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Genetic relationship between different populations of the *Gloydius halys caucasicus* (Nikolsky 1916) of mountainous areas of the Iran

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Abstract:

Gloydius halys caucasicus (Nikolsky 1916) is a venomous viper, distributed in China, N Iran, S/SW Russia, Kazakhstan (between Volga and Ural River), Uzbekistan, Tajikistan, Kyrgyzstan, E Afghanistan, Mongolia and Turkmenistan. This viper is biomedical and economically important snake in the Iran. Data presented here, is on the genetic structure and relationship of different populations of Caucasian viper, from mountainous areas of the Takht –E- Soleiman, Talagan in the Alborz province and Lar, in the Tehran province of the Iran. Thirty one snakes were collected during Khordad -Murdad (Jun- August) months in the year, 1392, and kept in an vivarium under conventional conditions. To do molecular studies, the

DNA genome were extracted from snake's blood samples (All procedures were carried out in accordance with ISIRI 7216-2 animal ethical guidelines) and used to perform the Nested PCR. In the PCR, a 755- bp fragment from the D- loop mitochondrial genome (used for sequencing) was amplified and the PCR product was sequenced. Nineteen variable nucleotide sites and ten haplotypes were identified. In all the 19 position variable substitution mutations had occurred and no In/Del were observed. These nucleotide changes indicate the presence of high genetic diversity in the sequences and show strong inclination to substitutional changes in the nucleotides. Phylogenetic tree based on genetic distance matrix was drawn. Cluster analysis showed some differentiations between populations while some individuals of different population grouped in one cluster with high similarity. AMOVA and phylogenetic tree analysis showed a significant differences in the populations of the *Caucasian viper* in the Iran ($P < 0.003$), which represents the genetic variation between populations. It seems that the genetic characteristics of a species in each region are affected by the geographical / ecological conditions.

Keywords: Mitochondrial genome, phylogeny, Pit Viper, Iran

Introduction

The snakes of the Iran, taxonomically are in the families of the Leptotyphlopidae (2 species), Typhlopidae (3 species), Boidae (6 species), Colubridae (42 species), Hydrophidae (9 species), Elapidae (2 species) ، Crotalidae (1 specie) and Viperidae (13 species).The Viperidae family including 14 genus are endemic of the Europe, Asia, Africa in the tropics and temperate climates.The recent researches on the genus level on the phylogeny of the vipers of the Middle East and the Near East lead us toward the cleavage of the previous genus and creation of the 9 present genus and the number of species has increased from 25 to 31. Molecular researches have shown the gens of the Pseudocerastes, Eristicophis, Daboia, Macrovipera and Montivipera are monoclade and the vipera has basal position. Echis and Cerastes are monoclades (Stumpel & Joger, 2009).

The Gloydius halys caucasicus (Nikolsky,) is a venomous snake, from Crotalidae family and viperidae sub family. The species main range is from the Northeast coasts of the Caspian Sea towards the steppes of Kazakhstan, North Turkmenistan, North Uzbekistan, Kyrgyzstan, North Tajikistan, and Northewest China, most of Mongolia, Northwest Manchuria, and South

Siberia. Isolated subranges in South Caspian Sea, Northeast Iran, South Turkmenistan and Northwest and Northeast Afghanistan, Gansu, Southeast Mongolia, North Shanxi and Hebei provinces in China. Chorotype is Turanian plus Eastern Palearctic. G.h.caucasicus is reported from Southeast Azerbaijan, North Iran, South Turkmenistan and Northwest Afghanistan(Fig.1).

Caucasian pit viper is a venomous, biomedical and economically important snake and the only pit viper in the Iran. This subspecies is widespread in Iran and recorded in Semnan, Tehran, Alborz, Qazvin, Gilan, Mazandaran, Golestan, North Khorasan and Khorasan Razavi Provinces (Latifi, 1999 &2000). It is also common in Lar, Damavand, Afjheh, Firooz Mountain, Khan Ahmad, Kandavan, Taleqan, Borqan, Roodbar, Chehel Dokhtar, Gonbad-Kavoos, Gorgan and Kalardasht. Caucasian pit viper has narrow snout and extended upward at the tip; large, distinct scales on dorsal surface of head; pupils vertical; tail short with vibratory movements similar to Rattle Snakes. Body yellow-grey, red or light brown; dorsally ocelli or dark transverse bars, laterally one or two series of dark spots; dark snout; posteriorly, head with two diagonal dark stripes, temporal region with pale-edged dark spot, venter dark or with grey or brown spots. The strongly keeled dorsal scales are arranged in 23 rows at midbody. Ventrals 149-174; anal plate entire; subcaudals 31-44, divided (paired).[6] Grows to a maximum total length of 59 cm (23 in), which was for a female, with an included tail length of 68 mm (2.7 in). The largest male on record measured 53 cm (21 in) in total length, which included a tail length of 80 mm (3.1 in). The body build is described as moderately stout with a snout that is slightly upturned when viewed from the side.[2]

