GLOBAL PHYLOGEOGRAPHIC COMPARISONS AND DIETARY ANALYSES OF GRAY WOLF AND RED FOX FROM SARIKAMIŞ AND YENİCE IN TURKEY USING GENETICS AND GENOMICS APPROACHES

by

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ABSTRACT

GLOBAL PHYLOGEOGRAPHIC COMPARISONS AND DIETARY ANALYSES OF GRAY WOLF AND RED FOX FROM SARIKAMIŞ AND YENİCE IN TURKEY USING GENETICS AND GENOMICS APPROACHES

In this study, the genetic diversity of Turkish gray wolf (*Canis lupus*) and red fox (*Vulpes vulpes*) from two locations in Turkey (Sarıkamış and Yenice), and their phylogeographic relationships with the rest of the world were evaluated based on D-loop sequences of mitochondrial DNA (mtDNA). In addition, the dietary profile of those two species was examined using an NGS-based DNA metabarcoding approach. Our gray wolf results showed that Kars-Caucasus and Kars-Karabük samples were genetically similar to each other. For red fox samples, haplotype and nucleotide diversities in Kars were found to be low. In addition, global distributions of haplotypes were detected in the haplotype network and phylogenetic trees, and the haplotypes from Kars/Turkey formed groups with these global haplotypes, without much local structuring. The results of dietary habits for gray wolves showed the following categories: mammals (red deer), amphibia (frogs), the family Aves (Passeriformes and non-Passeriformes), and plants (wheat, rose family, buttercup, beech family, the family Poaceae). On the other hand, the diet composition of red foxes included the family Aves (Passeriformes) and plants (wheat and rose family). Our results of the vertebrate and plant dietary content for the gray wolf and red fox are in concordance with the literature. In addition, the evidence of frog as prey item for gray wolves is evidence of sub-optimal feeding behaviour, potentially suggesting the scarcity of adequate food resources in Sarıkamış and Yenice Forests.

ÖZET

GENETİK VE GENOMİK YAKLAŞIMLAR KULLANILARAK SARIKAMIŞ VE YENİCE / TÜRKİYE'DEKİ GRİ KURT VE KIZIL TİLKİ'NİN GLOBAL FİLOGENETİK KARŞILAŞTIRMALARI VE DİYET ANALİZLERİ

Bu çalışmada, Türkiye'nin iki bölgesinden (Sarıkamış ve Yenice) Türk gri kurdu (*Canis lupus*) ile kızıl tilkinin (*Vulpes vulpes*) genetik çeşitliliği ve filocoğrafik ilişkileri mitokondriyal DNA (mtDNA) D-loop dizileri temel alınarak değerlendirilmiştir. Bununla yanında, bu iki türün diyet profili sonraki nesil dizileme temelli bir DNA metabarkodlama tekniği kullanılarak incelenmiştir. Gri kurt sonuçlarımız Kars-Kafkasya ve Kars-Karabük örneklerinin genetik olarak birbirine benzediğini göstermiştir. Kars bölgesindeki kızıl tilki örneklerinin haplotip ve nükleotit çeşitlilikleri düşük bulunmuştur. Ayrıca haplotip ağında ve filogenetik ağaçlarda haplotiplerin global dağılımları tespit edilmiş ve Kars/Türkiye'ye ait haplotiplerin fazla yerel yapılanma olmuşturmadığı ve bu global haplotiplerle gruplar oluşturduğu gözlenmiştir. Gri kurtların beslenme alışkanlıklarının aşağıdaki kategorilerde olduğu görülmüştür: memeliler (kızıl geyik), amfibi (kurbağalar), kuşlar (Passeriformes ve Passeriformes olmayanlar) ve bitkiler (buğday, gül ailesi, düğün çiçegi, kayın ağacı ailesi, Poaceae ailesi). Kızıl tilkilerin diyet kompozisyonu ise şu şekildedir: kuşlar (Passeriformes) ve bitkiler (buğday ve gül ailesi). Kızıl tilkiye ait omurgalı ve bitki besin içeriği sonuçlarımız literatür ile uyumlu bulunmuştur. Buna ek olarak, kurbağanın gri kurtlar için bir av maddesi oluşu, Sarıkamış ve Yenice Ormanları'nda yeterli besin kaynaklarının azlığı anlamına gelebilecek, optimal olmayabilecek bir beslenme davranışının kanıtıdır.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	**Explanation**
^{o}C	Degree Centigrade
µL	Microliter
u	Unit
π	Nucleotide Diversity
%	Percent

Abbreviation	**Explanation**
bp	Base Pair
COI	Cytochrome-c Oxidase Subunit I
CR	Critically Endangered
cyt-*b*	Cytochrome *b*
D-loop	Displacement Loop
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EMBL	European Molecular Biology Laboratory
EN	Endangered
ESU	Evolutionary Significant Unit
H	Number of haplotypes
Hap	Haplotype
H_d	Haplotype Diversity
ITS	Internal Transcribed Spacer
IUCN	The International Union for the Conservation of Nature
kg	Kilogram
km^2	Square Kilometer
kya	Thousand Year Ago
LC	Least Concerned
Mbp	Mega Base Pair
$MgCl_2$	Magnesium Chloride
Min	Minute

Abbreviation	Explanation
ML	Maximum Likelihood
mm	Millimeter
mM	Milimolar
mt-COI	Mitochondrially Encoded Cytochrome C Oxidase I
mtDNA	Mitochondrial Deoxyribonucleic Acid
Mya	Million Years Ago
N	Number of Sequences
ng	Nanogram
NGS	Next Generation Sequencing
NT	Near Threatened
Numt	Nuclear-mitochondrial DNA
NJ	Neighbour Joining
PCR	Polymerase Chain Reaction
PIS	Parsimony-informative Sites
PopART	Population Analysis with Reticulate Trees
PS	Polymorphic Sites
sec	Second
SV	Singleton Variable Sites
TLC	Thin layer chromatography
V	Volt
VU	Vulnerable

1. INTRODUCTION

1.1. Global Phylogeographic Comparisons and Dietary Analyses of Gray Wolf (*Canis lupus*) and Red Fox (*Vulpes vulpes*) from Sarıkamış and Yenice in Turkey Using Genetics and Genomics Approaches

Significant population declines and habitat destruction have been experienced globally by multiple carnivore species in the last few centuries for multiple reasons (Ceballos et al., 2002). For instance, humans commonly persecute African wild dogs, dingoes, coyotes and gray wolves due to their predation of livestock. Some of the smaller canids, especially some South American foxes and the Arctic fox have been hunted for their furs historically, even though there has been a decline of the fur trade more recently. These direct threats, which are usually limited in terms of their geographic scope are naturally rare, however human activities often lead to indirect threats due to habitat loss, persecution and diseases, as well (Sillero-Zubiri, 2009). An example to indirect effects is the local extinction of Rüppell's fox in the Negev Desert of Israel, as a result of the widespread increase in red fox populations due to increased levels of agricultural practices (Alves et al., 2010). Due to these direct and indirect effects, multiple canid species are now threatened. The International Union for the Conservation of Nature (IUCN) considers many of the Canidae species (61%) as endangered or threatened, at the risk of local or total extinction. Ethiopian fox (*Canis simensis*), African wild dog (*Lycaon pictus*), and Asiatic wild dog (*Cuon alpinus*) are the canids in the IUCN Red List, under the ‘endangered’ category (IUCN, 2018). Table 1.1 presents the list of all of the canids in the IUCN Red List, and their threat categories.

Table 1.1. Endangered Canidae species and their IUCN status, and population trends. LC-Least Concerned; CR-Critically Endangered; NT-Near Threatened; EN-Endangered; VU-Vulnerable.

Canidae Species	**Common Name**	**IUCN Status (Trend)**	**References**
Canis lupus	Gray wolf	LC (stable)	Mech, L.D., Boitani, L., 2010
Vulpes vulpes	Red fox	LC (stable)	Sillero-Zubiri, C., Hoffmann, M, 2016
Canis rufus	Red wolf	CR (increasing)	Sillero-Zubiri, C., Hoffmann, M., 2008
Chrysocyon brachyurus	Maned wolf	NT (unknown)	Hoffmann, M., Sillero-Zubiri, C., 2016
Lycaon pictus	African wild dog	EN (decreasing)	Hoffmann, M., Hilton-Taylor, C., 2012
Cuon alpinus	Dhole	EN (decreasing)	Hoffmann, M., Sillero-Zubiri, C., 2016
Canis dingo	Dingo	VU (decreasing)	Letnic et al., 2012
Canis simensis	Ethiopian wolf	EN (decreasing)	Hoffmann, M., Murdoch, J.D, 2011

1.1.1. General Characteristics of Canids

Canids belong to a diverse group of predatory mammals, Order *Carnivora*, which have one pair of canine teeth that increase cutting performance (Sillero-Zubiri et al., 2004). *Feliformia* and *Caniformia* are the suborders within *Carnivora*. *Feliformia* includes felids, mongooses, hyenas among others, while the *Caniformia* includes canids alongside bears, mustelids, pinnipeds etc. Canids constitute the Family *Canidae*, where diagnostic features include the presence of an inflated entotympanic bulla, and a bony chamber that surrounds the middle ear region (Macdonald et al., 2004). The family *Canidae* represents three subfamilies; the *Hesperocyoninae* (includes seven extinct genera), the *Borophaginae* (includes four extinct genera), and the *Caninae* (includes two extinct genera, dogs, and foxes, among others). Today, the canids are recognized in approximately 16 genera and 36 species, with a rich fossil record.

Canids form a broadly distributed family of the order Carnivora. They have members on each continent except Antarctica (Sillero-Zubiri et al., 2004). They show higher dispersal capabilities when compared to numerous other non-volant species (Macdonald et al., 2004). Moreover, many canids are generalists, thus they can cross even the most extremely anthropomorphized regions; as an example, the presence of red fox in urban areas can be given (Wandeler et al., 2003). Therefore, weak patterns of intraspecific distinction can be commonly found in geographically remote areas (Roy et al., 1994; Vilà et al., 1999). On the other hand, genetic structure was detected due to density effects

(Roemer et al., 2001), prey specialization and movement (Musiani et al., 2007), kinship (Girman et al., 2001), and natal habitat biased dispersal (Sacks et al., 2004, 2008; Musiani et al., 2007), among others.

Another important aspect of canid biology is intrigued animosity among sympatric species. Whether it is a result of overlapping niches or is directly through spatial displacement and intrigued predation, the outcomes of these unfriendly dynamics can change styles of habitat use, social structure, and eventually the size of populations (Tannerfeldt et al., 2002; Sillero-Zubiri et al., 2004; Roth et al., 2008). Numerous cases have been reported, and the bias is for species more similar in size and with overlapping ranges to have the strongest competition. Some of these cases include African wild dogs (*Lycaon pictus*) predating on the bat-eared foxes (*Otocyon megalotis*) (Rasmussen, 1996), red foxes excluding arctic foxes (*Vulpes lagopus*) (Rodnikova et al., 2011), and gray wolves (*Canis lupus*) excluding coyotes (*Canis latrans*) (Arjo and Pletscher, 1999). However, there are also cases where, contrary to the expectations, either no aggressive behavior was observed or the smaller species was dominant over the larger species (Loveridge and Macdonald, 2003).

1.1.2. Carnivore and Location Choice for This Study

Turkey, a natural land bridge connecting Europe and Asia, hosts various mammal species (Johnson, 2002), including 22 potential carnivore species (Şekercioğlu et al., 2011a). Among those, the family Canidae is represented by three species in Turkey, two of them being the gray wolf (*Canis lupus*) and the red fox (*Vulpes vulpes*) (Ambarlı et al., 2016). Predators play a major role in the maintaining of ecosystem balance, and research has shown the importance of wolves for the functioning and stability of various ecosystems (Ripple and Beschta, 2012). For instance, predation by wolves regulates ungulate distributions and group size, which affects levels of biodiversity (White et al., 2010). Similarly, the red fox is a significant component of the ecosystems it inhabits, a predator for various game species (Jensen, 1970), and an important control agent for others (Johnson, Sargeant and Greenwood, 1989).

Gray wolf and red fox showed severe declines in the past in Turkey, especially due to being hunted for fur trading, road accidents, habitat degradation, poaching and poisoning (Can, 2004; Şekercioğlu et al., 2011b). In this perspective, these two species comprise the species of interest for this thesis, with analyses of their biology undertaken in Yenice, Karabük and Sarıkamış, Kars in Turkey.

1.1.3. Importance of Yenice, Karabük Province in Terms of Wildlife Diversity and Conservation Biology

Yenice, Karabük is located in central/northern Turkey, and it comprises one of the largest intact natural habitats for mammals in Turkey (Morrison et al., 2007). Field studies show that 28 mammal species have been recorded in the province of Karabük, Turkey. When other mammal species in the literature are added, the total number goes up to 57 (CSB-Karabük, 2017). In addition, Yenice forests comprise one of the safe havens in the region against hunting for many species, such as the bear (*Ursus arctos*), red deer (*Cervus elaphus*), and roe deer (*Capreolus capreolus*) (Ünal, 2012).

1.1.4. Importance of Sarıkamış, Kars Province in Terms of Wildlife Diversity and Conservation Biology

Kars province is a montane area interspersed with agricultural fields, where water resources comprise critical habitats for wildlife. Considering mammals, 17 different species inhabit Kars, including wolf (*Canis lupus*), jackal (*Canis aureus*), wild boar (*Sus scroto*), mongoose (*Herpestos ichneumon*), rabbit (*Lepus capeosis*), fox (*Vulpes vulpes*), wild goose (*Capra aegagrus*), chamois (*Rupicapra asiatica*), lynx (*Felis Iynze*), bear (*Ursus*), squirrel (*Sciurus anomolus*), weasel (*Mustela navalis*), badger (*Meles meles*), otter (*Lutra lutra*), and hedgehog (*Erinacous concolar*) (CSB-Kars, 2017). Some of the isolated forests in Sarıkamış/Kars provide insufficient habitat for large mammals, increase these species' vulnerability, and decrease their genetic diversity. Insufficient carnivore habitat and illegal hunting of natural prey species of carnivores lead to wolves and brown bears feeding in garbage dumps and on livestock, increasing the levels of the human-carnivore conflict (Chynoweth, 2016), and making the province an interesting place to study canid ecology and evolutionary history.

