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Genetic Comparison of Iranian Asafetida (*Ferula assa-foetida* L.) Populations Based on cpDNA Ribosomal Protein L16 Intron

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Abstract **– DNA sequences are important sources of data for comparison analysis based on different types of data provide substantially conflicting hypotheses of relationships among plants. The Asafetida or Anghouzeh (*Ferula assa-foetida* L.) is an Iranian endemic medicinal plant that belongs to Apiaceae family. This plant is one of the most important among thirty species of Ferula distributed in Iran and This species is growing wild or recently cultivated in several areas of Iran. In current study, Samples were collected from Six Populations in Iran and DNA was extracted using a CTAB protocol. Gene was amplified and finaly product was squenced.We have analyzed nucleotide sequence variation in an approximately 712 bp of Intron of Asafetida ribosomal protein L16 (rpl16) gene of chloroplast DNA molecule. In current research, 4 distinct haplotypes with 7 polymorphic sites were found. The nucleotide frequencies were 0.249 (A), 0.448 (T/U), 0.157 (C), and 0.146 (G). The transition/ transversion rate ratios were k1 = 0.965 (purines) and k2 = 0.799 (pyrimidines). The overall transition/ transversion bias is R = 0.332. The Tajima's Neutrality Test (D) and nucleotide diversity (π) were estimated D= 0.895628 and π= 0.004416 for sequences, respectivly. The nucleotide diversity showed a diference (P<0.05) in genetic diversity of rpl16 gene sequences between all populations. The phylogram of genetic divergence In the all Phylogenetic methods revealed two major clusters. In the NJ and UPGMA methods, Tang Sorkh and Maymeh were clustered at one group and Also, the Bastak and Geno were clustered together. but In the PM methods, the Bastak was separated from Geno. The genetic distances were low in IranianAsafetida populations. Assuming neutrality, the low diferences were observed across populations.**

Keywords **– Asafetida, Anghouzeh, Endangered Species, Medicinal Plant, *Ferula Assa-Foetida* L.**

I. INTRODUCTION

The Plants have been a constant source of drugs and recently, much emphasis has been placed on finding novel therapeutic agents from medicinal plants. Today many people prefer to use medicinal plants rather than chemical drugs. Ferula is a genus of about 130 species of flowering plants in the family Umbelliferae, native to the Mediterranean region and central Asia [3 & 17]. The Iranian flora comprises of 30 species of Ferula genus, which some are endemic [27]. Several species of this genus have been used in folk medicines [49] and investigations on the ferula species have indicated antinociceptive, anti inflammatory and antipyretic effects [50], contraceptive action [31 & 41] and smooth muscle relaxant activity [4]. This genus is well documented as a good source of biologically active compounds such as sesquiterpene coumarins and sesquiterpene [26]. Also, the Ferula genus has been found to be a rich source of gum-resin [17]. Asafetida (*Ferula assa-foetida*), is a species of Ferula grows in Iran, Afghanistan and Kashmir. It has an unpleasant smell, is herbaceous and perennial and grows up to 2m height [1, 2 & 8], with stout, hollow, somewhat succulent stems 5-8 cm diameter at the base of the plant. The leaves are 30-40 cm long, tripinnate or even more finely divided, whit a stout basal sheath clasping the stem. The flowers are yellow, produced in large compound umbels [38]. *Ferula assa-foetida* has several medicinal properties including antispasmodic, aromatic, carminative, digestive, expectorant, laxative, sedative, nervine, analgesic, anthelminitic, aphrodisiac and antiseptic. Iranian *Ferula assa-foetida* contains gum extract (glucose, galactose, L-arabinose, rhamnose and glucuronic acid etc.), resin (ferulic acid esters, free ferulic acid, coumarin derivatives) and volatile oils (sulphur-containing compounds and various monoterpenes) [3, 4, 6 & 7]. Noncoding regions of cpDNA, such as introns and intergenic spacers, tend to evolve more rapidly than coding loci, both in nucleotide substitutions and in the accumulation of insertion and deletion events (indels), presumably because they are less functionally constrained [10, 12 & 30]. Because these noncoding regions can potentially supply more informative characters than coding regions of comparable size, they have become popular for phylogenetic studies among taxa that are recently diverged. The chloroplast gene rpl16, encoding the ribosomal protein L16 [15 & 34], is interrupted by an intron in many, but not all, land plants. In most flowering plants, this intron is 1 kb in length [9 & 32]. Pairwise comparisons of the 17 chloroplast introns shared between tobacco and rice indicate that the rpl16 intron is most divergent, with 64.5% sequence similarity [14 & 51] reported that this intron has an exceptionally high rate of sequence change when Spirodela is compared with tobacco and Small et al. (1998) concur that this intron is rapidly evolving, at least in the context of the seven noncoding cpDNA loci examined in a group of recently radiated tetraploid cottons [42]. Given its large size relative to other plastid introns and potential for much variation, we have chosen to examine the historical relationships of subfamily Apioideae and allied taxa using

