Evolutionary history and distribution of African wildcat, *Felis lybica* in Iran

Marzieh Mousavi*[1], Saeid Naderi[2], Hamid Reza Rezaei [3], Mohammad Ali Adibi[1]

1. Department of Environmental Sciences, Faculty of Natural Resources and Environment, Science and Research Branch, Islamic Azad University, Tehran, Iran

2. Department of Environment, Faculty of Natural Resources, University of Guilan, Rasht, Iran

3. Department of Environment, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

* Corresponding author's E-mail: mrmussavi@yahoo.com

ABSTRACT

Wildcat, *Felis lybica* is one of the least-known cat species in Iran, in spite of the widespread distribution in the country. The molecular overview of the species using concatenated two mitochondrial markers (Cyt b, NADH5) with sampling throughout the range of the species in the country was examined. Mitochondrial DNA analysis and geographical distribution of the haplotypes indicated two well-supported subclades in the study area, comprising Asiatic wildcat, *F. lybica ornata* and African wildcat, *F. lybica lybica* (FST = 0.65; p-value < 0.001). Time-calibrated Bayesian phylogenetic analysis revealed divergence time between *F. l. ornata* and *F. l. lybica* dates to 340,000 years ago (HPD 95%: 192,000-489,000 years ago). Based on the results of dating phylogenetic tree, Central Asia is the origin area for distribution of *Felis* genus (Domestic Cat lineage). On the other hand, wildcat moved out of Central Asia towards Western Asia, Europe and Africa. Given estimated divergence times of less than one million years ago among wildcat subspecies, it seems that Pleistocene climatic fluctuations may have led to the diversification of this taxon. As our study does not prove hybridization between wildcat and its domestic congeners, further investigations should focus on this remarkable threatening factor.

Keywords: Mitochondrial markers, Phylogeny, Speciation dates, wildcat.
Article type: Research Article.

INTRODUCTION

The wildcat, *Felis silvestris/Felis lybica* is a polytypic species with three main morphological forms including European wildcat, *F. silvestris silvestris* Schreber, 1777; the African wildcat, *F. lybica lybica* Forster, 1780; and the Asian wildcat, *F. lybica ornata* Gray, 1830 (Guggisberg 1975; Hemmer 1978a; Randi & Ragni 1991; Wozencraft 2005; Nowell & Jackson 1996; Johnson & O'Brien 1997; Pierpaoli *et al*. 2003; Mattucci *et al*. 2015). The European wildcat occupies deciduous and mixed forests in Europe and the Caucasus, while the African and Asiatic wildcats occupy semi-arid area and steppes in Africa, the Arabian Peninsula, Central Asia, into western India, western China and southern Mongolia (Driscoll *et al*. 2007; Yamaguchi *et al*. 2015; Kitchener *et al*. 2017). In the western Asia, two geographically-distinct subclades were recognized in Iran but their distributions overlap in some areas: Asian wildcat distributed in east of Iran, while the African wildcat in the west (Mousavi *et al*. 2019). Describing the genetic variation found in organisms is one of the most important aims of conservation geneticists that is fundamental to recalling the biodiversity found in species, populations and ecosystems (Mirzakhah *et al*. 2015). In the western Asia where the wildcat occurs, diversification events have been mainly attributed to climatic fluctuations and geological events (Bilgin *et al*. 2008). The impact of climatic fluctuations on the genetic structure of organisms during the Pleistocene epoch in the western Asia is well known (Kasapidis *et al*. 2005; Dubey *et al*. 2007; Gür 2013; Gür *et al*. 2018; Asadi Aghbolaghi *et al*. 2019; Asadi Aghbolaghi *et al*. 2020). Multiple glacial contractions and expansions have played an important role in the formation of biodiversity

patterns in the area (Taberlet *et al*. 1998; Bernatchez *et al*. 2001; Veith *et al*. 2003; Bilgin *et al*. 2008). Climatic fluctuations accompanying glacial and interglacial cycles, imposed species to either transfer to places that remain suitable, adapt to changing environmental conditions, or go extinct (Jansson & Dynesius 2002). Climate variability is causing shifts in the geographic spreading of the species (Root *et al*. 2003 & Parmesan 2006). These range shifts have left signs of high genetic diversity in the refugia (Hewitt 2000 & Stewart *et al*. 2010). The wildcat, *F. silvestris/F. lybica* is a conservation concern mainly due to hybridization with the domestic cat *F. catus* (Driscoll *et al*. 2007; Driscoll *et al*. 2011). The risk of continuous hybridization because of overlapping distribution of wildcat and domestic cats may cause genetic extinction of local wildcat populations, as reported in Hungary, Scotland and Poland (Beaumont *et al*. 2001; Pierpaoli *et al*. 2003; Driscoll *et al*. 2011; Zwijacz-kozica *et al*. 2017). Although it has been shown that fur characteristics can be used instead of molecular techniques in identifying species and hybrids, it is only reliable where extremely distinctive characteristics between wildcat and domestic cat are used (Ragni & Possenti 1996; Kitchener *et al*. 2005; Devillard *et al*. 2013). However, it seems that simultaneous use of morphological and molecular approaches will provide more confirmed consequences for identifying wildcat, domestic cat, and hybrids (Ragni and Possenti, 1996; Yamaguchi *et al*. 2004; Kitchener *et al*. 2005; Devillard *et al*. 2013; Kilshaw *et al*. 2014; Senn *et al*. 2018). Although the wildcat is a widespread felid species in Iran, the genetic structure of the species remains ambiguous. In a study conducted by Mousavi *et al*. (2019), some implications about wildcat in Iran obtained using only one mitochondrial DNA marker (Cyt b), however in the present study we conducted an extensive survey of mitochondrial DNA variation, using concatenated two markers, cytochrome b (Cyt b) and NADH5 dehydrogenase subunit 5 (NADH5) with two main objectives:

1. To clarify the genetic structure of wildcats across its wide distribution in Iran using mitochondrial (mt) DNA.
2. To elucidate the species' evolutionary history and the divergence time of Asiatic wildcat, *F. lybica ornata* and African wildcat, *F. lybica lybica*.