Caucasian pit viper favorite places are scrublands, mountains, hills, forests and even under rocks. Maximum length of Caucasian pit viper is 67 cm and tail is about 6 cm. Diet of this snake is quite typical: Amphibians (frogs), small rodents and lizards Amphibians (frogs), small rodents and lizards are the best choices.

There are many differences between Caucasian pit viper and their relatives: Rattlesnakes. Rattlesnakes are best known for, and most easily recognized by, their rattle. The rattlesnake babies are born with what is called a pre-button. The baby snake loses this piece when it sheds its skin for the first time. With the shedding a new button appears. But Caucasian pit viper doesn’t have this piece although it makes noises by different mechanism. Aside from this pair of simple eyes, Caucasian pit viper just like rattlesnakes is able to detect thermal radiation emitted by warm-blooded organisms in their environment. Functioning optically like a pinhole camera eye, thermal radiation, in the form of infrared wavelength light, enters, passes

through the opening of the pit and strikes the pit membrane located in the back wall, warming this part of the organ. Due to the extremely high density of these heat-sensitive receptors innervating this membrane, the rattlesnake can detect temperature changes of 0.003 ℃ or less in its immediate surroundings. Infrared cues from these receptors are transmitted to the brain by the trigeminal nerve, where they are used to create thermal maps of the snake's surroundings. Due to the small sizes of the pit openings, typically these thermals images are low in resolution and contrast. Nevertheless, rattlesnakes superimpose visual images created from information from the eyes with these thermal images from the pit organs to more accurately visualize their surroundings in low levels of light. This mechanism helps them not just for seeing but also for hiding better.

No comprehensive molecular phylogenetics has been provided so far for Gloydius. The genus Gloydius was for longtime included in Agkistrodon (now considered exclusive of North and Central America). Researchers by morphological character analysis (1981) and molecular tests confirmed Caucasian pit viper as a Gloydius halys caucasicus. So any other names aren't scientific. Population of Caucasian pit viper in the Iran, estimated average but it hasn't scientific basis. A comprehensive and detailed study about this subspecie is really important in the Iran because raises some novel issues at the intersection of scientific research, conservation efforts and antivenin production programs. Today, molecular methods, such as sequencing the mitochondrial genome is one of the most used methods to determine the phylogenetic relationship between the close species. Mitochondrial DNA (mtDNA) have certain advantages to identify species and drawing phylogenetic, including higher copy number per cell , smaller size , maternal heritability , being haploid , the absence of recombination , existance of non-protected areas such as the D-loop region to study the related species evolution (Ladloi, 2003). The D-loop occurs in the main non-coding area of the mitochondrial DNA molecule, a segment called the control region or D-loop (a displacement loop) region. D-loops occur in a number of particular situations, including in DNA repair, in telomeres, and as a semi-stable structure in mitochondrial circular DNA molecules. Since the mitochondria are of maternal origin and where recombination takes place so, this property leads to more genetic differences in the mitochondrial genome than nuclear DNA. It is therefore a good indicator for identifying groups that have been separated for 10, 100 or 1,000 years. The substitution rate of nucleotides in the mtDNA of higher animals is approximately 5 to 10 times higher than the nuclear genome that is changing 2% every million year. Speed of variations in the nucleotides in different regions of the

mitochondrial genome is different as compare with tRNA and rRNA. This area (D-Loop) shows enough variety with many distinctions in the population level. So this gene is a reliable marker and is considered suitable for reptile's phylogenetic studies. [Anderson, 2009, Dudud et al.,2011].Study of the genetic makeup of Caucasian viper, which has a significant population in the Central Plateau of the Iran, seems essential and the genetic identification of species is important for the overall management of the reptiles and venomous snakes of the country (Papasotiropoulos et al.,2002).