the rpl16 intron. Previous studies have already demonstrated the utility of this region for phylogenetic inferences in Lemnaceae [20], Poaceae [22], and Cactaceae [13]. The analysis of DNA sequence polymorphisms and SNPs (single nucleotide polymorphisms) can provide insights into the evolutionary significance of DNA polymorphisms and into the selective and demographic factors acting on populations and species. The objective of the present study was to Genetic comparison of Iranian Asafetida (*Ferula assa-foetida* L.) populations using cpDNA sequences for determination of genetic structure and population's variability. In this paper, we characterize the molecular variation of the rpl16 intron in Asafetida. This intron data are then combined and the resultant estimate of relationship compared to phylogenies for the group inferred using other characters such as nuclear ribosomal DNA ITS [14 & 21] and chloroplast matK [33] sequences, and chloroplast restriction sites [32].

II. Materials and Methods

2.1. Plants and Sampling

In current study, Random leaf tissues and seeds of The Asafetida were collected from six populations involve: Kerman, Zarand of Kerman Province, Tang Sorkh of Kohgiluyeh and Boyer-Ahmad Province, Maymeh of Ilam Province, Geno Biosphere Reserve of of Hormozgan Province and Bastak of Hormozgan Province in Iran (Fig 1).

Fig. 1. Geographical location of the studied populations

Young leaf samples from one-month-old field grown plants and seeds were collected and brought on ice to laboratory. The leaves were thoroughly washed with tap water and rinsed with distilled water, blot dried and weighed. The leaves were either stored at -80°C or used directly for extraction.

2.2. DNA Extraction and PCR Amplification

About 100-200 mg of fresh leaf tissue from 2-3 young leaf plants and seeds were sampled and immediately were frozen in liquid nitrogen and ground with mortar and pestle into powder. Genomic DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method [11] with minor modifications. Quantity was determined by measuring the absorbance at 260 nm and the concentration, purity and quality were determined by measuring the absorbance at 260/280 nm and 230/260 ratios using a NanoDropTM 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). DNA extractions were appropriately labeled and stored at-20°C for analysis. The Intron of Ferula assa-foetida ribosomal protein L16 (rpl16) gene of cpDNA was amplified. The study was concentrated on a 712 bp fragment of rpl16 gene. Primer sequence for PCR was designed using Oligo 7 software. The full sequence of primer: rpl16 F: 5'- GATGGTTGGTTCTGAATTCC -3' and rpl16 R: 5'- GATAAACGGAAGGGTGAAAG -3'. The amplification reaction was carried out in a volume of 25 µl containing: 1X PCR buffer (10 mM Tris–HCl (pH 8.4), 50 mM KCl), 1.5 mM MgCl2, 0.2 mM dNTPs, 0.4 µM of each primer, 100–200 ng of DNA template and 1 U Taq DNA polymerase. Amplification of DNA samples from individual isolates was carried out in a Thermocycler (eppendorf , Garmany) under the following conditions: denaturation at 94°C for 5 min (initial denaturation), followed by 35 cycles consisting of 94°C for 30 sec (denaturation), 56°C for 40 sec (annealing),72°C for 50 sec (extension) and a final extension step was at 72°C for 5 min. Confirmation of successful PCR amplifications was carried out by electrophoresis on a 1% (w/v) agarose gel for 1 h at 80V, stained with GelRed staining (Biotium, USA) and photographed using Gel–Doc XR (BioRad, USA).

