

Phylogenetic Status of the Turkish Red Fox (*Vulpes vulpes*), based on Partial Sequences of the Mitochondrial Cytochrome *b* Gene

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Abstract

Genetic diversity and multiple mitochondrial phylogroups of the red fox have been revealed from scattered locations in previous studies. There is a still lack of information about the genetic diversity and phylogeographic structure of the red fox in Asia Minor. We investigated the genetic diversity in the Turkish red fox using a part of the cytochrome *b* mitochondrial gene (375 bp), and attempted to evaluate the phylogeographic structure in various geographic ranges of the species with the use of sequences available from the GenBank from various geographic origins and our data. Bayesian and Network analyses of the cytochrome *b* sequences from Turkey and GenBank suggested that the red fox is divided into four main phylogroups. They are grouped accordingly: Group 1 (SW Anatolia, Turkey and Hokkaido, Japan), Group 2 (Eurasia and North America), Group 3 (only North America), and Group 4 (Vietnam). The majority of Turkish haplotypes grouped with those of Eurasia. Despite the great distance between the localities, two haplotypes from SW Anatolia, Turkey grouped with previously reported haplotypes from Hokkaido, Japan. The present study shows that the Turkish red fox is nested within two main phylogroups and exhibits a high genetic diversity.

Key words

Vulpes vulpes, Mitochondrial DNA, Cytochrome *b*, Turkey.

Introduction

The red fox *Vulpes vulpes* is one of the most widely distributed canid species throughout Northern Hemisphere (also introduced to Australia in the 1800's – WILSON & REEDER 2005, MACDONALD & REYNOLDS 2008). During the past two decades, *V. vulpes* has been one of the most investigated mammals from various regions. There are comprehensive studies dealing with the phylogeographic structure and pattern of genetic diversity in the red fox populations, using allozyme or isozyme (FRATI *et al.* 1998, SIMONSEN *et al.* 2003), RAPD (GACHOT-NEVEU *et al.* 2009), mitochondrial DNA cytochrome (Cyt) *b* (FRATI *et al.* 1998, INOUE *et al.* 2007, PERRINE *et al.* 2007,

FERNANDES *et al.* 2008, AUBRY *et al.* 2009, SACKS *et al.* 2010, 2011, STATHAM *et al.* 2011, 2012, TEACHER *et al.* 2011, EDWARDS *et al.* 2012, YU *et al.* 2012a), control region (INOUE *et al.* 2007, KIRSCHNING *et al.* 2007, AUBRY *et al.* 2009, SACKS *et al.* 2010, 2011, STATHAM *et al.* 2011, 2012, TEACHER *et al.* 2011, EDWARDS *et al.* 2012, KUTSCHERA *et al.* 2013, GALOV *et al.* 2014), tRNA-threonine and tRNA-proline (INOUE *et al.* 2007), microsatellite data (LADE *et al.* 1996, WANDELER *et al.* 2003, KUKKOVA *et al.* 2004, WANDELER & FUNK 2006; SACKS *et al.* 2010, 2011, OISHI *et al.* 2011) and single nucleotide polymorphism (SACKS *et al.* 2011).

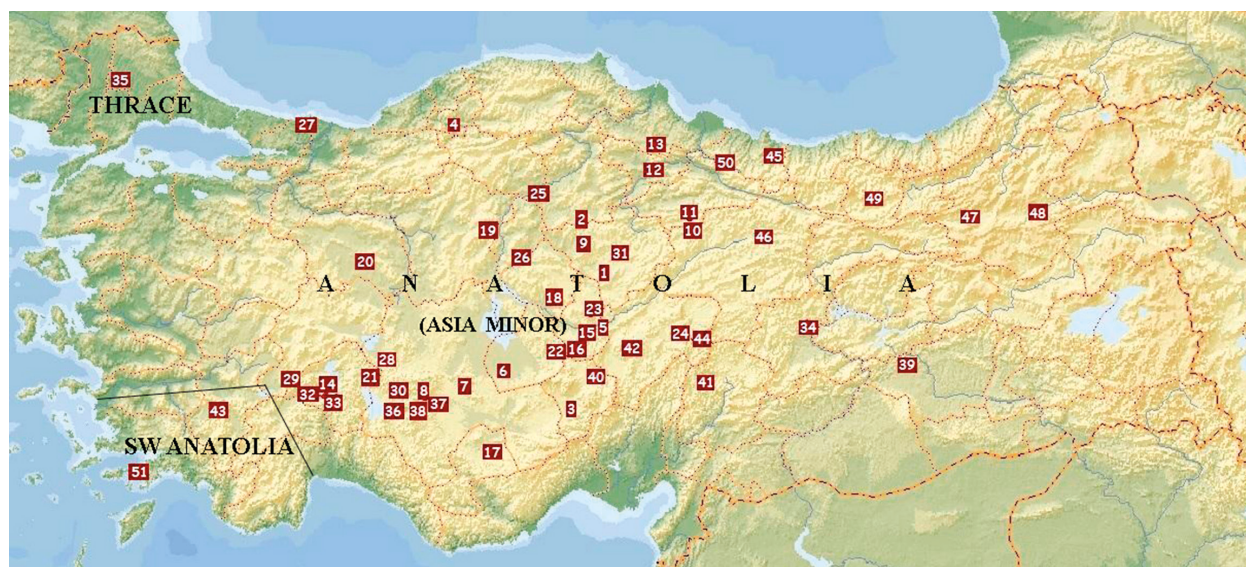


Fig. 1. Collection localities and distribution of red foxes (*Vulpes vulpes*) in Turkey. Map numbers are locality codes (see Table 1).