Fig.1. Map of the distribution of G.h.caucasicus in the world

Materials and Methods

Caucasian viper, were collected from mountainous areas of the Takht -E- Suleiman (average annual temperature of 9.5 ℃, maximum of 27.6 ℃, and minimum of -11.3 ℃), and Talegan, in the Talegan county from Alborz province and Lar, from the Tehran province of the Iran. Thirty one snakes were collected during Khordad -Murdad (Jun- August) months in the year, 1392, and kept in a vivarium under conventional conditions. To do molecular studies, the genome/ DNA were extracted from snake's blood samples (All procedures were carried out in accordance with ISIRI 7216-2 animal ethical guidelines[3]) on the 1% agarose gel and used to perform the Nested PCR. In the PCR, a 755- bp fragment from the D- loop mitochondrial genome were used for sequencing and amplified, the PCR product was sent to Bioner Co, South Korea for sequencing. All the sequences were arranged by Clustal X, (Thomson et al., 1997) in the application of the BioEdit. Phylogenetic tree of the nearest neighbor tree

(Neighbor Joining) type for the samples was drawn using software MEGA4. Genetic distance between the samples (Kumar et al., 2004) from different regions based on the Maximum Composite Likelihood model and average difference of base pair (Tamura et al., 2007) within and between the samples of the regions was obtained using the software MEGA4. Population genetic structure was evaluated using analysis of molecular variance (AMOVA) with software Arlequin, (Exoffier et al., 2005).

Results and discussion

DNA extraction

Genomic DNA extraction shown in Figure 2. As the bands indicate good quality of DNA extraction on the agarose gel.

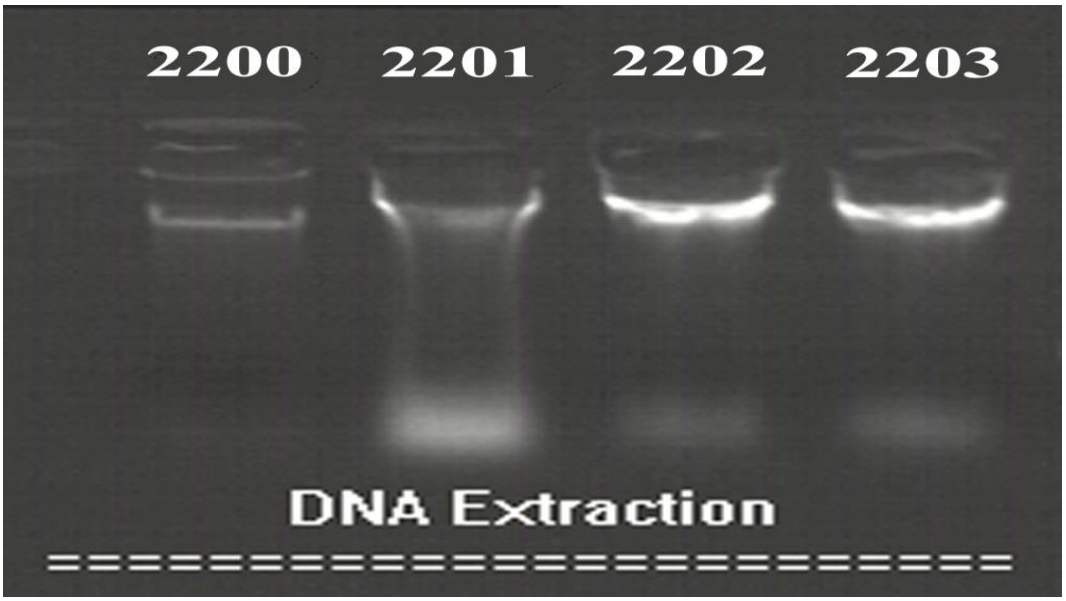

Fig. 2. DNA bands on agarose gel

The results of the first PCR is shown in figure 3.The product of first PCR was used for Nested PCR. Primers used in this reaction are shown below:

Gloydius CR F1030--- 5' -GGGGCGAAAGGCATTTATGAAACG- 3'

