

# A preliminary phylogenetic analysis of golden jackals (*Canis aureus*) (Canidae: Carnivora: Mammalia) from Turkey based on mitochondrial D-loop sequences

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## Abstract

In the present study, partial sequences (439 bp) of mitochondrial DNA including D-loop region were obtained from seven golden jackals, *Canis aureus*, collected in Turkey. They were compared to the D-loop sequences registered in the GenBank database under the name *Canis aureus*. We determined four D-loop haplotypes (333 bp) among the seven Turkish sequences. Despite the limited number of sequences, our analysis indicated that *Canis aureus* consists of two allopatric haplogroups (a major haplogroup representing Austria, Bulgaria, Croatia, Italy, Serbia and Turkey, and a minor haplogroup containing one haplotype from India) within the sampling area. Interestingly, one haplotype from Senegal was clustered close to grey wolves, used as out-group, and this haplotype might not belong to golden jackal as suggested in previous studies. Our work presented the important data obtained from the Turkish samples to reveal the phylogenetic relationships among golden jackals, and it has suggested that there is a relatively high genetic variability in Turkish golden jackals.

## Key words

*Canis aureus*, Mitochondrial DNA, D-loop, Turkey.

## Introduction

The golden jackal (*Canis aureus* L., 1758) is distributed in South-East Europe, the Levant, Arabian Peninsula, the Middle East region, the Indian subcontinent, and South-East Asia and Africa (WILSON & REEDER 1993, 2005, JHALA & MOEHLMAN 2008). However, populations of small canids occurring in Africa may all belong to the cryptic African wolf, *Canis lupus lupaster*, as have been confirmed already for Egypt, Eritrea and Senegal (RUE-NESS *et al.* 2011, GAUBERT *et al.* 2012).

Mitochondrial DNA has a tendency for a higher rate of evolution when compared to nuclear DNA, and is a useful marker for the elucidation of intraspecific genetic structure (SUNNUCKS 2000). The D-loop (control region) is the non-coding region of the mammalian mitochondri-

al DNA, which includes substitutions, indels of various lengths and copies of tandem repeats as well as others, and it is highly variable (SBISÀ *et al.* 1997).

Despite the fact that it is widely distributed, the golden jackal is known as the least investigated species using mitochondrial and nuclear markers. The genetic structure of the golden jackal populations was previously investigated using the mitochondrial control region sequences and microsatellite data from Austria and Serbia by ZACHOS *et al.* (2009), and from Bulgaria, Croatia, Eastern Italian Alps and Serbia by FABBRI *et al.* (2014), and recently from Poland by KOWALCZYK *et al.* (2015). To characterize the genetic structure of the golden jackal populations in Israel, COHEN *et al.* (2013) used micro-

satellite loci. Genetic differentiation among the Croatian golden jackals, grey wolves and dogs were investigated by using sequence variation in Y chromosome (GOMERČIĆ *et al.* 2013). GALOV *et al.* (2014) also tried to develop a Y chromosome marker to determine hybridization between the golden jackals and dogs. Interestingly, RUENESS *et al.* (2011) showed that mitochondrial DNA sequences of *C. aureus lupaster* from Egypt did not belong to the golden jackal, but to the cryptic African wolf, *C. lupus lupaster*. Furthermore, relying on a large data set including mitochondrial and nuclear DNA analysis of KOEPFLI *et al.* (2015) revealed that conspecific populations in Africa and Eurasia of golden jackal are different species. They also stated that African population of golden jackal probably belongs to a distinct species. However, no genetic analysis has hitherto been performed on samples of the Turkish golden jackal.

Turkey, consisting of both the European and Asian parts, is a zoogeographical land bridge among Africa, Asia and Europe, and currently hosts more than 150 mammalian species (JOHNSON 2002, KRYŠTUFEK & VOHRALIK 2001, 2009). Of these species, the golden jackal (*C. aureus*), “the Turkish coyote” (see JOHNSON 2002), is an inhabitant in the considerable part of Turkey (KRYŠTUFEK & VOHRALIK 2001, 2009, see JOHNSON 2002).