2.3. Sequencing and Analysis

The rpl16 PCR products were purified from any free dye dideoxynucleotides using the spin columns and finally were analyzed with an ABI PRISM 3100 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. The sequences were aligned initially using the program Clustal X 1.83 (Thompson et al, 1994) and the aligned sequences were edited using MEGA 5 (Tamura et al, 2011: http://www.megasoftware.net) software and realigned manually. Also, the reliability of the alignment was additionally checked. The Gaps were positioned to minimize nucleotide mismatches. In Current reasearch, all statistical parameters involve: Selection on Chloroplast haplotypes within samples using Tajima's D model [45], The Pattern of Nucleotide Substitution with MaximumComposite Likelihood method [47], the homogeneity of Substitution Patterns between Sequences (P-values and disparity Index test) using 1000 resample

data, Estimates of Base Composition Bias Difference between Sequences [24], Codon-based Test of Neutrality for analysis between sequences (Z-Test) and The variance of the difference using the analytical method, The numbers of synonymous and nonsynonymous differences between sequences (Fisher's Exact Test of Neutrality) using the Nei-Gojobori method [29], the nucleotide diversity and Tajima's Neutrality Test for sequences [45], Estimates of Evolutionary Divergence over Sequence Pairs between Groups and the Estimates of Net Evolutionary Divergence between Groups using the Maximum Composite Likelihood method, The evolutionary distances were computed using the Maximum Composite Likelihood method [47] and The evolutionary histories by the neighbor-joining method (NJ) [40], UPGMA method [43] were calculated using MEGA 5 [47]: http://www.megasoftware.net) software.

III. Results

In Current reasearch, DNA was obtained either directly from the field plants and seeds with high quality (Fig 2).

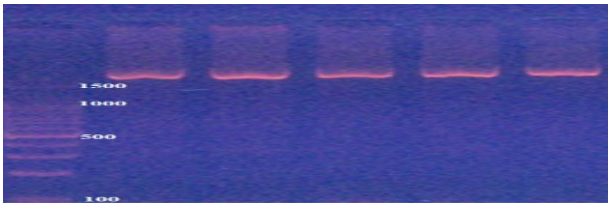

Fig. 2. Extrcted DNA from leavs and seeds

The 712 bp fragment of Intron of Asafetida ribosomal protein L16 (rpl16) gene of cpDNA was amplified (Fig 3).

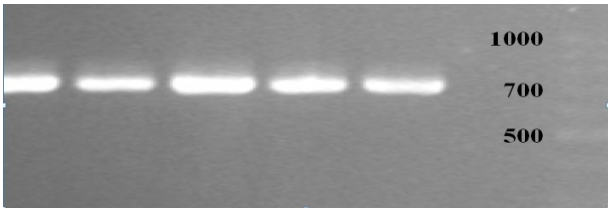

Fig 3. PCR products of rpl16 (Obtained size: 712bp)

In current research, 4 distinct haplotypes with 7 polymorphic sites were found within six populations of Iranian Asafetida. Each entry shows the probability of substitution from one base (row) to another base (column) instantaneously (Table 1) with Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution [46]. Only entries within a row should be compared. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in no bold. The nucleotide frequencies are 0.249 (A), 0.448 (T/U), 0.157 (C), and 0.146 (G). The transition/ transversion rate ratios are k1 = 0.965 (purines) and k2 = 0.799 (pyrimidines). The overall transition/ transversion bias is R = 0.332.