Gloydius CR R910--- 5' -TGCGTGGTTTTTTTGTATAGGATTTCGGG- 3″

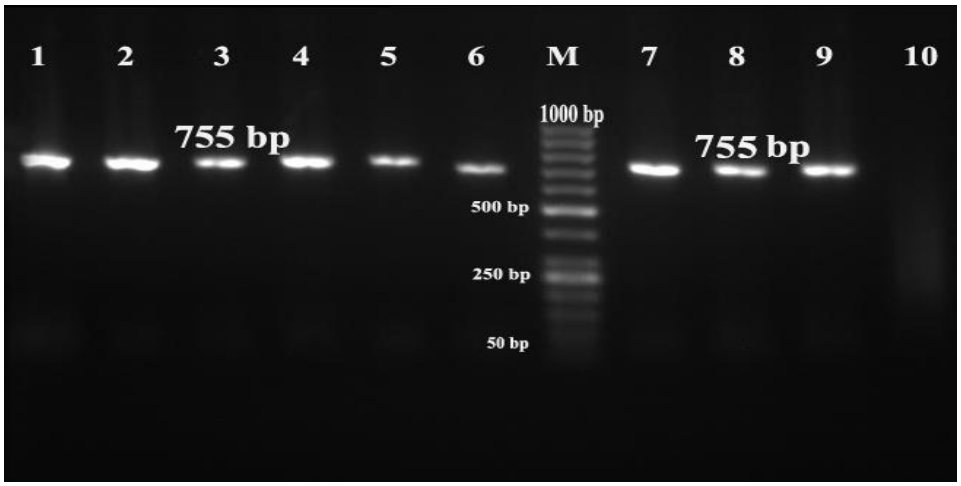

Fig 3. The DNA bands of the first PCR product on agarose gel

Nested-PCR

The increased amount of DNA by the second PCR reaction (Nested PCR) is shown in figure 4. At this stage, the following primers were used

Gloydius CR F10--- 5' -CCTACATCACCCAAAATTTAAAGCC- 3'
Gloydius CR R60--- 5' -CTGATTAAACCATAAAAAATAAC- 3'

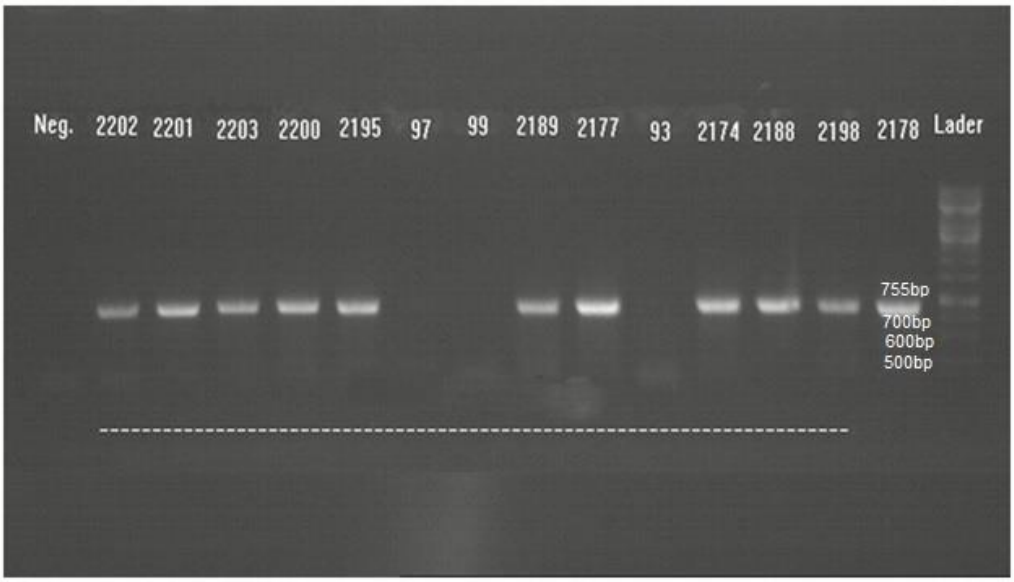

Fig. 4. DNA bands of the Nested-PCR product on agarose gel

Sequencing of the D-Loop region of mitochondria

Nested-PCR products amplified the D-Loop region of mitochondrial sequencing. Analysis on the results was sequenced, the following we see the sequence for one of the samples.