As genetic diversity and phylogenetic relationships of the Turkish golden jackal were unknown, in the present study we amplified a partial fragment of mitochondrial DNA including the D-loop region (control region) of seven golden jackals from the Black Sea region in the Asian part of Turkey. Furthermore, the Turkish D-loop sequences were compared to sequences obtained from the GenBank database.

## Materials and Methods

Tissues samples (ear, tail, muscle) were collected from seven road-killed individuals belonging to the Turkish golden jackal (Table 1, Fig. 1). All tissues were preserved at  $-20^{\circ}\text{C}$  and in 99% ethanol before total DNA extraction. To extract total DNA, we used a commercial extraction kit (The DNeasy Blood and Tissue Kit, Qiagen). Using the total DNA, the partial fragment of mitochondrial DNA included D-loop region (control region) was amplified with a specific PCR (The Polymerase Chain Reaction) primer pair (Forward: DLH 5'-CCTG-AAGTAAGAACCAGATG-3' and Reverse: LF15926F 5'-ATATAAATACTTTGGTC TTGTAACC-3') (KIRSCHNING *et al.* 2007).

PCR amplifications were performed in a total of 50  $\mu\text{l}$  reaction mixture; 10  $\times$  Taq buffer with  $(\text{NH}_4)_2\text{SO}_4$ : 5  $\mu\text{l}$ , dNTP mix: 1  $\mu\text{l}$ , Taq DNA polymerase (5 u/ $\mu\text{l}$ ) (Thermo Scientific): 0.3  $\mu\text{l}$ ,  $\text{MgCl}_2$ : 3  $\mu\text{l}$ , BSA: 3  $\mu\text{l}$ , 5  $\mu\text{l}$  of each primer, DNA extract: 1  $\mu\text{l}$ ,  $\text{dH}_2\text{O}$ : 26.7  $\mu\text{l}$ ). The PCR program comprised of a pre-denaturation procedure consisting of 5 min. at  $95^{\circ}\text{C}$  by 1 cycle, a denaturation step of

40 sec. at  $95^{\circ}\text{C}$ , an annealing step of 1 min. at  $54^{\circ}\text{C}$ , an extension step of 90 sec. at  $72^{\circ}\text{C}$  by 30 cycles and an ending step of 10 min. at  $72^{\circ}\text{C}$  by 1 cycle. To verify the quality of total DNA and PCR products, 1% agarose gel was run and stained with ethidium bromide. Purification of PCR products was carried out with the Macherey-Nagel Nucleospin Gel and PCR Clean-up kit. The purified products were sequenced in forward and reverse directions with PCR primers by using a sequencer (ABI 3100 Genetic Analyzer).

Geneious v.6.1 (accessible from <http://www.geneious.com>) and DnaSP ver. 5.10.01 (LIBRADO & ROZAS 2009) were used to align the mitochondrial DNA sequences and to calculate haplotype (*Hd*) and nucleotide diversities (*Pi*). Based on the K2P (Kimura 2-parameter) nucleotide substitution model (KIMURA 1980), genetic distances among the Turkish haplotypes were estimated by means of MEGA v.6.0 (TAMURA *et al.* 2013). The HKY (Hasegawa-Kishino-Yano) + I, which was used in BI and ML analyses was chosen to be the most suitable model of nucleotide substitution with the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) using jModeltest2 (DARRIBA *et al.* 2012).

Relying on mitochondrial D-loop sequences, phylogenetic relationships of *C. aureus* were revealed using Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods with MEGA6 (TAMURA *et al.* 2013). The bootstrap value for each branch on the ML and NJ trees was calculated with 10000 pseudoreplicates.

Bayesian analysis (BI: Bayesian Inference) using the MCMC (Markov Chain Monte Carlo) technique was carried out with MrBayes v.3.2 (RONQUIST *et al.* 2012), discarding the first 25% of samples as burn-in (The Average Standard Deviation of split Frequencies  $< 0.01$ ) and calculating the Bayesian posterior probabilities for 0.2 million generations with tree sampled every 100 generations. After discarding burn-in, the remaining samples were held to generate the consensus tree (50% majority rule), and to calculate 95% Bayesian credibility interval and posterior probability. Bayesian tree diagram was drawn using FigTree v1.3.1 (RAMBAUT 2009).