1.1.5. General Biology of the Gray Wolf (*Canis lupus*)

1.1.5.1. Physical characteristics. The gray wolf is the largest member of family Canidae, which is a lineage that includes jackals, foxes, coyotes and other dog-like mammals (Wilson and Reeder, 2005). The gray wolves generally use keen senses, they have large canine teeth and strong jaws. A male can reach about 2 meters in length, including a tail of about half a meter. They are on average 75 cm tall at the shoulder, and weigh about 45 kg. The range of weights has been observed to vary from 14 kg to 65 kg. depending on different geographical areas. The average female wolves are 20 percent

smaller than the males. Although they are usually gray, the fur of gray wolves can be brown, reddish, black or whitish. The underparts and legs of the body are generally yellow-white. Light-colored wolves are common in the Arctic regions (Britannica, 2018).

1.1.5.2. Distribution and evolution. Canids appeared in North America ca. 6 million years ago in the late Miocene period, and reached Asia in Beringia in a period of global warming, in early Pliocene about 4-5 million years ago (Wang and Tedford, 2008). During this period and later in North America and Eurasia, various species of the genus Canis began to appear. The gray wolves, extremely mobile carnivores, spread over the world to find new habitats and mates (Vilà et al., 1999). Morphological (Nowak, 1995) and genetic (Wayne et al., 1997) studies support that wolves and coyotes share common ancestory. This ancestral species is referred to as *Canis lepophagus* (or *Canis arnensis*) in the fossil record. *C. lepophagus* appeared in Northern America at the end of the Pliocene (ca. 2.6 Mya), and subsequently spread throughout Eurasia. The coyote and wolf have diverged from common lineages about 1.5 Mya (Nowak, 2003; Wang and Tedford, 2008). Based on the fossil record, gray wolves reached Europe ca. 800 kya (Wang and Tedford, 2008). The range of the Eurasian wolf (*Canis lupus lupus*) known to have the largest range among various gray wolf subspecies extends from Portugal to China (Vilà et al., 1999).

1.1.6. General Biology of the Red Fox (*Vulpes vulpes*)

Although the size of the red foxes is categorized to be medium among canids, they are regarded as the largest of the genus "*Vulpes"*, which includes 12 species. The species shows the widest geographical range within the order Carnivora, across the entire Northern Hemisphere, spreading to the areas of extreme cold or aridity. In Africa, they are found only in the Mediterranean area (Macdonald and Reynolds, 2008). This wide distribution is accompanied by considerable geographical variation in the body size of adults, e.g., red foxes from the Middle East and Algeria are smaller than their European counterparts (Sillero-Zubiri et al., 2004). Being a very resilient and adaptable species, it lives in a broad variety of habitats, through a wide range of altitudes and latitudes, including tundra, desert and forests, and also agricultural areas and city centers (Macdonald and Reynolds, 2008). Red foxes are known to flourish in regions of intense human presence (Macdonald et al., 2004). The deterioration, fragmentation and loss of habitat, and also the direct and indirect effects (e.g. legal hunting) comprise the most pressing threats for their survival. Still, its adaptability and opportunistic nature enables the red fox to successfully persist throughout its distribution area (Macdonald and Reynolds, 2008).

1.1.6.1. Main physical characteristics. Foxes have generally smaller bodies than the other members of the family Canidae, such as wolves, domestic dogs and jackals. Although foxes are characterized by some fundamental physical features, some of these characteristics vary based on habitat (Hildebrand, 1952). Red fox is a homoeothermic and endothermic species. Its fur varies from pale yellowish red to deep reddish brown on its dorsal side, and is whitish on the ventral side of its body. Its body length and head length can reach up to 900 mm and 455 mm, respectively, and its tail length varies between 300 - 555 mm. The body weight is between 3 - 14 kg. It has tail glands similar to other canid species, however it is above the root of the tail on the dorsal side. Its eye color is ordinarily yellow. The manus has five claws and the pes has four claws, where the dewclaw is primitive and does not touch the ground. Feeding habits of red fox are affected by their teeth row, which is more than half the length of the skull (MacDonald and Reynolds, 2005).

1.1.6.2. Distribution and evolution. The red fox has the widest distribution when compared to any carnivorous and terrestrial mammal (Schipper et al., 2008), inhabiting an area of over 70 million km^2, covering much of North America, Europe, Asia and North Africa (Lariviére and Pasitschniak-Arts, 1996). Its natural habitat conditions range from arctic tundra, forests, grasslands and temperate deserts to environments occupied by people.

Red foxes expanded into North America at least 500 kya, but were confined above the Arctic Circle for a considerable time before reaching the midcontinent in the last glacial cycle (ca. 100 kya) (Wang and Tedford, 2008). Despite the availability of mtDNA data in North America and Eurasia (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 still insufficient data on fox genetic diversity and phylogenetic configuration in Turkey and the Middle East.

1.1.7. Phylogeography and its Role in Conservation

The term ‘phylogeography’ can be defined as the “phylogenetic analysis of geographically contextualized genetic information for testing hypotheses with respect to the relationship between geographic phenomena, species’ distribution and the speciation driving mechanisms” (Hickerson et al., 2010). Currently, the term phylogeography refers to a wide range of methodologies and techniques, which combine phylogenies and statistical models that lets the formulation of models and a priori hypothesis testing for investigating the evolutionary histories especially at the intraspecific level, i.e. within species. This new branch of biological knowledge has contributed to the estimation

of demographic histories, migration rates, historical hybridization events, hybrid zones, introgression, all of which help to understand the key roles of biodiversity in ecological and evolutionary processes (Hickerson et al., 2010).

Studies of intraspecific phylogeography also have significant conservation implications, resulting in the designation of units of protection. For instance, Evolutionary Significant Unit (ESU) is such a unit of conservation below the species level, which is isolated by reproduction, and represents a significant element of evolutionary inheritance of a particular species (Moritz, 1994). The definition of ESUs in various national conservation legislations as well as in the European convention on biological diversity has rendered it invaluable for the conservation of natural and captive populations. It is important to define the ESU to maintain the evolutionary potential of natural populations (Moritz, 1995), and phylogeographic studies are important for their definition. Phylogeography can also help prioritize of fields of high value for conservation (Moritz, 1994). In this perspective, a literature review of the phylogeographic studies of the species of interest for this study, the gray wolf and the red fox, follows next.

1.1.8. Phylogeographic Studies on the Gray Wolf

There are multiple studies investigating phylogenetic relationships of gray wolf populations and species, based on mtDNA, effectively spanning the entire world (Table 1.2). The table provides information in terms of target DNA source, the geographic region of interest, the number of samples, markers used, and references.

Table 1.2. The studies of phylogenetic relationships of gray wolf populations based on mtDNA.

Target Sample	**Region of The Sample**	**Sample Quantity**	**Marker**	**References**
Organs and Whole Blood	North America, Europe, China and Middle East	350	mtDNA restriction site (500 bp)	Wayne et al., 1992
Tissue and serum samples	Sweden, Norway	36	mtDNA d-loop (257 bp) and nuclear DNA (Microsatellite)	Ellegren et al., 1996
Not available	North America, Asia, Europe (+**Turkey**)	162	mtDNA d-loop (261 bp)	Vila et al., 1997
Sequences from a previous study, **fecal**, blood and tissue	From 30 localities worldwide	259	mtDNA d-loop (350 bp)	Vila et al., 1999
Tissue	Italy, Bulgaria, Greece, **Turkey (Asia)**, Croatia, Finland, Israel, Slovakia, Spain	148	mtDNA d-loop (546 bp)	Randi et al, 2000
Teeth and Tissue	Norway and Sweden	55	mtDNA d-loop (229 bp)	Flagstad et al., 2003
Hairs with roots and tissue	Belarus and Poland	mtDNA(35)+nuclear (29)	Polymorphic mtDNA fragment (300 bp) + nuclear DNA	Jędrzejewski et al., 2005
Tissue	Poland, Lithuania, Latvia, Belarus, Ukraine, the European part of Russia, Slovakia, Bulgaria, Greece and **Turkey (Europe)**	643	HV1 domain of the mtDNA d-loop (257bp)	Pilot et al., 2006
Blood, hair, buccal swabs	Mongolia, China, Afghanistan, Romania, China, Yugoslavia, Russia, Canada, Spain, Sweden, Saudi Arabia	mtDNA d-loop (40)/mtDNA genome(8)	mtDNA d-loop (582 bp) and mtDNA d-loop genome (16,195 bp)	Pang et al., 2009
Blood and muscle	Croatia	91	mtDNA d-loop (281 bp)	Gomercic et al., 2010
Blood and tissue	North America	124	mtDNA d-loop (224 bp)	Fain et al., 2010
Tissue	Southern and Northern Europe	947 + 24 ancient	mtDNA d-loop (230 bp/661 bp/57 bp (ancient)	Pilot et al., 2010
Not available	North America and Eurasia	89	mtDNA d-loop (347 bp) and Atpase 6 & 8 genes (1067 bp)	Rutledge et al., 2010
Tissue	Pasific Northwest	307	mtDNA d-loop (380 bp) and mtDNA cyt-b gene (611 bp)	Weckworth et al., 2010
Tissue	Coastal Columbia and Southeast Alaska	310	mtDNA d-loop (335 bp) and mtDNA tRNAs (91 bp)	Weckworth et al., 2011

Table 1.2. Continued.

Target Sample	**Region of The Sample**	**Sample Quantity**	**Marker**	**References**
Tissue	Iran	22	mtDNA d-loop (544 bp)	Aghbolaghi et al., 2014
Skin and Blood	Saudi Arabia	103	mtDNA d-loop (300 bp) and mtDNA cytb gene (800/400 bp)	Bray et al., 2014
Muscle Tissue	Serbia, Macedonia, Bosnia and Herzegovina	87	mtDNA d-loop (192 bp)	Djan et al., 2014
Tissue or Blood	Croatia, Bosnia- Herzegovina and Italy	434	mtDNA d-loop (280 bp), STR and Y-STR	Fabbri et al., 2014
Bones, dry blood and tissue	Finland	114 ancient	mtDNA d-loop (450 bp)	Jansson et al., 2014
Bone powder	Japan	56 + 18 ancient	Complete mtDNA genome sequences	Matsumura et al., 2014
Fecal	Nepal	6	mtDNA d-loop (440 bp) + 200 bp for ancient samples	Chetri et al., 2016
Blood, Tissue, Hair	North America, Scandinavia,Russia/Siberia, Iran, China, Greenland	122 + 6 ancient	mtDNA d-loop (582 bp)	Ersmark et al., 2016
Bone powder	China, Russia and Japan	113 ancient	mtDNA d-loop (360 bp)	Ishiguro et al., 2016
Tissue (ear, skin tail, etc.)	**Asian Part of Turkey**	12	mtDNA d-loop (332 bp) and partial mtDNA seq. (440 bp)	İbiş et al., 2016
Not available	From 22 localities worldwide	95 + 10 ancient	Sequences of the 12 protein coding genes (mtDNA genomes)	Koblmüller et al., 2016
Tissue, hair	Morocco	3	mtDNA d-loop (680 bp) + Complete mtDNA + Cytb	Moliner et al., 2016
Fecal, Saliva, Hair	Denmark, Northern Zealand	145	mtDNA d-loop (250 bp) + Y chromosome	Nielsen et al., 2016
Hair	USA	22	mtDNA 16S rRNA (338 bp) + nuclear 18S rRNA	Sastre et al., 2016
Skin	Denmark	13	mtDNA d-loop (230 bp)	Thomsen et al., 2016
Fecal	Portugal	12	mtDNA d-loop	Quaresma et al., 2016
Tissue, blood	Italy, Spain, Portugal, Slovenia, Croatia, Greece, Bulgaria, Czech Rebublic, Slovakia, Poland, Estonia, Latvia and Finland	190	mtDNA d-loop (498 bp) + ATP6 (588 bp)	Montana et al., 2017
Tissue, blood	Iberian	56	mtDNA d-loop (420 bp) + autosomal STR + Y-SNPs + Y-STR	Pires et al., 2017
Fecal, Saliva, Hair	Portugal	93	mtDNA d-loop (442 bp) + Microsatellite	Torres et al., 2017

In five of the studies, the mtDNA d-loop region was used. Less than 250 bp long sequences were examined in five studies, and greater than 400 bp long sequences were amplified in 11 studies. The total number of the gray wolf species analyzed was less than 100 samples in 16 studies, and more than 300 in four studies. The phylogeographic relationships of the gray wolf populations were investigated using various sample types such as tissue (22 studies), blood (11 studies), hair (seven studies), bone (four studies), serum (one study), and feces (five studies).

Gray wolf samples studied by various researchers were collected from different regions of the world. Some studies had a regional focus, e.g. Turkey (Asia) (two studies) - Turkey (Europe) (one study), Northern Zealand (one study), and Balkans (seven studies). On the other hand, others were continental-scale studies such as in Europe (18 studies), Asia (six studies), US (five studies), Middle East (three studies), northern North America (one study), and western North Africa (one study). Vilà et al., (1999), investigated the phylogeographic relationships of gray wolf populations with worldwide sampling, based on partial mtDNA d-loop sequences. The result of this global study showed that the changing population sizes during the late Pleistocene have created a signature on the levels of genetic variation in gray wolves, and demonstrated little genetic partitioning at both continental and regional scales.

Phylogeographic analyses of a limited number of gray wolf samples from Turkey have also been performed by various researchers (Vilà et al., 1997, 1999; Randi et al., 2000; Pilot et al., 2006, 2010; İbiş et al., 2016). In the study of Randi et al., (2000), a set of Italian wolves were sequenced for the mtDNA d-loop, and the results were compared with data from other wolf and dog populations from Europe and the Near East, with two gray wolf samples collected from Anatolia. Wolf haplotypes clustered into five major lineages, with L3 lineage comprising Turkish, Bulgarian, Croatian and Israeli haplotypes. In another study, Pilot et al., (2006) investigated wolf DNA collected from Eastern Europe and the European part of Turkey. The populations showed non-random genetic structure in the absence of clear physical barriers to movement. The study showed that most of the local wolf populations located in Eastern Europe had more than one mtDNA haplotype, and most haplotypes were widely distributed. The distribution of the haplotypes did not show any obvious geographical pattern. Clades showed that the Eastern European wolf populations did not include any geographically distinct subunits. İbiş et al. (2016) studied 12 Turkish gray wolf samples, collected from the cities of Ardahan, Bolu, Çankırı, Erzurum, Iğdır, Kars, and Yozgat. They found seven d-loop haplotypes, five of which were shared with conspecific sequences belonging to other Eurasian

regions. On the other hand, the two haplotypes that were not recorded previously were unique for the Turkish population.