We supposed that Pleistocene climate variations created two refugial areas for Asian and African wildcats. After Pleistocene these species distributed in the Iranian terrain. Therefore, we expected to see genetically distinct lineages dating to the Pleistocene climatic fluctuations and geological events.

MATERIALS AND METHODS
### Sample collection, DNA extraction and sequencing
Overall, 38 tissue samples of wildcats were collected from specimens found throughout Iran. Samples were collected within the protected areas or vicinity of protected areas between 2015 and 2017 (Fig. 1; Table 1). All tissue samples were preserved in 96% ethanol. DNA from the samples was extracted using the DynaBio kit (Takapouzist Co.) following the manufacturer's protocol. The sequences of 1,140 bp of mtDNA Cyt b from Mousavi *et al*. (2019) used in current work (samples 1-23 in Table 1). For NADH5 gene the primers used for the 814 bp of the NADH5 region were; F2B (5'-TGCCGCCCTACAAGCAAT-3'), R3B (5'-TAAGAGACGTTTAATGGAGTTGAT-3'; Driscoll *et al*. 2007). A total volume (25 µl) of the PCR reaction mixture contained 2.5 µl extracted DNA (1-150 ng µl^{-1}), 12.5 µl prepared Master Mix (1.5 Mm MgCl2), 9.25 µl distilled water and 0.375 µl of each primer (5 pmol µl^{-1}). The PCR conditions were as follows: 94°C, 3 min; 35 cycles (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min); and 72 °C, 10 min (Tamada *et al*. 2005). Amplified PCR products were visualized by 1% agarose gel electrophoresis, and were subsequently sent to Bioneer Company (South Korea) for sequencing. The original chromatograph data were checked using SeqScape (v.2.6; Applied Biosystems). ClustalW was used to align sequences in MEGA 6 software (Tamura *et al*. 2013).

Phylogenetic analyses
We plotted two phylogenetic trees, one using only Iranian samples based on 1,954 bp comprising (1,140 bp of Cyt b and 814 bp of NADH5) and the other based on 1,022 bp, including (208 bp mtDNA Cyt b and 814 bp NADH5) with Iranian sequences and worldwide wildcat/domestic cat sequences in GenBank database (supplementary Table S1). Models of nucleotide substitution were chosen using Akaike's information criterion in JModelTest (v.0.1.1; Posada 2008). The corresponding sequence of (*Felis margarita*) was used as outgroups (Driscoll *et al*. 2007). Phylogenetic trees were inferred by Bayesian inference (BI) and maximum likelihood (ML) methods. Bayesian analysis was constructed the BI tree using the MrBayes (v.3.2.2; Ronquist & Huelsenbeck 2003) in four simultaneous runs of Monte Carlo Markov chains (MCMC). The analysis was run for 2×10^6 generations, retaining one tree per 100 generations.

Table 1. Sampling sites for Iranian wildcats (see Fig. 1) and results of two genes (Cyt b and NADH5) and their subclades for every sample. Samples No: 1 to 23 used in developing phylogentic trees, based on concatenated genes.