A mitochondrial D-loop haplotype network was generated by a Median-Joining method using the Network v.4.6.1.1 software (BANDELDT *et al.* 1999; <http://www.fluxus-engineering.com>).

Two sequences of the grey wolf (*Canis lupus*) (NC\_009686: ARNASON *et al.* 2007; NC\_008092: BJORNERFELDT *et al.* 2006) were included in phylogenetic analyses as out-group.

## Results

We amplified the 439 bp fragment (tRNA<sup>Thr</sup> + tRNA<sup>Pro</sup> + D-loop) of the mitochondrial DNA from the seven Turkish golden jackals, including 333 bp for D-loop (control region) (Table 1, Fig. 1). Genetic analysis revealed four

**Table 1.** The Turkish golden jackal samples and sequences obtained from the GenBank database.

Turkish samples No.	Haplotype/ Sequence code	Map number	Locality	Reference
1584	TrCa1	1	Karadeniz Sahili, Bafra, Samsun, Turkey	THIS STUDY
638	TrCa2	2	Kiyaslar Köyü, Devrek, Zonguldak, Turkey	THIS STUDY
856	TrCa3	3	Karadeniz Sahili, Arhavi, Artvin, Turkey	THIS STUDY
1252	TrCa3	4	Karakoca, Ulubey, Ordu, Turkey	THIS STUDY
1257	TrCa3	5	Karadeniz Sahili, 4 km. Batı, Fındıklı, Rize, Turkey	THIS STUDY
1271	TrCa3	6	Ovacık Köyü, Artvin, Turkey	THIS STUDY
1339	TrCa4	7	Efirlı, Ordu, Turkey	THIS STUDY
	KT268318-19		Poland	KOWALCZYK <i>et al.</i> 2015
	KJ490945-46		Caucasus	PILOT <i>et al.</i> 2014
	AF184048		Bulgaria	RANDI <i>et al.</i> 2000
	HQ845260; GU936680		Serbia	RUENESS <i>et al.</i> 2011; ZACHOS <i>et al.</i> 2009
	AY289996-97		India	AGGARWAL <i>et al.</i> 2007
	JQ088675-76		Senegal	GAUBERT <i>et al.</i> 2012
	NC_008092		Out-group	BJORNERFELDT <i>et al.</i> 2006
	NC_009686		Out-group	ARNASON <i>et al.</i> 2007

