The tree diagram of the ITS1 gene was drawn using MegAlign software using the UPGMA algorithm. According to the results, at the level of 70% similarity, the samples were divided into three main categories, with flax alone in one group, fenugreek and native fenugreek in one group, and the rest of the samples in the third group.

In addition, fenugreek and native fenugreek showed the highest affinity and closeness at the ITS1 gene sequence level (Table 3, Fig. 5). Cumin and flax also showed the highest difference (Fig. 2). The distribution

and abundance of organic bases were estimated by CLC Sequence Viewer 6 tool to check and accurately estimate the ratio of organic bases in the examined samples. In the ITS1 sequence, nucleotide G had the highest amount and nucleotide A had the lowest amount in fenugreek, waybread, flax, and fixweed plants, but in the native fenugreek plant, nucleotide A had the highest amount, and nucleotide C had the lowest amount The results showed that in relation to the abundance of organic bases in all examined samples regarding the ITS1 gene, the G~C content values were higher than the A~T values and in the waybread plant, the C value was higher than G, and in native fenugreek, A~T values were also higher than G~C values (Fig. 6 and 7).

3.2. ITS2 gene

The success rate of ITS2 gene replication was also 41.2% in the examined samples. In addition, the amplified sequence length of the ITS2 gene for fenugreek, cumin, native fenugreek, fixweed and sesame was obtained as 319, 317, 323, 214 and 311 nucleotides, respectively. When aligning the ITS2 sequences in the tested plants from nucleotide 15 to nucleotide 100, a strong nucleotide similarity was observed, showing a clear difference between the samples in terms of nucleotides (Fig. 8). The phylogenetic analysis of samples using MegAlign software showed that in terms of ITS2 gene sequence, between fenugreek, cumin, native fenugreek, fenugreek, and black sesame plants, the highest similarity with 67% was related to fenugreek and native fenugreek and the lowest similarity (17.5%) was related to fixweed and black sesame.

The tree diagram of the ITS2 gene using MegAlign software and based on the UPGMA algorithm showed that at a 70% similarity level, the samples were divided into three main groups. The first group included fenugreek, the second included sesame and cumin, and the third included fenugreek and native fenugreek. Also, fenugreek and native fenugreek showed the most similarity and were located in the same group. Besides, fenugreek and fixweed showed the least similarity (51.5%), related to different species (Table 4, Fig. 9). The results of the frequency of organic bases for the ITS2 gene in all investigated samples showed that G~C values were higher than A~T values (except for the fixweed plant, whose A~T value was higher). Also, the amount of base G in cumin and sesame plants was higher than that of C, and in fenugreek, native fenugreek and fixweed plants, the amount of base C was estimated to be higher than that of G. Except for the Fixweed sample, the A~T amount was higher than the G~C content amount, and the base A amount was lower than the base T. In the nucleotide sequence of the ITS2 gene, nucleotide C in fenugreek and native fenugreek plants, nucleotide G in cumin and sesame plants, and nucleotide T in fixweed plants showed the highest amount (Table 3, Fig. 10). Nucleotide A had the lowest value (amount) in all ITS2 plants. Next, the position of ITS1 and ITS2 genes in the genome can be seen (Fig. 11).

Table 3. Organic bases ratio in ITS1 nucleotide

Nucleotide	Plant	bases	Molecular weight		G~C	A~T	A		C		G		T	
			single	double			number	Mo1%	number	Mo1%	number	Mo1%	number	Mo1%
ITS1 31	Fenugreek	378	117498	235471	52.71	47.29	90	23.26	97	25.06	107	27.65	93	24.03
ITS1 61	Waybread	335	102402	204269	60.30	39.7	82	24.48	98	29.25	104	31.04	51	15.22
ITS1 101	Flax	387	117874	235658	55.56	44.44	95	24.55	103	26.61	112	28.94	77	19.90
ITS1 131	Local fenugreek	383	116402	232684	47.26	52.74	109	28.46	87	22.72	94	24.54	93	24.28
ITS1 141	Fixweed	411	1.0E+05	250146	53.8	46.2	98	23.80	105	25.60	116	28.22	92	22.38