No	Sample code	Locality	*Cyt b* (1140 bp) 23 samples	Accession number in GenBank (Mousavi *et al.* 2019)	NADH5(814bp) 38 samples	Subclade
1	IR4	Kh.Razavi3/Sarakhs	Yes	MN175476	Yes	*F. l. ornata*
2	IR18	Kh.Razavi9/Sabzevar	Yes	MN175493	Yes	*F. l. ornata*
3	IR15	Kerman/Sirch	Yes	MN175490	Yes	*F. l. ornata*
4	IR3	Zanjan	Yes	MN175477	Yes	*F. l. ornata*
5	IR23	Sistan /Chabahar	Yes	MN175495	Yes	*F. l. ornata*
6	IR16	Tehran	Yes	MN175491	Yes	*F. l. ornata*
7	IR14	Hamadan	Yes	MN175488	Yes	*F. l. ornata*
8	IR22	North Khorasan/Bojnurd	Yes	MN175492	Yes	*F. l. ornata*
9	IR12	Yazd/Ardakan	Yes	MN175485	Yes	*F. l. ornata*
10	IR13	Semnan/Turan	Yes	MN175486	Yes	*F. l. ornata*
11	IR7	Semnan/Abdolabad	Yes	MN175481	Yes	*F. l. ornata*
12	IR1	Chaharmahal	Yes	MN175475	Yes	*F. l. lybica/F. catus*
13	IR20	Esfahan	Yes	MN175497	Yes	*F. l. lybica/F. catus*
14	IR2	Kurdistan/Bijar	Yes	MN175480	Yes	*F. l. lybica/F. catus*
15	IR19	Shiraz	Yes	MN175496	Yes	*F. l. lybica/F. catus*
16	IR9	Sistan /Negor	Yes	MN175479	Yes	*F. l. lybica/F. catus*
17	IR21	North Khorasan/Shirvan	Yes	MN175487	Yes	*F. l. lybica/F. catus*
18	IR5	West Azerbaijan	Yes	MN175478	Yes	*F. l. lybica/F. catus*
19	IR17	Kerman/Nokohan	Yes	MN175494	Yes	*F. l. lybica/F. catus*
20	IR6	South Khorasan	Yes	MN175474	Yes	*F. l. lybica/F. catus*
21	IR10	Ilam/Jondollah	Yes	MN175483	Yes	*F.l. lybica/F. catus*
22	IR11	Yazd	Yes	MN175484	Yes	*F. l. lybica/F. catus*
23	IR8	Ilam2/Karzan	Yes	MN175482	Yes	*F. l. lybica/F. catus*
24	IR24	Kh.Razavi2	No		Yes	*F. l. ornata*
25	IR25	Kh.Razavi4	No		Yes	*F.l. ornata*
26	IR26	Kh.Razavi1	No		Yes	*F. l. ornata*
27	IR27	Kh.Razavi7	No		Yes	*F. l. ornata*
28	IR28	Kh.Razavi6	No		Yes	*F. l. ornata*
29	IR29	Kh.Razavi8	No		Yes	*F. l. ornata*
30	IR30	Qom	No		Yes	*F. l. ornata*
31	IR31	Golestan/Golestan NP	No		Yes	*F.l. ornata*
32	IR32	Golestan/Jahan-Nama	No		Yes	*F. l. ornata*
33	IR33	Yazd/Taft	No		Yes	*F. l. ornata*
34	IR34	Yazd/Bahabad	No		Yes	*F. l. ornata*
35	IR35	Semnan/Chahshirin	No		Yes	*F. l. ornata*
36	IR36	Semnan/Damqan	No		Yes	*F. l. ornata*
37	IR37	Kurdistan /Khoshmaqam	No		Yes	*F. l. lybica/F. catus*
38	IR38	Sistan /Iranshahr	No		Yes	*F. l. lybica/F. catus*
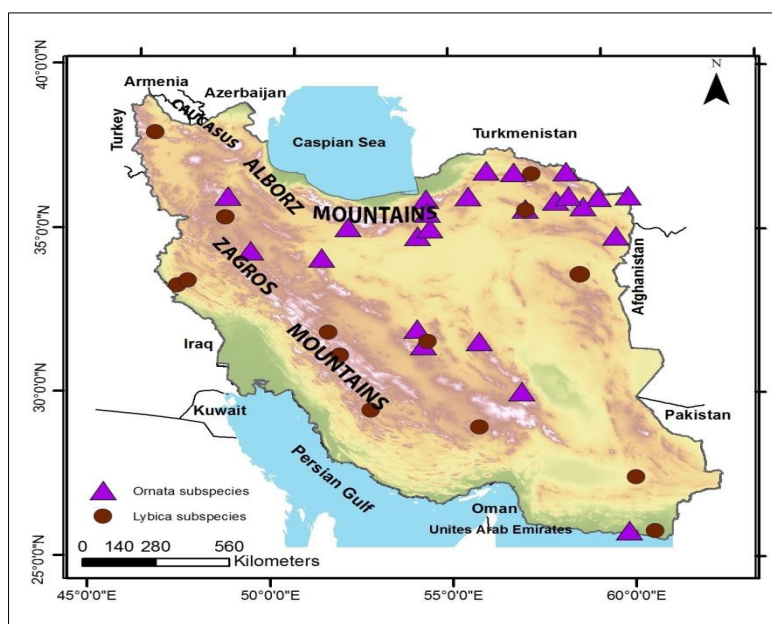
Fig. 1. Geographical distribution of the sampling sites for wildcat in the study area (see Table 1). Two subclades were indicated using different shapes: *F. lybica ornata* (Triangle) and *F. lybica lybica* (Dot).

The log likelihood values of the sample points were plotted against generation time and all the trees prior to reaching stationary (10%) were discarded, ensuring that burn-in samples were not retained. All remaining trees were combined in a 50% majority consensus tree. The ML analysis was implemented using RaxMl (v.3.1; Stamatakis 2014) with 1000 bootstrap replicates. The different genetic statistics, such as polymorphic sites and haplotype diversity between the two identified subclades of wildcats in Iran, neutrality test of Fu and Li's D* test (Fu 1997) and Tajima's D (Tajima 1989) were calculated with DnaSP 5.10 (Librado & Rozas 2009). We conducted the levels of population structure, analysis of molecular variance (AMOVA), and fixation index (FST; Weir & Cockerham 1984) using Arlequin 3.5 (Excoffier & Lischer, 2010) with 10,000 permutations for Iranian samples. Additionally, evolutionary relationships among haplotypes were represented by a median joining network generated with PopArt 1.7 (Leigh & Bryant 2015) and Network (v.10.1; Bandelt *et al*. 199).

Estimating divergence time

To assess the divergence time estimation, we plotted dated Bayesian tree using data sets, Cyt b (1,140 bp), because of low substitution rate for this gene, 0.02 (substitutions/site/Myr). Divergence times were estimated using 51 sequences from Iranian wildcats and the others consisting of Domestic Cat and Leopard Cat lineages in GenBank (Supplementary Table S2) with BEAST (v.2.5.2; Bouckaert *et al*. 2014). Calibrated Yule Model was used with normal distribution for the tree prior, and the HKY model applied for nucleotide substitution (Bouckaert *et al*. 2014). Posterior distributions of parameters were approximated using Markov chain Monte-Carlo (MCMC) run in BEAST (v.2.5.2) for 10 million generations with the first 10% of trees/parameters discarded as burn-in. The convergence of the MCMC chains was checked with TRACER v.2.5.2 (Bouckaert *et al*. 2014). The tree was calculated using Maximum clade credibility tree approach in Tree Annotator (v.2.5.2; Bouckaert *et al*. 2014). Node heights (i.e. node ages) were calculated as means of the posterior estimates and 95% highest posterior density intervals (HPD). The nodes were selected from *O'Brien et al*. (2008). We used normal distribution, because it produced more reliable results.