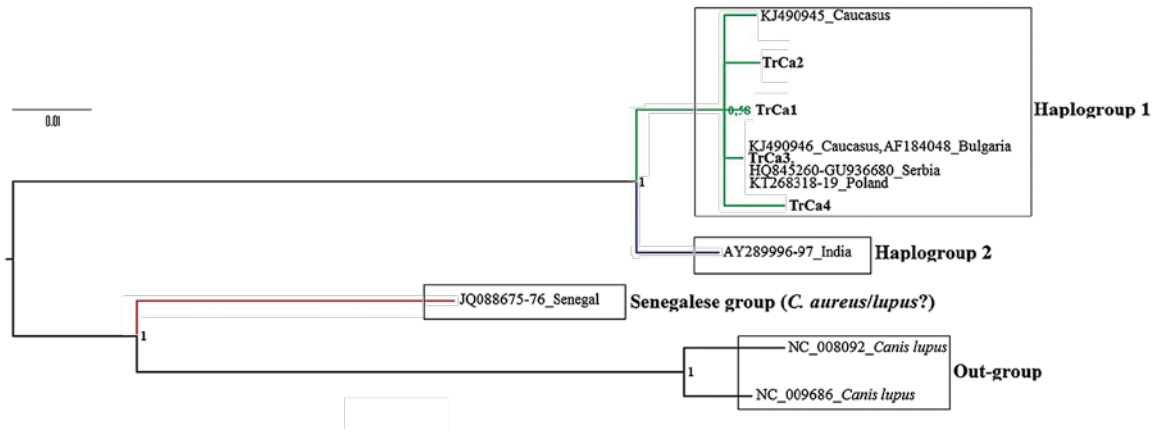


**Fig. 1.** Localities of the Turkish golden jackal samples (see Table 1 for map numbers).

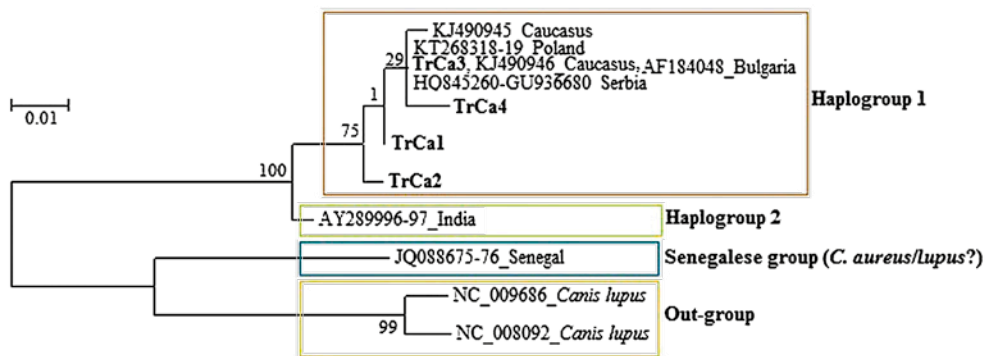
Turkish haplotypes (TrCa1-TrCa4) of the 439 bp fragments, including five variable (polymorphic) sites and two parsimony informative sites. The most common haplotype was TrCa3 (57.1%). Four haplotypes of the Turkish golden jackal (TrCa1–TrCa4) are deposited in the GenBank database (Accession numbers: KT988006–KT988009). Based on the 439 bp fragments, the haplotype (*Hd*) and nucleotide diversities (*Pi*) of the Turkish samples were 0.7143 and 0.00412, respectively. Sequence divergences of the four Turkish haplotypes ranged from 0.002 to 0.012, with a mean of 0.0066, relying on K2P.

When mitochondrial D-loop region (333 bp) of the seven Turkish golden jackals was analyzed, four D-loop haplotypes were determined as stated above, and the most common haplotype that was shared by four samples obtained from four localities was TrCa3 (Table 1, Fig. 1). Haplotype and nucleotide diversities for the mitochondrial D-loop region of Turkish golden jackal were *Hd*: 0.7143 and *Pi*: 0.00543, respectively.

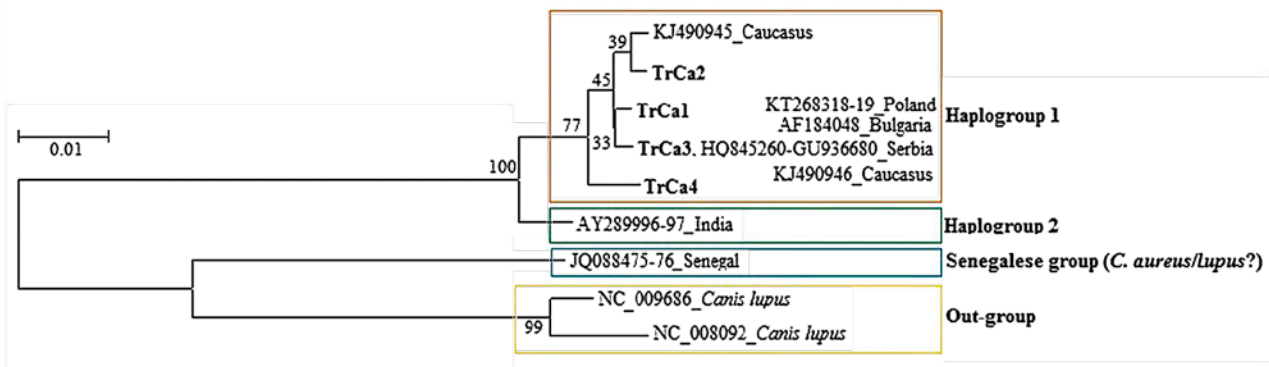
Three phylogenetic trees (BI, ML and NJ) (Figs. 2–4) were constructed using the mitochondrial D-loop sequences of 269 bp obtained from Turkey and the GenBank database (Table 1), including Senegal sequences, which were probably deposited under the name *Canis aureus*. The phylogenetic trees showed consistency in their branching topologies that are supported with high posterior probabilities (Fig. 2) and bootstrap values (Figs. 3–4). In these trees (Figs. 2–4), the samples of the golden jackal were separated into two haplogroups. From these haplogroups, Haplogroup 1 consisted of haplotypes from Austria, Bulgaria, Caucasia, Croatia, Italy, Poland, Serbia and Turkey, which did not show any geographical clustering. Haplogroup 2 contained a single haplotype, including two sequences of Indian golden jackal, and it composed a separate lineage (Figs. 2–4). Haplogroup 1 was diverged from Haplogroup 2 with an average distance value of 1.9%. However, Senegalese samples (Senegalese group), which consisted of a single