Table 4. Organic bases ratio in ITS2 nucleotide

Nucleotide	Plant	bases	Molecular weight		G~C	A~T	A		C		G		T	
			single	double			number	Mo1%	number	Mo1%	number	Mo1%	number	Mo1%
ITS2 32	Fenugreek	319	97357	194254	55.49	44.51	65	20.38	98	30.72	79	24.76	77	24.14
ITS2 82	Cumin	317	96209	193343	61.20	38.80	51	16.09	92	29.02	102	32.18	72	22.71
ITS2 132	Local fenugreek	323	98477	196669	55.11	44.89	67	20.74	96	29.72	82	25.39	78	24.15
ITS2 142	Fixweed	241	72896	146497	48.96	51.04	41	17.01	63	26.14	55	22.82	82	34.02
ITS2 162	Sesame	311	94365	189900	65.27	34.73	49	15.76	90	28.94	113	36.33	59	18.97

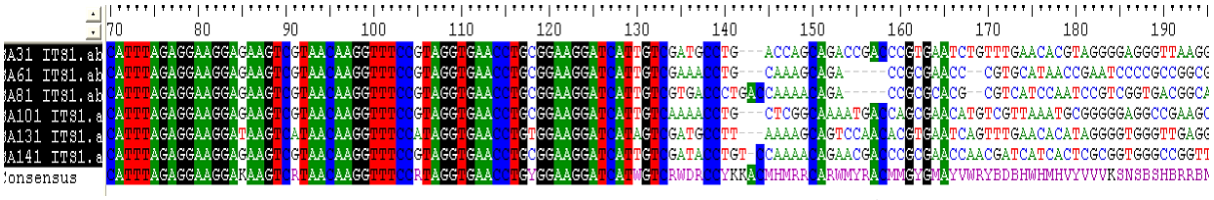

Figure 2. Alignment using CLUSTALW and producing consensus sequence for ITS1 barcode.

Figure 3. Sequencing report in ITS1 barcode

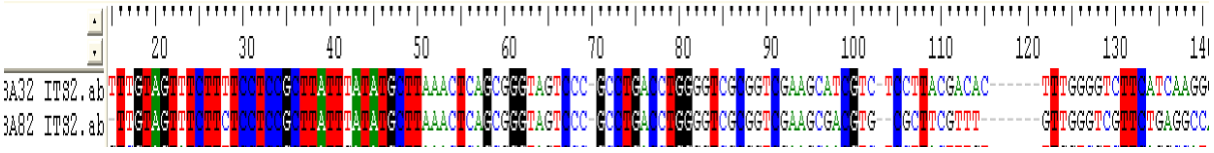

Figure 4. Sequencing via CLUSTALW and producing consensus sequence for ITS2 barcode

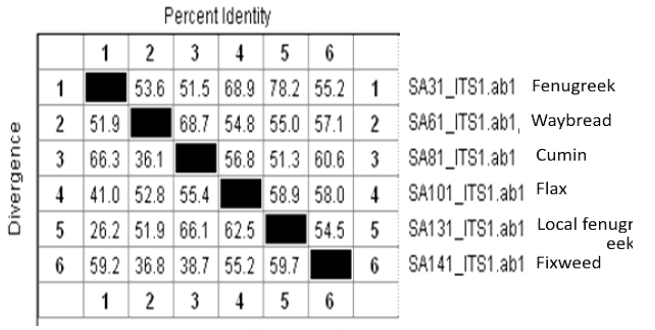

Figure 5. Similarity and difference of pants in ITS1 gene.

The biodiversity of any region is a precious and yet vulnerable resource. Biodiversity can be studied at three levels: genetic, species, and ecosystem diversity. In the genetic concept of species, species are recognized and differentiated based on DNA similarity in different individuals and populations. Considering the limitations of the morphological species identification methods and the illustrative advances in genetic science and the high accuracy of molecular techniques, these methods can be used in the field of identifying and managing the genetic diversity of plants and animals. (Khoshnamvand and Malekian, 2015).

Bioinformatics analysis, especially sequencing, is a useful and powerful tool for identifying samples at the DNA level. Therefore, due to the advances in molecular biology, DNA sequencing has been used in many phylogenetic studies due to changes in nucleotide bases over time (Hidayat and Pancoro, 2008). ITS regions are located in a region of ribosomal DNA that is transcribed. These regions include two regions: ITS1 between the small ribosomal subunit rDNA gene and 8.5S rDNA) and ITS2 (between 8.5S rDNA and the large ribosomal subunit rDNA gene) (Hebert et al., 2003). Previous studies have shown that ITS barcodes provide the possibility of identifying species well so

ITS-RFLP has been applied in determining the genotype of fungi of the genus Pleurotus (Van De Wiel *et al.*, 2009) and also the analysis of ITS and IGS regions was used in the study of the diversity of fungi (Haider, 2012).