1.1.9. Phylogeographic Studies on the Red Fox

Various phylogeographic studies based on mtDNA for red foxes underline the importance of taking a geographically broad snapshot of genetic variation from the entire range of the species (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). Different studies resulted in the definition of mitochondrial phylogroups categorized as Holoarctic and/or Eurasian, Nearctic, and Hokkaido/Japan (Inoue et al., 2007; Aubry et al., 2009; Yu et al., 2012; Kutschera et al., 2013), suggesting limited gene flow between these regions.

Considering Turkey, the information on genetic diversity and phylogeographic structure of the red fox is insufficient, with only one study (İbiş et al., 2014) having investigated the phylogenetic position and genetic diversity of the Turkish red fox populations. In this study, İbiş et al., used a partial fragment of the mitochondrial cyt-b gene to evaluate the phylogeographic structure of the red fox from 51 localities from Turkey. They compared the haplotype and nucleotide diversity in Turkish red foxes with data from other studies, with samples collected from the Mediterranean Basin, Northern Japan, North America and East Asia. Their results also indicated high genetic diversity within the Turkish red foxes.

There are multiple studies investigating phylogenetic relationships of red fox populations, based on mtDNA, effectively spanning the entire world. A summary of the studies on phylogeographic relationships of red fox populations from the literature is shown in Table 1.3. In a parallel manner to the summary for the gray wolf, this table provides information in terms of target DNA source, the geographic region of interest, the number of samples, markers used, and references for phylogeographic studies on the red fox.

Table 1.3. The studies of phylogenetic relationships of red fox populations based on mtDNA.

Target Sample	**Region of The Sample**	**Sample Quantity**	**Marker**	**References**
Tissue	Spain, Italy, Austria, Bulgaria, Israel	120	mtDNA cytb (375 bp)	Frati et al., 1998
Blood and tissue	Japan	88	mtDNA d-loop (397 bp) + mtDNA cytb (375 bp)	Inoue et al., 2007
Tissue	Northern Serbia	110	mtDNA d-loop (335 bp)	Kirschning et al., 2007
Fecal, Ear punches	California	85 (ancient+modern)	mtDNA cytb (354 bp)	Perrine et al., 2007
Fecal, Muscle, skin	Iberian Peninsula	Not available	mtDNA cytb (227 bp)	Fernandes et al., 2008
Turbinate bone	Northern America	220 (ancient)	mtDNA d-loop (324 bp) + mtDNA cytb (354 bp)	Aubry et al., 2009
Fecal, Bone, Skin snips	US	63 (modern + ancient)	mtDNA d-loop (342 bp) + mtDNA cytb (354 bp) + microsatellite	Sacks et al., 2010
Fecal, muscle tissue, blood	California	103	mtDNA cytb (354 bp) + nuclear genome + microsatellite + SNPs	Sacks et al., 2011
Blood	Russia	8	mtDNA d-loop (342 bp) + mtDNA cytb (354 bp)	Statham et al., 2011
Bone, tooth	Austria, Bulgaria, England, Germany, Israel, Italy, Portugal, Sardinia, Spain, Sweden, UK, Ukrain	165 (modern + ancient)	mtDNA d-loop (268 bp) + mtDNA cytb (250 bp)	Teacher et al., 2011
Tissue	Denmark, England, France, Germany, Holland, Ireland, Norway, Italy, Poland, Serbia, Scotland, Sweden, Wales	333 (modern + ancient)	mtDNA d-loop (409 bp) + mtDNA cytb (464 bp)	Edwards et al., 2012
Nasal, Bone, Tissues, Buccal swaps	Eurasia, Alaska, Norway, Russia, Canada, the northeastern US, Montane areas in the Western Contiguos US	148 (modern + ancient)	mtDNA d-loop (342 bp) + mtDNA cytb (354 bp)	Statham et al., 2012
Blood, muscle tissue, hair	North korea, Russia, China, Mongolia, Seoul, Korea	18	mtDNA cytb gene	Yu et al., 2012
Fecal, Muscle, hair, skin	Germany, Finland, Poland, Central Siberia	53	mtDNA d-loop (449 bp)	Kutschera et al., 2013
Tissue (liver, kidney, muscle)	Croatia	229	mtDNA d-loop	Galov et al., 2014
Tissue (ear, tail, skin, skeletal muscle)	51 different localities in Turkey	51	mtDNA cytb (375 bp)	İbiş et al., 2014
Tissue	North America	189	mtDNA d-loop (329 bp)	Langille et al., 2014
Blood, tissue, museum specimens	Southern Europe, Japan	1164 (76 red fox)	mtDNA d-loop (343 bp) + cytb (354 bp)	Statham et al., 2014
Tissue	North Africa, Europe and Middle East	39	mtDNA d-loop (226 bp) + cytb (364 bp)	Leite et al., 2015
Bone extracts, skin snips, frozen tissue	North America	126	mtDNA d-loop (601/1432) + cytb (100-200/399 bp)	Volkmann et al., 2015
Skin, blood	Poland, North America	170	mt-ATP6 gene (385 bp) + mtCO1 gene (646 bp)	Horecka et al., 2017

In terms of the genetic regions of interest, out of 21 studies in total, the mtDNA d-loop region was used in 14. Sequences shorter than 100 bp and longer than 300 bp long were used in nine studies, and 11 studies, respectively. The total number of the red fox samples analyzed was less than 100 samples in nine studies, and more than 200 in three studies. The phylogeographic relationships of the red fox populations were investigated using various sample types such as tissue (18 studies), blood (six studies), hair (three studies), bone (six studies), and feces (five studies).

Geographically speaking, red fox samples in these studies were collected from different regions of the world, including US, Balkans, Central Europe, UK, Northeastern Europe, Western Europe, Canada, Central-Eastern Asia, North Africa, Eastern Europe and Northern Asia, Southern and Northern Europe, and the Middle East. The only study where samples (n=51) from Turkey were investigated was that of İbiş et al. (2014). The results of the Bayesian and network analyses in this study showed that the red fox was divided into four phylogroups; one from Anatolia, Turkey and Hokkaido, Japan, another from Eurasia and North America, and one group each from North America and Vietnam. The study also showed that Turkish red foxes exhibited high levels of genetic diversity.

1.2. Dietary Habits through Genetics

Multiple hypotheses have been raised with regards to the causes of the global extinction crisis we are facing today; however, hunting and human caused habitat alterations stand out as the primary reasons (Laliberte and Ripple, 2004; Ripple et al., 2014). Specifically, there is a serious decline in large mammal carnivores (≥15 kg) populations (Ripple et al., 2014). These declines in large carnivores populations makes studies on their diet critical for their protection and management. Dietary investigations focus on determining the primary prey of carnivores, as well as the predator individuals or populations that depend on livestock, causing human-wildlife conflict (Gese, 2001; Shehzad et al., 2012b), both of which can be essential for setting up preservation and management priorities (Gese, 2001; Shehzad et al., 2012b). The dietary habits of species are also essential to examine complex ecosystem processes (Treves and Karanth, 2003; Razgour, 2011), as they help to better understand the prey-predator interactions at each trophic level (Deagle, 2010; Razgour, 2011). At the species level, the food habits and the predation risk help to understand phenomena such as resource selection, changes within the population, and physiological health (Mills, 1992; Deagle, 2010)

Traditionally, dietary scat studies have relied on morphological recognition of inedible prey remains (Symondson, 2002, Sheppard and Harwood, 2005). To obtain reliable dietary data of prey

species, morphological analyses and identification keys are used in standard protocols (Ciucci et al., 1996; Kennedy and Carbyn, 1981; De Marinis and Asprea, 2006). In the traditional method, the scat analysis is used to identify undigested biological material (Casper et al., 2007a), such as teeth, hair particles, feathers, scales and bones (Livaitis, 2000). However, the accuracy of the morphological approaches is considered to be limited due to the relative absence of identifiable hard parts (Casper et al., 2007a), and the difficulty of their distinction in closely related species (Spaulding, 2000; Zeale et al., 2011).

Although, morphological prey identification protocols have been improved in Europe (Ciucci et al., 1996), the approach is relatively labor intensive and may miss or underestimate prey (Casper et al., 2007b). It can also be influenced by observer bias when rigorous sampling protocols and observer training might be lacking or inadequate (Spaulding et al., 2000). Some prey remains may be unidentifiable due to hair length, digestion related processes and prey size (Spaulding et al., 2000). Investigations of predators also show that morphological dietary analyses can miss information on numerous trophic connections (Dennison and Hodkinson, 1983; Feller et al., 1985; Jarman et al., 2013). More recently, DNA-based dietary studies became a practical alternative technique for morphological analyses, and numerous studies have utilized the technique in studies of vertebrate dietary ecology. The use of PCR makes it possible to amplify trace amounts of degraded DNA in predator feces (King et al., 2008), and short mtDNA amplicons increase the success in the amplification process (Sutherland, 2000). Next generation sequencing techniques are also being used to define a wide range of prey species in generalist or rarely studied predators (Shehzad et al., 2012b; Jarman, 2013).

1.2.1. Traditional Methods for Carnivore Diet Analyses

1.2.1.1. Direct monitoring of predation. Various direct approaches have been used for dietary analysis, all with their potentials and pitfalls (Shrestha and Wegge, 2006). Observing predation in the field is probably the most straightforward method (Scheiffarth, 2001). This approach provides both accurate and direct data on diet of animals. Moreover, additional information on the prey animals, such as age and sex can be obtained. A caveat is that, the act of observation can cause changes in the predation behavior, due to the presence of the observer (Gordon, 1995). Another disadvantage is that only a limited number of individuals can be monitored at a time. It is also difficult to study the diets of nocturnal animals, as such studies require special night vision devices (Allison and Destefano, 2006). Furthermore, it may not be possible to directly follow prey intake if the animal is elusive and

inhabiting hard-to-access regions (McCarthy et al., 2008; Ale and Brown, 2009). As a variation for this method, video monitoring systems have been used to obtain information about the diets of both predator and prey species. Using this appraoch, the dietary data can be obtained without disturbing the animals (Varley et al., 1994; Merfield et al., 2004). However, video monitoring techniques can be inadequate in the field conditions, due to reasons such as low predator abundance and difficulty of capturing of foraging and hunting behaviour by camera (Grönberg, 2011).

1.2.1.2. Morphological diet analysis. Traditional morphological mammalian dietary analyses are through identification of prey remains in feces (Farrel, 2000). Feces represent an accessible and easily collected source of data, which can be obtained without disturbing the animal (Putman, 1984). This is a key characteristic, especially when investigating endangered and elusive animals (Kohn and Wayne, 1997). The essential limitation of this sampling procedure is that the characterization is for the short-term diet (Deagle, 2006). Carnivore feces include undigested components, such as bones, hairs or shells that assist with the identification of the devoured prey (Bowen, 2000, Pickering, 2001). However, the recovery of undigested remains and hard parts is not easy, e.g. large bones and teeth are fragmented and may not be easy to distinguish (Oli, 1993).

One of the most common techniques used in the study of the diet of endangered and elusive carnivores is the "hair mounting method", which includes the analysis of undigested prey hair in predator feces (Bianchi et al., 2011). This approach compares hair slides with reference samples (Oli, 1993; Mukherjee et al., 1994), however the method can be exhausting and tedious because it requires the preparation of a large number of slides from each sample to get reliable data (Oli, 1993), and hair from some closely related species can have indistinguishable characteristics (Hall-Aspland and Rager, 2007). The absence of the reference specimens may also limit accurate prey identification (Fernandez et al., 1997).

These shortcomings of the hair-slide approach have been bypassed through new technical developments that do not require recovery of digestion-resistant dietary components. Thin layer chromatography (TLC) of fecal bile acids can be used as an alternative in this regard (Fernandez et al., 1997). The fatty acids released from ingested lipid molecules are not degredad during digestion. The differentiation of dietary and non-dietary components is possible, as animals can synthesize a limited number of fatty acids. However, the main limitation of this method is that the signatures of fatty acids of prey species may be very close that of the predator, thus the identification may be difficult (Piche et al., 2010). Analysis of stomach content or stomach flushing (Wilson, 1984) from

animals killed due to natural causes can also be used for diet determination. Soft-body prey contents in fecal samples are mostly underestimated, but actual assessment of digesibility of consumable prey items provides an obvious advantage for this approach (Hyslop, 1980).

1.2.2. Molecular Methods for Carnivores Diet Analyses

1.2.2.1. PCR-based / DNA-based methods. Molecular diet analysis has certain advantages when compared to traditional methods, as they do not depend on morphological characteristics that may be damaged during capturing, ingestion and digestion (Jarman et al., 2002; King et al., 2008). The method also provides higher resolution for the identification of prey items, when compared to the traditional methods (Zeale et al., 2011).

Recently applied molecular techniques provide objective identification of both soft and hard materials in scats. Therefore, some of the challenges of the traditional morphological approaches can be eliminated or limited by the help of these molecular methods. However, there are some limitations of these molecular methods as well, such as the difficulty of amplification of DNA from higly degraded scat samples (Tollit et al., 2009), the high cost of labor and the need for special equipment. Researchers have used molecular techniques to study diets of various species from feces including pinnipeds (Deagle et al., 2005; Casper et al., 2007a, 2007b; Matejusová et al., 2008; Tollit et al., 2009; Bowles et al., 2011), fur seals (*Arctocephalus pusillus*; (Deagle, Kirkwood, and Jarman, 2009), bottlenose dolphins (*Tursiops truncatus*-Dunshea, 2009), penguins (Deagle et al., 2007, 2010), chamois (*Rupicapra rupicapra*—Rayé et al., 2011), domestic sheep (Pegard et al., 2009), snow leopards (*Panthera uncia*-Shehzad et al., 2012a), leopard cats (*Prionailurus bengalensis*-Shehzad et al., 2012b), and bats (Zeale et al., 2011).

The molecular analyses of diet exploit an extensive variety of samples from different sources such as stomach or gut contents (Marshall et al., 2010; Leray et al., 2015), coprolites of extinct mammals (Poinar, 2001; Hofreiter et al., 2003), whole body extracts (Shehzad et al., 2012b), and regurgitates (King et al., 2010, Waldner and Traugott, 2012).

