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Detecting Hybridization Between Iranian Wild Wolf (*Canis lupus pallipes*) and Free-ranging Domestic Dog (*Canis familiaris*) by Analysis of Microsatellite Markers

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The genetic threat due to hybridization with free-ranging dogs is one major concern in wolf conservation. The identification of hybrids and extent of hybridization is important in the conservation and management of wolf populations. Genetic variation was analyzed at 15 unlinked loci in 28 dogs, 28 wolves, four known hybrids, two black wolves, and one dog with abnormal traits in Iran. Pritchard's model, multivariate ordination by principal component analysis and neighbor joining clustering were used for population clustering and individual assignment. Analysis of genetic variation showed that genetic variability is high in both wolf and dog populations in Iran. Values of H_E in dog and wolf samples ranged from 0.75–0.92 and 0.77–0.92, respectively. The results of AMOVA showed that the two groups of dog and wolf were significantly different ($F_{ST} = 0.05$ and $R_{ST} = 0.36$; $P < 0.001$). In each of the three methods, wolf and dog samples were separated into two distinct clusters. Two dark wolves were assigned to the wolf cluster. Also these models detected D32 (dog with abnormal traits) and some other samples, which were assigned to more than one cluster and could be a hybrid. This study is the beginning of a genetic study in wolf populations in Iran, and our results reveal that as in other countries, hybridization between wolves and dogs is sporadic in Iran and can be a threat to wolf populations if human perturbations increase.

Key words: Pritchard's model, wolf, neighbor joining, PCA, genetic distance, *Canis lupus*

INTRODUCTION

The introgression of domestic genes into gene pool of wild wolves and the threats to the integrity of the gene pool of this species due to crossbreeding with free-ranging dogs (Boitani, 1984; Gottelli et al., 1994) could be a conservation problem in human-perturbed populations of wolves. Since wolves and dogs are closely related species and are isokaryotypic, they are able to mate successfully and produce fertile offspring in habitats in which they co-occur (Wayne et al., 1995). Hybridization between gray wolves and dogs is such a rare event that is hard to derive the level of concern (Vilà et al., 1999). There are occasional crossbreeding events between wolves and dogs in nature, as observed in Russia (Bibikov, 1985), Ukraine (Galaka, 1969), Latvia (Andersone et al., 2002), and Italy (Randi et al., 2002; Randi and Lucchini, 2002; Verardi et al., 2006; Lacolina et al., 2010).

Several authors have suggested that it is more likely that male dogs interbreed with female gray wolves (Vilà and Wayne, 1999). Such hybrids would not have been detected by genetic study using mtDNA makers (Vilà, 1998). Hybridization between these two species was reported mostly by microsatellite markers (Roy et al., 1994; Andersone et al., 2002; Vilà et al., 2003). The microsatellite markers are usually variable enough to be used for the identification of individual hybrids in mammalian populations (Paetkau et al., 1998; Randi and Lucchini, 2002; Lacolina et al., 2010; Verardi et al., 2006; Andersone et al., 2002).

The development of Bayesian methods helps to conduct simulations that are used in the identification and adscription of genetic profiles to different clusters. Hardy–Weinberg (HWE) and linkage equilibrium (LE) among different loci are the most important assumptions of Pritchard models (Pritchard et al., 2000).

The gray wolf is widely distributed in most parts of Iran (Ziaei, 2009; Fig. 1). There is no reliable estimation on wolf population size in Iran. Large habitat diversity and presence of this species in different habitats on the other hand, has caused variations in morphological traits, such as color and body size in different habitats. Habitat destruction, uncon-

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Fig. 1. Locations where wolf samples were collected. The darker areas indicate mountainous regions. Wolf is distributed in all over the country, except the true deserts.

trolled hunting, and decrease in the number of natural prey are the most important factors that threaten this species in Iran and eventually cause the Iranian wolf population to suffer severe persecution in some habitats in recent years. A factor that could play an important role in the decrease of the number of wolves is the negative attitude of the people in rural areas towards wolves due to livestock losses, mainly where no prevention methods are implemented to reduce attacks.