1 CCTATATATG TACTCTTTAC ATATATGGGT CCTCATATCG CTATGTATAA TAATACATTA

61 ATCGTTTTTC CCCATGCCTA ATAAACGGGA ATTAAACTTT AATGAATTGT ATATAAAACT

121 GGCTCACTAG CATAATTTCC TCCCCTCATT TCCTGGTCGT TCCATTTAAC AGAGGTTGTC

181 TATTATTAGT AACCATGGCT ATCTACTTCA AACCGGTGTC CCATGATTTA ACCCTTCCCG

241 TGAAATCCTC TATCCTTCCA CTGCAGGCAT ACAGTCCCGC TTTTCACGTC CATATACTGT

301 AACTCCTCCC GTTTATGTCC TTTCCAAGGC CGCTGGTTAC TCTTTCAGGA GCTTCTCAAT

361 GGTCCGGAAC CACCCCGCCT TACTTGCTCT TTCCAAGGCC TATGGTCGCA CCCTTTATAC

421 TGGTACATTT AACCTCATGT TCTTATCACG AATGCATGTT CCACCCCTGG TTGTCTTTTT

481 ATAGGTACCT TTCACCTGAC ACCCATATAT GCCCGTTACC GTCACCCCTC TCCGGGGTAG

541 ATCATTAGTC CAGGTGGAGC TATGTTCTTG GTCTTGCACT TTTCCCTATA GGGATACATC

601 TTCTTAATGC TTGTTATACA TATTATTACA TACTGCTAAA AATTTCATTA TTTTTTATTA

661 AAGAAATCCC GGTGTAAATA CATTTTTACA CCCGATTTTT TAAATTTTTA CCAAAATTAA

721 TACCACTTTT CTATACTAAA ATTACAAACC CGAAA

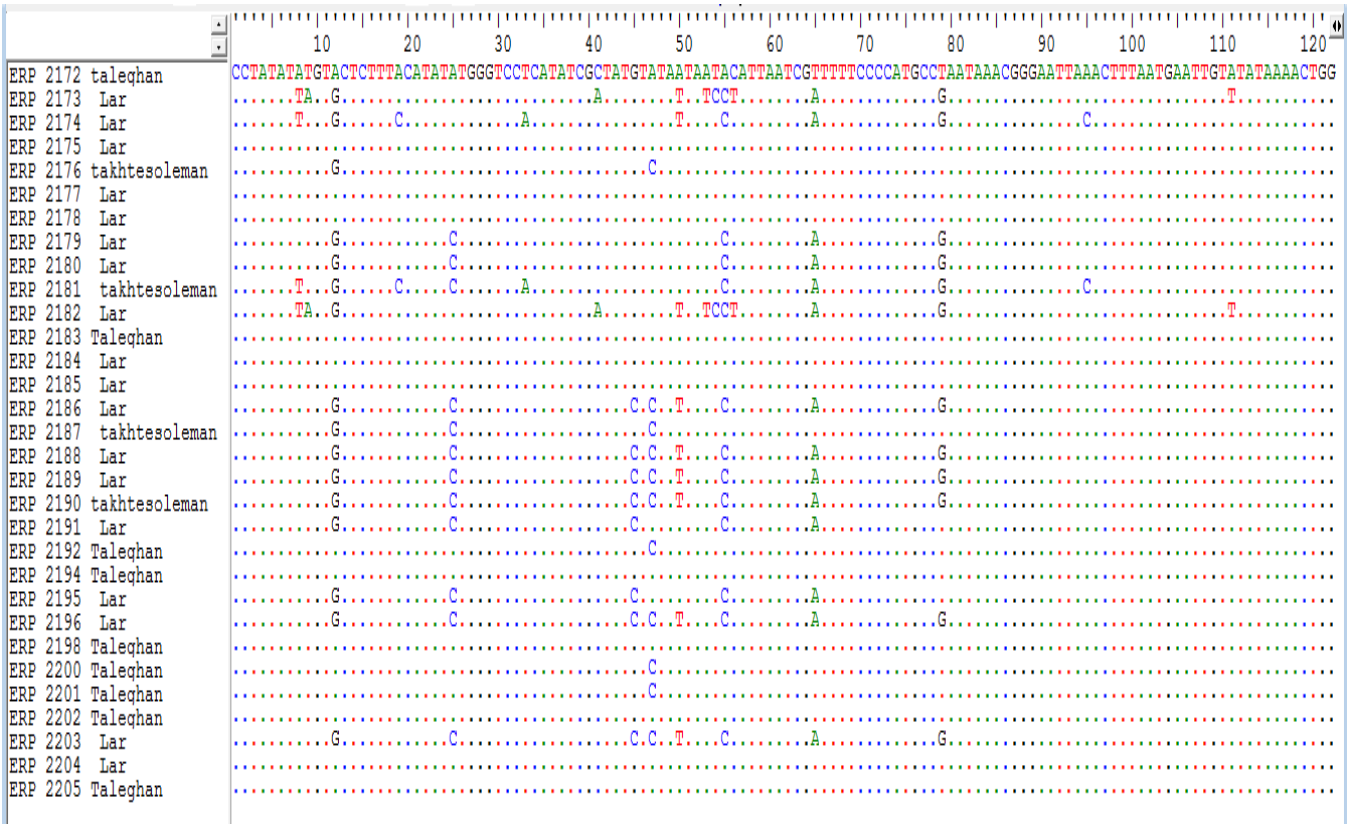

Fig. 5. Matched D-loop region sequences of the G.h.caucasicus vipers of three different regions of the Iran