ITS1 and IGS regions are suitable genomic sequences for investigating genetic relationships. The study of ITS sequence has played an important role in phylogeny studies at the genus and subgenus level and the diagnosis of macroscopic fungi (Hilu and Liang, 1997). In fact, ITS 1 and 2 barcodes have high replication power, and, also the ITS2 genomic region also presents fewer replication and sequencing problems (Chen *et al.*, 2010).

However, this research was conducted in order to use the DNA barcoding method containing ITS1 and ITS2 genes to identify and investigate the genetic relationships of numerous medicinal plants in the country and test the ability of these two genes to separate such species due to the use of molecular techniques can be a useful and effective tool to identify phylogeny relationships and protect genetic resources. In this regard, the valuable information obtained from DNA sequences of nuclear genomes or other plant organs can be used to investigate the genetic similarity and affinity of plant species.

Based on the statistical results (Tables 3 and 4, Fig. 5 and 10), the observed differences between the ITS positions in this research indicated the presence of the evolution phenomenon between these two genomic regions so that they can complete the differentiation of the plants under discussion. The sequencing information of the two ITS loci used in this research was used to draw the genotype classification diagram, which showed the ability of these two loci to investigate the phylogeny relationships of the studied species (Fig. 7 and 9). These two loci have a high potential for investigating the phylogeny relationships of diverse plant species, which has been mentioned in earlier reports (Shinwari *et al.*, 2018; Tang *et al.*, 2016).

As stated, this research was done using ITS barcodes to separate some medicinal plants, so that based on the ITS1 barcode, the flax plant was placed in a separate group and fenugreek and local fenugreek were placed in the second group (Fig. 7). According to the ITS2 barcode, the fixweed plant is placed in a separate category, and these two plants (fenugreek and its local form) were also located in the same category (Fig. 9). Three categories were formed in both barcodes. This issue shows the closeness of fenugreek and native fenugreek, which can be used in breeding researches such as cross-breeding.

In this regard, in a study, 114 samples of the legume family native to China (including 85 species from 49 different genera) (Gao *et al.*, 2010) and 51 samples belonging to 19 different genera of medicinal plants (Sun and Chen, 2013) were investigated, where ITS2 barcode was introduced as a suitable barcode that correctly shows the ability to separate the studied genera and species and provides the possibility to separate each sample based on morphological characteristics, which is in accordance with the present results. Also, Chase et al. (2005) also used ITS and rbcl barcodes to identify Moraea and Protea species and were able to successfully separate genera and species using DNA barcodes (Chase *et al.*, 2005). Also, Rai et al. (2012) were able to investigate Asparagus and *Decalepis hamiltonii* plants from Asparagaceae and Asclepiadaceae, and *Hemidesmus indicus* using ITS2 gene barcodes and obtained good results stressing that it shows the efficiency of this gene locus in the study of medicinal and other plant species.

DNA barcoding has been applied to identify medicinal plants. In this regard, Asadi et al. (2015) studied 8 medicinal plants from Ardabil province using this method and coded them as rbcL and ITS and found that the licorice plant was placed in the same group with plants of the same genus, confirming the current research. Since one of the conditions of a barcode is the ability to separate the samples well, and in this research the ITS1 and ITS2 barcodes worked well, it is therefore proposed that these barcodes, along with the barcodes from other regions of the genome such as rbcL, were identified as supplemental barcodes used to distinguish samples from medicinal plants. In fact, as Asadi et al. (2015) stated, using a combine of barcodes together will have more appropriate results. As applied in this study, among the approaches that can be employed to obtain advantageous results both in terms of the relevance and the impact of the contributing factors in the molecular or morphologic characteristics of plants, are simple or multivariate statistical methods (Arminian *et al.*, 2013; Kamel *et al.*, 2009) which are suggested to be adopted as a complement to molecular investigations.