The uncontrolled presence of dogs, not just those associated with humanized areas, is generally increasing in Iran. This appears to raise the risk of mating between wolves and dogs in many environments. Anomalous morphological traits such as the presence of completely dark wolves, in addition to the increase in wolf attacks to natives, are likely causes of mixing this species with domestic or feral dogs. Wolf-dog hybrids tend to exhibit synanthropic behaviors (Bibikov, 1985), especially when they could be dependent on human anthropogenic resources. This could have negative effects on the long-term conservation of wolves and public attitudes. In this study we analyzed 15 microsatellite loci to estimate the extent of genetic differentiation between wolves and dogs. To determine genetic structure and individual assignments based on the autosomal microsatellite data set, three different approaches were used: (1) Bayesian clustering assignments by assuming that all the samples may belong to a single indistinct population independently of any prior classification by Bayesian clustering and admixture model in STRUCTURE programs version 2.3.3 (Pritchard et al., 2000; Falush et al., 2003), (2) multivariate ordination method by principal component analysis (PCA) in PCAGEN, and (3) clustering of individuals by inter-individual genetic distances to construct neighbor-joining trees.

MATERIALS AND METHODS

Sample collection, DNA extraction and microsatellite genotyping

A total of 28 wolf tissue samples were collected from dead-found wolves, which had been killed accidentally on roads or were

illegally shot by natives across the entire species range in Iran (Fig. 1). Dog tissue samples (28) were obtained from feral individuals in western and central Iran, where they are sympatric with wolves. Also, four known wolf-dog hybrids (with identification nos H20, H21, H22, H24) were obtained in captivity in west part of Iran (Hamedan and Zanjan). These four known hybrids were crossed at home. We analyzed two unusual dark wolves (W4 and W7) in Hamedan and Zanjan, as well as a dog that was shot in the wild in Zanjan province and showed anomalous traits (D 32). We divided these samples into three groups according to their origins and morphological traits, namely, (1) wild-living Iranian wolves (labeled W throughout the text), (2) dogs, (labeled D), and (3) hybrids (labeled H).

Total DNA was extracted by AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea). Fifteen microsatellites were selected for their polymorphism and reliable scorability in wolves and dogs (Randi et al., 2002; Anderson et al., 2002; Verardi et al., 2006). Polymerase chain reaction (PCR) amplification of 15 microsatellite loci were performed in 12 µl volumes by AccuPower® PCR PerMix kit (Bioneer, South Korea) by using a Perkin Elmer 9600 thermal cycler. PCR was carried out through following steps: initial denaturation of five minutes at 95°C, 30 seconds at 95°C, 30 seconds at 55–64°C, one minute at 72°C, and a final extension of five minutes at 72°C. Cycling conditions were optimized for each primer pair. PCR products were separated on 8% polyacrylamide gels, visualized with silver-staining, and were photographed using the molecular imager® Gel Doc™ XR system. Images were analyzed with the Gel-Pro Analyzer 6 Software.

Analysis of genetic variation

The distribution of allele frequency distributions, presence of private alleles, values of observed (H_o) and expected heterozygosity (H_e), pairwise estimates of F_{ST} (Weir and Cockerham, 1984) and R_{ST} (Slatkin, 1995), coefficient of inbreeding (F_{IS} ; Weir and Cockerham, 1984) and difference in allele and genotype frequency distribution (Fisher's exact probability test; Raymond and Rousset, 1995) were computed separately for the Iranian wolves and dogs using the GENEPOP 4.1 program (Raymond and Rousset, 1995).

Differences in the number of alleles and heterozygosity between wolves and dogs were tested using t-test and Wilcoxon signed rank test, respectively. Estimators of F_{ST} , R_{ST} and F_{IS} can be used to test the deficit of heterozygosity and the extent of divergence among and within populations respectively (F_{ST} , R_{ST} and F_{IS} significantly > 0). Deviations from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium (LE) between loci pairs were tested for all locus-population combinations, using the Markov chain method (Guo and Thompson, 1992), as implemented in GENEPOP. Significance levels were adjusted using the sequential Bonferroni method to take into account multiple tests on the same data set (Rice, 1989). Differentiation and the extent of divergence between wolf and dog populations were estimated by using Analysis of Molecular Variance analysis (AMOVA; Excoffier et al., 1992) as implemented in ARLEQUIN 3.5.1.2 using F_{ST} and R_{ST} . For AMOVA we determined the significant deviation of F_{ST} and R_{ST} from zero by permuting the data 999 times.

Genotype assignment

ARLEQUIN was used to compute the log likelihood of the genotype of each individual in each sample (dog and wolf) assuming that each individual comes from a population having allele frequencies equal to those estimated for each sample (Waser and Strobeck, 1998). Log-likelihood of each individual in two populations was used to represent log-log plots for each pair of populations.

Bayesian clustering assignment

Admixture model (Pritchard et al., 2000) using unlinked loci and I model (with five repetitions of 10^5 iterations following a burn-in period of 10^4 iterations) were used to identify the number of genetically distinct clusters that maximize the likelihood of the data ($\ln P(D)$), and to assign the individuals to the inferred clusters in STRUCTURE 2.3.3 (MAXPOPS = 1–5; USEPOPIINFO = 0). For the selected K values, the average proportion of membership (Q_i) of the sampled populations to the inferred clusters was assessed. Value of $Q > 0.8$ was considered for the assignment of individual genomes to one cluster, and $Q < 0.8$ was considered when individuals were admixed and there was no single assignment (Verardi et al., 2006, Randi and Lucchini, 2002).