Multiple studies based on mitochondrial DNA of red fox emphasize that there has been a considerable importance to have a comprehensive picture of genetic variation in the entire range of the species, when new insights are added into phylogeographic and colonization patterns of red fox with a wider biogeographic context (FRATI *et al.* 1998, INOUE *et al.* 2007, PERRINE *et al.* 2007, AUBRY *et al.* 2009, TEACHER *et al.* 2011, EDWARDS *et al.* 2012, YU *et al.* 2012a, KUTSCHERA *et al.* 2013). Some of the studies resulted in the formation of mitochondrial phylogroups, which were classified as Holarctic and/or Eurasian, Nearctic, and Hokkaido (INOUE *et al.* 2007, AUBRY *et al.* 2009, YU *et al.* 2012a, KUTSCHERA *et al.* 2013). Although there is a substantial inventory of mitochondrial data from Eurasia and North America (FRATI *et al.* 1998, INOUE *et al.* 2007, PERRINE *et al.* 2007, AUBRY *et al.* 2009, TEACHER *et al.* 2011, EDWARDS *et al.* 2012, YU *et al.* 2012, KUTSCHERA *et al.* 2013), there is a still lack of information about the genetic diversity and phylogeographic structure of the red fox from Turkey and the Middle East, except Israel. In this sense, more genetic and phylogeographic studies are needed to place these new mitochondrial data in a wider context and to elucidate the current pattern of phylogeographic structure throughout the red fox' range. Therefore, it would not only be beneficial to determine the genetic structure of different red fox populations but also to focus on regional details, especially those in previously unsampled areas. Turkey is one of such areas. Because of its continental position, Turkey constitutes a mysterious and interesting area for mammalian biogeography due to its climate, vegetation, heterogeneous topography and geological history; together these unique factors produce a biodiversity hotspot (MÉDAIL & QUÉZEL 1999, BILGIN 2011). Currently, there are at least 150 or more mammalian species from Turkey (KRYŠTUFK & VOHRALIK 2001, 2009, WILSON & REEDER 2005). Although there are a few studies based on its morphology and distribution (ÖZKURT *et al.* 1998, TEMIZER 2001), the phylogenetic status and

genetic diversity of Turkish red fox have not been previously investigated. Therefore, there is a lack of information in this respect. In reference to the red fox dentition study, SZUMA (2008) suggested that the modern red fox evolved either in Asia Minor (Anatolia) or North Africa during the early Pleistocene. Consequently, to confirm this, the phylogenetic status of Turkish red fox should be revealed using different markers.

In this study, our primary purpose was to determine the magnitude of genetic diversity within the Turkish red fox by sequencing part of the Cyt *b* gene. With emphasis on this information we provide new insights into phylogeographic processes which may be essential for a better understanding of evolution.

Material and Methods

Localization, tissue sampling and DNA extraction

Various tissues (ear, tail, skin and skeletal muscle) of 51 red foxes, which were road kills, were collected from 51 different localities in Turkey (Table 1, Fig. 1) during 2002–2010. Tissue samples had been stored in the corresponding author's private collection at the Department of Biology, Faculty of Sciences, Erciyes University in Kayseri, Turkey.

Total genomic DNA was extracted by using the DNeasy Blood and Tissue Kit (QIAGEN), following the manufacturer's instructions. A segment of the mitochondrial cytochrome (Cyt) *b* (375 base pairs) from each Turkish sample was amplified by polymerase chain reaction (PCR) using the primer pair

L14724 (5' GATATGAAAAACCATCGTTG 3') and **H15149** (5' CAGAATGATATTTGTCCTCA 3')

Table 1. List of Turkish samples and sequences/haplotypes used in the present study (* Map numbers are locality codes in Figure 1).

Map number*	Cyt b haplotype	Tissue	Locality	Coordinate/Altitude
1	TR1	Tail	Özlüce/Boğazlıyan/Yozgat	N 39° 29.185' E 35° 04.298' H – 1099m
2	TR1	Muscle	Boğazkale/Çorum	N 39° 56.087' E 34° 42.963' H – 1215m
3	TR1	Tail	Kemerhisar/Bor/Niğde	N 37° 45.509' E 34° 34.032' H – 1128m
4	TR1	Tail	Karabük	N 41° 09.810' E 32° 38.428' H – 304m
5	TR1	Tail	Bozca/Avanos/Nevşehir	N 38° 44.331' E 35° 02.312' H – 1048m
6	TR1	Tail	Avanos/Nevşehir	N 38° 41.409' E 34° 48.850' H – 1012m
7	TR1	Tail	Konya	N 38° 01.340' E 32° 51.829' H – 1001m
8	TR1	Tail	Konya	N 37° 53.613' E 32° 09.022' H – 1565m
9	TR1	Tail	Yozgat	N 39° 48.504' E 34° 46.330' H – 1204m
10	TR1	Tail	Tokat	N 40° 03.833' E 36° 29.187' H – 1143m
11	TR1	Ear	Çamlıbel/Tokat	N 40° 04.261' E 36° 29.011' H – 1132m
12	TR1	Tongue	Amasya	N 40° 41.779' E 35° 48.765' H – 408m
13	TR1	Ear	Bekdiğın/Havza /Samsun	N 40° 59.861' E 35° 46.125' H – 725m
14	TR1	Muscle	Eğirdir/Isparta	N 37° 52.000' E 30° 15.000'
15	TR1	Tubercle	Acıgöl/Nevşehir	N 38° 32.691' E 34° 29.174' H – 1264m
16	TR1	Ear	Aksaray	N 38° 30.969' E 34° 21.098' H – 1227m
17	TR1	Ear	Kayaönü Mah./Karaman	N 37° 14.318' E 33° 21.128' H – 1039m
18	TR1	Ear	Kırşehir	N 39° 05.094' E 34° 16.287' H – 1200m
19	TR1	Ear	Elmadag/Ankara	N 39° 55.348' E 33° 15.923' H – 1072m
20	TR1	Ear	Eskişehir	N 39° 30.058' E 31° 13.424' H – 1028m
21	TR1	Muscle	Ilgın/Konya	N 38° 18.062' E 31° 39.356' H – 1080m
22	TR1	Ear	Eşmekaya/Aksaray	N 38° 15.152' E 33° 28.976' H – 971m
23	TR1	Ear	Çalış/Nevşehir	N 38° 58.846' E 34° 54.890' H – 1316m
24	TR1	Ear	Pınarbaşı/Kayseri	N 38° 43.000' E 36° 23.000'
25	TR2	Tail	Kızılırmak/Çankiri	N 40° 21.169' E 33° 58.767' H – 555m
26	TR2	Tail	Yeniyapan/Keskin/Kirikkale	N 39° 35.319' E 33° 42.013' H – 922m
27	TR3	Tail	Kandıra/Kocaeli	N 41° 04.907' E 30° 10.238' H – 41m
28	TR4	Tail	Isparta	N 39° 03.203' E 31° 25.615' H – 1200m
29	TR4	Tail	Dikici Köyü/Dinar/Afyon	N 38° 00.140' E 30° 11.522' H – 940m
30	TR4	Ear	Beyşehir/Konya	N 37° 54.691' E 31° 56.955' H – 1270m
31	TR5	Tail	Bahadin/Sarıkaya/Yozgat	N 39° 36.066' E 35° 15.707' H – 1123m
32	TR5	Tail	Isparta	N 37° 52.539' E 30° 30.730' H – 935m
33	TR5	Tail	Eğirdir/Isparta	N 37° 50.753' E 30° 51.579' H – 922m
34	TR5	Muscle	Arguvan/Malatya	N 38° 46.000' E 38° 16.000'
35	TR5	Tail	Taşköprü/Babaeski/Kırklareli	N 41° 27.000' E 27° 03.000'
36	TR5	Tail	Konya-Seydişehir yolu/Konya	N 37° 45.000' E 32° 15.000'
37	TR5	Muscle	Konya	N 37° 45.674' E 32° 12.683' H – 1328m
38	TR5	Ear	Afşar köyü/Beyşehir/Konya	N 37° 42.478' E 31° 44.470' H – 1138m
39	TR5	Skin	Ergani/Diyarbakır	N 38° 15.000' E 39° 46.000'
40	TR6	Tail	Çarıklı/Niğde	N 38° 11.639' E 34° 59.174' H – 1368m
41	TR6	Muscle	Kanlıkvak/Göksun/Kahramanmaraş	N 38° 08.000' E 36° 36.000'
42	TR6	Ear	Erciyes Dağı/Kayseri	N 38° 31.587' E 35° 31.566' H – 2215m
43	TR7	Tail	Tavas/Denizli	N 37° 35.000' E 29° 03.000'
44	TR8	Ear	Pınarbaşı/Kayseri	N 38° 41.848' E 36° 21.790' H – 1546m
45	TR9	Kidney	Karakoca/Ulubey/Ordu	N 40° 50.000' E 37° 46.000'
46	TR9	Ear	Zara/Sivas	N 39° 51.772' E 37° 37.428' H – 1340m
47	TR9	Cheek Skin	Ilıca/Erzurum	N 39° 58.751' E 40° 56.382' H – 1881m
48	TR9	Upper lip	Horasan/Erzurum	N 40° 00.299' E 41° 57.455' H – 1594m
49	TR9	Muscle	Kelkit/Gümüşhane	N 40° 14.022' E 39° 27.077' H – 1723m
50	TR9	Tongue, Ear	Gökçebayır/Akkuş/Ordu	N 40° 41.648' E 36° 59.460' H – 1028m
51	TR10	Skin	Kurucabük/Datça/Muğla	N 36° 45.598' E 27° 54.341' H – 22 m