1.2.2.2. DNA barcoding methods. DNA barcoding is an approach that allows an organism to be identified to the species level, based on short DNA fragments amplified from a small tissue sample (Kress and Erickson, 2012). The use of these short DNA sequences or DNA barcodes to individually identify organisms has contributed to various fields of biological research (Savolinen et al., 2005; Hajibabae et al., 2007). The main purpose of this method is to create a large-scale reference sequence

database against which unknown samples can be compared to for identification purposes (Arnot et al., 1993). In this perspective, DNA barcodes are helpful for identification of unknown specimens (Janzen et al., 2011; Chacon et al., 2013), to enable the investigation of complex interactions (Smith et al., 2006, 2011), and for identification of prey content (Wallace et al., 2012), among others. Mitochondrial protein-coding, cytochrome-c oxidase subunit I (COI) is considered as a standard DNA barcode for animals (Hebert et al., 2003; Hajibabaei, 2012). On the other hand, nuclear internal transcribed spacer (ITS) ribosomal DNA gene is used as fungi DNA barcode (Schoch et al., 2012), while the rbcL and matK genes are used as the DNA barcodes for plants (Holling Sworth et al., 2009).

Building DNA barcode libraries for all living organisms is the focus of multiple international efforts (Savolainen et al., 2005; Hajibabei et al., 2007), and increasing the species taxonomic coverage in these databases are strongly needed (Hajibabaei et al., 2005; Kwong et al., 2012; Kvist, 2013). The conventional methods for creating DNA sequence data to acquire a barcode for a species is done through PCR amplification and Sanger sequencing (Sanger et al., 1997). For species identification, a specimen does not require description; however, for inclusion in the databases, a species needs to be identified first.

1.2.2.3. Next generation sequencing (NGS) systems. Technological developments in sequencing systems have provided new approaches for tackling numerous ecological questions, including those related to diet (Deagle et al., 2009; Valentini et al., 2009). Through NGS technologies, millions of reads of short fragment DNA can be obtained in a single experiment, at low costs (Schadt et al., 2010; Hert et al., 2008; Schuster, 2008). These systems are very effective for characterizing environmental samples, as they can help sequence short and degraded DNA. Ecologists have exploited the NGS technologies in fecal diet studies of various species (Valentini et al., 2009; Raye et al., 2011; Zeale et al., 2011), because different PCR amplicons can be described without cloning (Valentini et al., 2009). Recently, the availability of NGS platform has enabled the execution of large-scale diet studies due to lowering of the costs (Deagle et al., 2009; Pegard et al., 2009; Valentini et al., 2009).

1.2.3. Gray Wolf Diet - A Brief Summary

Like other big carnivores, wolves are ‘keystone species' in numerous ecosystems, and have impacts on lower trophic levels (Estes et al., 2011). For instance, wolves regulate herbivore populations, and impact the distribution of mesopredators and herbivores, which in turn can affect plant communities (Estes et al., 2011; Ripple and Beschta, 2012). Being highly opportunistic

carnivores, their dietary behavior can change depending on local dietary components (Mech and Boitani, 2003; Imbert et al., 2016; Newsome et al., 2016). The primary prey of gray wolves are wild boars and ungulates (Mech and Boitani, 2007), and also includes roe, fallow and red deer, smaller mammals and fruits (Madsen et al, 2015, Nowak et al, 2011).

In the areas, especially where the number of livestock is high, the conflict between human and wolf has increased (Ambarlı, 2013). In southern Europe, such as the Iberian Promontory and Italy, wolves normally devour livestock such as goats, sheep and horses (Peterson and Ciucci, 2003; Alvares, 2011; Lopez-Bao et al., 2013), which regularly causes conflict with local human populations (Boitani, 2000; Chapron et al., 2014; Lopez-Bao et al., 2015). Where both wild and domestic prey are found, wolf diet choice relies upon their relative availability, and on the influence of climatic or demographic factors. Predation on livestock increases during the grazing period, while wild prey is more vital during the rest of the year (Peterson and Ciucci, 2003).

1.2.4. Red Fox Diet - A Brief Summary

Red foxes are nocturnal omnivores like other fox species (Manivannan, 2013). Foxes ordinarily gather a wide variety of food items like snakes, scorpions, fish, insects, berries, fruits, and other kinds of small animals. They are mainly opportunistic feeders that chase live prey, particularly rodents (Fedriani et al., 2000). They feed on small mouse-like rodents like mice, ground squirrels, hamsters and deer mice. They target animals up to 3 kg in weight and require around half kg of food per day. Red foxes can also feed on plant material and they have been recorded to live on a fruit-only diet in some areas, particularly in autumn (Manivannan, 2013).

1.3. Objectives

In the perspective provided above, the main goal of the phylogeographic section of the thesis is to reveal genetic variability and the phylogenetic relationships of gray wolf (*Canis lupus*) and red fox (*Vulpes vulpes*) samples from Sarıkamış and Yenice Forests in a global perspective, by generations d-loop sequences, and comparing them with those obtained from databases. The dietary analysis, undertaken using the same fecal samples that were used for the phylogeographic analysis targets to determine the dietary profile of gray wolf and red fox populations in Sarıkamış and Yenice Forests. Specifically, the aim is to determine the dietary profile of these two species in terms of vertebrate, invertebrate and plant material, using an NGS-based DNA metabarcoding approach.

2. MATERIALS AND METHODS

2.1. Genetic Diversity and Phylogeographic Analyses

2.1.1. Sampling

mtDNA control region sequences (147 bp) were generated for 49 gray wolves from Sarıkamış, Kars and Yenice, Karabük, and four red foxes (134 bp) from Sarıkamış, Kars (Figure 2.1). Karabük. The wolf samples were collected on forest paths from Sarıkamış (n=46) by members of the local environmental organization KuzeyDoğa Society and Yenice Forests (n=3) by Ayşe Mergenci, Nehir Sevim, and a local team from the Directorate of Water Affairs and Forestry in Karabük. Red fox samples (n=4) were collected from Sarıkamış Forests by the same team.



Figure 2.1. The sampling localities for this study: Sarıkamış/Kars and Yenice/Karabük in Turkey.

2.1.2. Total DNA Extraction and Amplification

DNA was extracted from the gray wolf and red fox scat samples with Qiagen QIAamp® DNA Stool Mini Kits (Hilden, Germany) according to manufacturer's protocols. A partial fragment of the mtDNA control region was amplified by using the primers L15562: 5'-

CCATGCATATAAGCATGTACAT-3' and H15790: 5'-AGATGCCAGGTATAGTTCCA-3' (Kopaliani et al., 2014). Polymerase Chain Reaction (PCR) amplifications (in total volumes of 20 μL) were carried out with 2 μL of 10x Buffer, 0.3 μL of 100 mM $MgCl_2$, 0.3 μL of 10 mM dNTPs, 0.3 μL of each primer (10 μM stock), 0.3 μL of Maximo Taq DNA Polymerase (5u/μL), 3 μL of template DNA, and sterile distilled water to the full reaction volume. The temperature requirements were 95ºC for 3 minutes, followed by 46 cycles at 30 secs at 94ºC, 1 minute at 56ºC, 72ºC for 1 minute, and a final extension for 10 minutes at 72ºC (Kopaliani et al., 2014). After the analyses PCR products, if any, were visualized on a 1% agarose gel. The PCR amplifications were carried out multiple times for some of the samples with degraded DNA, where there were no positive results. At the end, 53 samples with positive PCRs were sequenced commercially at Macrogen Inc., Korea.

2.1.3. Data Analyses

Sequencher 5.4.1 (GeneCodes Corporation, Michigan) was used to edit and align the sequences (35 samples were sequenced in forward and reverse directions, 14 in one direction only). After aligning with sequences available in GenBank and trimming, the sequences were assembled into contigs of 147 bp and 134 bp, for the gray wolf and the red fox, respectively.

Maximum likelihood (ML) and neighbor-joining (NJ) trees were constructed using the software MEGA v.7 (Kumar et al., 2015) to evaluate the relationships between species, bootstrapping 1000 times to determine the confidence in branching patterns. Additionally, PopART v.1.7 (Population Analysis with Reticulate Trees) (Bandlet et al., 1999) was used to build a haplotype network, using the Kimura-2 parameter model. In addition, genetic diversity and differentiation analyses were performed. The number of polymorphic sites (including parsimony informative and singleton variable), haplotype diversity (Hd), nucleotide diversity (π), and haplotype counts were calculated using program DNAsp 5.10.1 (Librado and Rozas, 2009). For these analyses, the following regions were defined based on sequence data available from the literatüre: Caucasus, Croatia, Afghanistan, Iran, Mongolia, Poland, Russia, Saudi Arabia, Thrace/Turkey, Ukraine, Yugoslavia, Eastern Balkans, Asia (Southern - Eastern), Northeastern Europe for the gray wolf, and US, UK, Canada, Turkey, Russia, Australia, Asia (Eastern, Western and Central), Africa (Northeastern - Northwestern), Europe (Central, Western and Northeastern), Balkans for the red fox.

2.2. Dietary Habits through Genetics

2.2.1. Summary of the Genetics Based Diet Analysis Method

The method adopted for the carnivorous diet analysis in this study builds on a previous DNA metabarcoding study on the diet of brown bear (*Ursus arctos*) (De barba et al, 2014). Following DNA extraction; extraction products were amplified using spesific primers for vertebrates, invertebrates and plants, with three blocking oligonucleotides (homo, wolf and mam) to inhibit host, mammal and wolf DNA amplification. For the diet analysis, 20 different samples were selected out of 138 samples for which DNA was originally extracted from. These included 51 gray wolf DNA extractions from Yenice, 57 gray wolf DNA extractions from Sarıkamış, and 30 red fox DNA extractions from Sarıkamış. DNA concentrations of these 138 sequences were measured using a Qubit® Fluorometer, and 20 samples with the highest DNA concentrations were chosen for the downstream metabarcoding library preparation. In terms of replication, two wolf samples from Yenice and three wolf samples from Sarıkamış included three replicate library preparations, and the remaining 15 samples had two replicates, for a total of 46 samples (Table 2.1).

For identifying the vertebrate, invertebrate and plant material in scat, the same regions that were studied in De barba et al., (2014) were used (Table 2.2). The Illumina libraries for diet characterization were prepared with a two-step PCR strategy. The first PCR step (barcoding PCR) was performed to amplify three universal primers and variable sections of the vertebrate, invertebrate and plant components of the diet, with an overhang. The second PCR (index-PCR) was undertaken after first multiplex PCR, where the overhang from the first PCR was used to anneal the Illumina adaptors to the final libraries. In addition, different pairs of index primers were added to individual samples in the second thermocycling step. This step allowed multiplexing of samples together and sequencing them in a single Illumina lane, followed by post-sequencing demultiplexing to map the reads to individual samples.

Table 2.1. The 46 pooled sequences, their replicates (n), sample types and localities.

Sample name	Replicate(n)	Sample Type	Locality
1	3	Wolf	Yenice
2	2	Wolf	Yenice
3	2	Wolf	Yenice
4	3	Wolf	Yenice
5	2	Wolf	Yenice
60	3	Wolf	Yenice
116	2	Wolf	Yenice
232	2	Fox	Sarıkamış
233	2	Fox	Sarıkamış
234	2	Fox	Sarıkamış
236	2	Fox	Sarıkamış
240	2	Fox	Sarıkamış
2257	2	Wolf	Sarıkamış
2341	2	Wolf	Sarıkamış
2408	3	Wolf	Sarıkamış
2416	2	Wolf	Sarıkamış
3153	2	Wolf	Sarıkamış
3442	3	Wolf	Sarıkamış
4138	3	Wolf	Sarıkamış
4144	2	Wolf	Sarıkamış

2.2.2. DNA Extraction, PCR and Library Construction

A subset of the same DNA extractions used for phylogeographic analyses were used for the diet analyses. 15 gray wolf samples collected from two regions (seven from Yenice / Karabük – eight from Sarıkamış / Kars) and five red fox samples (from Sarıkamış / Kars) were used for the diet characterization.

Scats of wolf and fox were processed in different pre- and post-PCR rooms, with separate equipment for DNA extraction, PCR, and post-PCR processing. The standard 16S illumina bacterial metabarcoding protocol (see www.illumina.com) (including the adapters and indexes) was used, with modifications to use the vertebrates, invertebrates and plant primers for the target regions of interest, instead of the 16S bacterial primers. The expected sizes of the PCR products of the target regions were 271 bp, 210 bp, and 226 bp, for vertebrates, invertebrates and plants, respectively. The size of the PCR products were controlled through gel electrophoresis, to potentially confirm correct target region amplification. The final libraries for each sample include includes Illumina i5 adapter, i5 index, read 2 primer, forward region primer, region of interest, reverse region primer, read 1 primer, i7 index, and Illumina i7 adaptor (Figure 2.2). Figure 2.2 also shows the actual base composition for libraries built for the three groups of interest.

Vertabrate: (98 bp region of interest, 116 bp with the forward primer in single-end read, 116 bp with the reverse primer in the paired-end read)

CAAGCAGAAGACGGCATACGAGATTAAGGCGAACCAAGGTCTCGTGGGCTCGGA
GATGTGTATAAGAGACAGTTAGATACCCCACTATGCNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNCTAGAGGAGCCTGTTCTACTGTCTCTTATACACATC
TGACGCTGCCGACGATGTGTCCTAGTACGGTGTAGATCTCGGTGGTCGCCGTATCATT

Invertebrate: (36 bp region of interest, 55 bp with the forward primer in single-end read, 57 bp with the reverse primer in the paired-end read)

CAAGCAGAAGACGGCATACGAGATTAAGGCGAACCAAGGTCTCGTGGGCTCGGA
GATGTGTATAAGAGACAGCCAACATCGAGGTCRYAANNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNCTGTTATCCCTANRGTAAYTCTGTCTCTTATACACATCTGAC
GCTGCCGACGATGTGTCCTAGTACGGTGTAGATCTCGGTGGTCGCCGTATCATT

Plant: (51 bp region of interest, 69 bp with the forward primer in single-end read, 74 bp with the reverse primer in the paired-end read)

CAAGCAGAAGACGGCATACGAGATTAAGGCGAACCAAGGTCTCGTGGGCTCGGA
GATGTGTATAAGAGACAGGGGCAATCCTGAGCCAANNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGATAGGTGCAGAGACTCAATGGCTG
TCTCTTATACACATCTGACGCTGCCGACGATGTGTCCTAGTACGGTGTAGATCTCGGTG
GTCGCCGTATCATT

i5 index

i7 index (reverse complement of)

NNNNNNNNN (region of interest)

Forward region primer

Reverse region primer (reverse complement of)

Read 2 primer

Read 1 primer (reverse complement of)

Illumina i5 adaptor

Figure 2.2. Designed forward and reverse primer sequences for the Illumina sequencing libraries.

Table 2.2. Sequences of the blocking oligonucleotides and primer pairs used in the study.