**Fig. 2.** Bayesian tree constructed from haplotypes of the mitochondrial D-loop region (269 bp) of golden jackals, rooted with *C. lupus*. The bold haplotypes indicate the Turkish golden jackal.



**Fig. 3.** ML tree constructed from haplotypes of the mitochondrial D-loop region (269 bp) of golden jackals, rooted with *C. lupus*. The bold haplotypes indicate the Turkish golden jackal.

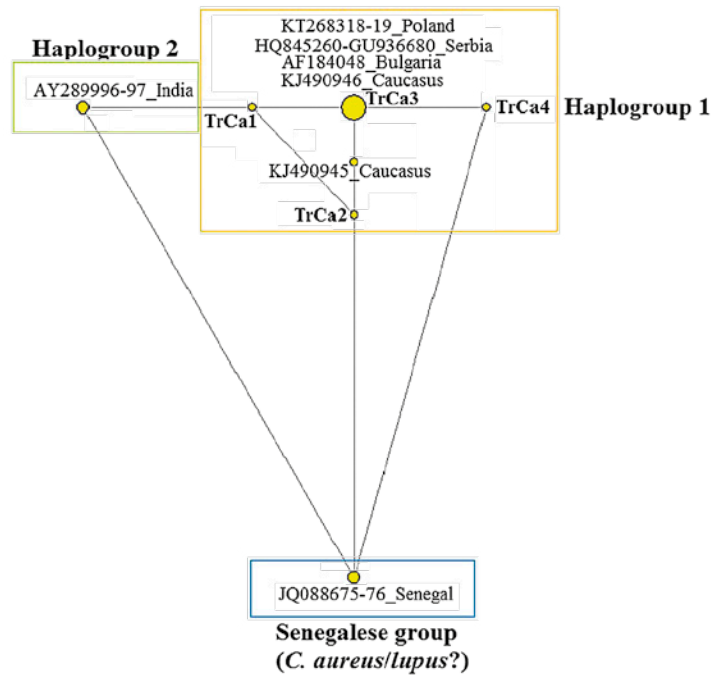


**Fig. 4.** NJ tree constructed from haplotypes of the mitochondrial D-loop region (269 bp) of golden jackals, rooted with *C. lupus*. The bold haplotypes indicate the Turkish golden jackal.

haplotype including two sequences, clustered together with an out-group containing the *Canis lupus* sequences (Figs. 2–4).

Besides the three phylogenetic trees (Figs. 2–4), a median-joining network with the seven mitochondrial D-loop haplotypes (269 bp) of *C. aureus* from Turkey and the GenBank database was shown in Figure 5. The haplotype network (Fig. 5) also depicted that there was

a relatively similar relationship among the Turkish and Austrian, Bulgarian, Caucasian, Croatian, Italian, Polish and Serbian samples, as shown the three phylogenetic trees (Figs. 2–4). In both trees (Figs. 2–4) and network (Fig. 5), a single haplotype obtained from the Senegalese sequences registered in the GenBank database under the name *C. aureus* showed a different grouping from the other golden jackal haplotypes.