Neighbor-joining clustering

POPULATION 1.2.32 was used to calculate Cavalli-Sforza and Edwards (Dc; 1967) and Goldstein (d_{mu}^2 ; Goldstein et al., 1995) inter-individual pairwise genetic distances averaged over loci. The neighbor-joining (NJ) algorithm (Saitou and Nei, 1987) was employed to cluster each set of genetic distances. A distance matrix was constructed with the Neighbor in PHYLIP 3.69 and POPULATION programs (<http://softlinks.amnh.org/microsatellites>).

Multivariate ordination by principal component analysis (PCA)

To visualize the inter-population differences between wolf and dog, principal component analysis (PCA) was performed based on microsatellite allele frequencies in PCAGEN1.2 (Goudet, 1995). PCA clusters individuals only on the basis of their genotypes and makes no assumptions regarding Hardy–Weinberg equilibrium or linkage equilibrium (Rutledge, 2010). Each individual was scored as 0.0 (the allele was not observed), 0.5 (the individual was heterozygote), or 1.0 (the individual was homozygote). Then, the data was ordinated in a multidimensional space by PCA using PCAGEN.

RESULTS

Analysis of genetic variation

All microsatellites were polymorphic showing five (locus CPH22 in dogs) and 17 (CPH8 in wolves) allele per locus with an overall average of nine alleles per locus. The number of alleles was not significantly different between wolves and dogs ($P > 0.05$). The number of private alleles varied between two populations (34 in wolves and 14 in dogs). Values of H_E and H_O in total samples were 0.75–0.92 and 0.28–1.0, respectively (Table 1). The results of the Wilcoxon signed rank test showed that H_O and H_E were not significantly different between wolves and dogs ($P > 0.05$).

The results of Fisher's exact probability test showed that allele frequency distribution was significantly different between two groups ($P < 0.05$) except CPH2, CPH6, CPH8, and CPH9. Also genotype frequency distribution was significantly different between wolves and dogs except CPH2 and CPH6. Significant departure from HWE ($P < 0.001$) can be due to heterozygote excess, population substructuring (Wahlund's effect), and a high mutation rate in microsatellite DNA (Aminafshar et al., 2008). Linkage disequilibrium was estimated in a total of 105 locus combinations in each wolf and dog samples. The number of significant comparisons ($P < 0.05$) before Bonferroni correction in wolf and dog sample groups were $10/105 = 9.5\%$ and $11/105 = 10.5\%$, respectively. After sequential Bonferroni correction, the occurrence of significant comparisons decreased in both sample groups and were $1/105 = 0.95\%$ and $2/105 = 1.9\%$ in dog and wolf groups, respectively. Average F_{IS} was low in both dogs and wolves samples ($F_{IS} = -0.122$ and $F_{IS} = -0.148$, respec-

Table 1. Summary of allelic variation in wolves and dogs genotyped at 15 microsatellite loci.

Locus ID	Alleles	Allele size	H_E in wolves	H_E in dogs	H_O in wolves	H_O in dogs
CPH2	9	91–115	0.82	0.78	1	1
CPH3	13	153–198	0.85	0.85	1	1
CPH4	13	107–169	0.88	0.82	1	1
CPH5	9	110–141	0.81	0.82	1	1
CPH6	12	111–154	0.88	0.85	1	1
CPH7	16	155–218	0.89	0.89	1	1
CPH8	17	201–262	0.92	0.89	1	1
CPH9	13	142–185	0.85	0.89	1	1
CPH12	15	181–239	0.85	0.85	1	1
CPH16	13	152–200	0.89	0.75	1	1
CPH22	7	106–136	0.82	0.75	1	1
FH2004	15	139–269	0.77	0.82	1	0.28
CXX.213	16	135–197	0.85	0.92	1	1
C20.253	9	122–172	0.85	0.82	1	1
C09.250	11	99–133	0.84	0.82	0.84	1

Microsatellite ID is indicated according to Mellersh et al. (1997).

tively). Microsatellite variability was significantly partitioned between wolves and dogs ($F_{ST} = 0.05$ and $R_{ST} = 0.36$; $P < 0.001$; AMOVA). The allele frequency distributions varied across loci and showed differences between wolves and dogs (Fig. 2).