RESULTS AND DISCUSSION

Phylogenetic analyses (Cyt b and NADH5)

The best substitution models were estimated for concatenated genes (Cyt b and NADH5), HKY for first phylogenetic tree i.e., only Iranian samples and HKY+1 for second tree i.e., Iranian sequences along with worldwide sequences of wildcat. Both inference methodologies (ML and BI) supported two discrete subclades within the distribution area in Iran (Fig. 2). The first subclade (G1) belonged to the Asiatic wildcat, *Felis lybica ornata* and the second subclade (G2) included African wildcat, *F. lybica lybica*. Subclade G1, comprised samples from some parts of Iran including northeast and east (Golestan, Khorasan), southeast (Sistan) and some areas in center and north (Yazd, Kerman, Qom, Semnan, and Tehran). Subclade G2, comprised samples from northwest

(West Azerbaijan), west (Kurdistan, Ilam), southwest (Chaharmahal, Shiraz) and some areas in center and southeast (Esfahan, Yazd, Kerman, Sistan). Two defined subclades overlap in some localities as well. Table 2 displays the genetic statistics for the Iranian wildcats, between the two subclades based on 1,954 bp comprising (1,140 bp of Cyt b and 814 bp of NADH5). Fifteen haplotypes were identified for the Iranian wildcats. The ornata subclade exhibited higher haplotype diversity (0.93 ± 0.07) than the lybica (0.82 ± 0.12). The average number of nucleotide differences (7.24) in ornata was higher than in lybica (3.82). Given these genetic statistics, it can be concluded that in Iran Asiatic wildcat has more genetic diversity than African. The estimated amount of the Fu and Li's D* test and Tajima'D were negative for the whole population and each of the subclades (Table 2), however the values were not statistically significant ($p > 0.01$). The AMOVA analysis results for the Iranian wildcats revealed that the variance among wildcat subclades is 60.37% and within populations is 55.52%. The fixation index results (0.65) confirmed a significant difference in the genetic structure of the two Iranian subclades (Table 3).

Table 2. The genetic statistics of wildcat in Iran based on the analysis of 1,954 bp (1,140 bp of Cyt b and 814 bp of NADH5).

Group	n	h	Hd (SD)	Pi (SD)	K	P	Fu	Tajima's D
G1(ornata)	10	8	0.93(0.07)	0.004(0.0006)	7.24	25	-0.61	-0.69
G2(lybica)	11	7	0.82(0.12)	0.002(0.0007)	3.82	16	-1.77	-1.342
wildcat	21	15	0.94(0.04)	0.006(0.0004)	10.96	44	-1.08	-0.44

Note: n: number of individuals; h: number of haplotypes; Hd: haplotype diversity; SD: standard deviation; Pi: nucleotide diversity (per site); K: average number of nucleotide differences; P: variable (polymorphic) sites. $p > 0.01$ for all Fu and Tajima's D.

Table 3. AMOVA results for Iranian wildcat based on the 1,954 bp (1,140 bp of Cyt b and 814 bp of NADH5), between two Iranian subclades.

Source of Variation	d.f.	Sum of squares	Percentage of variation
Among Populations	1	60.37	65.35
Within Population	20	55.52	34.65
Total	21	115/89	
FST	0.65	Significance tests (10100 permutations), p-value < 0.001	

The resulting phylogenetic tree based on 1,022 bp of concatenated genes (208 bp of Cyt b and 814 pb of NADH5) demonstrated two distinctive clades: one clade including the European wildcat, *F. s. silvestris* and another, the African wildcat, *F. lybica spp* which comprised three subclades: The first subclade belonged to the African (North Eastern) wildcat, *F. lybica lybica* and *F. catus*, the second subclade to South African wildcat, *F. lybica cafra* and the third to the Asiatic wildcat, *F. lybica ornata*, respectively (Fig. 3). The Iranian samples were placed within two subclades, African (or Near Eastern) wildcat, *F. l. lybica* and Asiatic wildcat, *F. l. ornata.* For the haplotype network, we conducted two analyses; the first conducted on the basis of 1,954 bp of concatenated mtDNA cytochrome b and NADH5 for the Iranian sequences. Fifteen Iranian haplotypes were assigned into two identified groups in the phylogenetic tree. No common haplotype was shared between the two groups (Table 1, Fig. 4). The second was performed based on 1,022 bp of concatenated (mt)DNA cytochrome b and ND5 for the Iranian wildcats and retrieved sequences of wildcat/domestic cat from GenBank. The 67 sequences from Iran and the wildcats/domestic cats from GenBank harbour 36 haplotypes. These 36 haplotypes were divided into four subclades represented by different colors (Fig. 5): European wildcat, Asiatic wildcat, African (Near Eastern) wildcat together with domestic cats and South African wildcat. Iranian samples shared common haplotypes with Asian and African sequences. Haplotype 14 was the most frequent one including wildcat samples (*F. l. lybica*), domestic cat samples from France and England together with Iranian sequences (subclade: *F. l. lybica*). The second most frequent haplotype was haplotype 19 including samples from Asiatic wildcat from Mongolia, Turkmenistan and India along with Iranian samples (subclade: *F. l. ornata*).