The genetic diversity of the D- loop region

By examining the 755 bp fragment of D_Loop region of the mitochondrial genome in Caucasian viper, 19 variable nucleotide sites at 120 bp was obtained (Figure 5). From the 19 positions in all of the variable substitutional mutations had occurred. Based on the analysis made on the results of nucleotides displacements, frequencies of nucleotides (A) was equal to 0.252, (T / U) 0.353, (C) 0.259 and (G) 0.136. In this study, the displacement of transition to transversion K1 = 0.737 is for purine and K2 = 0.128 for pyrimidine. Optionally transition to transversion ratio equal to R = 0.148 (transition / transversion) is that R is calculated from the following formula.

$$R= [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$$

Table 1. Displacement of nucleotides transition and transversion (italicized numbers show the shift transversion and highlighted numbers show the shift transition)

Maximum composite likelihood estimate of the pattern of nucleotide substitution				
	A	T	C	About
A	-	*14.93*	*10.93*	4.24
T	*10.67*	-	1.4	*5.75*
C	*10.67*	1.92	-	*5.75*
About	7.87	*14.93*	*10.93*	-

Analysis showed that there was no singleton variable site, while the 19 nucleotides position variables were observed and are set forth below:

Site positions: 8 , 9 , 12 , 19 , 25 , 33 , 41 , 45 , 47 , 50 , 53 , 54 , 55 , 56 , 65 , 79 , 95 , 111 , 123.

The 10 haplotype with haplotype genetic diversity equal to 0.812 haplotype diversity variance, P =0.003, SD = 0.055) of the 31 samples were as follows:

Number of haplotypes, h: 10

Haplotype diversity, Hd: 0.8129

Variance of Haplotype diversity: 0.00305

Standard Deviation of Haplotype diversity: 0.055

Nucleotide diversity, Pi: 0.00741

Hap_1: including 2 individuals: [1- 15]

Hap_2: including 6 individuals: [2-3 6-7 18-19]

Hap_3: including 2 individuals: [4-5]

Hap_4: including 12 individuals: [8 10-12 16-17 23-24 27 29-31]

Hap_5: including 1 individuals: [9]

Hap_6: including 2 individuals: [13-14]

Hap_7: including 1 individuals: [20]

Hap_8: including 1 individuals: [21]

Hap_9: including 1 individuals: [22]

Hap_10: including 3 individuals: [25-26 28]

Detailed studies on the haplotypes revealed that the haplotypes 1, 3, 5 and 6 included only population Lar , while haplotypes 7 , 8 and 9, each comprise one individual of population of Takht-e Soleiman and haplotype 10 includes only population Taleghan . Other haplotypes include haplotypes 2, and 4 were present in all the three populations. Data are presented below:

Hap_1: 2 [ERP_2173_Lar; ERP_2182__Lar]

Hap_2:6[ERP_2188__Lar;ERP_2189__Lar;ERP_2196__Lar;ERP_2203__Lar; ERP_2186__Lar; ERP_2190_takhtesoleman]

Hap_3: 2 [ERP_2191__Lar; ERP_2195__Lar]

Hap_4: 12 [ERP_2204__Lar ; ERP_2175__Lar ; ERP_2177__Lar ; ERP_2178__Lar ; ERP_2184__Lar ; ERP_2185__Lar ; ERP_2205_Taleghan ; ERP_2202_Taleghan ; ERP_2198_Taleghan ; ERP_2194_Taleghan ; ERP_2183_Taleghan ; ERP_2172_taleghan]

Hap_5: 1 [ERP_2174__Lar]

Hap_6: 2 [ERP_2179__Lar; ERP_2180__Lar]

Hap_7: 1 [ERP_2176_takhtesoleman]

Hap_8: 1 [ERP_2181__takhtesoleman]

Hap_9: 1 [ERP_2187__takhtesoleman]

Hap_10: 3 [ERP_2201_Taleghan; ERP_2200_Taleghan; ERP_2192_Taleghan]

Genetic analysis between and within populations

The number of different nucleotides between populations, the displacement average number of nucleotides in each position and the number of common mutations are presented in the table 2. Based on the analysis, the most common mutations were observed among the population of Lar and Takht e soleman, while just one common mutant was seen in the Lar and Taleghan populations and the maximum number of different nucleotides existed in these populations (0.5, 704). The number of base substitution in each position of all the individuals sequences in every population is shown in the table2.