Assignment of individual genotype to population

The log-likelihood of the individual samples in dogs and wolves is plotted in Fig. 3. The separation of wolves and dogs into the two clusters suggests that 15 loci are sufficient to provide a clear-cut separation between wolf and dog populations.

Bayesian clustering assignment

The number of (K) for all samples was estimated by running STRUCTURE 2.1 with $K = 1–5$. Results of this analysis are shown in Table 2. Values of $\ln P(D)$ were minimum for $K = 5$ ($\ln = -4773.3$), and maximum for $K = 3$ ($\ln = -3914.9$). These results indicate that all samples (wolves, dogs and hybrids) may contain three genetically distinct clusters. By using two inferred clusters, the (Q_i) of each predefined group (wolves, dog and hybrid) into two clusters were estimated. Cluster I and II included all dogs ($q = 0.893$) and all wolves ($q = 0.927$). Pritchard's model at 15 unlinked microsatellites showed that wolf and dog were split into two distinct clusters with average $Q > 0.80$. But four known hybrids samples (H20, H21, H22, and H24) were intermediate between two groups and therefore, assigned to the wolf and dog clusters with average $q_I = 0.427$ and $q_{II} = 0.572$, respectively. With $k=2$, two wolves with dark coat color (W4 and W7) were also assigned to wolves group (cluster I). But D32 was partially assigned to both clusters with $Q_I = 0.251$ and $Q_{II} = 0.749$. There were also two samples (D30 and D31) that were partially assigned to both clusters with $q_i < 0.80$. Figure 4 shows bar plotting of the results obtained from STRUCTURE using $K = 2$ and 3 with the I-model (Table 3).

In the second approach the individuals were assigned into three predefined groups by assuming $K = 3$ and I-model. We also estimated the value of membership of three