Molecular dating

The estimated divergence time inferred from (mt)DNA data (Cyt b) revealed the first diverged species in *Felis* genus (Domestic Cat lineage) is Black-footed cat, *F. nigripes* in Pliocene (~3.34 Mya), then Jungle cat, *F. chaus*

in Pliocene (~2.48 Mya). Sand cat, *F. margarita*, as sister taxa of wildcat was separated in Pleistocene (~740,000 year ago). Three species including (*F. silvestris*, *F. lybica*, and *F. bieti*) had a common ancestor in Pleistocene (~430,000 years ago). Estimated intraspecific divergence times for the wildcat in Iran were less than 1 Mya. In the Pleistocene, African wildcat (*F. lybica lybica*; G2) and Asian wildcat (*F. lybica ornata*; G1), diverged about 340,000 years ago. On the other hand, the estimated age for the ancestor of two specified subclades of wildcat in current study is ca. 340,000 years ago (HPD 95%: 192,000-489,000 years ago; Fig. 6).
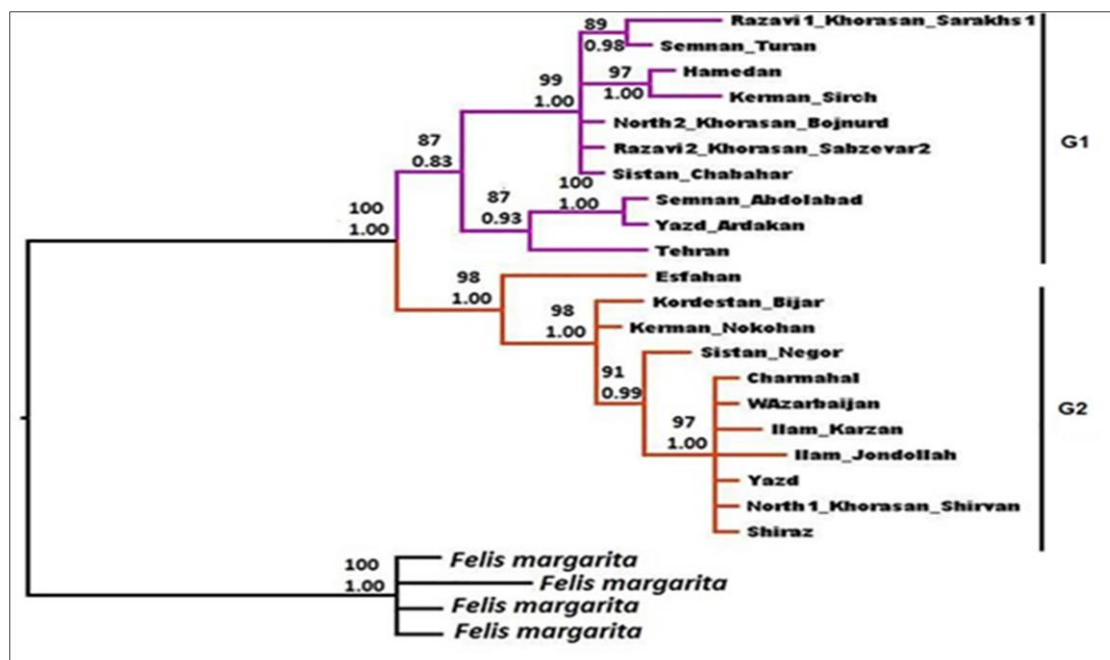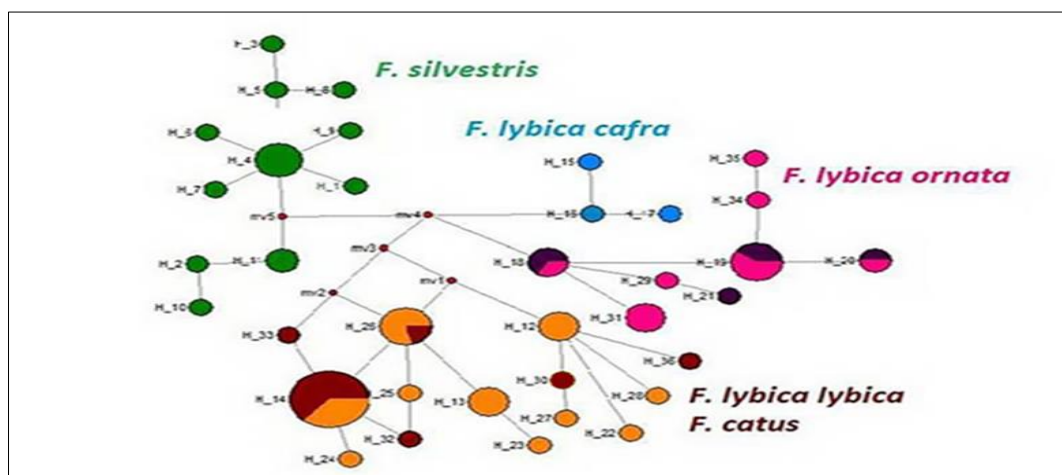