**Fig. 5.** Median-joining network constructed from haplotypes of the mitochondrial D-loop region (269 bp) of golden jackals. The bold haplotypes indicate the Turkish golden jackal.

## Discussion

The golden jackal, *Canis aureus*, is known as a widespread species of the family Canidae (WILSON & REEDER 1993, 2005, JHALA & MOEHLMAN 2008). Although the golden jackal is widely distributed, genetic investigations are relatively scarce (RANDI *et al.* 2000, AGGARWAL *et al.* 2007, ZACHOS *et al.* 2009, GAUBERT *et al.* 2012, COHEN *et al.* 2013, GOMERČIĆ *et al.* 2013, FABBRI *et al.* 2014, GALOV *et al.* 2014, KOEPFLI *et al.* 2015, KOWALCZYK *et al.* 2015). Owing to this fact, the genetic diversity and phylogenetic relationships of golden jackals from Turkey are still unclear.

Mitochondrial DNA has been used as a suitable genetic marker to reveal phylogenetic relationships and population genetic structure of canid species (FRATI *et al.* 1998, VILÀ *et al.* 1999, RANDI *et al.* 2000, SHARMA *et al.* 2004, AGGARWAL *et al.* 2007, INOUE *et al.* 2007, KIRSCHNING *et al.* 2007, ZACHOS *et al.* 2009, GOMERČIĆ *et al.* 2010, TEACHER *et al.* 2011, GAUBERT *et al.* 2012, COHEN *et al.* 2013, GOMERČIĆ *et al.* 2013, FABBRI *et al.* 2014, İBIŞ *et al.* 2014, GALOV *et al.* 2014, PILOT *et al.* 2014, STATHAM *et al.* 2014, KOEPFLI *et al.* 2015, KOWALCZYK *et al.* 2015, etc.).

In this study, we investigated the genetic diversity and phylogenetic relationships of golden jackal from Turkey using the partial mitochondrial DNA sequences, including D-loop (control region). In search of available data from the GenBank database, we could not find a satisfactory number of sequence to compare with our data (RANDI *et al.* 2000, AGGARWAL *et al.* 2007, ZACHOS *et al.* 2009, RUENESS *et al.* 2011, GAUBERT *et al.* 2012, FABBRI

*et al.* 2014, PILOT *et al.* 2014, KOWALCZYK *et al.* 2015). This study suggests only that the golden jackal (*Canis aureus*) consists of two allopatric haplogroups (Figs. 2–4) within the sampling area; besides the sequences from Turkey Haplogroup 1 including sequences from Austria, Bulgaria, Caucasia, Croatia, Italy, Poland and Serbia (RANDI *et al.* 2000, ZACHOS *et al.* 2009, FABBRI *et al.* 2014, PILOT *et al.* 2014, KOWALCZYK *et al.* 2015, THIS STUDY), and Haplogroup 2 comprising one haplotype from India (AGGARWAL *et al.* 2007, RUENESS *et al.* 2011). However, one haplotype from Senegal (GAUBERT *et al.* 2012) was clustered close to grey wolves, which were used as out-group (Figs. 2–4). Furthermore, the haplotype network (Fig. 5) also depicted that there was a similar relationship among the Turkish and Austrian, Bulgarian, Caucasian, Croatian, Italian, Polish and Serbian samples (RANDI *et al.* 2000, ZACHOS *et al.* 2009, FABBRI *et al.* 2014, PILOT *et al.* 2014, KOWALCZYK *et al.* 2015, THIS STUDY), as shown in Figures 2–4. In all analyses, which based on the 269 bp D-loop sequences, the Turkish haplotype (TrCa3, the most common haplotype) was identical to other sequences (ZACHOS *et al.* 2009, FABBRI *et al.* 2014, KOWALCZYK *et al.* 2015) (Figs. 2–5). In a study comparing genetic variability of the Caucasian grey wolves to those of Europe and The Middle East, PILOT *et al.* (2014) reported two haplotypes belonging to golden jackal from Caucasia region. In their report, the two haplotypes from Caucasia, one of which was shared with one of the Turkish haplotypes and where is neighboring to the Northeast part of Turkey, were grouped in Haplogroup 1 (Figs. 2–5).