(see KOCHER *et al.* 1989, IRWIN *et al.* 1991, FRATI *et al.* 1998). PCR amplifications were performed in 50 µl reaction mixture (1 × Taq buffer with (NH₄)₂SO₄, 200 µM dNTP mix, 2 u Taq DNA polymerase (Fermentas), 2 mM MgCl₂, 1 µM of each primer, 1 µl DNA extract). The PCR condition was 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C, and 7 min at 72°C. The quality of the total DNA and the PCR products were verified by electrophoresis in 0.8% and 2% agarose gels, respectively, and stained with ethidium bromide. PCR products were purified using High Pure PCR Product Purification Kit (Roche). Sequencing was performed in both directions (Forward and Reverse) using the PCR primers, resulting in sequences readable for 375 bp. The purified PCR products were sequenced with an ABI 3100 Genetic Analyzer (RefGen, METU, Technopark-Ankara, Turkey).

Genetic diversity estimation for the Turkish red fox

Sequences were checked and edited using the program BioEdit v.7.0.9.0 (HALL 2007), yielding a sequence of 375 bp. The number of segregating sites (*S*), haplotype diversity (*h*), nucleotide diversity (π) and frequency of each haplotype were calculated using DnaSP v. 5.10.01 (LIBRADO & ROZAS 2009). Genetic distances were estimated under the Kimura 2-parameter (K2P) nucleotide substitution model (KIMURA 1980) in MEGA5 v. 5.01 (TAMURA *et al.* 2011).

Phylogeographic analyses of mtDNA haplotypes of red fox

To place these new mitochondrial DNA data in a wider context, we included all previously published red fox Cyt *b* gene sequences in the phylogenetic analyses. To the best of our knowledge, this represented all matrilineal phylogeographic studies from various parts of red fox range in the North Hemisphere (LEDJE & ARNASON 1996: X94929; FRATI *et al.* 1998: Z80957–Z80997; PALOMARES *et al.* 2002: AJ441335; ARNASON *et al.* 2006: AM181037; KOEPFLI *et al.* 2006: AY928669; INOUE *et al.* 2007: AB292741–AB292765; PERRINE *et al.* 2007: EF064207–EF064220; FERNANDES *et al.* 2008: EF689058–EF689062; AUBRY *et al.* 2009: FJ830756–FJ830776; SACKS *et al.* 2010: GU004541; ZHONG *et al.* 2010: GQ374180; TEACHER *et al.* 2011: JN232446, JN232448–72, JN232474–79; STATHAM *et al.* 2012: HM590010–11; YU *et al.* 2012a: JN652603–17, JQ0033577–78; YU *et al.* 2012b: JN711443). This analysis also included some unpublished sequences (QUINTEIRO & REY-MENDEZ 2004: AY586403–06; YOO *et al.* 2006: DQ498124–27; DANG *et al.* 2011: JN709944) downloaded from GenBank (NCBI: The National Center for Biotechnology Information) that were added to max-

imize the context of the present investigation. The abbreviations for sequences from GenBank were shown in Table 2.

Common sequences shared by different studies including ours have been based on 375 bp of Cyt *b* gene. However, sequences taken from PERRINE *et al.* (2007), AUBRY *et al.* (2009), TEACHER *et al.* (2011) and STATHAM *et al.* (2012) were slightly shorter (354 bp and 250 bp). Therefore, the sequences were truncated to align to the shorter published sequences (354 bp and 250 bp).