Table 1. Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

Pattern of Nucleotide Substitution	A	T	C	G
A	-	15.62	5.48	**4.92**
T	8.70	-	**4.38**	5.10
C	8.70	**12.48**	-	5.10
G	**8.40**	15.62	5.48	-

The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences (Disparity Index test). A Monte Carlo test (1000 replicates) was used to estimate the P-values [23, 24 & 48]. *P*-values smaller than 0.05 are considered significant (Marked with yellow bold highlights). The estimates of the disparity index per site are shown for each sequence pair above the diagonal (Table 2).

Table 2. Test of the homogeneity of Substitution Patterns between Sequences (the P-values are shown below the diagonal and disparity index per site are shown for each sequence pair above the diagonal)

P-values and disparity Index test	M	T. S	Z	K	G	B
Maymeh (M)		0.00	0.01	0.00	0.00	0.00
Tang Sorkh (T)	1.00		0.01	0.00	0.00	0.00
Zarand (Z)	0.11	0.12		0.01	0.01	0.01
Kerman (K)	1.00	1.00	0.11		0.00	0.00
Geno (G)	1.00	1.00	**0.01**	1.00		0.00
Bastak (B)	1.00	1.00	**0.02**	1.00	1.00	

The difference in base composition bias per site is shown in matrix (Table 3). Note that even when the substitution patterns are homogeneous among lineages, the compositional distance will correlate with the number of differences between sequences [24].

Table 3. Estimates of Base Composition Bias Difference between Sequences

compositional distance	M	T. S	Z	K	G	B
Maymeh (M)						
Tang Sorkh (T)	0.00					
Zarand (Z)	0.01	0.01				
Kerman (K)	0.00	0.00	0.01			
Geno (G)	0.00	0.00	0.01	0.00		
Bastak (B)	0.00	0.00	0.01	0.00	0.00	

The probability of rejecting the null hypothesis of strict-neutrality (dN = dS) (below diagonal) is shown (Table 4). Values of P less than 0.05 are considered significant at the 5% level and are Bolded. The test statistic (dN - dS) is shown above the diagonal. dS and dN are the numbers of

synonymous and non synonymous substitutions per site, respectively. The variance of the difference was computed using the analytical method [28].

Table 4. Codon-based Test of Neutrality for analysis between sequences (Z-Test)

Neutral Evolution	M	T. S	Z	K	G	B
Maymeh (M)		0.00	1.54	1.54	2.06	2.06
Tang Sorkh (T)	1.00		1.54	1.54	2.06	2.06
Zarand (Z)	0.13	0.13		0.00	1.42	1.42
Kerman (K)	0.13	0.13	1.00		1.42	1.42
Geno (G)	**0.04**	**0.04**	0.16	0.16		0.00
Bastak (B)	**0.04**	**0.04**	0.16	0.16	1.00	

The probability (P) of rejecting the null hypothesis of strict-neutrality in favor of the alternative hypothesis of positive selection is shown for each sequence pair (Table 5). P values smaller than 0.05 are considered significant (Zhang et al, 1997). The numbers of synonymous and nonsynonymous differences between sequences were estimated using the Nei-Gojobori method [28].

Table 5. Fisher's Exact Test of Neutrality for Sequence Pairs

Fisher's Exact Test of Neutrality	M	T. S	Z	K	G	B
Maymeh (M)						
Tang Sorkh (T)	1.00					
Zarand (Z)	0.62	0.62				
Kerman (K)	0.62	0.62	1.00			
Geno (G)	0.38	0.38	0.62	0.62		
Bastak (B)	0.38	0.38	0.62	0.62	1.00	

Assuming neutrality, any diferences in genetic diversity observed across populations. The effect of the mutational model was explored by estimating nucleotide diversity for data under two substitution models. These models difered in their transition bias. The Tajima test statistic (D) and nucleotide diversity (π) [45] were estimated D= 0.895628 and π= 0.004416 for sequences (Table 6).