Name	DNA Type/DNA Region	Primer sequence (5'-3')	References
Blocking PCR Oligonucleotides			
HomoB2	Mitochondrial/12S mtDNA	CTATGCTTAGCCCTAAACCTCAACAGTTAAATCAACAAAACTGCT-C3	De barba et al., 2014
Wolf12SFBL	Mitochondrial/12S mtDNA	CCACTATGCTTAGCCCTAAACATAGATAATTTTACAAC-C3	This study
MamMAVB1	Mitochondrial/16S mtDNA	CCTAGGGATAACAGCGCAATCCTATT-C3	De barba et al., 2014
Primers used for the target sequence amplification			
Vertebrate			
12SV5-F	Mitochondrial/12S mtDNA	TTAGATACCCCACTATGC	Riaz et al., 2011
12SV5-R	Mitochondrial/12S mtDNA	TAGAACAGGCTCCTCTAG	Riaz et al., 2011
Invertebrate			
16SMAV-F	Mitochondrial/16S mtDNA	CCAACATCGAGGTCRYAA	De barba et al., 2014
16SMAV-R	Mitochondrial/16S mtDNA	ARTTACYNTAGGGATAACAG	De barba et al., 2014
Plant			
g	Chloroplast/trnL(UAA)	GGGCAATCCTGAGCCAA	Taberlet et al., 2007
h	Chloroplast/trnL(UAA)	CCATTGAGTCTCTGCACCTATC	Taberlet et al., 2007

2.2.3. First PCR

Thermocycler conditions for the first PCR were as follows: initial denaturation step at 95ºC for 15 min, followed by 55 cycles of 94ºC for 30 sec, 55ºC for one min, and 30 sec of extension. All of the PCR amplifications were carried out using QIAGEN Multiplex PCR kit, with a total volume of 25 uL as follows:

2.2.3.1. Vertebrate PCR. 12. 5 µL of QIAGEN Multiplex PCR Master Mix, 2.5 µL Q-solution, 0.2 µL of 12SV5-F (10 mM) and 12SV5-R primers (10 mM), 0.4 µL of HomoB2 (10mM) blocking oligonucleotide, 0.4 µL of Wolf12SFBL (10mM) blocking oligonucleotide, 6.4 µL of water, and 2 µL of DNA template.

2.2.3.2. Invertebrate PCR. PCR reactions were carried out in 25 µL mixtures containing: 12.5 µL of QIAGEN Multiplex PCR Master Mix, 2.5 µL Q-solution, 0.5 µL of 16SMAV-F (10mM) and 16SMAV-R (10mM) primers, 0.4 µL each of HomoB2 (10mM), Wolf12SFBL (10mM), MamMAVB1 (10mM) blocking oligonucleotides, 4.8 µL water, and 2 µL of template DNA.

2.2.3.3. Plant PCR. PCR reactions were carried out in 25 µL mixtures containing: 12.5 µL of QIAGEN Multiplex PCR Master Mix, 2.5 µL Q-solution, 0.25 µL each of g primer (10mM) and h primer (10mM), 7.5 µL water, and 2 µL of template DNA.

After the PCRs, 4 µL of loading buffer was added into each PCR tube, containing 25 µL of the first PCR product, and the mix were run on a 2% gel for about 120 min at 130-140 V. PCR products of the correct size were excised from the gel, and purified using the QIAquick Gel Extraction Kit (Qiagen).

2.2.4. Index PCR

The Index PCR reactions were carried out in 25 µL mixtures containing 0.2 mM final concentration of each indexed primer (1 µL – 10mM), 2x QIAGEN Multiplex PCR Master Mix (12.5 µL), 8 µL of RNase-free water, and 2.5 µL of template DNA.

Thermocycler conditions were as follows: initial denaturation step at 95 ºC for 3 min, followed by 20 cycles of 95ºC for 30 sec, 60.5ºC for 30 sec, and 72ºC for 30 sec, and an extension step at 72ºC for 5 min.

2.3. Pooling of PCR Products and Sequencing

The concentrations of the 46 individual DNA libraries were measured using a Qubit® Fluorometer, before the final pooling. After measurements, 200 ng DNA from each sample was used for subsequent equimolar pooling. 46 sequences were pooled by adjusting their concentrations (ng/µl), and all libraries with similar concentrations were pooled together (Table 2.3). In total, 46 Illumina diet libraries were sequenced, and the numbers including the replicates were 17 from Yenice and 19 from Sarıkamış for the gray wolf, and 10 from Sarıkamış for the red fox.

Next generation sequencing of the 46 PCR products generated a total of about 864.48 Mbp paired-end sequence reads (see Appendix B for individual library based data sizes). All data were analysed and filtered using various sub-programs in the OBITools-1.01.22 pipeline (http://metabarcoding.org/obitools), run using the default parameters.

The pipeline includes using solexaPairEnd as a first step to align and merge direct and reverse reads. ngsfilter was used to identify primers and tags. Some sequences showed a perfect match on tags and some sequences showed a few errors, which would not affect the analysis; however, some showed errors on primer sites, and therefore were not included in the subsequent analyses. The amplified regions, excluding primers and tags, were kept for the next step, where obiuniq was used to cluster together strictly identical sequences, and retain information about their distribution among samples. Sequences shorter than 80 bp for vertebrates, 36 bp for invertebrates, and 51 bp for plants, corresponding to target sites of interest were excluded using the obigrep sub-program. For this step, a minimum read count of three was used to keep a particular sequence for the next step. Taxon assignment was made using ecoTag (Pegard et al., 2009). ecotag basically employs a global alignment algorithm (Needleman and Wunsch, 1970) to find sequences in a reference database, which we created in April 2018 for this analysis. This database was retrieved from the EMBL (European Molecular Biology Laboratory) nucleotide library using ecopcr, by extracting the sections of the mitochondrial 12S,16S genes, and the P6 loop of chloroplast trnL (UAA) intron (Bellemain et al., 2010; Ficetola et al., 2010) corresponding to our sequences for vertebrates, invertebrates, and plants, respectively.

Table 2.3. Samples and library concentrations used in this study. The number of samples also include the replica numbers.

Name	Species/Locality	Concentrations (ng/µl)	Code of I & B	Barcode and Index Sequences (i7-i5)	
Vertebrate					
1-1	Wolf/Yenice	11.9	I5B3	GTAAGG	cgtactagTTCTGC
2-2	Wolf/Yenice	28	I4B6	AGAGTA	cgtactagCATGCC
4-1	Wolf/Yenice	14.8	I4B8	AGAGTA	cgtactagCCTCTC
5-1	Wolf/Yenice	25.4	I4B7	AGAGTA	cgtactagGTAGAG
60-1	Wolf/Yenice	16.8	I1B1	TAGATC	cgtactagTCGCCT
60-3	Wolf/Yenice	31.8	I3B3	TATCCT	cgtactagTTCTGC
240-2	Fox/Sarıkamış	27.2	I2B6	CTCTCT	cgtactagCATGCC
2416-1	Wolf/Sarıkamış	11.2	I2B1	CTCTCT	cgtactagTCGCCT
2416-2	Wolf/Sarıkamış	22.6	I3B5	TATCCT	cgtactagAGGAGT
3153-1	Wolf/Sarıkamış	20.2	I4B2	AGAGTA	cgtactagCTAGTA
4138-3	Wolf/Sarıkamış	26.2	I2B7	CTCTCT	cgtactagGTAGAG
4144-1	Wolf/Sarıkamış	20.4	I3B7	TATCCT	cgtactagGTAGAG
Plant					
2-1	Wolf/Yenice	26.2	I3B2	TATCCT	cgtactagCTAGT
3-2	Wolf/Yenice	25.8	I5B6	GTAAGG	cgtactagCATGCC
4-2	Wolf/Yenice	13.6	I2B3	CTCTCT	cgtactagTTCTGC
4-3	Wolf/Yenice	25.6	I4B3	AGAGTA	cgtactagTTCTGC

(continued)

Table 2.3. Continued.

Name	Species/Locality	Concentrations (ng/µl)	Code of I & B	Barcode & Index Sequences (i7-i5)	
Plant					
5-2	Wolf/Yenice	22.8	I6B6	ACTGCA	cgtactagCATGCC
60-1	Wolf/Yenice	11.5	I1B1	TAGATC	cgtactagTCGCCT
60-2	Wolf/Yenice	26.6	I2B4	CTCTCT	cgtactagGCTCAG
60-3	Wolf/Yenice	32	I3B3	TATCCT	cgtactagTTCTGC
116-1	Wolf/Yenice	12.8	I3B1	TATCCT	cgtactagTCGCCT
232-1	Fox/Sarıkamış	20.2	I1B4	TAGATC	cgtactagAGGAGT
232-2	Fox/Sarıkamış	24	I6B5	ACTGCA	cgtactagAGGAGT
233-1	Fox/Sarıkamış	13	I1B2	TAGATC	cgtactagCTAGTA
234-1	Fox/Sarıkamış	17	I5B8	GTAAGG	cgtactagCCTCTC
234-2	Fox/Sarıkamış	16.4	I6B2	ACTGCA	cgtactagCTAGTA
236-2	Fox/Sarıkamış	24	I5B1	GTAAGG	cgtactagTCGCCT
240-1	Fox/Sarıkamış	15.6	I6B1	ACTGCA	cgtactagTCGCCT
2257-1	Wolf/Sarıkamış	21.8	I2B2	CTCTCT	cgtactagCTAGTA
2257-2	Wolf/Sarıkamış	22.6	I5B2	GTAAGG	cgtactagCTAGTA
2341-1	Wolf/Sarıkamış	20.6	I1B6	TAGATC	cgtactagCATGCC
2341-2	Wolf/Sarıkamış	14.7	I5B5	GTAAGG	cgtactagAGGAGT
2408-1	Wolf/Sarıkamış	26.2	I1B7	TAGATC	cgtactagGTAGAG

(continued)

Table 2.3. Continued.

Name	Species/Locality	Concentrations (ng/µl)	Code of I & B	Barcode & Index Sequences (i7-i5	
Plant					
2408-2	Wolf/Sarıkamış	24.6	I4B6	AGAGTA	cgtactagTCGCCT
2408-3	Wolf/Sarıkamış	24.8	I3B4	TATCCT	cgtactagGCTCAG
2416-1	Wolf/Sarıkamış	28.6	I2B6	CTCTCT	cgtactagTCGCCT
2416-2	Wolf/Sarıkamış	22.6	I3B5	TATCCT	cgtactagAGGAGT
3153-2	Wolf/Sarıkamış	23.8	I1B8	TAGATC	cgtactagCCTCTC
3442-1	Wolf/Sarıkamış	20.2	I1B4	TAGATC	cgtactagGCTCAG
3442-2	Wolf/Sarıkamış	24.6	I4B4	AGAGTA	cgtactagGCTCAG
3442-3	Wolf/Sarıkamış	27	I3B6	TATCCT	cgtactagCATGCC
4138-1	Wolf/Sarıkamış	23.2	I3B8	TATCCT	cgtactagCCTCTC
4138-2	Wolf/Sarıkamış	24.8	I2B5	CTCTCT	cgtactagAGGAGT
4138-3	Wolf/Sarıkamış	11	I2B7	CTCTCT	cgtactagGTAGAG
4144-1	Wolf/Sarıkamış	25.8	I3B7	TATCCT	cgtactagGTAGAG
4144-2	Wolf/Sarıkamış	26	I4B5	AGAGTA	cgtactagAGGAGT

3. RESULTS AND DISCUSSION

3.1. Genetic Diversity and Phylogeographic Analyses

3.1.1. Gray Wolf

3.1.1.1. Patterns of genetic diversity. As a result of the mtDNA analyses, six haplotypes from Kars (out of 46 samples) and two haplotypes from Karabük (out of three samples) were obtained from sequences generated in this study. The summary statistics of genetic diversity and differences of gray wolf mtDNA d-loop sequences are given in Table 3.1. Comparing one of the regions of interest for this study, Kars, with Northeastern Europe (two regions with similar sample sizes, N=46 and N=44, respectively) the nucleotide diversity, haplotype diversity and haplotype numbers were seen to be similar to each other.

Considering Karabük in terms of its genetic diveristy, the haplotype diversity had the maximum value (H_d=1). The same pattern (of H_d=1) was seen in Saudi Arabia and Mongolia, with similar samples sizes. The nucleotide diversities for these three regions were found to be 0.01814, 0.00730 and 0.00680, with the value for Karabük being the highest. This is probably due to the higher number of polymorphic sites (four) in Karabük c.f. Saudi Arabia (one) and Mongolia (one).

Table 3.1. Summary statistics of genetic variability of gray wolf mtDNA d-loop sequences. N: Number of sequences; π: Nucleotide diversity; h: Number of haplotypes; H_d: Haplotype diversity; SV: Singleton variable sites; PIS: Parsimony-informative sites; PS: Polymorphic sites.

Geographic Region	N	Π	h	Hd	SV	PIS	PS
Kars / Turkey	46	0.00952	6	0.669	0	5	5
Karabük / Turkey	3	0.01814	2	1.000	4	0	4
Northeastern Europe	44	0.00838	9	0.548	2	4	6
Saudi Arabia	2	0.00730	2	1.000	1	0	1
Eastern Balkans	7	0.01350	4	0.810	2	2	4
Caucasus	8	0.13850	4	0.893	0	4	4
Russia	4	0.01142	3	0.833	2	1	3
Spain	3	0.00454	2	0.667	1	0	1
Eastern Asia	12	0.01225	4	0.803	6	2	8
Yugoslavia	3	0.00907	2	0.667	2	0	2
Iran	4	0.01134	3	0.833	2	1	3
Ukraine	5	0.02740	5	1.000	4	0	4
Mongolia	2	0.00680	2	1.000	1	0	1

3.1.1.2. The haplotype network, neighbor-joining and maximum likelihood trees. 49 gray wolves were evaluated by building haplotype network, neighbor-joining tree and maximum likelihood tress, as shown in Figure 3.1, 3.2, and 3.3, respectively. 10 individuals from Kars and two individuals from the Caucasus shared the same haplotype (Hap 22). In addition, haplotype 23 from Kars and haplotype 24 from the Caucasus formed a clade. This pattern indicates some level of local grouping and connectivity of gray wolves in Kars and Caucasus.

In addition, Hap 26 from Karabük and Hap 22 from Kars being in the same clade indicates their common ancestry and/or connectivity in Turkey. The same pattern was also observed for Hap19 and Hap20 from Kars, and Hap18 from Karabük. Vilà et al. (1999) obtained similar results to ours that mtD

NA d-loop sequences of gray wolves were often shared between neighbouring localities. Our results showing that Kars-Caucasus and Kars-Karabük samples were genetically similar are in parallel with these findings of Vilà et al. (1999).