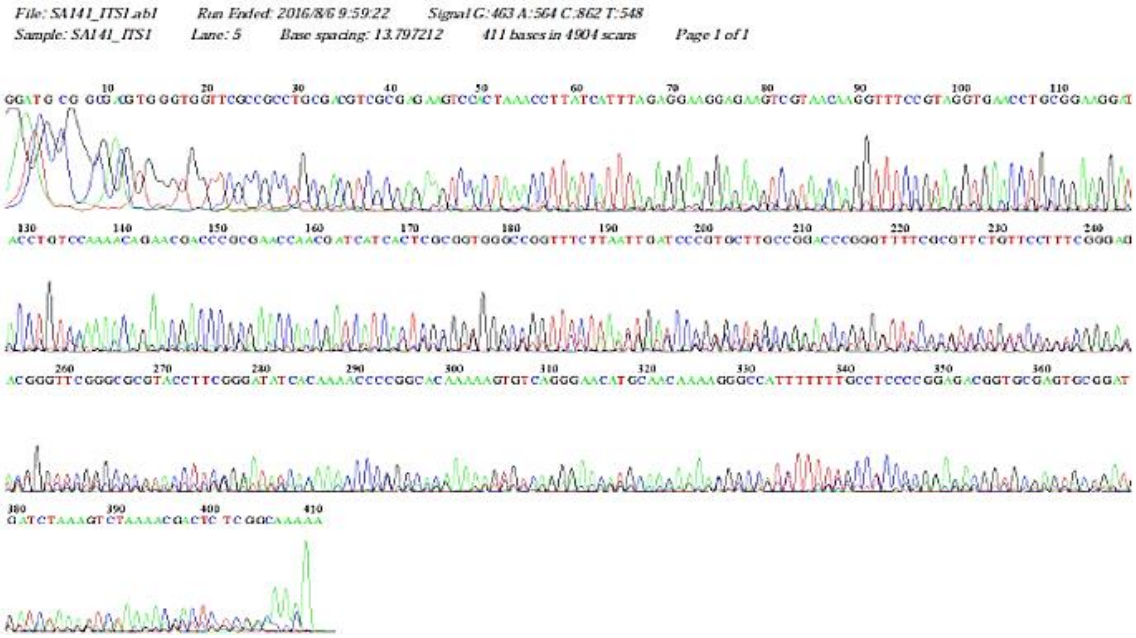

Figure 6. A part of the ITS1 gene of Fixweed (*Descurainia sophia)*

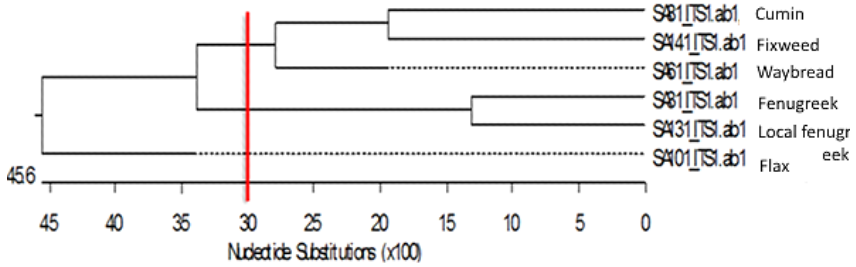

Figure 7. Phylogenetic tree designed via CLC Sequence viewer 6 tool using UPGMA algorithm in DNA level for ITS1 barcode