Table 6. Tajima's Neutrality Test for sequences

S	p_s	Θ	π	D
7	0.010853	0.003594	0.004416	0.895628

(The abbreviations used are as follows: S = Number of segregating sites, ps = S/m, Θ = ps/a1, and π = nucleotide diversity. D is the Tajima test statistic)

The number of base substitutions per site from averaging over all sequence pairs between groups is shown (Table 7). All results are based on the pairwise analysis of sequences. Analyses were conducted using the Maximum Composite Likelihood method [46].

Table 7. Estimates of Evolutionary Divergence over Sequence Pairs between Groups

Evolutionary Divergence	M	T. S	Z	K	G	B
Maymeh (M)						
Tang Sorkh (T)	0.00					
Zarand (Z)	0.00	0.62				
Kerman (K)	0.01	0.01	0.00			
Geno (G)	0.01	0.01	0.00	0.01		
Bastak (B)	0.01	0.01	0.00	0.01	0.00	

The number of base substitutions per site from estimation of net average between groups of sequences is shown (Table 8).

Table 8. Estimates of Net Evolutionary Divergence between Groups of Sequences

Net Evolutionary Divergence	M	T. S	Z	K	G	B
Maymeh (M)						
Tang Sorkh (T)	0.00					
Zarand (Z)	0.00	0.00				
Kerman (K)	0.01	0.01	0.00			
Geno (G)	0.01	0.01	0.00	0.01		
Bastak (B)	0.01	0.01	0.00	0.01	0.00	

In Neighbor-Joining cluster (Fig 4), the optimal tree with the sum of branch length = 0.01089662 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances are in the units of the number of base substitutions per site.

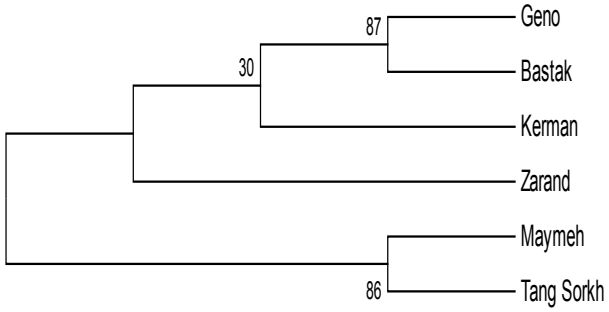

Fig. 4. Evolutionary relationships using the Neighbor-Joining method

In UPGMA method cluster (Fig 5), the optimal tree with the sum of branch length = 0.01102684 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances are in the units of the number of base substitutions per site.

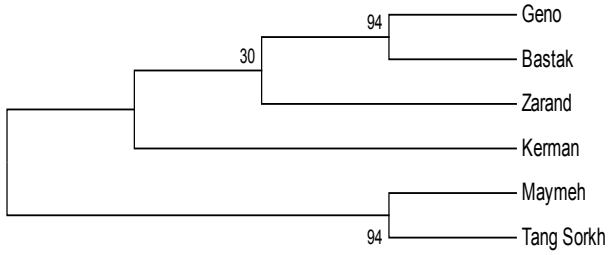

Fig. 5. Evolutionary relationships using the UPGMA method

In Maximum Parsimony cluster (Fig 6), Tree #1 out of 3 most parsimonious trees (length = 7) is shown. The consistency index is (1.000000), the retention index is (1.000000), and the composite index is 1.000000 (1.000000) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm [29] with search level 3 (16 & 29] in which the initial trees were obtained with the random addition of sequences.