The most common haplotypes of Hap 1, Hap 6, Hap 7, Hap 20 and Hap 22 being found extensively in multiple regions is evidence of connectivity between these different regions. Considering our data set, the sharing of a haplotype (Hap 25) between Kars and east Asia (China) indicates the possibility of long distance connectivity/dispersal or retention of ancestral polymorphism, similar to the wide geographic distribution of the most common haplotypes.

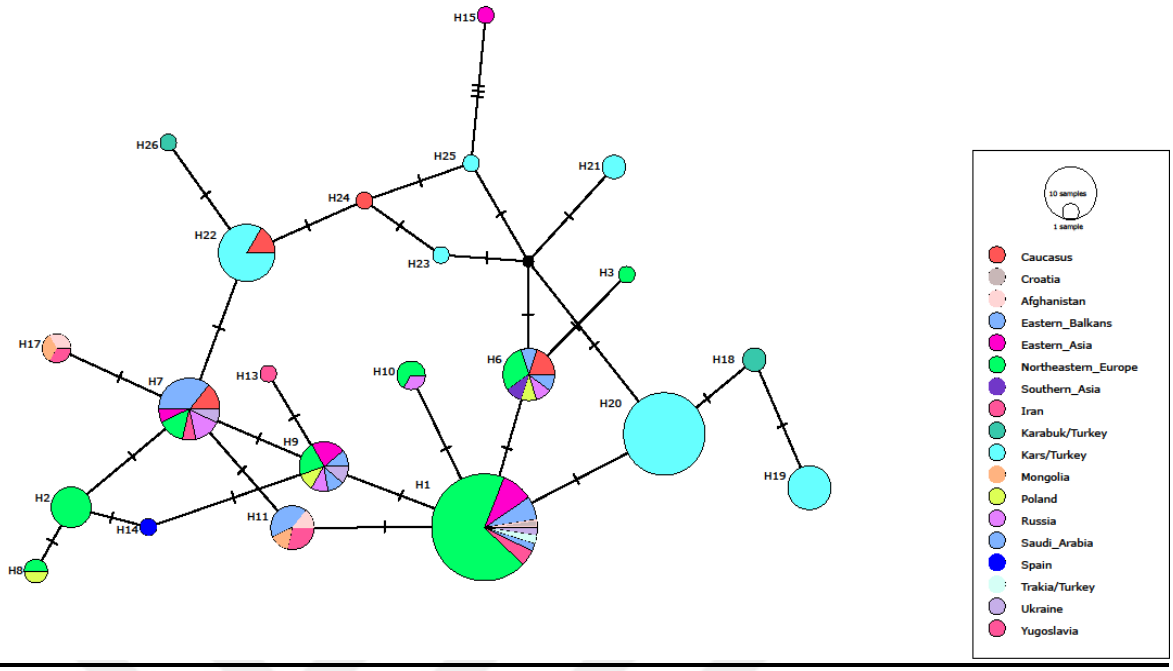


Figure 3.1. Haplotype Network of Turkish gray wolf haplotypes including 99 GenBank sequences.

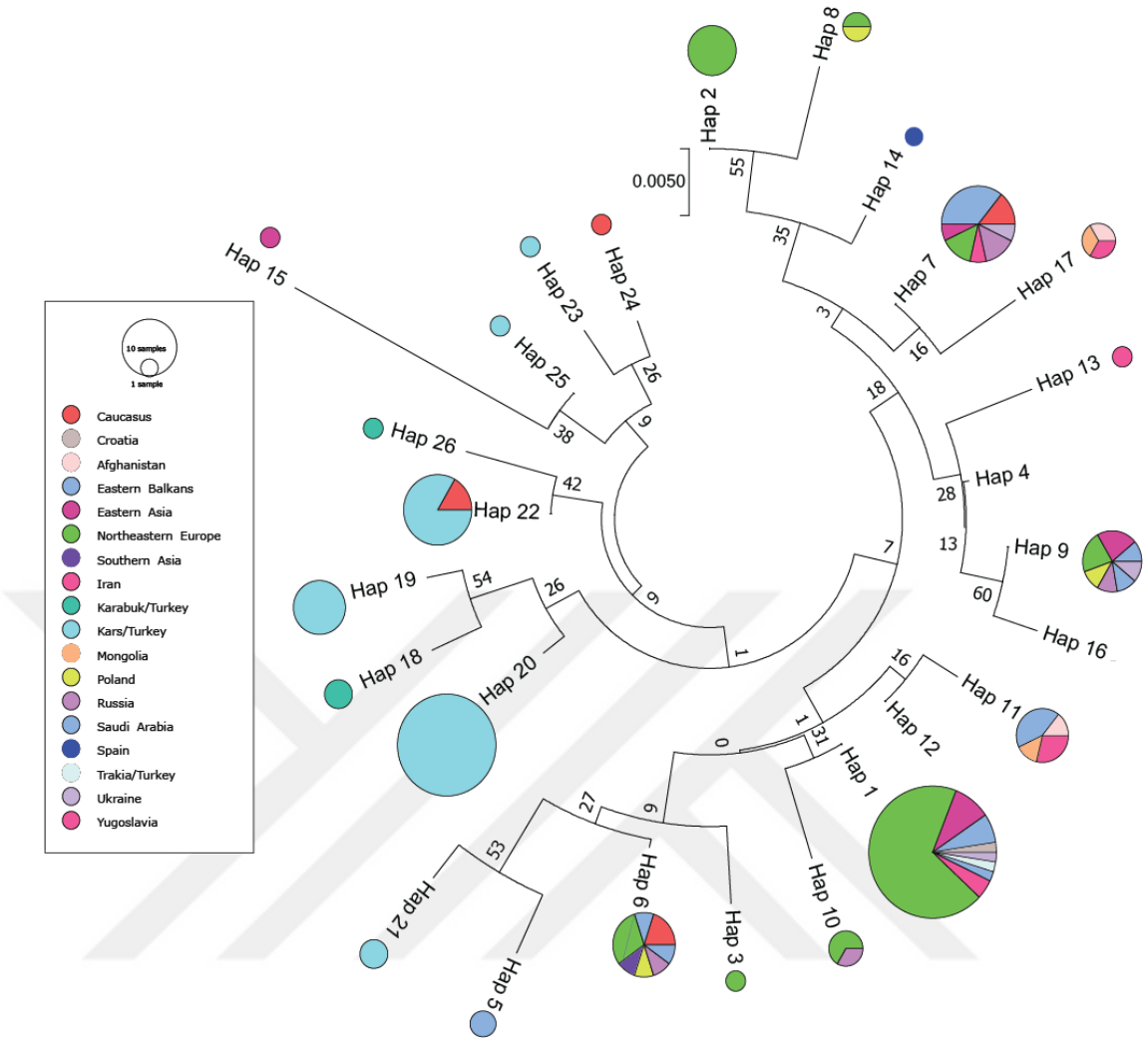


Figure 3.2. Wolf sequences - The combination of haplotype network and neighbor-joining tree.

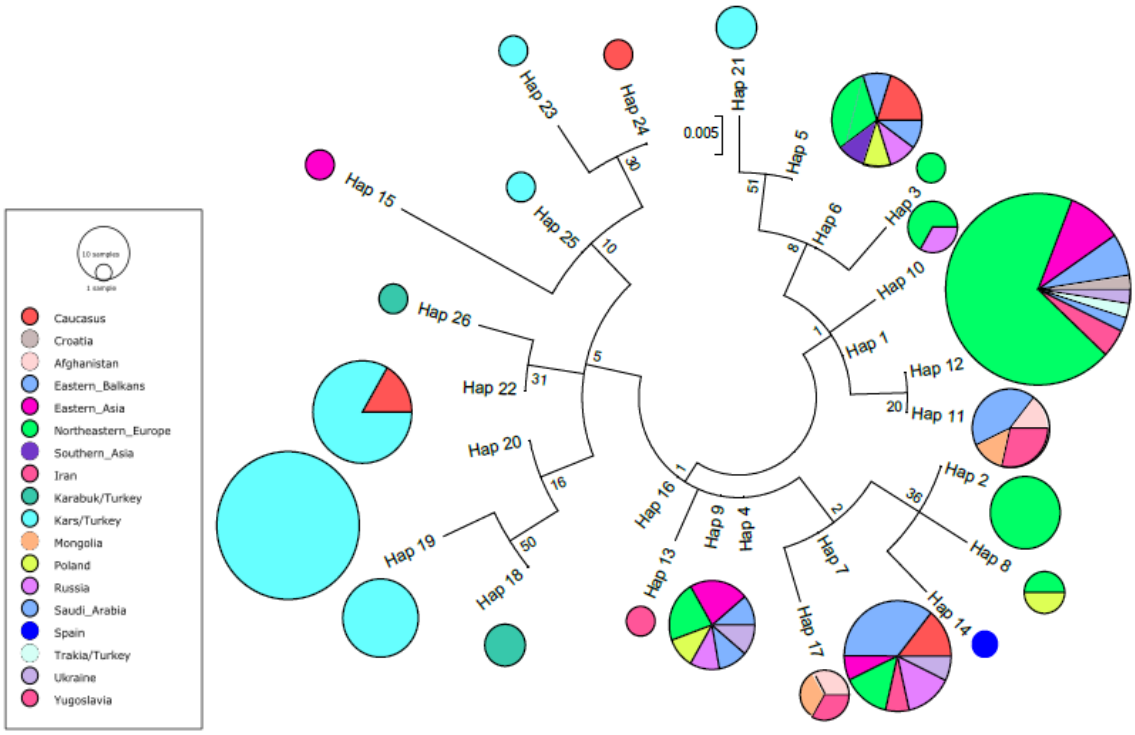


Figure 3.3. Wolf sequences - Maximum likelihood tree were constructed using The Kimura 2-parameter model.

3.1.2. Red Fox

3.1.2.1. Patterns of genetic diversity. As a result of the mtDNA analyses, two haplotypes were obtained out of four sequences from Kars. The global summary statistics of genetic diversity and differences of red fox mtDNA d-loop sequences are given in Table 3.2.

Table 3.2. Summary statistics of genetic variability of red fox mtDNA d-loop sequences. N: Number of sequences; π: Nucleotide diversity; h: Number of haplotypes; H_d: Haplotype diversity; SV: Singleton variable sites; PIS: Parsimony-informative sites; PS: Polymorphic sites.

Geographic Region	**N**	**Π**	**h**	**Hd**	**SV**	**PIS**	**PS**
Kars / Turkey	4	0.00373	2	0.500	1	0	1
Turkey	2	0.00746	2	1.000	1	0	1
Northeastern Europe	32	0.00928	7	0.716	5	2	7
Western Europe	8	0.00693	4	0.643	2	1	3
Central Europe	73	0.01042	9	0.764	6	5	11
US	155	0.00345	9	0.364	3	4	7
Canada	4	0	1	0	0	0	0
Russia	23	0.01162	7	0.791	3	3	6
Western Asia	29	0.01766	13	0.921	1	6	7
Central Asia	8	0.01546	7	0.964	4	2	6
Eastern Asia	25	0.01453	10	0.877	4	5	9
Northeastern Africa	17	0.02023	8	0.860	5	5	10
UK	7	0.00355	2	0.476	0	1	1
Balkans	38	0.00912	6	0.706	1	3	4

Haplotype and nucleotide diversities in Kars were found to be low, possibly due to the small sample size. For geographic regions with sample sizes above 15, the highest nucleotide and haplotype diversity values were in eastern Asia (π= 0,01453, H_d= 0,877), western Asia (π= 0,01766, H_d= 0,921), and northeastern Africa (π= 0,02023, H_d= 0,860). On the other hand, the US had the lowest nucleotide and haplotype diversities (π= 0,00345, H_d= 0,364). These global results suggest that the New World red fox populations have their origins in the Old World, as also indicated by Churcher (1959).

3.1.2.2. Haplotype network, neighbor-joining and maximum likelihood trees. Red fox sequences were evaluated by building haplotype network, neighbor-joining and maximum likelihood trees as shown in Figure 3.4, 3.5, and 3.6, respectively. We observed 46 different haplotypes, of which the majority were found in western Asia. In this study, two haplotypes (Hap12 and Hap14) were observed in Kars/Turkey. Hap 12 was the most common global haplotype with a very widespread geographic distribution. Hap 14 is a new haplotype not previously recorded in the literature, found in only one sample in Karabük. Global distributions of haplotypes were detected in the haplotype network and phylogenetic trees, and the haplotypes from Kars/Turkey formed groups with these global haplotypes, without much local structuring. The lack of structuring for the haplotypes from Turkey contradicts the study of İbiş et al. (2014) who detected structuring for samples Turkey and Eurasia, where two distinct phylogroups formed in 1) southwestern Anatolia, Turkey and Japan, and 2) Eurasia (e.g.

Bulgaria, Spain, Italy, Austria, Israel, Turkey, Japan). The reason for this discrepancy could be due to a shorter fragment of mtDNA region having been used in this study (134 bp) providing lower levels of resolution when compared to the study of İbiş et al., (2014) where a longer fragment of mtDNA (375 bp) was used.