For all datasets, phylogenetic relationships between haplotypes of the Cyt *b* gene were revealed by using median-joining (MJ) network within the software Network 4.6.1.1 (BANDELT *et al.* 1999, <http://www.fluxus-engineering.com>) to represent the intra-specific genealogy of the haplotype datasets. To solidify statistical analysis for the mitochondrial Cyt *b* phylogroups, we also constructed phylogenetic trees with Bayesian phylogenetic analysis (BI: Bayesian inference of phylogeny) as implemented in the program Mr.Bayes v.3.2.1 (RONQUIST & HUELSENBECK 2003). For all datasets, the HKY (Hasegawa-Kishino-Yano) + G (gamma) model of nucleotide substitution was determined as the most appropriate model according to Bayesian Information Criterion (BIC) using the program jModeltest v.0.1.1 (POSADA 2008). The four Monte Carlo Markov chains were used to calculate the Bayesian posterior probabilities for 2 million (for 250 bp), 4 million (for 354 bp) and 2 million (for 375 bp) generations with trees sampled every 100 generations; the first 25 % of samples were discarded as burn-in (average standard deviation of split frequencies <0,01). After discarding burn-in trees and evaluating convergence, the remaining samples were retained for generating consensus trees (50 % majority rule), calculating 95 % Bayesian credibility intervals and posterior probabilities. Bayesian tree diagrams were obtained with tree figure drawing tool, FigTree v1.3.1 (RAMBAUT 2009). Additionally, we obtained genetic divergence among the phylogroups by using K2P (Kimura's 2-parameter distance) estimator (KIMURA 1980) implemented in MEGA5 (TAMURA *et al.* 2011). An approximate estimation of divergence time among the phylogroups was calculated on the basis of genetic divergence obtained with K2P, using AVISE (2000)'s formula:

$$P_{\text{net}} = P_{\text{AB}} - 0.5(P_{\text{A}} + P_{\text{B}}),$$

where P_{net} is the corrected distance between the A and B phylogroups, P_{AB} is the mean genetic divergence in pairwise comparisons of haplotypes A versus B, and P_{A} and P_{B} are the mean genetic divergence among haplotypes within these phylogroups. We also used the Cyt *b* divergence rate of 2 % per million years as large mammals (BROWN *et al.* 1979, AVISE *et al.* 1998). The arctic fox, *Alopex lagopus*, [Accession number: AY598511 (DELISLE & STROBECK 2005)] was used as out-group following TEACHER *et al.* (2011).

Table 2. The sample abbreviations for haplotype information of the previous reports used in median-joining networks and Bayesian trees.

FRATI <i>et al.</i> (1998)	A*, B*, C*, D*, E*, F*, G*, H*, I*, J*, K*, L*, M*, N*, O*, P*, Q*, R*
ARNASON <i>et al.</i> (2006)	AM: AM181037
INOUE <i>et al.</i> (2007)	C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14
PERRINE <i>et al.</i> (2007)	A, B, C, D, E, F, G, H, I, J, K, M, N, O, P
AUBRY <i>et al.</i> (2009)	A, A3, A4, A6, C, D, E2, F, F2, F3, G, G2, J, N, O, R, T, U, U2, U4, U5, U6, U7, V, V2, W, Z, Z2, Z3
STATHAM <i>et al.</i> (2012)	A8: HM590010; F4: HM590011
Yu <i>et al.</i> (2012a)	JN1: JN711443
Yu <i>et al.</i> (2012b)	NK02, NK03, KW5, KS8, KS10, CD10, RW1328, RW1651, RW1601, KS2, KW2, KS3, KS4, KW4, CD5, CD6, CD7, CD8, MO06, MO09
FERNANDES <i>et al.</i> (2008)	FS: EF689058-62
SACKS <i>et al.</i> (2010)	D2
ZHONG <i>et al.</i> (2010)	GQ: GQ374180
TEACHER <i>et al.</i> (2011)	JS1, JS2, JS3, JS4, JS5, JS6, JS7, JS8, JS9, JS10, JS11, JS12, JS13, JS14, JS15, JS17, JS18, JS19, JS20, JS21, JS22
KOEPFLI <i>et al.</i> (2006)	AYK: AY928669
QUINTEITO and REY-MENDEZ (2004)	QR1: AY586403; QR2: AY586404; QR3: AY586405; QR4: AY586406
Yoo <i>et al.</i> (2006)	DQ1: DQ498125-27; DQ2: DQ498124
DANG <i>et al.</i> (2011)	JN2: JN709944
LEDJE and ARNASON (1996)	UL: X94929
PALOMARES <i>et al.</i> (2002)	AJ: AJ441335

Results

Sequence data of the Turkish red fox samples

The mitochondrial Cyt *b* sequences (375 bp) from 51 individuals of Turkish red fox exhibited 10 haplotypes (Table 1, Fig. 1). These 10 haplotypes (TR1–10) are deposited in GenBank (Accession numbers: KM068792–KM068801).

Haplotypes TR1 and TR5 were shared by 24 (47 %) and 9 (17.6 %) samples, respectively, which were geographically the most widespread Turkish haplotypes (Table 1). The remaining haplotypes were not widely distributed as TR1 and TR5 (Table 1, Fig. 1). Haplotypes TR1, TR2, TR4–6, and TR8–10 were new and so far had not been reported in any red fox population (Table 1, 2).

Within the 375 bp length sequences, 23 polymorphic segregating sites (6.1%), of which 21 (91.3%) and 2 (8.7%) were transitions and transversions, respectively, were detected. Haplotype diversity (*h*) and nucleotide diversity (π) were 0.7380 and 0.01029, respectively. The sequence divergences among the Turkish haplotypes ranged from 0.003 to 0.044, with an average of 0.020.

Phylogenetic relationships among the red fox haplotypes

We identified a total of 54, 79 and 45 haplotypes in the three data sets: 250 bp, 354 bp and 375 bp the Cyt *b* sequences, respectively, including sequences from GenBank and Turkey (51 samples). The three data sets were

analyzed by using the Bayesian phylogenetic analysis as the probabilistic method and the MJ network analysis. The two datasets, 250 bp and 354 bp, displayed similar topology in trees and networks and, therefore, here we presented only the Bayesian tree and MJ network for 250 bp sequences.