Fig. 2. Phylogenetic tree of the Iranian wildcats based on the 1,954 bp mtDNA Cyt b and NADH5 genes. Branches are including G1 (*F. lybica ornata*) and G2 (*F. lybica lybica*). The tree is rooted with Sand Cat (*Felis margarita*). The numbers on the branches are bootstrap support (above) and posterior probabilities (below) in the maximum likelihood and Bayesian inference, respectively.
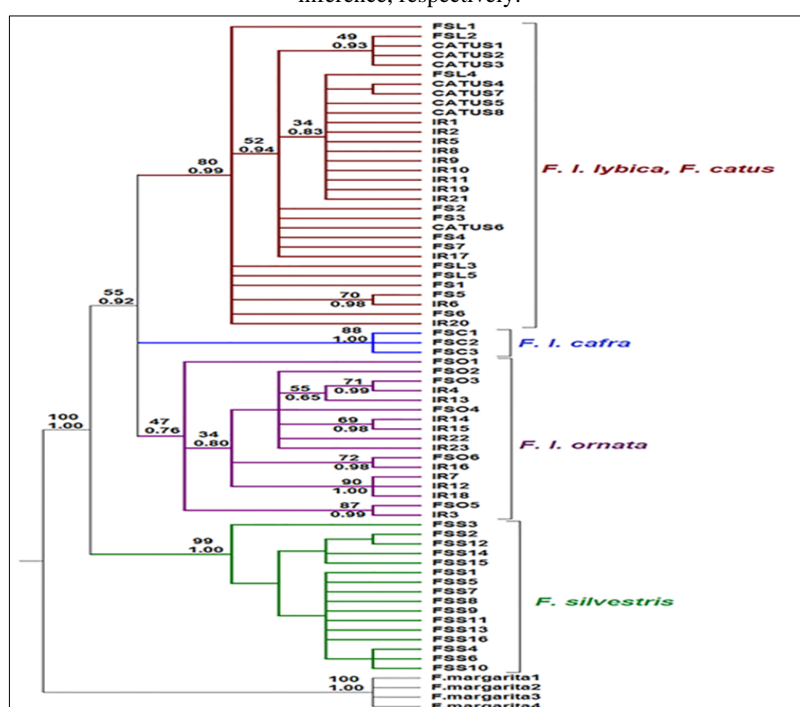
Fig. 3. Phylogenetic relationships of Iranian sequences with world range wild/domestic cats based on the 1,022 bp of mtDNA Cyt b and NADH5genes. The tree rooted with *Felis margarita*. The numbers on the branches are bootstrap support (above) and posterior probabilities (below) in the maximum likelihood and Bayesian inference, respectively.
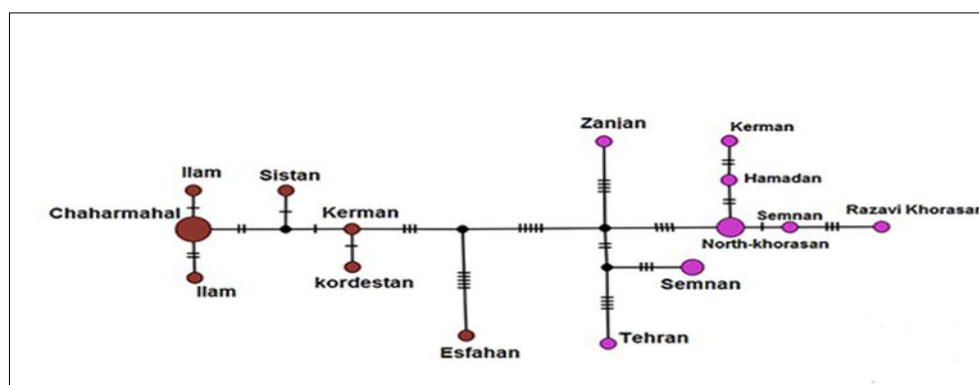
Fig. 4. Haplotype network for Iranian wildcats based on 1,954 bp of the mtDNA two genes (Cyt b and NADH5). Distinct groups were identified using different colored dots; *F. lybica ornata* (purple, G1) and *F. lybica lybica* (brown, G2).


Fig. 5. Phylogenetic relationships (median joining network) of Iranian wildcats and other wildcat/domestic cats based on 1,022 bp of the mtDNA two genes (Cyt b and NADH5). Distinct subspecies were identified using different colored dots: *F. silvestris* (green), *F. lybica cafra* (blue), *F. lybica ornata* (purple) and *F. lybica lybica/ F. catus* (brown). Light purple and dark brown represent Iranian haplotypes. The size of the dots reveals the number of samples in the haplotype.

DISCUSSION

Phylogenetic analyses of wildcats in Iran using concatenated two mtDNA genes including cytochrome b (Cyt b) and NADH5 as well as indicating divergence time for identified subclades of wildcat in Iran were the main objectives of this study. Findings suggested that Iranian samples were placed within two subclades i.e. African or Near Eastern wildcat, *F. lybica lybica* and Asiatic wildcat, *F. lybica ornata* and two subclades diverged from each other 340,000 years ago. Current study emphasized the fact that the phylogeographic analysis using Cyt b gene, in Mousavi *et al*. (2019) is now further supported by this new molecular data (concatenated two genes, NADH5 and Cyt b). Meanwhile, topology of the molecular clock phylogenetic tree is supported by that of O'Brien *et al*. (2008), Wardelin *et al*. (2010); Nyakatura & Bininda-Emonds (2012), that performed the whole genome of cat species in the GenBank to study the divergence times of extant wild felids. Archaeological and genetic evidence suggest that all domestic cats derived from the Near Eastern populations of wildcat, *F. lybica lybica* (Pierpaoli *et al*. 2003; O'Brien *et al*. 2009; Driscoll *et al*. 2007; Ottoni *et al*. 2017). There is a monophyletic condition of the domestic cat, *F. catus* and the African Wildcat sequences, *F. l. lybica*. Thus sequences of domestic cats from GenBank were assigned to the subclade of African wildcats together with the Iranian samples and probable domestic cat's specimens in this study were not recognized using this method as well. Given introgression between wildcat and domestic cat, in molecular research of this species, simultaneous use of mitochondrial and nuclear markers will provide more comparable and reliable outputs that could be considered in further works. The estimated coalescent date based on the mtDNA data for all *F. silvestris* (including *F. s. bieti*) subspecies is 230,000 years ago in Driscoll *et al*. (2007), whereas in present study this date is around 430,000 years ago, additionally in Leopard Cat lineage the first diverged species is Pallas's cat, *Otocolobus manul* in (~ 4.03 MYA) as well as two lineages, Domestic Cat and Leopard Cat in (~4.74 MYA) had a common ancestor. However, O'Brien *et al*. (2008)