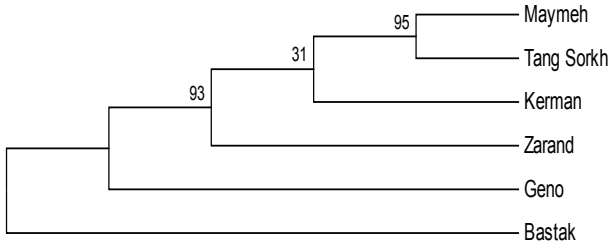

Fig. 6. Evolutionary relationships using the Maximum Parsimony method

The evolutionary history of genetic divergence in the all methods revealed two major clusters. In the Neighbor-Joining and UPGMA methods, Tang Sorkh and Maymeh were clustered at one group and Also, the Bastak and Geno were clustered together (Fig 4 and Fig 5). but In the Maximum Parsimony method, the Bastak was separated from the Geno (Fig 6).

IV. Discussion

The comparative analysis of DNA sequence data reveals much about plant evolution. We have used many important algorithms based on sequence comparisons for homologous chloroplast-encoded gene, to investigate Asafetida variation. The nucleotide diversity showed a difference ($P<0.05$) in genetic diversity of rpl16 gene sequences between all populations. However, genetic variation was significantly reduced across all populations. Genetic variation in a population is derived from a wide assortment of genes and alleles. The persistence of populations over time through changing environments depends on their capacity to adapt to shifting external conditions. The Asafetida is one of the most endangered plants in Iran, having been reduced to single remnant populations in the last years. Sometimes a population can have many members but still undergo paucity or loss of genetic diversity. This can happen through geographical isolation. However, in fact, many influences disturb HWE, including non-random mating, mutations, selection, limited population size, random genetic drift, gene flow, and meiotic drive can be and Captive breeding programs can provide large numbers of plants in the native regions. Natural hybridization and introgression occur widely in plants and play important roles in their evolution [5, 18 & 37]. Introgressive hybridization is of great interest for plant evolutionary studies because it produces considerable numbers of new genotypes, thereby increasing genetic diversity, which may lead to new adaptations [37 & 39] and the formation of new ecotypes [25, 35 & 36] or species [44]. Although the extent and significance of natural hybridization/introgression is uncertain, the new combinations of genes resulting from hybridization and introgression between wild or weedy relatives and their crop cultivars have been important in the evolution of domesticated crop species [19]. The phylogram of genetic divergence in the all Phylogenetic methods revealed two major clusters. The grouping patterns of sequences were slightly different when the two results developed by different clustering methods were compared. In the NJ and UPGMA methods, Tang Sorkh and Maymeh were clustered at one group and Also, the Bastak and Geno were clustered together. But In the PM methods, the Bastak was separated from Geno. If a new geographical block is imposed (e.g. the new course of a river or the development of a subdivision creating houses, roads, and sidewalks on a previously empty meadow) a population of plants or animals may become separated into two groups. When this happens, the pool of gene variants in the two separated populations may differ from one another. The genetic distances were low in Iranian Asafetida populations. Assuming neutrality, low differences were observed across populations.

V. Conclusion

This study demonstrated the existence of sequence variability in the rpl16 gene sequence among Asafetida from different populations and geographical regions. We have analyzed nucleotide sequence variation in an approximately 712 bp of Intron of Asafetida ribosomal protein L16 (rpl16) gene of chloroplast DNA molecule. In current research, 4 distinct haplotypes with 7 polymorphic sites were found. The nucleotide diversity showed a diference in genetic diversity of the sequences between all populations. The phylogram of genetic divergence In the all Phylogenetic methods revealed two major clusters. The genetic distances were low in Iranian Asafetida populations. Assuming neutrality, low

diferences were observed across populations. Present results showed that sequencing is appropriate tools for evaluating genetic variability. We conclude from these analyses that DNA sequence comparisons of chloroplast-encoded genes will be particularly useful in phylogenetic analyses of higher taxonomic categories. this study highlights the importance of sourcing plants directly from remnant populations in order to maintain genetic diversity.

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