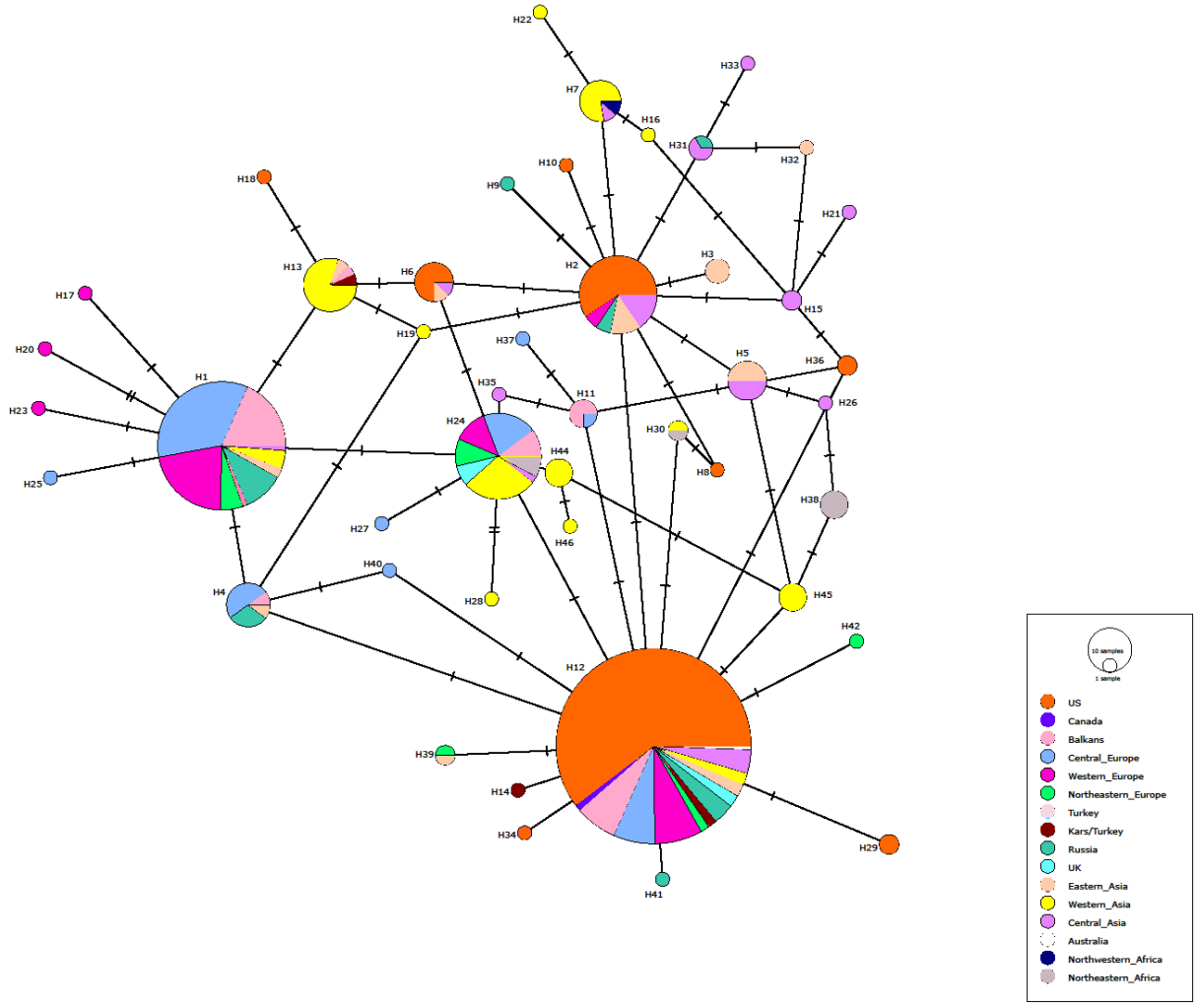


Figure 3.4. Haplotype network of Turkish red fox haplotypes including 424 GenBank sequences.

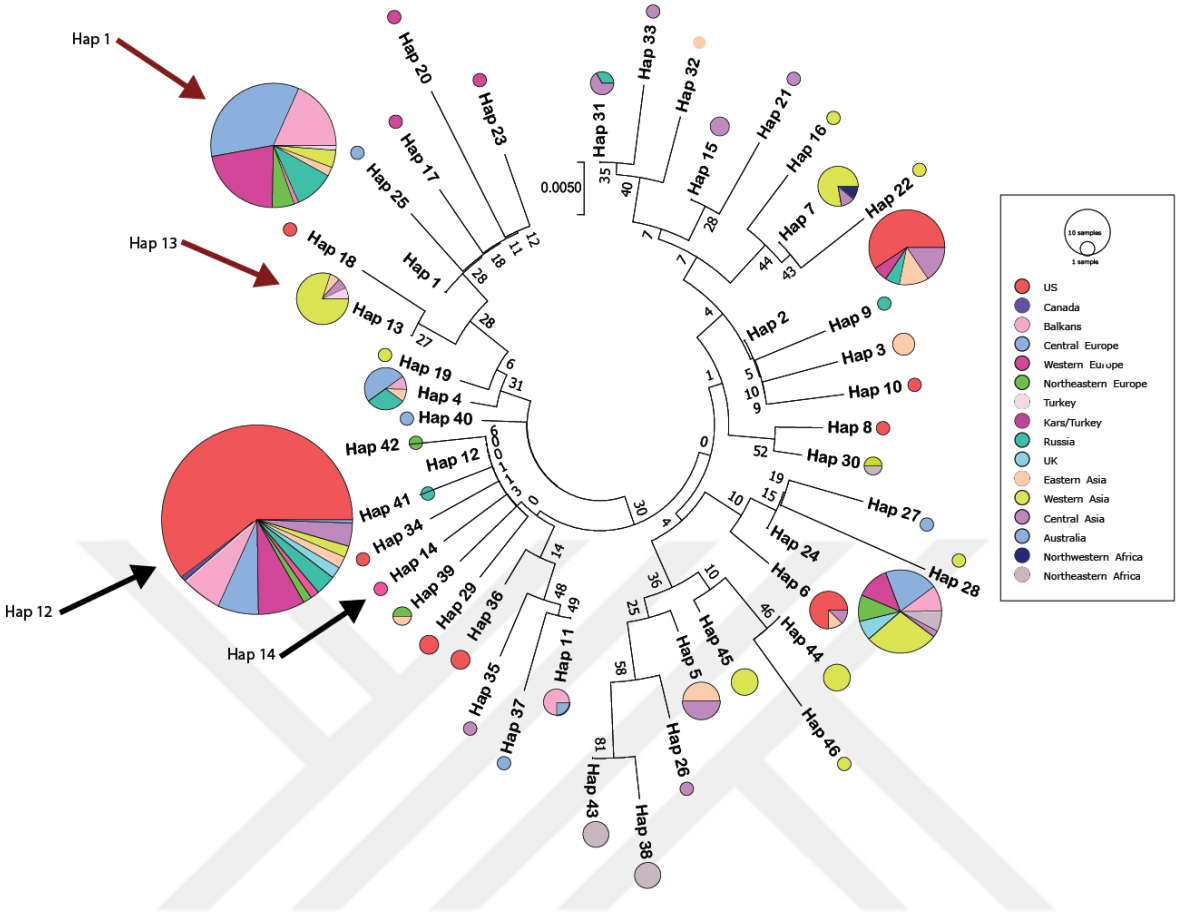


Figure 3.5. Fox sequences - The combination of haplotype network and neighbor-joining tree.

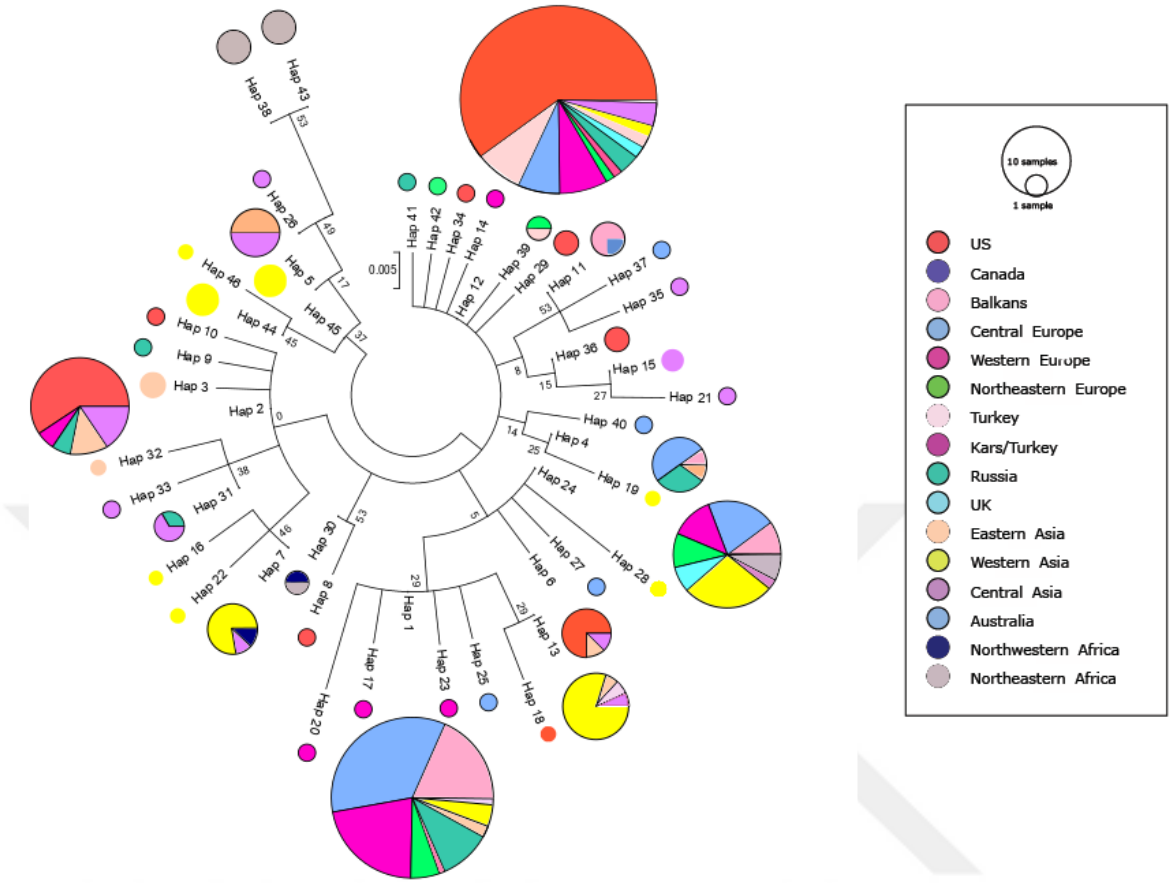


Figure 3.6. Fox Sequences-Maximum likelihood tree were constructed using the Kimura 2-parameter model.

3.2. Dietary Habits through Genetics

Sequences retained after applying the Obitools pipeline were matched to various taxa. Vertebrates, plant and invertebrate matches were found in 12, 34 and 0 samples, respectively. Analysis of 20 scat samples (46 samples including the replicas) documented a total of 11 food items, which were assigned to four vertebrate food categories and seven plant food categories. Table 3.3 and Table 3.4 show the vertebrate and plant diet composition for gray wolf and red fox, in Sarıkamış / Kars and Yenice / Karabük.

Table 3.3. Vertebrate diet composition for the two carnivore species in the two study areas.

Classification	**Taxon**	**Common name**	**Sample type**	**Localities**
Mammals				
Cervidae	*Cervus elaphus* Red deer		Gray wolf	Yenice
Amphibia	*Rana japonica*	Japanese brown frog		Yenice
	Rana kunyuensis	Unknown	Gray wolf	and
	Rana coreana	Korean brown frog		Sarıkamış
	Rana chensiensis	Chinese brown frog		
Aves				
Passeriformes	*Tangara mexicana*	Turquoise tanager	Gray wolf	
	Motacilla cinerea	Gray wagtail	and	Sarıkamış
	Uragus sibiricus	Finches	Red fox	
	Geospiza fortis	Finches		
	Tiaris olivaceus	Yellow-faced grassquit		
Non-Passeriformes	Anatidae	Duck-Greese or Swan	Gray wolf	Sarıkamış

Table 3.4. Plant diet composition for the two carnivore species in the two study areas.

	Taxon	**Common name**	**Sample type**	**Localities**
	Fagaceae	Beech family	Gray wolf	Yenice
	Rosacaea prunus	Rose family	Gray wolf	Yenice
	Rosaceae rosidaea	Rose family	Gray wolf	Yenice
	Fabaceae	Poaceae	Gray wolf	Yenice
	Rosaceae prunus	Rose family	Gray wolf	Sarıkamış
	Fagaceae	Beech family	Gray wolf	Sarıkamış
	Ranunculaceae (clematis)	Buttercup	Gray wolf	Sarıkamış
	Poaceae (Pooideae)	Wheat/other grain crops	Gray wolf	Sarıkamış
	Poaceae (Pooideae)	Wheat/other grain crops	Red fox	Sarıkamış
	Rosaceae (Potentilla)	Rose family	Red fox	Sarıkamış

3.3. Diet composition of Gray Wolves

For gray wolves, food items were later aggregated in the following categories and numbers: mammals (n=4 in Yenice), amphibia (n=2 in Yenice, n=2 in Sarıkamış), Aves (n=2 in Sarıkamış), and Passeriformes (n=1 in Sarıkamış). The plant diet composition of the gray wolf was as follows: Magnoliopsida (n=5 in Sarıkamış; n=6 in Yenice) and Liliopsida (n=5 in Sarıkamış). In this respect, vertebrate and plant diet compositions of gray wolf (Table 3.3 and Table 3.4) are discussed in greater detail below.

3.3.1. Vertebrate Diet Composition

3.3.1.1. Red deer. The results showed that the red deer (*Cervus elaphus*) (four samples) was the most frequently detected prey species for gray wolves in Yenice/Karabük. The literature also shows the predominance of the red deer as a main prey item for the gray wolf. Food remains in scats were analysed morphologically and prey remain analyses revealed red deer as the most commonly utilised prey species in Hungary (Lanszki et al., 2012). In another study in the Italian Alps, Gazzola et al. (2004) showed that red deer was the most abundant species in the ungulate community, and that the most important prey of wolves was Cervids (74.2%). Using microscopic methods, Jedrzejewski et al. (2000) was able to distiguish only red deer as a prey item from scat samples of Polish wolves, where bone or hoof remains were found. In Latvia, Zuma et al. (2009), who studied fresh stomach contents of wolves using the hair slide method, showed that Cervids comprised the largest proportion (64.7%) of the prey content for gray wolves. On the other hand, red deer was not recorded as a prey item for wolves in Sarıkamış. A camera trap study by Chynoweth et al. (2016) showed that red deer was not present in Sarıkamış Forest, and the absence of this species in the diet of wolves as shown by this study is parallel to the results of Chynoweth et al. (2016).

Sarıkamış forests and neighbouring forests provide habitat for gray wolf populations. However, in these forests, large carnivores are facing increasing threats due to human activity. Most human activities such as supplying firewood from the forest or using grazing areas reduce and fragment habitats of carnivores, decrease the natural prey base, and cause animals to suffer various risks such as vehicle collision, poaching, and direct persecution (Chynoweth et al., 2016).

One reason for not having detected any mammal prey items in Sarıkamış may be because of gray wolves potentially feeding at garbage dumps near the Sarıkamış forest. If they are able to find adequate food items in garbage dumps, wolves may not need to prey on other mammals in the forest.

Based on the study of Peirce and Van Daele (2006), wolves can be seen feeding at the municipal garbage dump located approximately 3 km from the Sarıkamış city center, showing that this garbage dump comprises an important food source for gray wolves.