suggests Domestic Cat and Leopard Cat lineages are two recent lineages that diverged in 6.2 MYA. We hypothesized that Central Asia is the origin area for distribution of *Felis* genus (Domestic Cat lineage). About 3.5 MYA common ancestor of wildcat, *F. silvestris/F. lybica* and Jungle cat, *Felis chaus* separated from black footed cat, *Felis nigripes*. We concluded that the divergence of wildcat, *F. silvestris/F. lybica* from the ancestor of Jungle cat, *Felis chaus* occurred in the Central Asia at the beginning of Pleistocene period about 2.5 MYA, the ancestor of wildcat, *F. silvestris/F. lybica* then moved out of Central Asia towards western Asia, Africa and Europe. Although according to Yamaguchi *et al.* (2004) the modern wildcat, *F. silvestris* probably descended from Martelli's wild cat, *F. (s.) lunensis* Martelli, 1906 which is known from Europe and may date back to as early as the late Pliocene ca. 2 MYA. Estimated divergence times of less than 1 MYA among wildcat subspecies in current study, suggests that Pleistocene climatic changes may have caused to diversification, on the other hand, distribution of wildcat in Central Asia, western Asia, Africa and Europe is affected by alternating series of glacial and interglacial periods in Pleistocene.
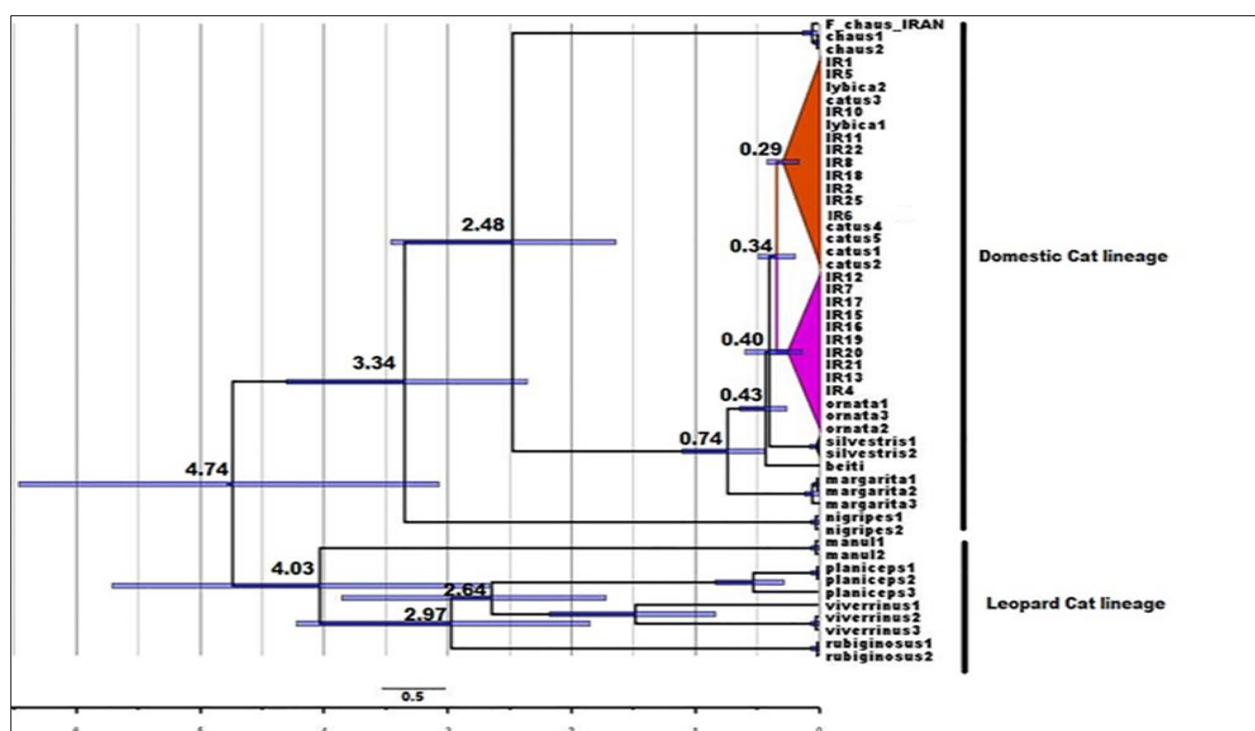
Fig. 6. Phylogenetic relationships and divergence time estimate within Domestic Cat and Leopard Cat lineages. Nodes are individually numbered, with node bars indicating 95% confidence intervals on the divergence time estimates (in million years ago: MYA). Brown branch is *F. l. lybica* and purple is *F. l. ornata*; two subclades of the African wildcat.