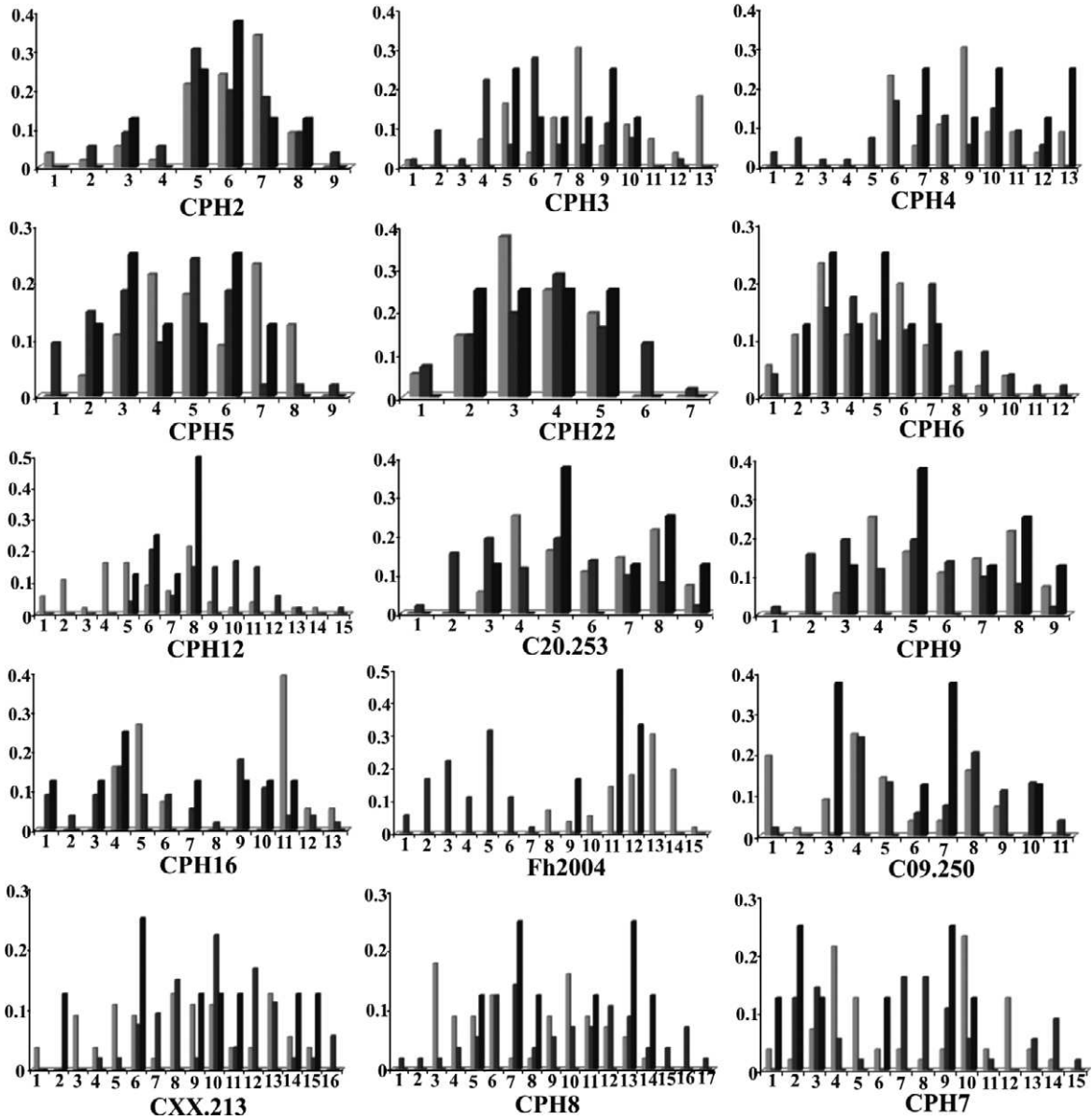


Fig. 2. Distribution of allele frequencies at 15 microsatellite loci in wild-living Iranian wolves (gray bars), dogs (bright gray bars), and hybrids (black bars).

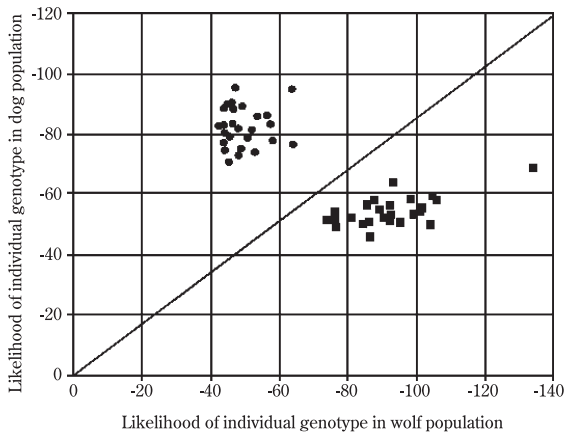


Fig. 3. Log likelihood plot of genotype assignment for dogs (circles) and wolves (squares).

Table 2. Ln P(D) data in different K for dog and wolf samples.

Number of K	Ln P (D) data
1	-4093.4
2	-3928.8
3	-3914.9
4	-4624.5
5	-4773.3

Table 3. Proportion of membership of each pre-defined population in two inferred clusters.

Predefined group	K = 2	
	1	2
Dog	0.893	0.107
Wolf	0.072	0.927
Hybrid	0.427	0.572

Table 4. Proportion of membership of each pre-defined population and some samples in three inferred clusters (K = 3).

Sample	K = 3		
	1	2	3
Dogs		Qi > 0.8	
Wolves	Qi > 0.8		
Hybrids			Qi = 0.771
D30	0.013	0.027	0.960
D31	0.039	0.242	0.719
W5	0.811	0.059	0.130
W12	0.810	0.070	0.120
W4	0.931	0.028	0.041
W7	0.818	0.016	0.166
D32	0.041	0.028	0.931

samples that were assigned to both clusters in the previous approach (K = 2) (Table 4). The results of this approach showed that all dogs and wolves were assigned to cluster I and II (with $Q_i > 0.08$), respectively. Also with K = 3 four hybrid samples were assigned in cluster III with $Q_i > 0.771$. Two wolves with black coat color (W4 and W7) which were placed in wolves cluster in K = 2, were also assigned to wolves cluster with $Q_i > 0.80$ even when K = 3. D32, which were assigned to cluster III with K = 2, and D30 and D31, which were partially assigned to both clusters with K = 2, were placed in cluster III when K = 3. Therefore these samples could be hybrids.

Neighbor-joining clustering

Neighbor-joining cluster analysis of the two studied populations revealed that the wolf samples were separated from the dog group. The NJ tree clustering using Cavalli-Sforza (Dc) is shown in Fig. 5. Iranian wolves and dogs were split into two distinct clusters. Three (H20, H21, and H22) of the four known hybrids were intermediate between wolves and dogs, but H24 was included in wolf samples. W4 and W7 were also included within the wolf cluster. But D32 was located intermediate between two groups. As well as the W13, D10, D30, and D31 were intermediate between two groups. NJ dendrogram based on Goldstein inter-individual genetic distance (d_{mu^2}) is shown in Fig. 6.