On the other hand, a recent study by Chynoweth et al. (2016) indicated that Sarıkamış forests host some notable mammalian carnivores such as Eurasian brown bear (*Ursus arctos arctos*), gray wolf (*Canis lupus*), Caucasian lynx (*Lynx lynx dinniki*), wildcat (*Felis silvestris*), and beech marten (*Martes foina*). However, none of these are prey species, which were exceptionally rare and included wild boar (*Sus scrofa*), Eurasian hare (*Lepus europaeus*), and roe deer (*Capreolus capreolus*). In addition, half of Turkey's forest area (Şekercioğlu et al., 2011b) including Sarıkamış forests has been degraded due to forest fires and deforestation for residential development (Gross, 2012), which might have further decreased the mammal diversity and abundance in the region. This rarity of mammalian prey species in Sarıkamış forests are in concordance with our dietary results in terms of detection of prey items. Due to the lack of adequate mammalian prey in Sarıkamış forests, the prey preference of gray wolves might have had to shift towards birds, amphibia, and plants.

Prey availability and human-carnivore conflict are strong factors that lead to the distribution and abundance of large carnivores and determine the suitability of regions for their conservation (Winterbach et al., 2015). Gray wolf and red fox that inhabit major ecosystems of Turkey experience many different threats, and they are known to be at the center of human-wildlife conflict (Ambarlı et al., 2016). In Sarıkamış forest, the scarcity of natural prey items leads to increased human-wolf conflict (Capitani et al., 2016). In our study, the lack of deer as food items for gray wolves and red foxes is likely to result in increased human-wildlife conflict in Sarıkamış forest.

3.3.1.2. Rana. In Sarıkamış and Yenice, frog DNA (of genus *Rana*) was detected in two samples each. Although there is no data on foxes preying on frogs of the genus *Rana* specifically, in harsh environmental conditions where it might be hard to find enough and preferred food items to feed on, wolves have been observed to eat frogs (https://www.wolfworlds.com). The results of this study point out that in Sarıkamış and Yenice Forests, there is such evidence of sub-optimal feeding behaviour, potentially suggesting the scarcity of food for wolves in these forests.

3.3.1.3. Aves (Passeriformes and Non-Passeriformes). Both Passeriformes and non-Passeriformes birds were detected in the diet of gray wolf samples in Sarıkamış. One Passeriformes prey item was detected in the diet of gray wolf. In addition, the sequencing results showed that non-Passeriformes (Anatidae) (two samples) were the vertebrate prey for gray wolves in Sarıkamış. Again, non-Passeriformes were detected in the diets of gray wolves in other studies as well. In Canada, non-Passeriformes were identified in gray wolf diet through detection of feathers, claws, and cuticles (Bryan et al., 2006). The results of this study are in concondance with the literature in terms of occasional preying of birds by gray wolves.

3.3.2. Plant Diet Composition for Gray Wolves

In Sarıkamış/Kars, plants of the rose family (Rosaceae) (three samples), beech family (Fagaceae) (one sample), buttercup family (Ranunculaceae) (one sample) and wheat family (Poaceae) (five samples) were identified in the gray wolf diet. On the other hand, rose family (Rosaceae) (four samples), grass family (Fabaceae) (one sample) and beech family (one sample) were detected in Yenice/Karabük.

These findings are paralel to the literature where, identifiable plant items found in scats of gray wolf included members of the beech family (Wagner et al., 2012) and rose family (Wagner et al., 2012; Meriggi et al., 1996). In addition Stahler et al. (2006) showed that in the dietary profile of gray wolves in Canada, plant material was prevalent with 74% of scats analyzed including some type of plant material, largely of the grass family. The plants such as grasses, unidentified seeds, and pine particles can also be ingested accidentally along with prey items that were on the ground (Jędrzejewski et al., 2002). The finding of wheat in the diet of wolf, which was not previously recorded in the literature could potentially be due to contamination because of the study of wheat in the laboratory that the study was undertaken in.

3.4. Diet Composition of Red Foxes

For the red fox, morphological approaches were used to analyze prey content in scats (e.g. Cavallini and Volpi, 1996; Lanszki, 2005; Prigioni et al., 2008; Pagh et al., 2015) and stomach (Cavallini and Volpi, 1996; Kidawa and Kowalczyk, 2011; Pagh et al., 2015). Prey identification for red foxes was generally performed by microscope and prey species were distinguished by defining

some particles such as hair, feather, dentition, wing, cuticle and leg (Lanszki, 2005; Prigioni et al., 2008).

In this study, the vertebrate diet composition of red fox included Passeriformes (n=1 in Sarıkamış) and the plant diet composition included Liliopsida (n=5 in Sarıkamış). In this respect, vertebrate and plant diet compositions of red fox (Table 3.3 and Table 3.4) are discussed in greater detail below.

3.4.1. Vertebrate Diet Composition for Red Foxes

3.4.1.1. Aves (Passeriformes). Passeriformes birds were detected in the diet of red fox samples in Sarıkamış. One red fox sample showed the presence of Passeriformes. Passeriformes birds were detected in the stomachs of red foxes as identified from feathers and other remains in Denmark (Pagh et al., 2015). Lanszki (2005), through microscopic analysis of basis of feather, bone, dentition and hair detected eight small Passeriformes spp. as prey items for red foxes in Hungary. In Eastern Italian Alps, diet composition of red foxes also showed that Passeriformes were preyed upon by red foxes, though to a small extent (3.7%) (Prigioni et al., 2008).

3.4.2. Plant Diet Composition for Red Foxes

3.4.2.1. Magnoliopsida. In the diet of red fox, wheat (Poaceae) (two samples) and rose family (Rosaceae) (three samples) were the plant content from red fox scat in Sarıkamış (Table 3.6). Evidence from the literature suggests that red foxes eat plant materials such as wild fruits, cultivated fruits (Cavallini and Volpi, 1996), maize zea mays (Lanszki, 2005), cereal, seed, sunflower seed, apple, pear, raspberry, bilberry, grass (Kidowa and Kowalczyk, 2011), rose-hips and whitebeam berries (Prigioni et al., 2008), leaves from herbs and trees, twigs, hay, seeds, or remains of fruits (Pagh et al., 2015).

In this study, as mentioned above, some plant dietary content for the red fox belongs to the rose family, and this finding is in line with the literature. Studies by Patalano and Lavori (1993) in Mediterranean Mountain Area, and Balestrieri et al., (2011) in Western Italian Alps showed Rose family as a plant food item for the red foxes. In addition, Papageorgiou et al., (1988) in Greece and Remanti et al., (2005) in Northwestern Italian Alps and Serafini and Lavori (1993) in Central Italy revealed similar plant content (Rose family). In all those studies, researchers examined the food

remains in scats through microscopic analyses of seeds. On the other hand, the detection of wheat could be due to contamination, as discussed above for the gray wolf.

3.5. Invertebrate Diet Composition for Gray Wolf and Red Fox

In this study, invertebrate prey content was not detected in gray wolf and red fox scat samples. The literature shows that the gray wolves eat invertebrate materials such as marine invertebrates (e.g. crabs, bivalves), as identified by shell fragments and cuticles. The study by Bryan et al., (2006) in Coastal British Columbia, revealed invertebrates were one of the most common prey items of grey wolves. In addition, other studies showed invertebrates were eaten by gray wolves (Ciucci et al., 1996 - Northern Apennines, Italy; Meriggi et al., 1996 - Northern Italy; Lanszki et al., 2012 - Hungary), with the prey items determined by microscopic analyses.

Similarly, the literature shows that the red foxes eat invertebrate materials such as carabid beetles *Corabidae spp*., cockhafer *Metolontha spp*. (Lanszki, 2005 - Hungary), dung beetles, ground beetles, earthworm, crab and larvae of diptera (Pagh et al., 2015 - Denmark), and ground beetles (Carabidae) (Kidawa and Kowalczyk, 2011 – Northeastern Poland), as determined by microscopic analyses. In our study, the inability to detect any invertebrate DNA could indicate the lack of a preference for this kind of food item. However, it is also possible that this could be a Type-1 error, where the presence of invertebrates was not detected, even though they were a part of the diet of the foxes and wolves in Sarıkamış and Yenice Forests. It is possible that even though the 1st PCR to amplify the invertebrate DNA was successful, the region amplified could have been a nuclear-mitochondrial insertion (numt), resulting in a failure to match any sequences in the invertebrate database created. There is evidence that numts are found in invertebrate genomes (Pamilo et al., 2007; Hakzani-Covo et al., 2003).

4. CONCLUSIONS

4.1. Genetic Diversity and Phylogeographic Analyses

The mitochondrial DNA d-loop sequences acquired in this study is an important tool to reveal genetic divergence and phylogeographic relationships, and to identify population boundaries within gray wolves and red foxes, particularly on the basis of non-invasively collected samples (fecal remains). This thesis is one of the limited number of studies which provides molecular data on *Canis lupus* and *Vulpes vulpes* in Turkey. The gray wolf results revealed that Kars-Caucasus and Kars-Karabük samples were genetically similar to each other, and these populations from Turkey and Caucasus might need to be protected together as management units at local geographic scales. For the red fox samples, haplotype and nucleotide diversities in Kars were found to be low. In addition, global distributions of haplotypes were detected in the haplotype network and phylogenetic trees, and the haplotypes from Kars/Turkey formed groups with these global haplotypes, without much local structuring. Global genetic diversity comparisons suggested that the New World red fox populations have their origins in the Old World, in line with the literature.

4.2. Dietary Habits through Genetics

The diet of gray wolf and red fox has not been studied previously using DNA barcoding method in Turkey. The results of dietary habits for gray wolves showed the following categories: mammals (red deer), amphibia (frogs), Aves (Passeriformes and non-Passeriformes), and plants (wheat, rose family, buttercup, beech family, the family Poaceae). The diet composition of red foxes included the family Aves (Passeriformes) and plants (wheat and rose family). Our results of the vertebrate and plant dietary content for the red fox are in concordance with the literature. The evidence of frog as prey item for gray wolves is evidence of sub-optimal feeding behaviour, potentially suggesting the scarcity of adequate food resources in Sarıkamış and Yenice Forests. On the other hand, invertebrate prey content was not detected for the gray wolves and red foxes. In our study, the inability to detect any invertebrate DNA could be due to amplification of numts during the diet library preparation for this group.

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APPENDIX A: LIBRARY CONSTRUCTION

First PCR

Total first PCR product will be equal to **[Forward Primer with Adapter] + [Region of interest] + [Reverse complementary of Reverse primer with adapter].**

Vertebrate Forward Primer: TTAGATACCCCACTATGC

Vertebrate Reverse Primer: TAGAACAGGCTCCTCTAG

Forward region primer with adapter:

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTAGATACCCCACTATGC

Reverse region primer with adapter:

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTAGAACAGGCTCCTCTAG

First PCR Product (5' to 3'):

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTAGATACCCCACTATGC

+ **[Region of interest, 98 bp]**

+CTAGAGGAGCCTGTTCTACTGTCTCTTATACACATCTGACGCTGCCGACGAC

Second PCR

Forward Sequencing primer: GTGGGCTCGGAGATGTGTAT

Reverse Sequencing primer: GGCTCCTCTAGAAGGGGTTG

Second PCR Product (5' to 3'):

Primer Totals [5' to 3']

Adapter sizes

Forward

CAAGCAGAAGACGGCATACGAGATTAAGGCGA[i5index]GTCTCGTGGGCTCGGAGATG
TGTATAAGAGACAG[Forward region primer]

66 bp+Barcode

Reverse

AATGATACGGCGACCACCGAGATCTACACCGTACTAG[i7index]TCGTCGGCAGCGTCAG
ATGTGTATAAGAGACAG[Reverse region primer]

70 bp+Barcode
CAAGCAGAAGACGGCATACGAGATTAAGGCGA**[i5index]** **+ First PCR Products +**

[Reverse complementary of [i7index]

+CTAGTACGGTGTAGATCTCGGTGGTCGCCGTATCATT **[Reverse complementary of reverse region primer]**

APPENDIX B: TOTAL NUMBER OF READS GENERATED, THEIR SAMPLE TYPES AND LOCALITIES

Sample Names	Sequence reads (Mbp)	Sample Type-Locality	
1-1	20.46	Wolf	Yenice
1-2	17.29	Wolf	Yenice
1-3	6.84	Wolf	Yenice
2-1	23.63	Wolf	Yenice
2-2	23.42	Wolf	Yenice
3-1	29.17	Wolf	Yenice
3-2	25.84	Wolf	Yenice
4-1	14.66	Wolf	Yenice
4-2	9.39	Wolf	Yenice
4-3	21.15	Wolf	Yenice
5-1	8.06	Wolf	Yenice
5-2	11.60	Wolf	Yenice
60-1	23.32	Wolf	Yenice
60-2	31.44	Wolf	Yenice
60-3	30.67	Wolf	Yenice
116-1	24.73	Wolf	Yenice
116-2	15.12	Wolf	Yenice
2257-1	19.90	Wolf	Sarıkamış
2257-2	11.74	Wolf	Sarıkamış
2341-1	12.83	Wolf	Sarıkamış
2341-2	1.32	Wolf	Sarıkamış
2408-1	10.55	Wolf	Sarıkamış
2408-2	26.80	Wolf	Sarıkamış
2408-3	36.88	Wolf	Sarıkamış

(continued)

APPENDIX B. CONTINUED.

Sample Names	Sequence reads (Mbp)	Sample Type-Locality	
2416-1	20.09	Wolf	Sarıkamış
2416-2	16.04	Wolf	Sarıkamış
3153-1	16.38	Wolf	Sarıkamış
3153-2	17.44	Wolf	Sarıkamış
3442-1	25.75	Wolf	Sarıkamış
3442-2	15.26	Wolf	Sarıkamış
3442-3	38.43	Wolf	Sarıkamış
4138-1	15.86	Wolf	Sarıkamış
4138-2	14.24	Wolf	Sarıkamış
4138-3	19.09	Wolf	Sarıkamış
4144-1	26.60	Wolf	Sarıkamış
4144-2	1.25	Wolf	Sarıkamış
232-1	14.06	Fox	Sarıkamış
232-2	17.96	Fox	Sarıkamış
233-1	24.95	Fox	Sarıkamış
233-2	15.04	Fox	Sarıkamış
234-1	20.35	Fox	Sarıkamış
234-2	8.11	Fox	Sarıkamış
236-1	20.41	Fox	Sarıkamış
236-2	25.42	Fox	Sarıkamış
240-1	16.11	Fox	Sarıkamış
240-2	16.83	Fox	Sarıkamış
Total reads:	**862.48**		