Based on the both 250 bp and 354 bp Cyt *b* sequences, the MJ networks formed four distinct phylogroups (Fig. 2): Group 1 (Southwest Anatolia, Turkey and Hokkaido, Japan), Group 2 (Eurasia and North America), Group 3 (only North America) and Group 4 (Vietnam). Based on the 375 bp Cyt *b* sequences from Eurasia and Turkey, the MJ network formed in two main distinct phylogroups (Fig. 3): Group 1 (The SW Anatolia, Turkey and Hokkaido, Japan) and Group 2 (Eurasia).

In addition to the MJ network analysis, we also used Bayesian phylogenetic analyses for the three data sets (250 bp, 354 bp and 375 bp). Bayesian analyses revealed that the general picture of red fox phylogeography was in agreement with those of previous studies.

In the two data sets (250 bp and 354 bp) that were analyzed, the majority of red fox haplotypes were divided into three major phylogroups as reported by INOUE *et al.* (2007), AUBRY *et al.* (2009), YU *et al.* (2012a) and KUTSCHERA *et al.* (2013) (Fig. 4). Of the 10 Turkish haplotypes, TR7 and TR10 clustered within the SW Anatolia–Hokkaido phylogroup (Group 1), and the remaining Turkish haplotypes grouped into Group 2 (Eurasia and North America). Furthermore, the Bayesian analysis based on the 375 bp Cyt *b* haplotypes formed two distinct groups; Group 1 (SW Anatolia, Turkey and Hokkaido, Japan) and Group 2 (Eurasia) (Fig. 5). In all Bayesian trees (Figs. 4, 5), the phylogeographic patterning of haplotypes in the three data sets exhibited that

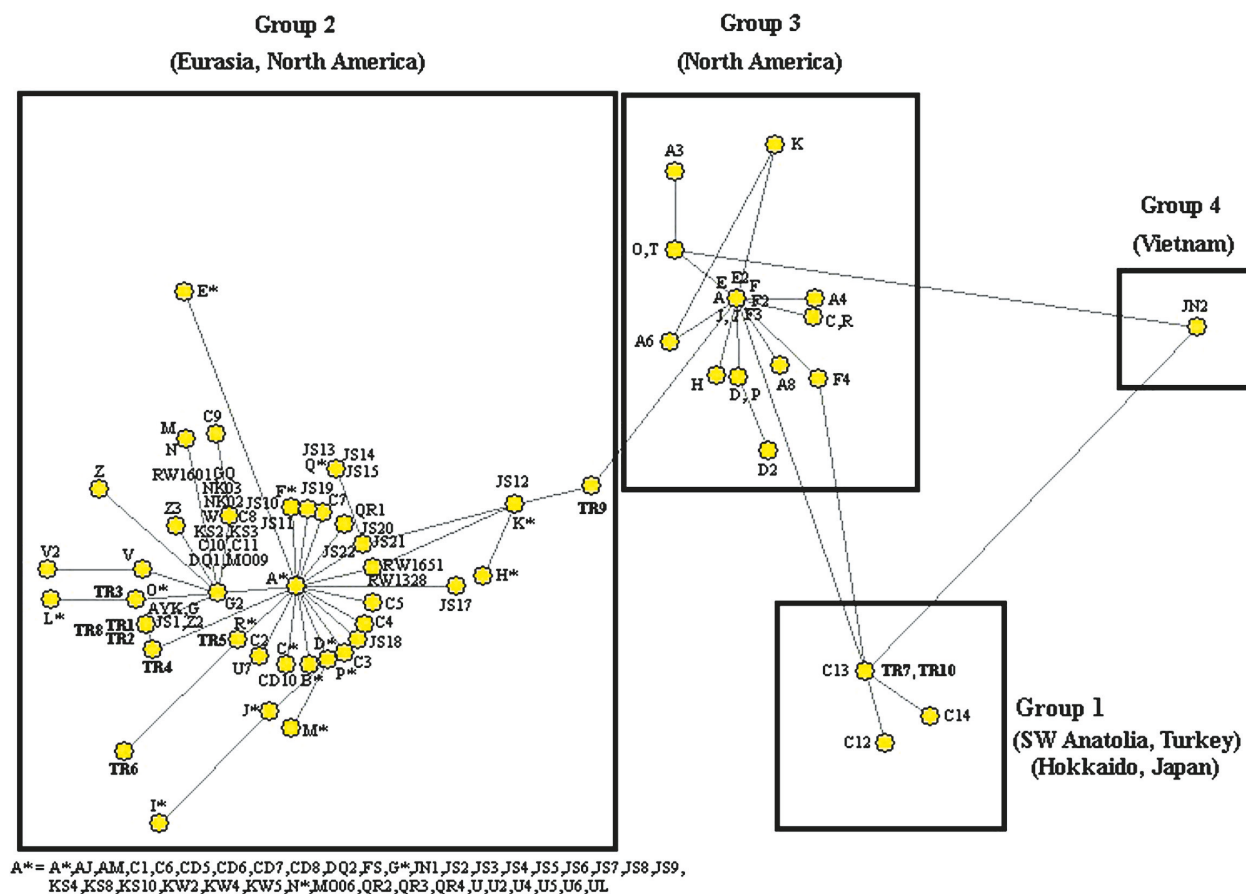


Fig. 2. Median-joining network reconstructed from haplotypes of *Vulpes vulpes* in the North Hemisphere, based on 250 bp sequences of the mitochondrial cytochrome *b*. Bold indicates the Turkish haplotypes. See Tables 1, 2 for the sample abbreviations.

Group 2 possessed numerous named and unnamed sub-phylogroups. As shown in both the MJ networks (Figs. 2, 3) and the Bayesian trees (Figs. 4, 5), the distribution pattern of red fox haplotypes did not exhibit a strong geographical dispersion at continental level.

By using the mean genetic distances calculated by K2P estimator (KIMURA 1980) and by taking the suggested Cyt *b* divergence rate of 2% per million years as large mammals (BROWN *et al.* 1979, AVISE *et al.* 1998), we estimated that the divergence time on the basis of the 250 bp Cyt *b* sequences was around 1.68 million years ago (mya) between Group 1 and 2, and 0.99 mya between Group 1 and 3 (Figs. 2, 4), on the basis of the 354 bp Cyt *b* sequences was around 1.27 mya between Group 1 and 2, and 0.71 mya between Group 1 and 3 (not shown Figure), and on the basis of the 375 bp cyt *b* sequences was around 1.22 mya between Group 1 and 2 (Figs. 3, 5).