Table 2. Comparison of number of the different nucleotides, number of nucleotides displacement and common mutations in the populations

Population	Lar	Taleghan	Takht –e-soleiman
Lar	---		
Taleghan	-Average number of nucleotide differences between populations: 5.704 - Average number of nuc. subs. per site between populations, Dxy: 0.00755 -Shared Mutations: 1	----	
Takht-e -soleiman	- Average number of nucleotide differences between populations: 6.194 -Average number of nuc. subs. per site between populations, Dxy: 0.00820 - Shared Mutations: 12	-Average number of nucleotide differences between populations: 5.833 - Average number of nuc. subs. per site between populations, Dxy: 0.00773 -Shared Mutations: 1	---

The average genetic distance within each population (Maximum Composite Likelihood method) is provided in the table 3 so that the population of Takht-e Soleiman has the greatest divergence or diversity (0.009) among its own individuals.

Table 3. Evolutionary divergence average on the pair sequences within the populations

Populations	d
Lar	0.008
Taleghan	0.009
Takht-e -soleiman	0.001

According to the analysis of molecular variance of the genetic differences between and within three populations, 25 % of the total variance was related to genetic differences between populations . While 75 % of the total variance related to genetic differences within

populations. AMOVA analysis table 4 showed significant genetic differences between the populations (P = 0.003).

Table 4.The AMOVA analysis of molecular variance in population

AMOVA					
Source	Df	SS	MS	Est. Var.	%
Among Pops	2	18.380	9.190	0.786	25%
Within Pops	28	65.556	2.341	2.341	75%
Total	30	83.935		3.128	100%
Stat	Value	P(rand >= data)			
PhiPT	0.251	0.003			

Analysis of the Cluster dissociation

Similarity matrix based on the similarity coefficient of theTamura and Nei (2007) on the data obtained from the sequencing of the 31 caucasian viper samples of the three populations was drawn. Method NJ (Neighbor Joining) by (100 % bootstrap) was used for data clustering . NJ tree is given in Figure 6. Generally, NJ tree is divided into three clusters that in the first cluster individuals of three populations are present, while one individual of the Takht e Soleimon, one individual of the Lar population apart from the rest of the population were in the second cluster.The third cluster consists of two individuals of the Lar population that is located far from other individuals. Of the 31 samples taken from three different populations 10 different hoptypes were grouped, as it is shown in the above analysis.