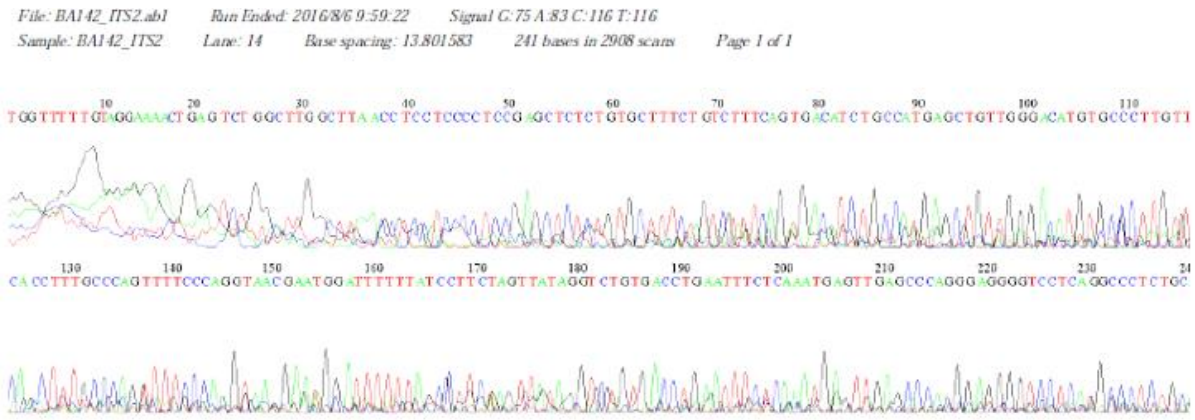

Figure 8. A part of the ITS2 gene sequence in Fixweed plant

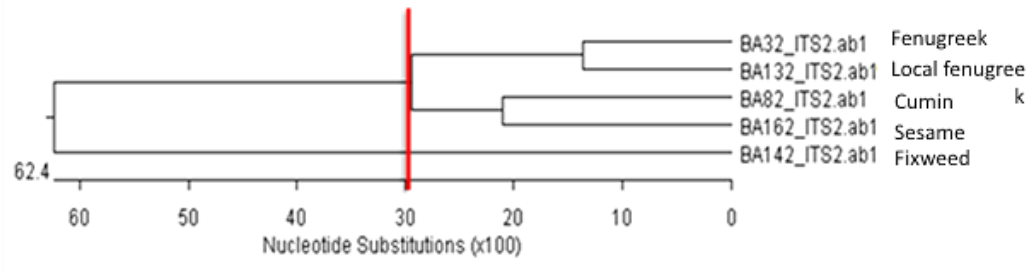

Figure 9. Phylogenetic tree designed via CLC Sequence viewer 6 tool using UPGMA algorithm in DNA level for ITS2 barcode

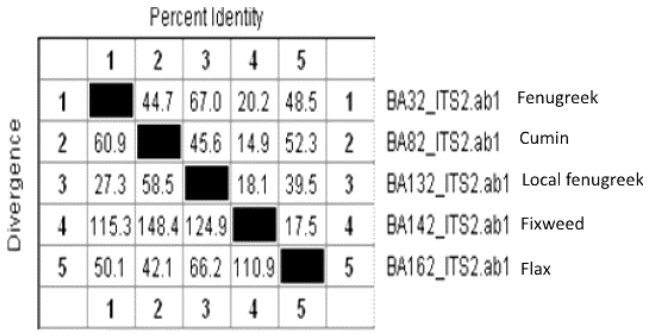

Figure 10. Similarity and difference of plants for ITS2 barcode

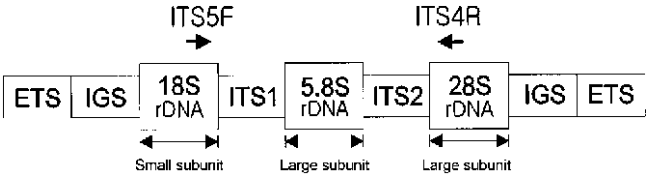

Figure 11. Nuclear rDNA region map and the position of primer pairs used for PCR. Internal transcribed spacer (ITS), External transcribed spacer (ETS), and Intergenic spacer (IGS) as reported by Yang et al. (2001).

4. Conclusion

In this research, with the help of primers designed based on ITS nuclear barcodes (ITS1 and ITS2 genes), the similarity and affinity of 7 different types of medicinal plants from Ilam and Khuzestan provinces, Iran, were investigated. The results of the sequences obtained from these barcodes showed the significant differences between the reproduced segments and sequences in the samples and their high similarities with the sequences in the NCBI database, as the closely related species were in the same groups. In addition, the amount of 4-nucleotide compounds in the species indicated the amount in the species can be used in breeding programs. According to the results, the DNA barcoding technique can be used as a reliable method to group and verify the diversity of medicinal plant species.

Conflict of Interests

The authors declare no conflict of interest.

Ethics approval and consent to participate

The authors declare that they do not use humans or animals in their research.

Consent for publications

The authors have read and approved the manuscript for publication.

Availability of data and material

The authors have embedded all data in the manuscript.

Authors' contributions

Bavi, M. performed the research as his M.Sc. thesis; Fazeli, A. the supervisor of the thesis and proposed the idea; Arminian, A. the advisor and wrote the final draft; Rostmai, Z. wrote the original draft.

Informed Consent

The authors declare not to use any patients in this research.

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