Discussion

By using a molecular marker, mitochondrial DNA, and a large number of samples from Turkey, we were able to present the first considerable work to assess the ge-

netic diversity and phylogenetic status of the Turkish red fox. Our results (Figs. 2–5) verified all phylogroups (clades) of red fox described by previous studies (INOUE *et al.* 2007, AUBRY *et al.* 2009; YU *et al.* 2012a): (i) Group 1 was spread within Hokkaido, Japan and SW Anatolia, Turkey; (ii) Group 2 in Eurasia and North America; (iii) Group 3 in North America, and (iv) Group 4 consisted of one haplotype from Vietnam. Our study showed that the Turkish red fox was nested within two main phylogroups (Figs. 2–5). The divergence time between phylogroups of red fox estimated on the basis of the mitochondrial Cyt *b* variations were found to be around 0.71–1.68 mya, and had occurred during different periods of the Pleistocene. Except for one data set (250 bp), compared to previous studies (INOUE *et al.* 2007, YU *et al.* 2012a, KUTSCHERA *et al.* 2013), our estimations were compatible with those of INOUE *et al.* (2007) using Cyt *b* and control region, and YU *et al.* (2012a) using Cyt *b*, but in fact quite different from those of KUTSCHERA *et al.* (2013), who used the mitochondrial control region. A similar depiction of divergence times was drawn for some large animals *e.g.*, brown bear and red deer as in the studies carried out by MATSUHASHI *et al.* (1999, 2001), LEONARD *et al.* (2000), MAHMUT *et al.* (2002) and SKOG *et al.* (2009). The above mentioned mammals (*Ursus arctos* and *Cervus elaphus*) and red fox (*Vulpes vulpes*) hereby are assumed to share

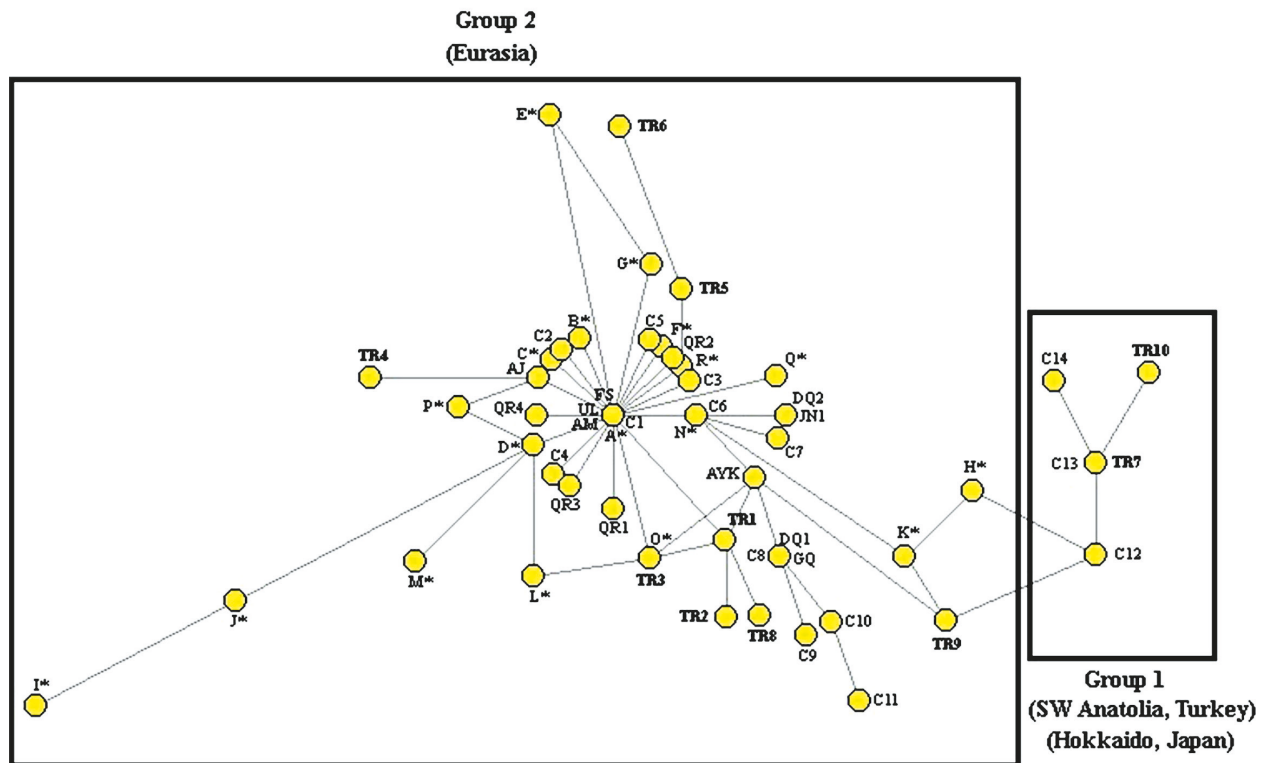


Fig. 3. Median-joining network reconstructed from haplotypes of *Vulpes vulpes* in the North Hemisphere, based on 375 bp sequences of the mitochondrial cytochrome *b*. Bold indicates the Turkish haplotypes. See Tables 1, 2 for the sample abbreviations.

a similar evolutionary history during the Pleistocene (YU *et al.* 2012a).

By comparing the haplotype and nucleotide diversity in Turkish samples from the studied region with data from other parts of the species range, we found ten distinct haplotypes (375 bp) in the 51 Turkish samples on the basis of partial sequences of the mitochondrial Cyt *b*, (with the average one different haplotype over $51/10=5.1$ samples) and haplotype diversity=0.7380. FRATI *et al.* (1998) found 18 distinct haplotypes (375 bp) in 41 samples from the Mediterranean Basin (with the average one different haplotype over 2.28 samples). Although INOUE *et al.* (2007) found 14 different haplotypes (375 bp) in 88 samples from Northern Japan (with the average one haplotype over 6.28 samples), YU *et al.* (2012a) determined nine haplotypes (338 bp) in 22 samples from East Asia (with the average one different haplotype over 2.44 samples). By analyzing the North American red foxes, PERRINE *et al.* (2007) and AUBRY *et al.* (2009) found 14 haplotypes (354 bp) in 73 samples (with the average one different haplotype over 5.21 samples), and 29 haplotypes (354 bp) in 220 samples (with the average one different haplotype over 7.58 samples), respectively. In conclusion, the Turkish red fox exhibits a high genetic diversity. If study areas are compared, the scale of our study area was geographically much smaller than those of FRATI *et al.* (1998), AUBRY *et al.* (2009) and YU *et al.* (2012a), but much larger than those of PERRINE *et al.* (2007) and INOUE *et al.* (2007). Of the 10 Turkish haplotypes from the 51 samples using a part of the Cyt