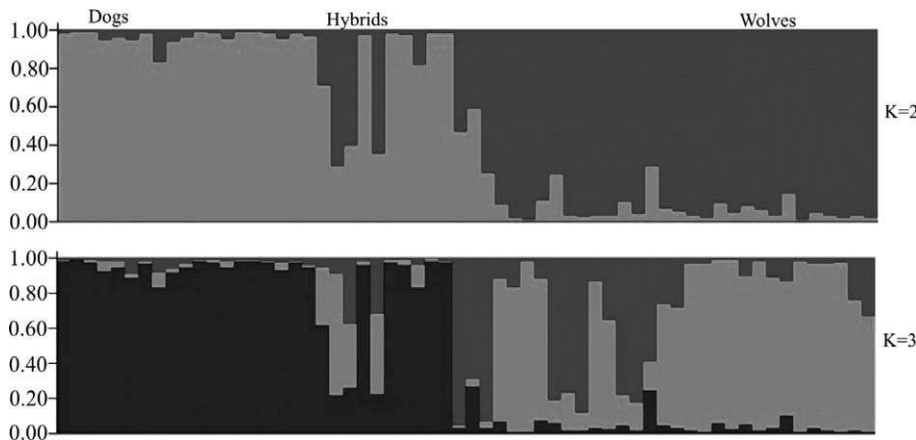


Fig. 4. Bar plotting of the results obtained from structure using K = 2 and 3 with the I-model.

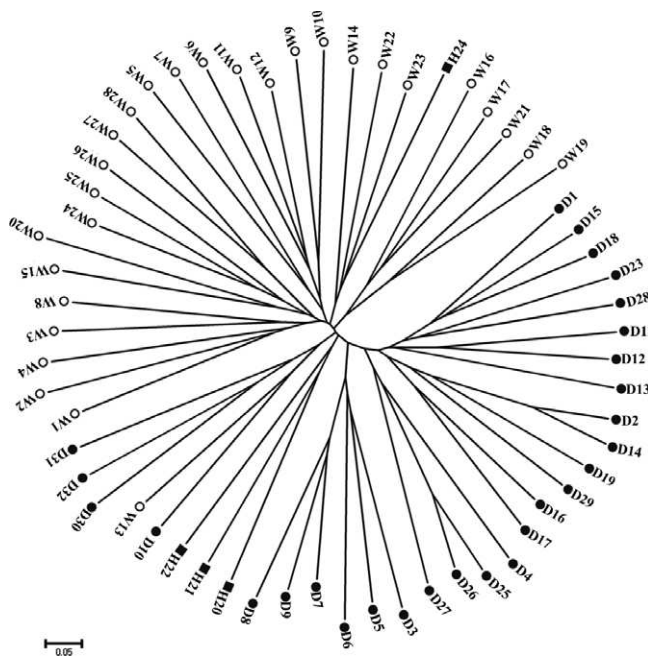


Fig. 5. Network of dog and wolf samples computed using Dc distances and neighbor-joining algorithm (wolves: white circles, dogs: gray circles, hybrids: squares).

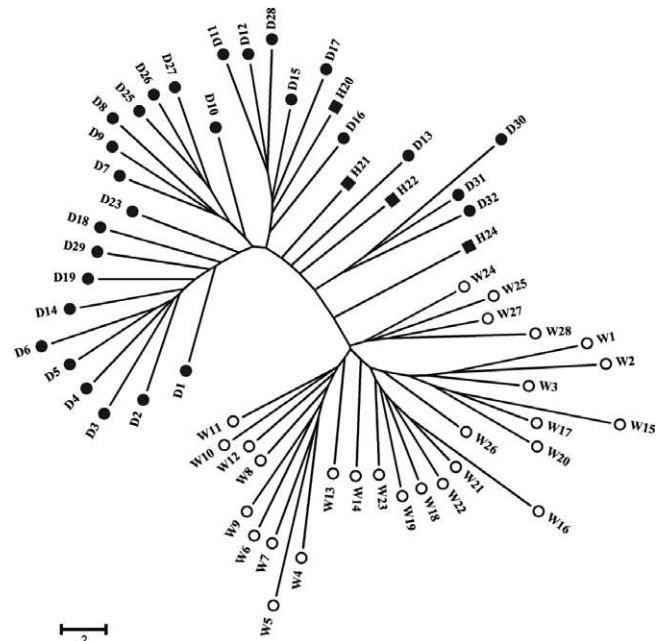


Fig. 6. Network of dog and wolf samples computed using d_{mu^2} distances and neighbor-joining algorithm (Wolves: white circles, Dogs: gray circles, Hybrids: squares).