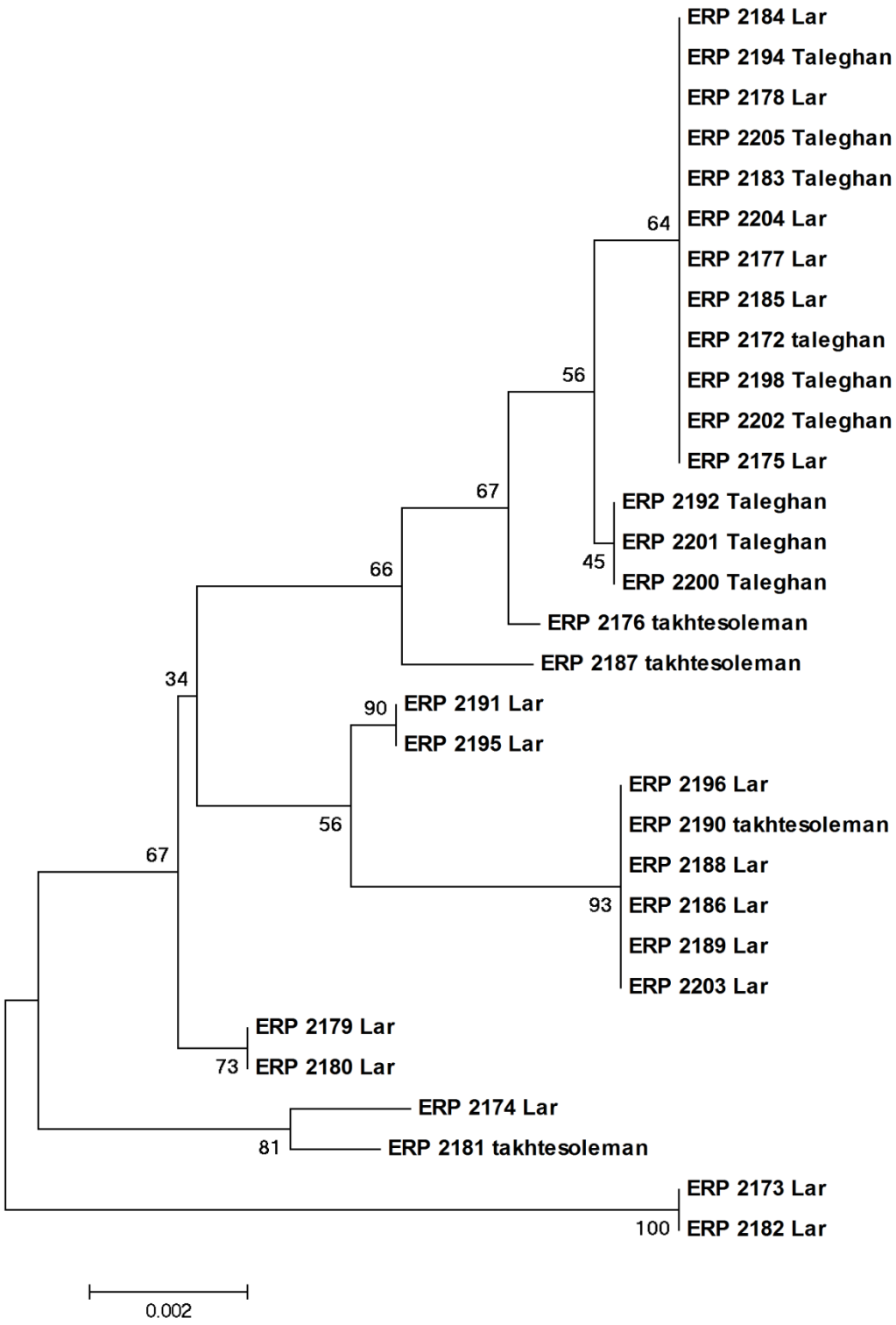

Fig. 6. Phylogenetic relationship (Neighbor Joining tree) of the Caucasian viper from three different populations of the Iran

The phylogenetic relationships of the Caucasian vipers were investigated using mitochondrial DNA sequences from cytochrome b, ND2 and 16S. The associations of the species conformed to subgeneric designations of Nilson et al. (1994). Vipera ammodytes was resolved as the sister group of all other Vipera included in our evaluations. Sequentially, the groups branched off as follows: V. latastei + V. aspis; V. seoanei, V. berus, V. sachalinensis + V. nikolskii; V. dinniki; V. orlovi, + V. kaznakovi; and finally V. eriwanensis, V. lotievi, V. renardi + V. ursinii (Murphy et al., 2006b).

Zinenko et al. 2016 reported both V. magnifica and V. orlovi (small vipers that have high conservation status due to their rarity and restricted distributions in an area of the Caucasus region) have relatively low numbers of private alleles, but observed heterozygosity that is higher than expected when compared to *V. kaznakovi* and *V. renardi*. Finally both observed heterozygosity and allelic richness are highest in *V. orlovi*.

Murphy et al.(2006) investigated polyandry within six clutches of Armenian Vipera eriwanensis. The clutches contained from 5 to 11 embryos. A suite of 11 hyper-variable microsatellite DNA loci was developed. These loci were variable among all species of Caucasian Vipera, and most were variable within species. Ten of these loci were consistently resolved in the embryos of V. eriwanensis, of which seven loci varied within at least some clutches. The necessity of sequencing alleles to confirm their homologies was revealed at one locus that appeared to exhibit little variation. Two alleles produced identical pherograms but these masked variation. One allele had a repeat sequence of (CT)5(CA)9AA(CA)3 and the alternative allele appeared as (CT)4(CA)10AA(CA)3 (Murphy et al., 2006a).

However in this study the phylogenetic tree analysis showed a significant differences in the populations of the *Caucasian viper* in the Iran ($P < 0.003$), which represents the genetic variation between populations. It seems that the genetic characteristics of a species in each region are affected by the geographical / ecological conditions.

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