b gene (375 bp), TR1, TR2, TR4, TR5, TR6, TR8, TR9 and TR10 were new haplotypes for red fox, and may be local haplotypes found in Turkey. TR1 was a common haplotype in Turkey (Table 1; Fig. 1), with an ecologically monotonous (step area and plateau) region (KOSSWIG 1955), where there are no effective barriers to prevent gene flow between populations. TR3 corresponded to the O* haplotype (see Table 2 and FRATI *et al.* 1998), which is distributed in Bulgaria. Furthermore, TR7 corresponded to the C13 haplotype (see Table 2 and INOUE *et al.* 2007), which is distributed in Hokkaido, Japan.

Based on three different data sets of the mitochondrial Cyt *b* sequences (250 bp, 354 bp and 375 bp), network analyses suggested that the majority of Turkish red fox, including 49 samples, belonged to Eurasia phylogroup, and that two haplotypes (TR7 and TR10) from SW Anatolia, Turkey clustered together with three haplotypes from Hokkaido, Japan (INOUE *et al.* 2007) (Figs. 2, 3). The existence of different haplotypes within Turkey may be due to the maternal gene flow, a founder effect or mutations accumulated throughout time. The Eurasian haplotypes in the Asian part of Turkey (Anatolia) are a signal of maternal gene flow via the European part of Turkey (Turkish Thrace) in the past. As stated by many authors (TABERLET *et al.* 1998, HEWITT 1999, INOUE *et al.* 2007, AUBRY *et al.* 2009), this admixture of Eurasian haplotypes and local haplotypes in Anatolia may be a result from dispersal or migration of red foxes during the glacial and interglacial periods of the Pleistocene. However, the close relationship between red fox haplotypes from

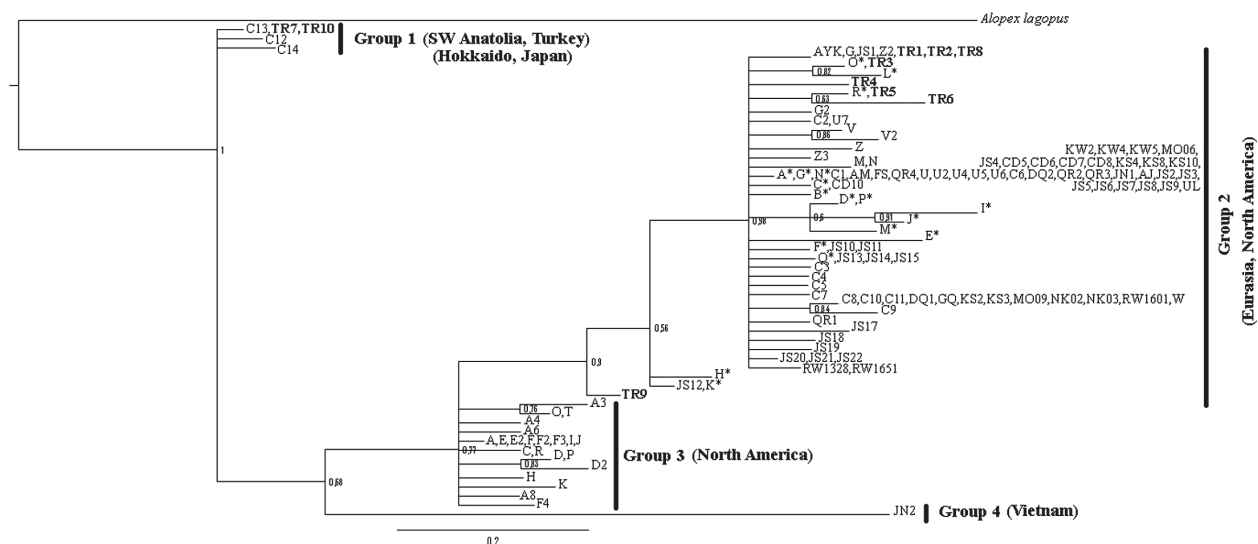


Fig. 4. Bayesian phylogenetic tree reconstructed from haplotypes of *Vulpes vulpes* in the North Hemisphere, based on 250 bp sequences of the mitochondrial cytochrome *b*. Numbers above branches show the Bayesian posterior probabilities. Bold indicates the Turkish haplotypes. See Tables 1, 2 for the sample abbreviations.