Based on the phylogenetic results, our analyses revealed that wildcat populations are divided into two main subclades in Iran, the first subclade (G1) belonged to the Asian wildcat, *F. lybica ornata* and the second subclade (G2) included African wildcat, *F. lybica lybica* and domestic cat, *F. catus*. Two groups; Asian wildcat, *F. lybica ornata* and African wildcat, *F. lybica lybica* separated from each other ca. 0.34 MYA. In some semi-desert areas in the center of Iran both subclades exist. However, it seems desert areas are big barrier to distribution of these subclades in the center of Iran (Lut and Markazi deserts). Most probably Iranian terrain is contact zone for two proposed subclades, though our results did not show shared haplotype between them.

CONCLUSION

In conclusion, diversification of the Asiatic wildcat, *F. lybica ornata* and African wildcat, *F. lybica lybica* across its current range can potentially be attributed to Pleistocene glacial-interglacial cycles. Domestic cats are widespread throughout the whole range of the wildcat, therefore, the risk of continuous hybridization due to overlapping distribution of wildcat and domestic cats may lead to changing genetic structure of the wildcat's populations (Beaumont *et al*. 2001; Pierpaoli *et al*. 2003). For the purpose of discriminating wildcat from domestic cat using morphological features in the study area, providing a protocol that helps us to identify wild samples from domestic cats or hybrids in the field is necessary. More extensive sampling as well as the use of nuclear

genome data will increase our knowledge about the taxonomic status and genetic introgression of wildcat in Iran. To improve management of the wildcat in the study area, we have to increase our knowledge about ecological requirements of the species, the status and the rate of hybridization. Continued rigorous monitoring and robust systematic filed surveys in the large and isolated protected areas that cover pure wildcats is also recommended.

ACKNOWLEDGEMENTS

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Supplementary Table Sl: Sequences were retrieved from GenBank database based on 1,022 bp of Cyt b and NADH5 genes.

No.	Accession number GenBank	subspecies	No.	Accession number in GenBank	Subspecies
1	EF587177	*F. silvestris*	29	EF587026	*F. l. ornata*
2	EF587176	*F. silvestris*	30	EF587032	*F. l. ornata*
3	EF587175	*F. silvestris*	31	EF587089	*F. catus*
4	EF587174	*F. silvestris*	32	EF587111	*F. catus*
5	EF587173	*F. silvestris*	33	EF587040	*F. catus*
6	EF587172	*F. silvestris*	34	EF587042	*F. catus*
7	EF587171	*F. silvestris*	35	EF587119	*F. catus*
8	EF587170	*F. silvestris*	36	EF587087	*F. catus*
9	EF587169	*F. silvestris*	37	EF587121	*F. catus*
10	EF587168	*F. silvestris*	38	EF587123	*F. catus*
11	EF587167	*F. silvestris*	39	EF587128	*F. silvestris/lybica*
12	EF587166	*F. silvestris*	40	EF587134	*F. silvestris/lybica*
13	EF587165	*F. silvestris*	41	EF587144	*F. silvestris/lybica*
14	EF587164	*F. silvestris*	42	EF587145	*F. silvestris/lybica*

15	EF587163	*F. silvestris*	43	EF587060	*F. silvestris/lybica*
16	EF587162	*F. silvestris*	44	EF587141	*F. silvestris/lybica*
17	MG813967	*F. l. lybica*	45	EF587146	*F. silvestris/lybica*
18	MG813966	*F. l. lybica*	46	EF587036	*F. margarita*
19	MG813961	*F. l. lybica*	47	EF587035	*F. margarita*
20	MG813960	*F. l. lybica*	48	EF587034	*F. margarita*
21	KP202275	*F. l. lybica*	49	EF587033	*F. margarita*
22	EF587018	*F. l. cafra*			
23	EF587016	*F. l. cafra*			
24	EF587025	*F. l. cafra*			
25	EF587031	*F. l. ornata*			
26	EF587030	F. l. ornata			
27	EF587029	F. l. ornata			
28	EF587028	F. l. ornata			

Supplementary Table S2: Sequences were retrieved from GenBank database (Cyt b gene) for plotting Dated Bayesian tree.

No.	Accession number GenBank	subspecies	No.	Accession number GenBank	Subspecies
1	U20753.1	*Felis catus*	20	NC_028309	*Felis nigripes*
2	NC_001700	*Felis catus*	21	KP202277	*Felis nigripes*
3	AB194812	*Felis catus*	22	NC_028323	*Otocolobus manul*
4	KU253483	*Felis catus*	23	KP202295	*Otocolobus manul*
5	KU253482	*Felis catus*	24	KR135742	*Prionailurus viverrinus*
6	KP202273	*Felis beiti*	25	KP202270	*Prionailurus viverrinus*
7	KP202275	*Felis lybica lybica*	26	NC_ 028305.1	*Prionailurus viverrinus*
8	MG813966	*Felis lybica lybica*	27	KY682741	*Prionailurus planiceps*
9	EF587029	*Felis lybica ornata*	28	KY682742	*Prionailurus planiceps*
10	EF587031	*Felis lybica ornata*	29	KY682747	*Prionailurus planiceps*
11	EF587028	*Felis lybica ornata*	30	NC_028304	*Prionailurus rubiginosus*
12	NC_028310	*Felis silvestris*	31	KP202266	*Prionailurus rubiginosus*
13	KP202278	*Felis silvestris*			
14	KP202276	*Felis margarita*			
15	KR132580	*Felis margarita*			
16	NC_028308	*Felis margarita*			
17	MN175473	*Felis chaus*			
18	KP202274	*Felis chaus*			
19	NC_028307	*Felis chaus*			