Of samples sequenced, the Austrian, Bulgarian, Croatian, Italian and Serbian golden jackals that were report-



ed to not display a genetic variation at the mitochondrial control region (D-loop) (ZACHOS *et al.* 2009, FABBRI *et al.* 2014). In contrast to the above-mentioned samples of golden jackal, the Turkish samples were relatively polymorphic, as it contained different D-loop haplotypes, all of which were linked to Haplogroup 1 in phylogenetic trees (Figs. 2–4).

In previous phylogenetic studies focused on grey and/or African wolves from Asia and Africa, a few samples of golden jackals (AGGARWAL *et al.* 2007, RUENESS *et al.* 2011, GAUBERT *et al.* 2012, PILOT *et al.* 2014) were included. Initially, AGGARWAL *et al.* (2007) used two sequences of golden jackal from Central India to construct a phylogenetic tree of the relationships among the Indian wolves. Haplogroup 2 of the present study consisted of the Indian haplotype found in the two sequences obtained from South Asia (AGGARWAL *et al.* 2007), which was basal to Haplogroup 1 (Figs. 2–4). In the study of RUENESS *et al.* (2011), which included two Indian sequences, the *Canis* species occurring in Egypt, formerly named *C. aureus lupaster*, was found to be related to wolves rather than the golden jackal (*C. aureus*). The systematic position of this cryptic African wolf (*Canis lupus lupaster*) was later confirmed by GAUBERT *et al.* (2012). These authors revealed also that the Senegalese samples previously identified as golden jackal belonged to the African wolf lineage. Recently, based on a large data set obtained from mitochondrial and nuclear genomes, KOEPFLI *et al.* (2015) revealed that African and Eurasian populations of golden jackal are genetically different lineages, and that African population of golden jackal probably belongs to a different species. Our study showed that the Senegalese sequences registered in the GenBank database under the name *C. aureus* (GAUBERT *et al.* 2012) grouped together with the wolves used as out-group (Figs. 2–4).

In conclusion, our work contributed to the understanding of genetic diversity and the phylogenetic relationships of golden jackals based on the variability in their mitochondrial D-loop region. As a result of the mitochondrial DNA analysis, we found that there were four haplotypes in the seven Turkish golden jackals (one different haplotype over  $7/4 = 1.75$  samples). In oppose to the studies of ZACHOS *et al.* (2009) ( $121/1 = 121$  samples) and FABBRI *et al.* (2014) ( $120/1 = 120$  samples), value found in this study (one different haplotype over  $7/4 = 1.75$  samples) was higher than the values found in the Bulgarian, Croatian, Italian and Serbian golden jackals. In this context, haplotype (*Hd*) and nucleotide (*Pi*) diversities were also much higher in the Turkish golden jackal (0.7143 and 0.00412, respectively) than in the Bulgarian, Croatian, Italian and Serbian golden jackals (both were zero). When comparing the genetic diversity of the Turkish golden jackal to the Austrian, Bulgarian, Caucasian, Croatian, Italian, Polish and Serbian golden jackals, it appeared that the Turkish golden jackal had higher genetic diversity. In genetic diversity, the Caucasian population also has resemblance to Turkish population therewithal.

As an indication of higher genetic diversity, the mitochondrial D-loop sequence polymorphism might have

resulted from difference of biotic and abiotic factors that affect the golden jackals in Turkey and the other geographical regions (Austria, Bulgaria, Caucasus, Croatia, Italy, Poland and Serbia). In order to further confirm this, more advanced genetic analyses need to be performed by using different mitochondrial and nuclear DNA markers.

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