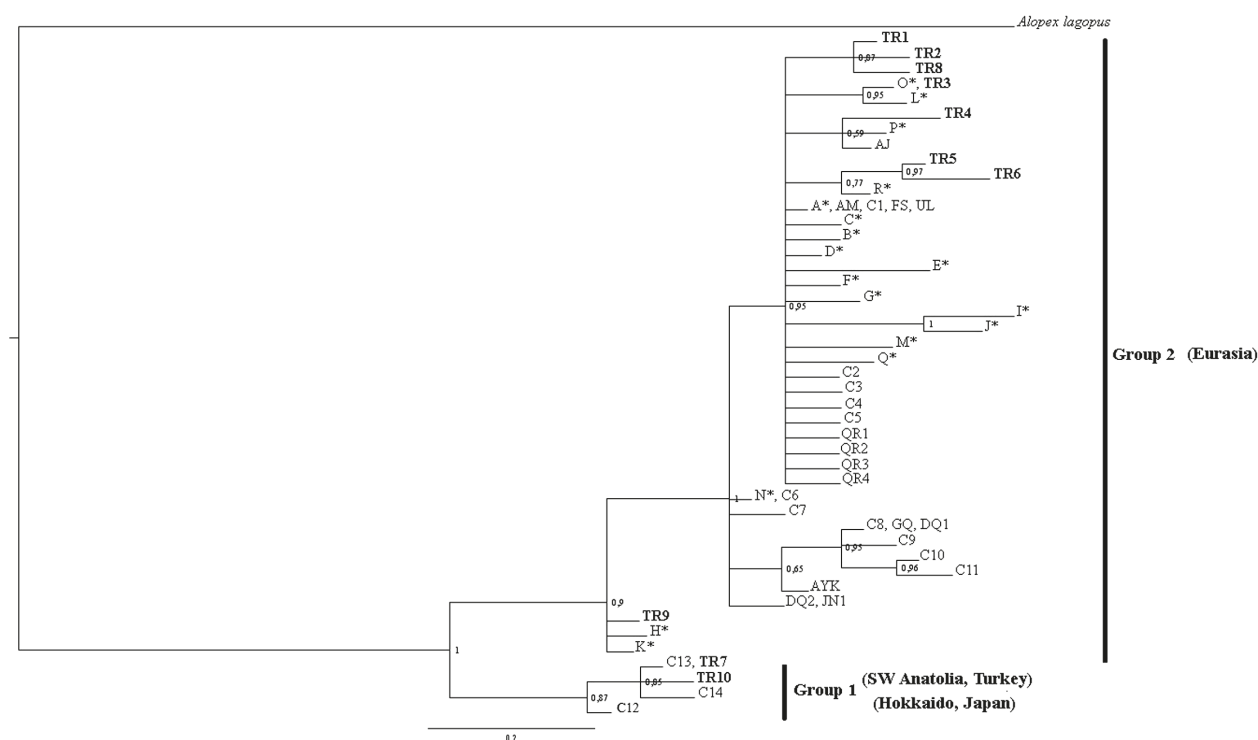


Fig. 5. Bayesian phylogenetic tree reconstructed from haplotypes of *Vulpes vulpes* in Eurasia, based on 375 bp sequences of the mitochondrial cytochrome *b*. Numbers above branches show the Bayesian posterior probabilities. Bold indicates the Turkish haplotypes. See Tables 1, 2 for the sample abbreviations.

SW Anatolia, Turkey and Hokkaido, Japan may be a result of random homoplasy. Therefore, our results indicate that the Turkish red fox also clusters within two or perhaps more phylogroups, as reported by INOUE *et al.* (2007) for the Japanese red fox (Hokkaido) and by YU *et al.* (2012a) for the Korean red fox. Based on three dif-

ferent data sets of the Cyt *b* sequences (250 bp, 354 bp and 375 bp), Bayesian analyses also suggest that Turkish red fox belongs to two highly different phylogroups and shows an unclear geographical clustering (Figs. 4, 5), similar to the haplotype networks (Figs. 2, 3). This phylogeographic pattern of Turkish red fox is compatible

with previous studies (INOUE *et al.* 2007, AUBRY *et al.* 2009, OISHI *et al.* 2011, YU *et al.* 2012a). The occurrence of two distinct phylogroups was reported by INOUE *et al.* (2007) and OISHI *et al.* (2011) using mitochondrial DNA (Cyt *b* and control region) and microsatellites in Japanese red fox, respectively, by AUBRY *et al.* (2009) using mitochondrial DNA (Cyt *b* and control region), by YU *et al.* (2012a) using the cyt *b* in Korean red fox.

There are many studies that provided useful data which assists to understand the phylogeographic patterns of numerous species across Turkey and the surrounding regions of the Mediterranean Basin. These publications cover various species like red deer (LUDT *et al.* 2004), brown trout (BARDAKCI *et al.* 2006), reptiles (JÖGER *et al.* 2007), free-living mice (MACHOLAN *et al.* 2007), house mouse (RAJABI-MAHAM *et al.* 2008), ground squirrels (GÜNDÜZ *et al.* 2007, KRYŠTUFÉK *et al.* 2009), Middle Eastern tree frogs (GVOZDIK *et al.* 2010), voles (THANOU *et al.* 2012), orthoptera (ÇIPLAK *et al.* 2005, KAYA *et al.* 2013) and herptiles (POULAKAKIS *et al.* 2013) from Holoarctic region that used to study the historical processes influencing their current distribution. Additionally, it has been suggested that the modern red fox (*V. vulpes*) originated from Asia Minor (Anatolia) or North Africa around the Mediterranean Basin (HERSTEINSSON & MACDONALD 1982, SZUMA 2008), or China (KURTÉN 1968). However, the origin of the modern red fox is discussed controversially, since the North African and Turkish red foxes are not investigated in detail thus far. Although we aimed to add some insights into the current discussion, our study has not showed a sufficient evidence that the modern red fox originated from Asia Minor (Anatolia) due to the clustering positions of Turkish haplotypes in all analyses (Figs. 2–5).

When it comes to the remarkable clustering of two haplotypes from SW Anatolia with haplotypes from Hokkaido, the indicated close relationship between haplotypes from the both areas is hardly comprehensible from the zoogeographic point of view, and it may be the result of independent evolution (random homoplasy). Given that the level of haplotype diversity in Turkey and the position of TR9 in haplotype networks (Figs. 2, 3), the northern part of Turkey, where the Turkish haplotype TR9 is distributed, may be considered as the Pleistocene refugium for red fox (Table 1, Fig. 1). The north Anatolian part of Turkey was also accepted to be a possible refugium for several species (SANTUCCI *et al.* 1998, HEWITT 1999, 2000, WIELSTRA *et al.* 2013). In contrast, recent studies, not including Turkey, suggest that red foxes in Eurasia survived the last glacial maximum as a single, large, interbreeding population (TEACHER *et al.* 2011, EDWARDS *et al.* 2012, KUTSCHERA *et al.* 2013).

In conclusion, compared with other parts of the red fox's range, the occurrence of at least two main phylogenetic groups and more than one subphylogroups (Figs. 2–5) and the finding of high genetic variation in red foxes from Anatolia confirmed the importance of the studied region. In this sense, to elucidate in depth the Pleistocene zoogeography of the Turkish red fox and the phylogeographic pattern of the red fox through-

out the species range, one should use much larger data sets from mitochondrial DNA and nuclear DNA markers and, in the meantime, collect more red fox samples from the other locations of Turkey, North Africa, North-east Europe, Caucasus and the Central Asia as well as South Asia including Iran, Southern China, Pakistan, northern India. Additionally, samples from Australia, introduced in the 1800's, may also add data sets since mitochondrial D-loop sequences are deposited in GenBank.

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