The results of this genetic distance were similar to the dendrogram obtained with individual genotypes based on Dc distance matrix, except for W13, D13, and D10. W13 and D10 in Dc distance matrix had been placed in the intermediate location between two groups, whereas they were included in wolf and dog samples, respectively, in dmu^2 methods. But, D13, which had been placed in the dog sample in DC method, was placed intermediate between the dog and the wolf groups in dmu^2 distances. The results of grouping samples based on inter-individual genetic distance showed that wolves and dogs remain genetically distinct and individuals with hybrid origin were placed intermediate between wolf and dog populations.

Multivariate ordination by principal component analysis (PCA)

The results of ordination by PCA of the wolves and dogs genotypes are shown in Fig. 7. The First two principal axes (PC-I and PC-II) cumulatively explain 18% of the total genetic diversity. The assessment by PCA indicated that wolves and dogs were separated into two groups. All the hybrids (H20, H21, H22, and H24) were intermediate between wolves and dogs. W4 and W7 were included within the distribution of wolves like neighbor-joining clustering and Bayesian clustering, while D32 was completely intermediate between the wolves and the dogs clusters. Also some feral dogs and wolves (D30, D31, and W12) were intermediate between wolves and dogs. Therefore, excluding prior information on the origin of individual samples, PCA would have correctly identified wild-living wolves and dogs as belonging to two clearly distinct groups. Although D5, D6, and D8 which had been grouped under dog samples in NJ clustering, in PCA ordination were placed intermediate between the wolf and the dog samples. Therefore, it can be said that placing these sample clustered apart in the lower right side of dogs distribution could be due to geographical distances, but not hybridization.

DISCUSSION

Due to physiological, ethological, and mating differences in wolves and dogs, as well as directional hybridization (male dog cross with female wolf), hybridization between the two species is sporadic and does not appear to be a threat to the genetic composition of wolf populations (Vilà et al., 1997; Vilà and Wayne, 1998; Randi and Lucchini, 2002). Our results revalidate the concept that despite of occasional crossbreeding between wolves and dogs in western Iran, these species remain genetically distinct. Vilà (1999) and Boitani (1995) suggested that since male dogs provide limited care, high pup mortality in feral dog's offspring reduces the probability of survival of offspring and backcrossing rates of first generation hybrids. Directional hybridization between wolves and dogs would not have been detected in genetic surveys using mtDNA markers and was documented mainly by microsatellite markers (Roy et al., 1994; Randi and Lucchini, 2002; Verardi, 2006). mtDNA analysis provides information about maternal lineage, and may help facilitate the assignment of uncertain individuals as second

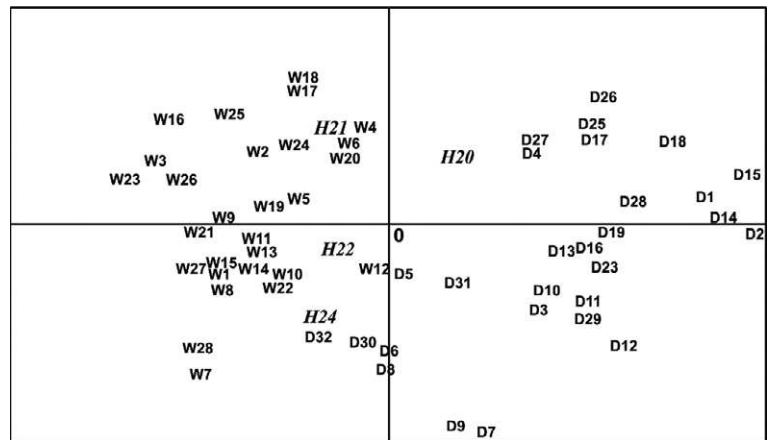


Fig. 7. First and second components of a principal components analysis (PCA) of 15-locus microsatellite genotypes (Wolves: W, Dogs: D, Hybrids: H).

generation hybrids.

Recent development in identifying hybrids among species that are closely related based on allelic distributions in the parent populations offers new methods for detecting extent of introgression of domestic gene to wild populations (Paetkau et al., 1995; Pritchard et al., 2000). In this study we used morphological traits to pre-classified wolf and dog samples and classified all samples into three groups (wolves, dogs and hybrids). We also analyzed two wolves with black coat color (W4 and W7), and one dog with anomalous traits (D32) in the western part of Iran. The black phenotype is not considered as a typical characteristic in Iranian wolves, as it has never been reported in the past, but it has been considered a sign of crossbreeding with free-ranging dogs. Nevertheless, Anderson et al. (2009) has showed that modern American black wolves DNA carry the evidence of an ancient introgression of dog genes, but this does not imply that they are hybrids or that have a recent hybrid ancestor.

Departure from HWE in studied loci can be due to heterozygote excess, high mutation rate in microsatellites (Aminafshar et al., 2008), and population substructuring (Wahlund's effect) due to active home range defense and resistance against intruding dispersers from outside the packs in wolves. Wolf samples in this study were selected across the entire wolf distribution range in Iran, and they could include genetically distinct subpopulations. Genetic differentiation between wolves and dogs is surveyed by values of $R_{ST} = 0.36$ and $F_{ST} = 0.05$. The consequences of domestication and demographic fluctuations have led dogs and wolves to diverge significantly in their microsatellite allele frequencies (Randi and Lucchini, 2002). Taking into account the limited sample size in this study, the large number of private alleles (34 in wolves and 14 in dogs) could indicate that there has been little gene introgression of domestic dogs to wild wolves, high genetic diversity in both populations, and geographically well separated populations within each species.

PCA, NJ and Bayesian methods clustered dogs and wolves into two separate groups. But there were some samples in dogs and wolves that were assigned differently from the prior phenotypic classification and were intermediate

between wolves and dogs. Two wolves showing black coat color (W4 and W7) were placed in wolf cluster, while D32 was intermediate between wolves and dogs in PCA and NJ and Bayesian methods. Samples with intermediate positions may have admixed ancestry.

These findings show that black color has not been considered a possible sign of crossbreeding with the domestic dog and may have derived from a natural combination of wolf alleles or the effect of ecological factor and adaptation to habitat conditions. The amount of gene flow and genetic structure in a population is not only explained by geographical limitations and historical events, but also is influenced by ecological processes (Sponer and Roy, 2002; Spinks and Shaffer, 2005). The high mobility of wolves could reduce the effect of topographic factors and historical events on gene flow between individuals in different habitats and thus the effect of ecological factors may be important for understanding the genetic and morphological differences that could occur in different regions (Pilot et al., 2006). Morphological differences between species or between individuals in different regions can be due to changes in developmental processes, different functional roles, and responses to selective pressures (Barrow and Macleod, 2008; Zelditch et al., 2004).

The Alborz and Zagros Mountains act as a natural barrier for humidity, creating cold and mountainous forest wolf habitats in northern and western Iran. The lowlands of south-central Iran are warmer and less forested, suggesting that changes in habitat factors could contribute to some morphological changes in those different ecoregions. In fact, instances of black coat color wolves may be attributable to a combination of various factors that could play an important role in morphological differences. These factors include geographical, ecological, historical factors, evolutionary processes in a long-term perspective, and point mutations.

Different factors might have affected extent of hybridization between wolves and dogs in Iran. One of these factors might be the large number of free-ranging dogs in western Iran. Intensive agriculture and high density of residential areas around wolf habitat areas in the west provide habitat conditions that are favorable for dogs. Most of these dogs are free-ranging dogs belonging to farmers. Native farmers and dog owners are not aware of the possible consequences of keeping free-ranging dogs. Unfortunately a common practice among natives in the western Iran is keeping wolf pups or wolf-dog hybrids at home. People consider a wolf-dog to be an aggressive guard dog. Farmers let these individuals roam freely near their farms. These individuals might mate with wild living wolves and threaten the integrity of the wolf genome. However, the most effective way of solving the problems related to uncontrolled dogs is by influencing people's attitudes toward dog-keeping, and reducing access to human anthropogenic resources (garbage, etc.). Also, in recent years due to increasing conflict between wolves and dogs, intensive wolf hunting occurred that could also favor crossbreeding with dogs. Such a severe hunting pressure causes a sharp population decline and therefore extent of crossbreeding could be higher in wolf populations at low density (Randi and Lucchini, 2001). Overhunted, isolated and low-density wolf populations that coexist with a larger number of domestic dogs are at higher risk of hybrid-

ization. If their social structure is disrupted, wolves can crossbreed with free-ranging dogs (Boitani, 1983; Bibikov, 1985; Anderson et al., 2002).

Finally, Bayesian admixture analysis showed that Iranian wolves and dogs are significantly differentiated and individual genotypes can be assigned to the two groups and this model allows detection of individuals with admixture ancestry. These methods would help to survey the extent of introgression of domestic gene in wild-wolf population and conservation strategies for this species. Despite the distribution of gray wolves in most parts of Iran, wolves are threatened throughout most of their range due to habitat destruction, uncontrolled hunting, loss of natural prey populations, and conflict with livestock and hybridization with dogs. A long-term study of wolf-dog hybridization is needed to understand the extent of introgression of domestic gene to wild wolf population better, and therefore, larger sample sizes of both wolves and genes should be analyzed to obtain accurate estimates of the rate of crossbreeding and the identification of second-generation hybrids. DNA mitochondrial analysis could help us to improve the assignment of the maternal lineage of uncertain individuals.

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